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Ki-67 labeling index of neuroendocrine tumors of the lung has a high level of correspondence between biopsy samples and surgical specimens when strict counting guidelines are applied

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Abstract

Optimal histopathological analysis of biopsies from metastases of neuroendocrine tumor (NET) of the lung requires more than morphology only. Additional parameters such as Ki-67 labeling index are required for adequate diagnosis, but few studies have compared reproducibility of different counting protocols and modalities of reporting on biopsies of lung NET. We compared the results of four different manual counting techniques to establish Ki-67 LI. On 47 paired biopsies and surgical specimens from 22 typical carcinoids (TCs), 14 atypical carcinoids (ACs), six large cell neuroendocrine carcinomas (LCNECs), and five small cell carcinomas (SCCs) immunohistochemical staining of Ki-67 antigen was performed.We counted, in regions of highest nuclear s t a i n i n g (HSR), a f u l l °—40-high-power f i e ld (diameter = 0.55 mm), 500 or 2000 cells, or 2 mm^2 surface area, including the HSR or the entire biopsy fragment(s). Mitoses and necrosis were evaluated in an area of 2 mm^2 or the entire biopsy fragment(s). Between the four counting methods, no differences in Ki-67 LI were observed. However, a Ki-67 LI higher than 5% was found in only four cases when in an HSR, 500 cells were counted (18%), five (23%) when in an HSR 2000 cells were counted, four (18%) when 2 mm^2 were counted, and one (5%) TC case when the entire biopsy was counted. A 20% cutoff distinguished TC and AC from LCNEC and SCC with 100% specificity and sensitivity, while mitoses and necrosis failed to a large extent. Ki-67 LI in biopsy samples was concordant with that in resection specimens when 2000 cells, 2 mm^2, or the entire biopsy fragment(s) were counted. Our results are important for clinical management of patients with metastases of a lung NET.

Keywords KI-67 antigen . Immunohistochemistry . Lung . Neuroendocrine . Tumors . Typical . Atypical . Carcinoid . Large . Small . Cell . Carcinoma . Methodology

Introduction

Neuroendocrine tumors (NETs) of the lung are a heterogeneous family of neoplasms comprising four histologic variants: typical carcinoid (TC), atypical carcinoid (AC), largecell neuroendocrine carcinoma (LCNEC), and small-cell carcinoma (SCC) [1–6]. Classification criteria include cytological and histological features, the occurrence and extent of necrosis, the number of mitoses per 2 mm^2, and the immunohistochemical (IHC) expression of pan-NE markers [1–3]. Although in comparison with previously proposed diagnostic schemes these criteria have been consolidated in the three most recent WHO classifications [3, 7–12], interindividual diagnostic reproducibility for these tumors remains disappointing, likely due to inconsistency in recognizing necrosis, mitoses, and cytological details [13–19]. Furthermore, diagnostic criteria have been established on surgical specimens but not validated on biopsies of lung NET, which therefore remain a diagnostic challenge with important clinical implications [1, 20–23]. Gastroenteropancreatic NE tumors (GEP-NETs) are graded on biopsies and resection specimens using both mitotic count and Ki-67 antigen labeling index (Ki-67 LI, the percentage of labeled nuclei after IHC staining), with clinical implications [24–27]. Ki-67 is a generally accepted proliferation marker [28, 29], and the Ki-67 LI is clinically relevant in terms of guiding treatment decisions as supported by studies on a variety of tumors with confirming data [24–27]. The role of Ki-67 LI in

lung NET has been the subject of several independent investigations, with potential diagnostic, prognostic, and grading implications (recently reviewed by Pelosi et al., 2014) [30]. Although differences in Ki-67 LI are reported between the four histological variants [3, 30], its significance as diagnostic criterion is contested [3] as there is overlap between the histological categories and Ki-67 LI are not perfectly congruent with other defining criteria [4–6, 30]. Currently, in lung NET, Ki-67 LI can only be used to distinguish between TC/AC from SCC on cytology and/or biopsy samples, notably when the volume of material is limited [3] and/or crush artifacts are present [31]. According to histology, TC, AC, and LCNEC/SCC indicate low-, intermediate-, and high-grade tumors, respectively [3]. However, the proportion of lung NET with similar histology that behave differently is not negligible, which justifies further grading. As prognostic capability has been attributed to Ki-67 LI in several studies [4–6, 19, 30], it can be used for grading [32], complementary to conventional terminology [33]. At variance with the wide experience gained in performing Ki-67 LI in GEP-NET [24–27, 34], methodological studies on biopsy samples of lung NET are currently lacking, although they may be potentially helpful in planning treatment for metastatic tumors [22, 23]. On challenging material mitotic count and necrosis assessment tend not to be more informative, and NE marker IHC may provide disappointing results [21, 31]. However, before authorizing diagnostic use of Ki-67 LI on biopsy samples, the accuracy and reproducibility of different counting methods are needed. In this study, we compared four techniques for manual counting of Ki-67 stained biopsy samples of lung NET and their corresponding surgical specimens.We found that identification of hot spot regions (HSR) and counting 2000 cells or a 2-mm2 surface area including HSR or the entire biopsy fragment(s), reduces variability in counting results. Counting of mitoses and estimation of necrosis did not contribute to provide more reproducible results.

