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Lung Carcinoid Tumors: Histology and Ki-67, The Eternal Rivalry

Short title: Lung Carcinoid Tumors: Histology or Ki-67?

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Abstract

Background: WHO classification of Thoracic Tumors defines lung carcinoid tumors (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 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.

Methods and results: 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% tumors were significantly associated with AC histology, stage III-IV, smoking, vascular invasion, tumor 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 *vs* <3) and histology (AC *vs* 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 *vs* <3, p adj=0.01) to TC and AC histological distinction, age, lymph node involvement, residual tumor and OTP.

Conclusions: Ki-67≥3% plays a potentially pivotal role in LCT prognosis, irrespective of histological grade.

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

1. Introduction

Lung neuroendocrine tumors (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 2mm^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 tumors 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 tumors (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).

2. Materials and Methods

2.1. 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 tumor 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 hematoxylin-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 Tumors (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.

2.2. Histologic Analysis and Immunohistochemistry

Morphologic analysis considered: a) well differentiated neuroendocrine morphology; b) architectural pattern of the tumor 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 tumor 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) tumor 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 tumor cells counted in areas of strongest nuclear labeling ("hot spots") as indicated in the WHO 2019 Digestive System Tumors; thyroid transcription factor 1 (TTF-1), CD44, orthopedia homeobox protein (OTP), somatostatin receptor 2A (SSTR-2A), mammalian achaete-scute homolog 1 (Ascl1), and p53 using the antibodies listed in Supplementary Table 1.

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 tumor cells), heterogeneous (variable expression in 21-60% of tumor cells) and overexpressed (strong p53 staining in more than 60% of tumor 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.

2.3. Statistical Analysis

Data were analyzed by descriptive statistics. Associations between demographic characteristics, clinicopathological features and Ki-67 groups (≥3% vs. <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 tumor-associated death, respectively. DFS was assessed from the date of diagnosis to the date of first relapse, tumor-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.

3. Results

3.1. 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 Supplementary Table 2, respectively. Overall, pathologic review identified 297 (83.7%) TCs and 58 (16.3%) ACs. The whole cohort comprised more females than males (62.3% *vs* 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 tumors. The most advanced surgicaly-resected tumors (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.

3.2. 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). Tumors 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%, p<0.0001) and solid morphologic pattern (*n*=39, 70.9%, p<0.0001).

3.3. 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 Supplementary Figure 1 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.

3.4. Immunohistochemical markers

The distribution of all investigated IHC markers were reported in Table 2 and Supplementary Table 3. 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.

3.5. Overall, Cancer-Specific and Disease-Free survival

Overall, 58 patients (16.3%) showed tumor-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-3B, 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).

In the entire cohort, 79 (22.3%) patients experienced a tumor-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-4B). 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 tumors 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 tumor stage ($p<0.0001$), positive STAS ($p=0.008$), solid architectural pattern ($p=0.01$) and residual tumor ($p=0.01$). 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.

3.6. 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 increase), tumor stage (III-IV *vs* I-II) and OTP (present *vs* 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 *vs* 0), residual tumor (R1/2 vs R0) and OTP (present *vs* 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 alone (≥3 *vs* <3, p adj=0.0008) or histology alone (AC *vs* 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 *vs* <3, p adj<0.0001) or histology alone (AC *vs* 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-$\Delta\chi2$=6.3, p adj=0.01) to center, period of diagnosis, age, lymph involvement, residual tumor, OTP and histology, while addition of histology (LR-$\Delta\chi2$=0.7, p adj=0.40) to a model containing Ki-67 did not improve significantly prognostic information (Table 5).

4. 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 tumors 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 tumors. 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 tumor 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 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 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, tumors with Ki-67 ≥3% are strongly associated with AC histology. Indeed, both WHO tumor histology (AC *vs* TC) and Ki-67 (≥3 *vs* <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 observed improved statistical significance by adding Ki-67 (≥3 *vs* <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 unfavorable prognosis.[22] Our study confirms this observation. Therefore, due its prognostic role as well as high sensitivity and specificity for pulmonary carcinoid tumors,[23] OTP IHC marker should be included in the diagnostic workup.

5. Conclusion

In conclusion, although Ki-67 index is not considered in the 2021 WHO Classification of Thoracic Tumors 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.

