Ki-67 Antigen in Lung Neuroendocrine Tumors

Unraveling a Role in Clinical Practice

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Abstract: Classification of lung neuroendocrine (NE) tumors is a step-wise process with four tumor categories being identified by morphology, namely typical carcinoid (TC), atypical carcinoid, large-cell NE carcinoma, and small-cell lung carcinoma (SCLC). Ki-67 antigen or protein (henceforth simply Ki-67) has been largely studied in these tumors, but the clinical implications are so far not clear. A well-defined role has regarded the diagnostic use in the separation of TC and AC from SCLC in nonsurgical specimens, with monoclonal antibody MIB-1 resulting in the most used reagent after antigen retrieval procedures. Uncertainties, however, have arisen in its assessment, usually expressed as Ki-67 labeling index, because of some variability in obtaining either value of the fraction. A diagnostic role is currently lacking, even though there are significant differences in most cases between TC and AC, less so between large-cell NE carcinoma and SCLC. In addition, the prognostic role of Ki-67 is debated, likely due to methodological and biological reasons. The last challenge would be to identify an effective lung-specific grading system based on Ki-67 labeling index. In this review article, five relevant issues to Ki-67 have been addressed by using a question-answer methodology, with relevant key points discussing major interpretation issues. The conclusion is that Ki-67 is a feasible and potentially meaningful marker in lung NE tumors, but more data are needed to determine its ideal function in this setting of tumors.

Key Words: Ki-67, Antigen, MIB-1, Labeling index, Lung, Neuroendocrine, Carcinoid, Large-cell neuroendocrine carcinoma, Small-cell lung carcinoma, Diagnosis, Immunohistochemistry, Prognosis, Therapy.

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In the lung, neoplasms with neuroendocrine (NE) morphology and differentiation encompass four histologically defined variants, namely typical carcinoid (TC), atypical carcinoid, large-cell NE carcinoma (LCNEC), and small-cell carcinoma (SCLC).[1,2] According to epidemiologic, genetic, and clinical data, pulmonary NE tumors may be assembled for prognosis and therapy purposes into a three-tier clinicopathological scheme, according to which TC are low-grade malignant tumors with long life expectation and usual surgical treatment; AC, intermediate-grade malignant tumors with more aggressive clinical course and multimodality therapy; and LCNEC and SCLC, high-grade malignant tumors with overlapping dismal prognosis and multimodality or exclusive medical treatment.[1–8]

Subtyping pulmonary NE tumors is a step-wise process in which the four histologic variants are primarily separated by the number of mitoses per 2 mm^2 and the presence of necrosis. Immunohistochemistry (IHC) for NE markers along with NE morphology is required to separate LCNEC from conventional non–small-cell lung carcinoma (NSCLC), and the distinction from SCLC is primarily based on cytological characteristics, including cell size.[1–3,9–11] NSCLC lacking NE morphology such as adenocarcinoma or squamous cell carcinoma but with NE differentiation by IHC or electron microscopy has not been shown consistently to have different prognosis or response to treatment, so this is not accepted as a distinct class of lung cancer.[1,2]

Ki-67 antigen, also known as simply Ki-67 or MKI67 antigen identified by monoclonal antibody Ki-67, is a 359-kD non-histone nuclear protein with short half-life, which is encoded by the 15 exon-spanning *MKI67* gene mapping to chromosome 10q26.2. This protein plays an essential role in the control and timing of cell proliferation,[12–17] which undergoes a complex mechanism of post-translational phosphorylation and dephosphorylation by cell cycle key regulators leading to its subcellular redistribution from the interior of the nucleus/nucleolus to the perichromosomal layer and heterochromatin during mitosis and meiosis and vice versa[18–22] Functionally, Ki-67 expression is finely tuned by specific microRNAs[23] and produced during the entire cell cycle with a maximum in the G2 and M phases.[24,25] However, it may be found at sites linked to the ribosomal RNA transcription machinery with a tight chromatin-associated function in both interphase and mitotic cells, although this finding does not argue against its valuable function as proliferation marker.[26,27] The name Ki-67 derives

TABLE 1. Literature Data on pKi-67 Immunostaining in Pulmonary Neuroendocrine Tumors