Materials and methods

Patients and tumors

For this retrospective and observational investigation, a series of 47 consecutive biopsies of lung NET and paired surgical specimens were identified in the archives of two participating Pathology Institutes (Milan and Turin), during the period from January 1983 to December 2014. This timeframe was due to the rarity of lung NET and our rather selective inclusion and exclusion criteria. These included the availability of corresponding biopsy and resection specimens, exclusion of unrepresentative biopsies (exclusive fibrosis, necrosis or inflammation with no tumor components) or samples with fixation artifacts on which IHC turned out suboptimal, lack of a previous history of NET elsewhere in the body, and absence of chemotherapy before biopsy or surgery, to minimize changes in proliferation rates or amount of necrosis. The cohort comprised 45 lung and two thoracic lymph node biopsies (39 bronchoscopy biopsies and eight computed tomography-guided thoracic biopsies), with corresponding surgical segmentectomy, lobectomy or pneumonectomy specimens along with radical mediastinal lymph node dissection to ensure accurate staging. Biopsy and resection specimens had been fixed in 4% buffered formaldehyde solution for up to 24 h and embedded in paraffin according to standard laboratory methods. All original hematoxylin and eosin (H&E) and IHC-stained sections were revised by two pairs of pathologists applying WHO classification-based criteria (for 27 Milan cases GP and MC; for 20 Turin cases LR and GG) without knowledge of patient identity or original tumor classification [35]. Assessment of interobserver variability was not a goal of the present study but significant differences were not observed between the two groups of pathologists in any assay.

Immunohistochemistry

Four micron sections were reacted for 30 min with antibodies to Ki-67 antigen, chromogranin A, and synaptophysin, and then incubated with a commercially available detection kit (DAKO EnVision Plus-HRP, Dako, Glostrup, Denmark) according to the manufacturer's instructions and previously refined IHC methods [31, 36]. Technical details on IHC procedures are shown in Supplement a l Mate r i a l A. Immunoreactivity was assessed semiquantitatively as the percentage of tumor cells showing nuclear (Ki-67) or cytoplasmic (chromogranin A and synaptophysin) decoration by visual scanning of the entire tumor area of stained sections for NE markers and discrete areas of highest nuclear labeling (hotspot regions, HSR) for Ki-67 antigen [30]. A manual cell counter was used to count Ki-67 staining nuclei to establish a LI, the percentage of labeled tumor cells. We used four counting approaches: the entire HSR at °—40 magnification (field

Table 1 Clinicopathologic characteristics on the 47 lung neuroendocrine tumors under evaluation

Characteristics	Variable	All histologic subtypes	Typical carcinoid ($n = 22$)	Atypical carcinoid ($n = 14$)	Large cell neuroendocrine carcinoma ($n = 6$)	Small cell carcinoma ($n = 5$)	p Value
Age	Mean ± SD	53.3 ± 22.1	50.5 ± 16.0	48.0 ± 32.9	66.1 ± 11.6	65.2 ± 4.2	$p = 0.09$[a]
Gender	M	20	5	6	4	5	$p = 0.01$[b]
	F	27	17	8	2	0	
pT	1A	19	11	4	2	2	$p = 0.23$[b]
	1B	9	7	2	0	0	
	2A	14	3	5	3	3	
	2B	1	0	1	0	0	
	3	4	1	2	1	0	
pN	pN0	27	14	7	3	3	$p = 0.79$[b]
	pN1	15	7	5	2	1	
	pN2	5	1	2	1	1	
Tumor stage[a]	IA	22	14	4	2	2	$p = 0.40$[b]
	IB	5	2	1	1	1	
	IIA	10	4	4	1	1	
	IIB	4	1	3	0	0	
	IIIA	6	1	2	2	1	
Type of materials	CT-guided biopsy	8	2	3	3	0	$p < 0.01$[b]
	Bronchoscopy biopsy	39	20	11	3	5	
Site	Lung	45	22	14	5	4	$p = 0.05$[b]
	LN	2	0	0	1	1	
Necrosis on biopsy samples	Absent	39	22	12	2	3	$p < 0.01$[b]
	Present	8	0	2	4	2	
Necrosis on resection specimens	Absent	29	22	7	0	0	$p < 0.01$[b]
	Present	18	0	7	6	5	
Necrosis percentage (mean ± SD)	Biopsy	2.5 ± 8.7	0	3.6 ± 10.8	2.3 ± 2.3	12.0 ± 17.9	$p = 0.09$[a]
	Resection	7.4 ± 13.1	0	3.4 ± 5.3	29.2 ± 11.1	25.0 ± 16.6	$p < 0.01$[a]
Histologic pattern on biopsy samples	Lobular	18	13	4	1	0	$p < 0.01$[b]
	Solid	16	4	3	5	4	
	Trabecular	13	5	7	0	1	
Histologic pattern on resection specimens	Lobular	17	15	4	1	0	$p < 0.01$[b]
	Solid	17	4	3	5	5	
	Trabecular	13	3	7	0	0	
Number of biopsy fragments per case	Mean ± SD	3.0 ± 2.3	2.6 ± 1.7	2.9 ± 3.2	3.2 ± 1.5	4.4 ± 2.3	$p = 0.21$[a]
Mean size in millimeter per biopsy sample	Mean ± SD	4.1 ± 3.5	3.7 ± 4.2	3.4 ± 2.3	7.5 ± 3.1	3.8 ± 1.3	$p = 0.01$[a]