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List of abbreviations:

AC, atypical carcinoid

Ascl1, mammalian achaete-scute homolog 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, hematoxylin-eosin

IHC, Immunohistochemical

LCTs, lung carcinoids tumors

LR-$\Delta\chi 2$, changes in likelihood ratio values

Lu-NET, lung neuroendocrine tumor

MC, mitotic count

NEN, neuroendocrine neoplasm

NET, neuroendocrine tumor

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

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Author's note: 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.

List of online Supporting information:

1. **Supplementary Table 1.** Antibody sources and dilutions.
2. **Supplementary Table 2.** Characteristics of patients with LCTs according WHO classification.
3. **Supplementary Table 3.** Association between selected tumor biomarkers and WHO classification in patients with LCTs.
4. **Supplementary Figure 1.** Ki-67 labeling index of the LCTs and duration of block storage.

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Figure legends

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 tumors.

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 tumors; WHO, World Health Organization; OTP, orthopedia homeobox protein; SSTR-2A, somatostatin receptor 2A.

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 tumors; WHO, World Health Organization; OTP, orthopedia homeobox protein.

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**
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 Tumor				
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
Tumor associated deaths				
No	262 (82.7)	225 (86.5)	37 (64.9)	
Yes	55 (17.4)	35 (13.5)	20 (35.1)	**0.0003**

Note: # patients where Ki-67 was evaluable, * p-value based on the Fisher's exact for categorical

variables and the Wilcoxon test for continuous variables. Abbreviation: LCTs, lung carcinoids tumors; STAS, spread trough air spaces.

Table 2. Association between selected tumor 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**

Note: # patients where Ki-67 was evaluable, * p-value based on the Fisher's exact Test for categorical variables. Abbreviation: LCTs, lung carcinoids tumors; TTF-1, thyroid transcription factor 1; SSTR-2A, somatostatin receptor 2A; OTP, orthopedia homeobox protein; Ascl1, mammalian achaete-scute homolog 1.

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 vs 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 vs 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 vs 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 vs 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 vs 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 vs <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 vs 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 vs 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 vs 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 vs 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 vs 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 vs 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 vs 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 Tumor (R1/2 vs 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**
TIF1 (Present vs 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 vs 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 vs 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 vs 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 vs 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 vs 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**

Note: *Adjusted for center and period of diagnosis categorized in decades (<1998, 1998-2007, 2008-2018); # Evaluated on Stage I-II-II patients only. Abbreviation: LCTs, lung carcinoids tumors; OS, overall survival, CCS, cancer-specific survival; DFS, disease-free survival; STAS, spread trough air spaces; TTF-1, thyroid transcription factor 1; OTP, orthopedia homeobox protein; SSTR-2A, somatostatin receptor 2A; Ascl1, mammalian achaete-scute homolog 1.

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

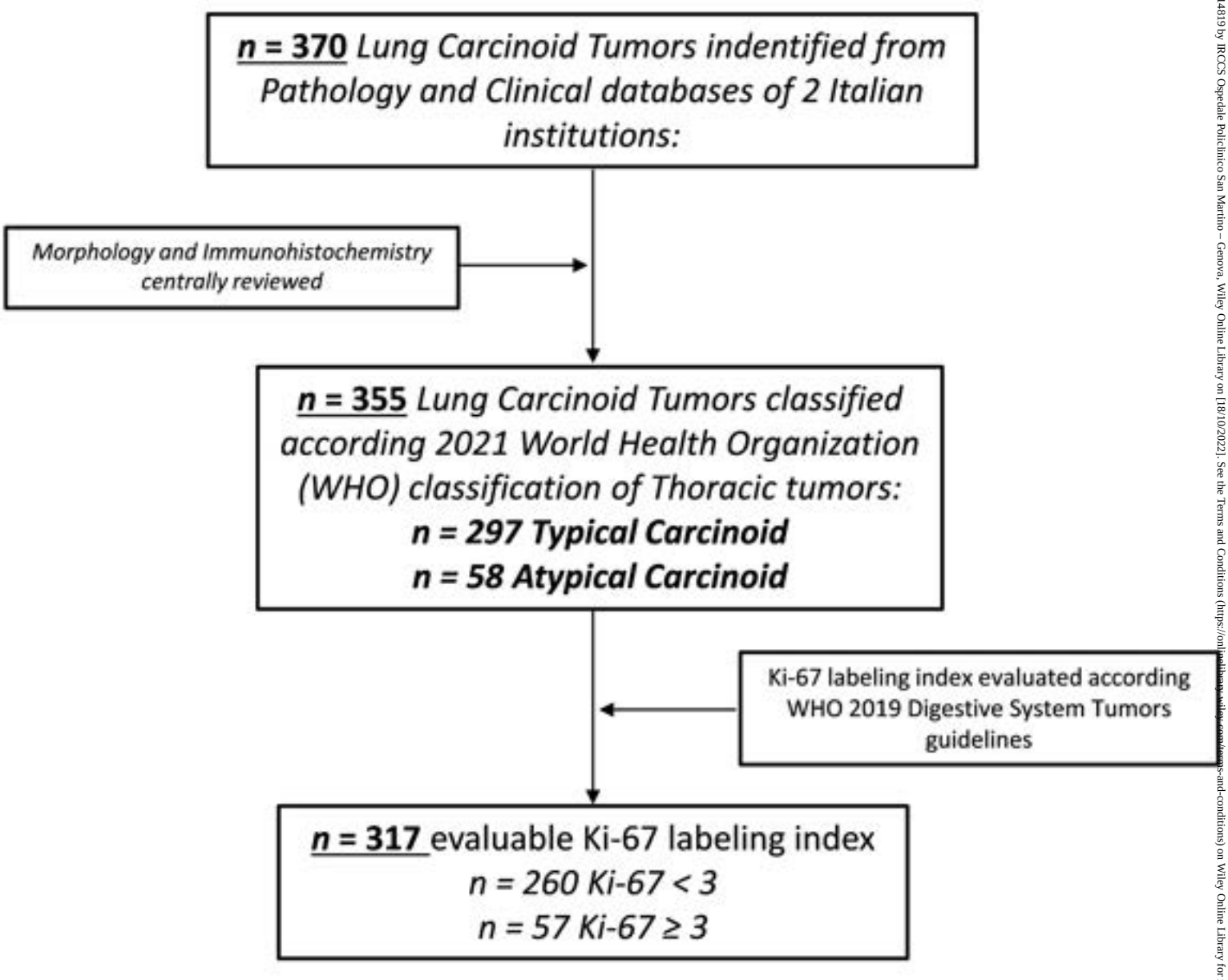
Variable	OS Multivariable Model I HR (95% CI)	P-value	OS Multivariable Model II HR (95% CI)	P-value
Age (10-years increase)	2.15 (1.66-2.79)	**<0.0001**	2.20 (1.69-2.86)	**<0.0001**
Histotype (AC vs TC)	-		3.38 (1.83-6.25)	**<0.0001**
Stage (III-IV vs I-II)	2.94 (1.60-5.40)	**0.0005**	2.82 (1.54-5.19)	**0.0008**
Ki-67 (≥3 vs <3)	3.41 (1.78-6.54)	**0.0002**	-	
OTP (Present vs 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 vs TC)	-		3.68 (1.91-7.08)	**<0.0001**
Stage (III-IV vs I-II)	2.71 (1.44-5.10)	**0.002**	2.58 (1.37-4.86)	**0.003**
Ki-67 (≥3 vs <3)	3.35 (1.72-6.53)	**0.0004**	-	
OTP (Present vs 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 vs TC)	-		4.15 (2.19-7.88)	**<0.0001**
N (1/2/3 vs 0)	3.61 (1.94-6.72)	**<0.0001**	2.87 (1.54-5.35)	**0.0009**
Ki-67 (≥3 vs <3)	5.23 (2.73-10.02)	**<0.0001**	-	
OTP (Present vs Absent)	0.47 (0.26-0.85)	**0.01**	0.50 (0.27-0.93)	**0.03**
Residual Tumor (R1/2 vs R0)	1.93 (0.84-4.43)	0.12	2.58 (1.13-5.88)	**0.02**

Note: *Adjusted for center and period of diagnosis categorized in decades (<1998, 1998-2007, 2008-2018); # Evaluated on Stage I-II-III patients only. Abbreviation: LCTs, lung carcinoids tumors; OS, overall survival, CCS, cancer-specific survival; DFS, disease-free survival; OTP, orthopedia homeobox protein.