Tumor stage was performed according to VII ed. TNM/AJCC system

M male, *F* female, *LN* lymph node

[a] Kruskal-Wallis test

[b] Fisher exact or chi squared test

diameter = 0.55mm; high-power field area = 0.237mm2), 500 or 2000 cells in the same HSR(s), and a 2-mm2 area including HSRs (on average 8.4) or the entire tumor fragment(s), when this was smaller. Mitoses were counted in a 2-mm2 area as is recommended in current diagnostic guidelines [3]. Necrosis was noted as being absent or present regardless of extent. Statistical analysis Differences in the distribution of clinicopathological characteristics across the various histological tumor subtypes [3], and comparison between biopsies and surgical specimens or among different ways to count Ki-67 LI was assessed using the Fisher exact test for categorical variables (gender, site, pT, pN, stage), the non-parametric Kruskal-Wallis test for continuous variables (age), the non-parametric Wilcoxon signedrank test (biopsies vs. surgical specimens), and the repeated measures analysis (Ki-67 LI). Distribution of Ki67 LI for the various histological subtypes and according to the four counting techniques was also graphically represented using boxplots. Correlation and agreement between biopsies and surgical specimens for Ki-67 LI, mitotic count, and NE markers were also assessed using Deming regression, intraclass correlation coefficient (ICC), and Bland-Altman. graphical analysis [37]. Briefly, interobserver (within material type) agreement was graphically assessed by plotting the differences between the two measurements against the mean of the two values for each sample [37]. Limits of agreement, defined as twice the standard deviation of the differences between measures, were also calculated according to 95% confidence intervals. Differences between KI-67 LI in biopsies vs. surgical specimens were assessed using the Wilcoxon signed-rank test, while differences between the four different manual counting techniques were assessed by repeated measure analysis, assuming unstructured covariance matrix based on a random effect model. Sensitivity and specificity of KI-67

LI and chromogranin A to predict histological subtype was determined. Association of the presence of necrosis with histological and immunohistochemical variables was carried out using Fisher exact test for categorical variables and McNemar's test. All analyses were carried out using the SAS statistical software version 9.2 (SAS Institute, Inc., Cary, NC). All p values were two-sided and p values <0.05 were considered as significant.

Results

Our patient cohort consisted of 20 males (mean ± SD 58.5 ± 11.8; range 32–81 years) and 27 females (mean ± SD 51.7 ± 17.4; range 17–78 years). Of the tumors, 22 were stage IA, five stage IB, 10 stage IIA, four stage IIB, and six stage IIIA [27]. Histological types are listed in Table 1, along with the type of material, presence, and quantity (as percentage) of necrosis; main histologic patterns (lobular, solid, or trabecular); and for biopsies, mean number and size of tissue fragments. There were no differences between biopsy samples and surgical specimens in Ki-67 LI for lung NET as a whole or stratified by histology, except for the TC group when counting 500 tumor cells in an HSR (Table 2). No significant differences were observed either between biopsies and surgical specimens by counting 2000 cells and 2 mm^2 compared to 500 cells (Table 3). However, Ki-67 LI was higher than 5% in only four TC cases (18%) when 500 cells were counted in HSR, five (23%) when 2000 cells were counted in HSR, four (18%) when 2 mm^2 were counted, and one (5%) when the entire tissue fragment was counted. Distribution of Ki-67 LI using 500 cell or 2 mm^2 counting according to histological subtype in biopsies and surgical specimens is plotted in Fig. 1a, b and Fig. 2a–h. We found for Ki-67 LI and neuroendocrine markers with all counting approaches an excellent correlation between biopsies and corresponding resection specimens by means of intraclass correlation coefficient analysis. For the NET subtypes, this correlation was weaker with a wider 95% confidence interval, likely due to the small

Table 2 Ki-67 labeling index as a function of the different material type according to the four ways to count

Parameter	Material type	All tumors ($n = 47$)	TC ($n = 22$)	AC ($n = 14$)	LCNEC ($n = 6$)	SCC ($n = 5$)
500 cells	Biopsy sample	21.0 ± 32.6	2.8 ± 1.6	6.7 ± 4.2	64.7 ± 27.8	88.7 ± 9.9
	Surgical sample	21.0 ± 31.1	3.9 ± 1.8	8.4 ± 4.8	57.9 ± 30.3	86.9 ± 15.2
	ICC (95% CI)	0.97 (0.94–0.98)	0.19 (−0.23 to 0.54)	0.38 (−0.13 to 0.73)	0.84 (0.37–0.97)	0.31 (−0.54 to 0.85)
	p Value[a]	0.37	0.02	0.27	0.56	0.44
Hotspot region (HSR)	Biopsy sample	21.8 ± 33.8	2.9 ± 1.7	6.6 ± 4.2	69.5 ± 26.0	90.7 ± 9.2
	Surgical sample	21.7 ± 31.9	3.6 ± 1.7	8.4 ± 5.2	65.1 ± 26.3	86.7 ± 13.7
	ICC (95% CI)	0.98 (0.96–0.99)	0.36 (−0.04 to 0.67)	0.41 (−0.09 to 0.75)	0.96 (0.81–0.99)	−0.04 (−0.75 to 0.71)
	p Value[a]	0.67	0.09	0.36	0.31	1.00
2000 cells	Biopsy sample	19.1 ± 30.9	2.8 ± 1.8	6.6 ± 4.2	50.5 ± 32.2	88.1 ± 11.1
	Surgical sample	20.2 ± 29.5	3.2 ± 1.6	8.3 ± 6.0	60.8 ± 23.5	79.7 ± 14.7
	ICC (95% CI)	0.94 (0.89–0.96)	0.36 (−0.04 to 0.66)	0.40 (−0.11 to 0.74)	0.62 (−0.12 to 0.91)	0.34 (−0.52 to 0.86)
	p Value[a]	0.78	0.34	0.67	0.69	0.06
2-mm^2-spanning HSR or entire biopsy fragment(s)	Biopsy sample	20.6 ± 32.3	2.7 ± 1.7	6.3 ± 3.9	63.4 ± 26.2	88.1 ± 11.1
	Surgical sample	20.1 ± 30.3	2.6 ± 1.4	8.0 ± 6.0	62.5 ± 25.6	80.4 ± 14.1
	ICC (95% CI)	0.98 (0.97–0.99)	0.60 (0.26–0.80)	0.44 (−0.06 to 0.76)	0.97 (0.85–0.99)	0.36 (−0.50 to 0.86)
	p Value[a]	0.50	0.63	0.75	0.84	0.19

All values are expressed as mean ± standard deviation of percentage values

TC typical carcinoid, *AC* atypical carcinoid, *LCNEC* large cell neuroendocrine carcinoma, *SCC* small cell carcinoma, *ICC* intraclass correlation coefficient, *CI* confidence intervals

[a] Signed-rank test

number of tumors evaluated (Supplemental Material B). These findings were confirmed using Deming regression and Bland-Altman plot analysis, which show that differences between biopsy samples and surgical specimens were consistent across the range of data (constant bias) (Supplemental Material C). On biopsy samples, a cutoff value for Ki-67 LI of 20% discriminated optimally between TC/AC and LCNEC/ SCC with 100% sensitivity and specificity, regardless of the used counting approach. For chromogranin A, this was less than perfect, with 82% sensitivity and 86% specificity (Table 4). Mitoses were recognizable in 38 out of 47 surgical specimens but in only 14 out of 45 biopsy samples (p = 0.000002) regardless of histological subtype, probably due to crush artifacts which prevented mitosis detection in two biopsies. For mitotic counts, correlations assessed with intraclass correlation coefficient, Deming regression, and Bland-Altman plot analyses were low (Supplemental Material B and C). Necrosis was absent or present in both specimen types or only in surgical specimen or biopsies in 30, eight, nine, and no cases, respectively (p =

Table 3 Ki-67 labeling index comparison according to the four ways to count tumor cells