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	**<.00001**	**< .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	**<.00001**	**< .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	**<.00001**	**< .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	**<.00001**	**< .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 Tumor + OTP	669.4	50.8	7	**<.00001**	**< .00001**
Center + Period of diagnosis + Age + N + Residual Tumor + OTP + Ki67	645.7	23.7	1	**<.00001**	**< .00001**
Center + Period of diagnosis + Age + N + Residual Tumor + OTP + Ki67 + Histotype	645.0	0.7	1	0.40	0.40
Without covariates	720.2	-			
Center + Period of diagnosis + Age + N + Residual Tumor + OTP	669.4	50.8	7	**<.00001**	**< .00001**
Center + Period of diagnosis + Age + N + Residual Tumor + OTP + Histotype	651.3	18.2	1	**0.0002**	**0.0003**
Center + Period of diagnosis + Age + N + Residual Tumor + OTP + Histotype + Ki67	645.0	6.3	1	**0.01**	**0.01**

Abbreviation: OS, overall survival, CCS, cancer-specific survival; DFS, disease-free survival; OTP, orthopedia homeobox protein; LR-Δχ2, changes in likelihood ratio values; DF, degrees of freedom. †Correction for multiple comparisons according to Benjamini–Hochberg



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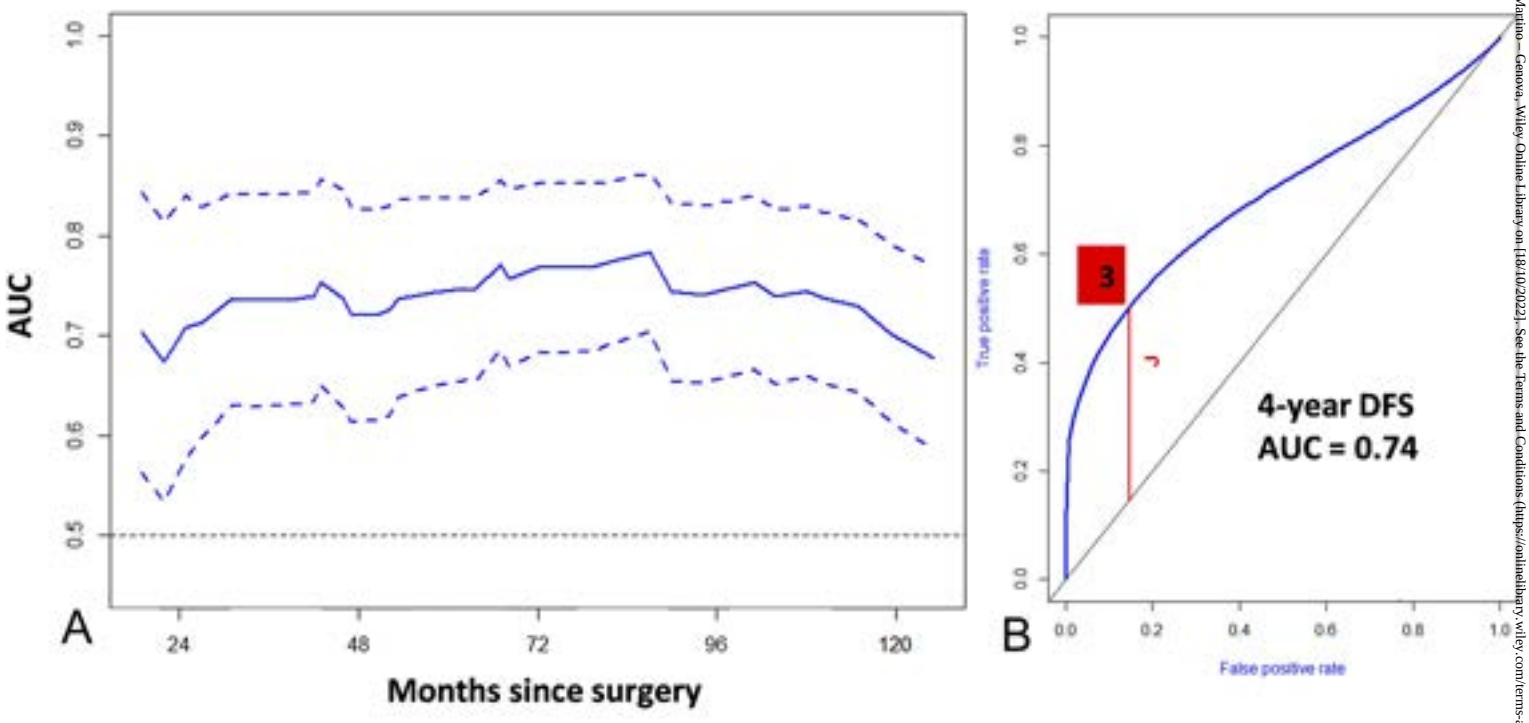