Material type	Parameter	All histologic subtypes ($n = 47$)	Typical carcinoid ($n = 22$)	Atypical carcinoid ($n = 14$)	Large cell neuroendocrine carcinoma ($n = 6$)	Small cell carcinoma ($n = 5$)
Biopsy sample	500 cells	21.0 ± 32.6	2.8 ± 1.6	6.7 ± 4.2	64.7 ± 27.8	88.7 ± 9.9
	Hotspot	21.8 ± 33.8	2.9 ± 1.7	6.6 ± 4.2	69.5 ± 26.0	90.7 ± 9.2
	2000 cells	19.1 ± 30.9	2.8 ± 1.8	6.6 ± 4.2	50.5 ± 32.2	88.1 ± 11.1
	2 mm^2	20.6 ± 32.3	2.7 ± 1.7	6.3 ± 3.9	63.4 ± 26.2	88.1 ± 11.1
	p Value[a]	*0.33*	*0.57*	*0.64*	*0.29*	*n/v*
Surgical sample	500 cells	21.0 ± 31.1	3.9 ± 1.8	8.4 ± 4.8	57.9 ± 30.3	86.9 ± 15.2
	Hot-spot	21.7 ± 31.9	3.6 ± 1.7	8.4 ± 5.2	65.1 ± 26.3	86.7 ± 13.7
	2000 cells	20.2 ± 29.5	3.2 ± 1.6	8.3 ± 6.0	60.8 ± 23.5	79.7 ± 14.7
	2 mm^2	20.1 ± 30.3	2.6 ± 1.4	8.0 ± 6.0	62.5 ± 25.6	80.4 ± 14.1
	p Value[a]	*0.04*	*0.0004*	*0.40*	*0.41*	*0.46*

All values are expressed as mean ± standard deviation of percentage values

ICC intraclass correlation coefficient, *CI* confidence intervals, *n/v* not valuable;

[a] *p* Value obtained from repeated measure analysis assuming unstructured covariance matrix

0.00008, Fisher exact test; p = 0.002, McNemar's test). Necrosis in biopsies predicted in 9 of 47 cases (47% sensitivity, 100% specificity, 19% false negative results) its presence in resection specimens.

Discussion

In this paper, we describe methods to assess Ki-67 LI in biopsy specimens of lung NET and compare the results to those obtained on corresponding surgical specimens. We show that once HSRs have been identified, when counting 2000 cells, 2 mm^2 including HSR(s) or the entire biopsy, fragment(s), all potential discrepancies due to sampling, biopsy size, or intratumor heterogeneity of Ki-67 antigen distribution are eliminated, in all histological types. More specifically, Ki-67 LI provided the same level range in biopsy samples as in surgical specimens, thus allowing low- to intermediate-grade and high-grade tumors to be accurately separated when using a 20% cutoff threshold. Conversely, the presence of necrosis and mitotic activity failed to distinguish between low-, intermediate-, and high-grade tumors. This methodological study paves the way to the use of Ki-67 LI in a daily diagnostic setting to characterize metastatic lung NET [21, 33, 38]. On surgical specimens of lung NET, some methodological studies on quantification of Ki-67 staining to generate a Ki-67 LI by manual or automated analysis systems have been performed [17, 19, 32, 39–41]. None, however, compared biopsies with corresponding surgical specimens, probably because in these tumors, the Ki-67 LI is not used for diagnosis or grading or as a prognostic factor [4, 6, 30], as morphology remains the favored approach [3]. However, when diagnosing lung NET, a distinction has to be made between low- to intermediate- and high-grade NET even when biopsies are small or crushed, to avoid errors in patient management and provide appropriate treatment adapted to the intrinsic aggressiveness of the disease which often cannot be done by morphology only [1, 20, 21, 31, 33, 38]. TC or AC is treated with somatostatin analogs, m-TOR pathway inhibitors, and/or peptide receptor radionuclide therapy (PRRT) [1, 4, 42–45], once imaging, symptoms, tumor burden, individual risks of evolving disease, and actionable targets have been accounted for [1, 21]. Once SCC has been ruled out, metastatic NETs are treated with PRRT or alkylating-based chemotherapy, to avoid the side effects of platinum/etoposide [42, 45]. Highly aggressive lung NETs, of which behavior and therapy would be similar to that of SCC, need to be distinguished from tumors with less predictable behavior based upon morphological characteristics, as they would merit biological treatment or nonplatinum-based chemotherapy [38, 42, 45]. In metastatic lung NET, the importance of the Ki-67 LI on biopsy samples is not so much the diagnosis as such (which paradoxically matters less once high-grade NET has been excluded) but rather to guide subsequent therapy choice [33]. This molecular, morphological, and clinical heterogeneity of lung NET [20, 39, 46–50] is similar to that of G3 GEP NET for which the Ki-67 LI is of equal diagnostic importance [26, 51–56]. Of note, LCNECs of the lung show a wide range of histological and molecular features, as some cases are morphologically close to SCCs [13, 14, 16, 47], while others are closer to conventional non-small cell carcinoma [47, 57–59] and yet others to AC [3, 8, 47]. As a result, this is the tumor category with the lowest diagnostic reproducibility among lung NETs [18, 60, 61].We earlier reported values of Ki-67 LI in LCNECs ranging from 26 to 90% or higher [3, 30]. Along with the dilemmas in therapy choice, LCNECs are a waste-basket category with different genotypes and phenotypes (SCC-like LCNECs, NSCLC-like LCNECs, carcinoid-like LCNECs,

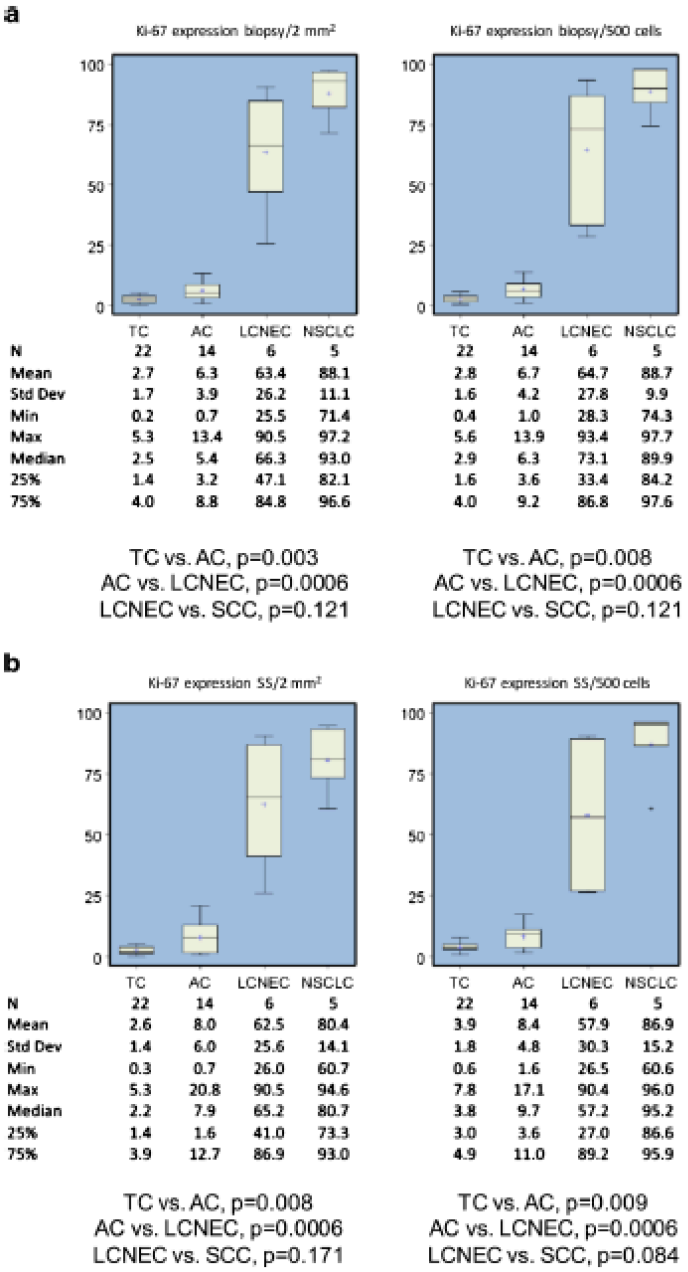

Fig. 1 a, b Distribution of Ki-67 LI across the four different histologic variants of lung NET on biopsy samples (a) and surgical specimens (b), evaluated on either 2 mm^2 or 500 tumor cells. All differences are statistically significant, apart from those between LCNECs and SCCs, likely due the small number of tumors under assessment. SS stands for surgical specimen