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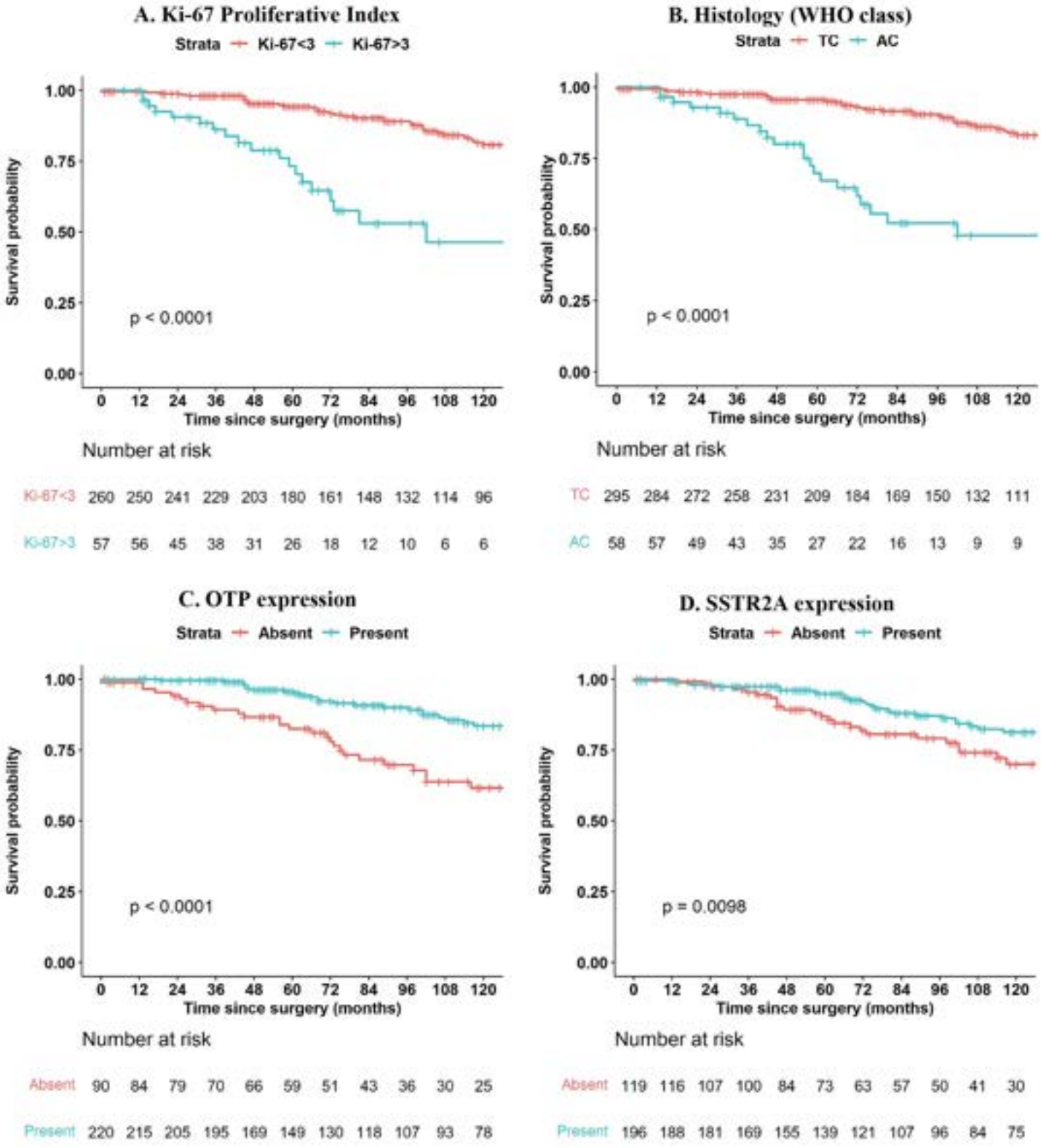

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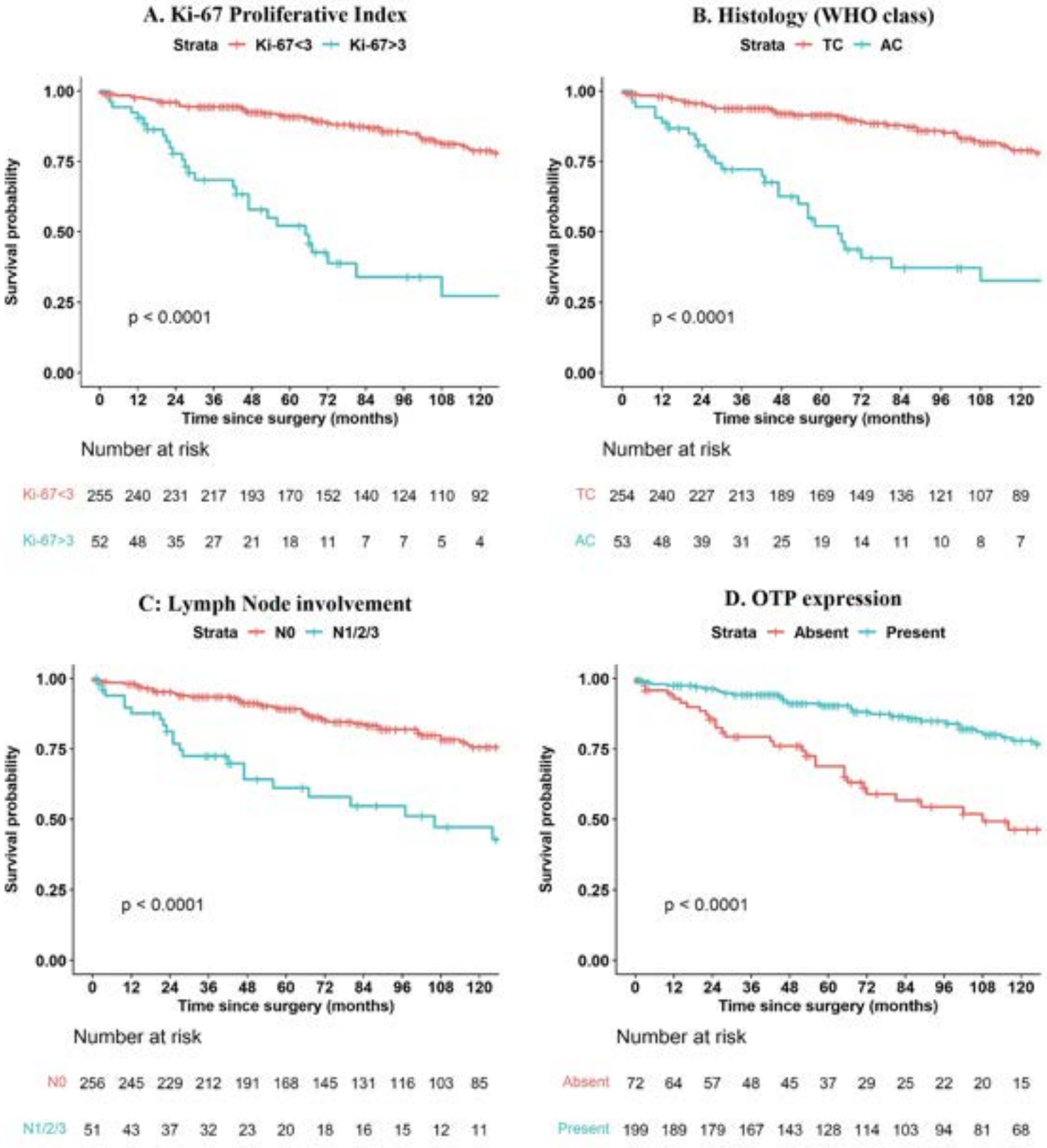

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