as recently proposed) putatively reflecting plasticity of a cancer stem cell rather than a single entity with a characteristic profile [47].We contend that integrated classification of lung NET, merging morphology [62] with grading which includes Ki-67 LI [32], might prove to be useful for clinical purposes [33]. In order to assess clinical usefulness, we assessed how on lung NET biopsies and corresponding resection specimens Ki-67 LI might be established and explored potential sources of variability. A Ki-67 LI can be established on small biopsy or cytology samples [31, 63–69], but studies comparing different counting methods are still lacking. We found that when on a biopsy 2000 cells, 2 mm^2 , or the entire biopsy fragment(s) is counted, the result was the same as that on the surgical specimen, independent of tumor type, which implies that potential confounders such as biopsy size, tumor sampling, heterogeneity in distribution of Ki-67 staining, or subjective interpretation no longer interfere with the result. We only observed differences for TC, as Ki-67 LI exceeded 5% in only four (18%), five (23%), four (18%), and one (5%) TC case for counting in an HSR 500 or 2000 cells or 2 mm^2 /entire tissue fragment(s), but this is without consequences as all values remaining inside the category of well-differentiated lung NET [3]. Of note, we first proposed 2000 cells for pancreatic NET [28] and this is now the accepted standard in the ENETS grading system and WHO classification of neuroendocrine tumors [25–27]. The excellent correlation between Ki-67 LI on biopsies and surgical specimens was confirmed by intraclass correlation coefficient, Deming regression and Bland-Altman plot analyses, in spite of the large 95% confidence intervals on subtypes, which is probably due to the small number of cases or lung NET with low proliferative activity [28, 29, 70] (Supplemental Material B). The basis for the establishment of a Ki-67 LI is the identification of HSR, which remains a

matter of perception. We did not conduct an interobserver reproducibility study, but significant differences between two sets of 27 and 20 cases counted by two different pathologists in the two participating institutions were not found (data not shown). Counting 2000 cells (average 4.5 quantified fields) yielded the same result as counting 2 mm^2 (average 8.4 quantified fields), which we take as evidence of a plateau effect, indicating that beyond 2000 cells, no further improvement can be obtained. Quantifying Ki-67 LI in HSR, as has become customary [19, 28, 30, 31, 40, 71–76], is

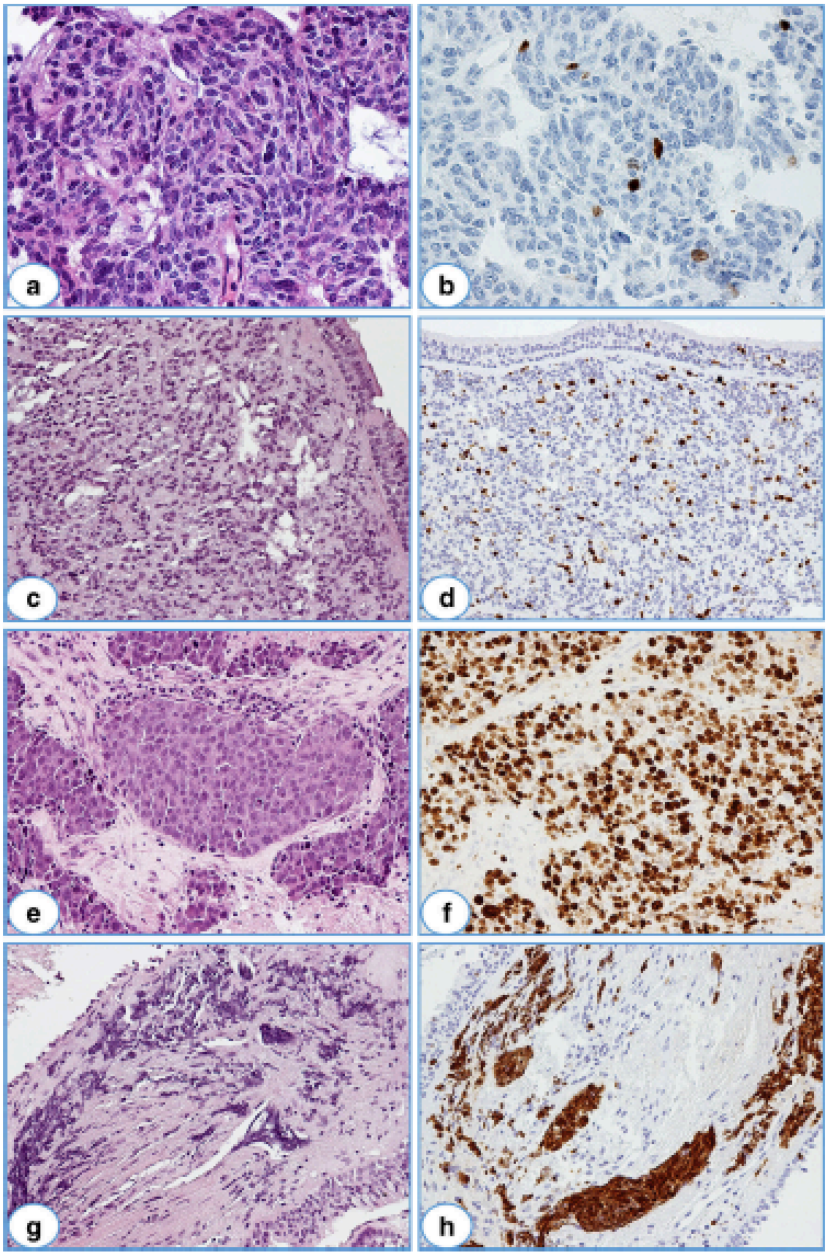

Fig. 2 a–h Representative pictures taken from biopsy samples belonging to the four histologic variants of lung NET showing differential Ki-67 LI distribution in typical carcinoid with polygonal to spindle cell features (a, b), in atypical carcinoid with trabecular pattern (c, d), LCNECs with organoid architecture and palisading cells (e, f), and SCC with extensive crush artifacts (g, h). Pathological evaluation refers to the surgical specimens paired to the relevant biopsies

methodologically sound and biologically relevant. Clinical relevance might be found in using Ki-67 LI on biopsies in a grading system of lung NET, as we recently proposed on surgical specimens by introducing the categories of Lu-NET G1, Lu-NET G2, and Lu-NET G3 [33]. We proposed a distinction between TC/AC and LCNEC/SCLC using a 20% cutoff value, which had 100% sensitivity and specificity in confirmation of our previous observations based on common sense [31]. Of note, this cutoff was very close to the 25% value, we recently proposed in a three-tier grading system of lung NET with the intention to highlight the intermediateprognosis G2 tumor category, which accommodated even some LCNEC [32]. Ki-67 LI ranged between 26 to 90% in LCNEC, in confirmation of its inherent biological heterogeneity. To distinguish between TC/AC and LCNEC/ SCLC, chromogranin A was less reliable as Ki-67 LI (Table 4), which further credits Ki-67 LI as a valuable marker for lung NET with clinical implications. It is not surprising that necrosis and mitotic activity were unreliable criteria in biopsy samples, because these may be very focal or obscured by tissue artifacts due to sampling or processing, as reflected in significant differences in mitotic activity between biopsies

Table 4 Ki-67 labeling index and chromogranin immunoreactivity according to histology

Presurgical biopsy	Variable	Surgical specimens			*Sensitivity*
		TC/AC	LCNEC/SCC	*p* Value	Specificity
	Ki-67 LI < 20%	36	0	5.7×10^{-11}	*100%*
	Ki-67 LI ≥ 20%	0	11		*100%*
	Chromogranin A < 90%	31	2	6.2×10^{-5}	*82%*
	Chromogranin A ≥ 90%	5	9		*86%*

TC typical carcinoid, *AC* atypical carcinoid, *LCNEC* large cell neuroendocrine carcinoma, *SCC* small cell carcinoma

and resection specimens (p = 0.000002) and 19% false-negative results on biopsies for necrosis when compared to the corresponding resection specimen. We contend that the lower diagnostic specificity of chromogranin A was more related to variable expression in morphologically similar high-grade NET [77] than to inadequate tumor sampling. A similar study has recently compared biopsy and surgical specimen results in GEP NET, based on virtual tissue microarray cores totaling a surface of 0.84 to 2.52 mm2 and whole tumor sections, with concordant results [78]. The authors used an image analysis system to generate a labeling index, while we chose visual counting using a manual counter to arrive at an approach applicable in daily practice where image analysis might not be available. Our procedure for counting tumor cells. on lung NET was not more time-consuming than were counting mitoses or apoptotic bodies or assessment of vascular invasion and avoided time-consuming setting up of image analysis [78, 79].

Conclusion

On biopsies of lung NET, a morphological diagnosis of carcinoid, SCLCs, and putatively LCNEC can be made [1, 3], when the Ki-67 LI is included as parameter but not necrosis, mitoses, or NE markers. This implies that Ki-67 LI can be used reliably on biopsies or surgical specimens of metastases, for comparison with the primary tumor, either synchronous or metachronous. Manual counting in HSR of 2000 cells, 2mm2, or the entire tissue fragment(s) reduced biological and methodological intratumor heterogeneity due to sampling, tumor size, or subtype and provides a rationale for a grading system based upon Ki-67 LI. Acknowledgements This work was supported by Novartis Novartis Farma Italia, Milan, Italy. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript, which are the responsibilities of the authors only. The paper has been professionally proofread by PRS (Proof-Reading-Service.com Ltd., Devonshire Business Centre, Works Road, Letchworth Garden City, Herts SG6 1GJ, UK). This work is dedicated to the memory of Carlotta, an extraordinarily lively girl who untimely died of cancer in the prime of life. Compliance with ethical standards Conflict of interest The authors declare that they have no conflicts of interest. Ethics The study was approved by the independent ethics committee of the National Tumor Institute IRCCS Foundation, Milan, Italy (accession number INT-18/16). All patients gave written consent for diagnosis and research activities when they were admitted to the hospital.

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