Study	Authors	Specimen Type	No. Cases	Antibody	Source	Dilution	Retrieval	Analysis System	Output Value	Referring Value	Cutoff Value %	Diagnostic Implications	Clinical Implications on Outcome
1	Wart, Virchows Arch 2013	Resection	20	MIB-1	Dako	1:400	Yes	Manual count and automated evaluation on ×40-digitalized slides	% as labeling index	Median	Range, 0.1%–20%	Differences between TC and AC; close correlation between KI-67 hotspot and overall evaluation; better interobserver agreement than mitotic count; close correlation of mitosis vs. hotspot or overall Ki-67	—
2	Zheng, Acta Cytol 2013	Cytology	55	30.9	Ventana	Prediluted	Yes	Manual count	% as labeling index	Mean	No	Avoiding misdiagnosis of TC/AC as poorly differentiated NE tumors	—
3	Walts, Mod Pathol 2012	Resection	101	30.9	Ventana	i.n.a.	Yes	Automated system	% as labeling index	Mean	5	Differences between TC and AC	Prognostic stratification, not independent of histology
4	Zahel, Virchows Arch 2012	Resection	193	MIB-1	Dako	1:200	Yes	Manual count	% as labeling index	Mean	2.5 and 5.8	Differences between TC and AC	Prognostic stratification, better G1/G2 than histology
5	Grimaldi, Front Endocrinol 2011	Resection	106	MIB-1	Dako	i.n.a.	i.n.a.	Manual count	% as labeling index	Mean	4	Differences between TC and AC	Prognostic stratification, independent of histology
6	Li, Appl Immunohistochem Mol Morphol 2011	Resection and biopsy	32	MIB-1	Dako	1:100	Yes	Manual count	Ki-67 cumulative score (intensity 1 to 3+ by percentage of tumor cells in each grade)	Mean	No	Differences across the entire spectrum, but not between TC and AC or between LCNEC and SCLC	—
7	Tsuta et al., Hum Pathol 2011	Resection	15	MIB-1	Dako	1:100	i.n.a.	Manual count	% as labeling index	Mean	No	—	—
8	Tsuta et al., Am J Clin Pathol 2011	Resection	113	MIB-1	Dako	1:100	Yes	Manual count	% as labeling index	Mean	No	No differences between TC and AC or between LCNEC and SCLC; correlation of KI-67 labeling index with mitosis and anti-phosphohistone H3 immunohistochemistry	—
9	Skov, J Thorac Oncol 2010	Resection and biopsy	276	MIB-1	Dako	1:1000	Yes	Manual count	% as labeling index	Mean	>8	Differences between TC and AC	No prognostic stratification for TC and AC or LCNEC and SCLC

10	Das-Neves-Pereira, Eur J Cardiothorac Surg	Resection	330	MIB-1	Dako	1:1800	i.n.a	Manual count	Staining index (density of stained cells in areas of tumor tissue)	i.n.a.	No	—	Predictor of metastasis at univariate but not multivariate analysis
11	Rugge, Clin Cancer Res 2008	Resection	67	MIB-1	Dako	1:100	Yes	Manual count	% as labeling index	Mean	5.4	Differences between TC and AC	Prognostic stratification, independent of histology
12	Aslan, Am J Clin Path 2005	Resection and biopsy	20	MM1	Ventana	Prediluted	Yes	Manual count	% as labeling index	Median	No	Avoiding misdiagnosis of TC/AC as SCLC in crush artifacts	—
13	Pelosi, Am J Surg Pathol 2005	Resection	220	MIB-1	Dako	1:200	Yes	Manual count	% as labeling index	Median	No	Differences between TC and AC and LCNEC and SCLC	—
	Pelosi, Am J Surg Pathol 2005	Resection and biopsy	16	MIB-1	Dako	1:200	Yes	Manual count	% as labeling index	Mean	20	Avoiding misdiagnosis of TC/AC as SCLC in crush artifacts	—
14	Igarashi, Mod Path 2004	Resection	111	MIB-1	Dako	1:100	Yes	Manual count	% as labeling index	Mean	No	Differences between TC and AC but not LCNEC and SCLC	Worse prognosis within overall but not individual tumor categories
15	Iyoda, Ann Thor Surg 2004	Resection	20	MIB-1	Dako	i.n.a.	i.n.a.	Manual count	% as labeling index	Mean	No	—	—
16	Lin, 2003	Cytology	40	MIB-1	Immunotech	i.n.a.	Yes	Manual count	% as labeling index	i.n.a.	25 (LG) and >50 (HG)	Avoiding misdiagnosis of TC/AC as SCLC in crush artifacts	—
17	Pelosi, Cancer 2003	Resection	11	MIB-1	Dako	1:100	Yes	Manual count	% as labeling index	Mean	No	—	Worse prognosis as tumor category compared with conventional NSCLC
18	Pelosi, Lung Cancer 2003	Resection	128	MIB-1	Immunotech	1:400	Yes	Manual count	% as labeling index	Mean	5 (LG) and 55 (HG)	—	Association with LN metastasis-promoting fascin expression in TC/AC group
19	Van Eeden, Hum Pathol 2002	Resection and biopsy	10	Rabbit polyclonal	Dako	1:1200	Yes	Manual count	% as labeling index	Categorical	<25% (negative) and >25% (positive)	Correlation between KI-67 and mitotic count in both tumor groups	No worse prognosis within the group of TC and AC
20	Arbiser, Mod Path 2001	Resection	20	MIB-1	Immunotech	1:50	Yes	Manual count	% as labeling index	Mean	No	No distinction between TC and AC or between SCLC and LCNEC	—
21	Helpap, Virchows Arch 2001	Resection	31	MIB-1	Dianova	1:50	Yes	Manual count	% as labeling index	Mean	No	Distinction between TC and AC; avoiding misdiagnosing TC/AC as high-grade NEC	—

(*Continued*)

TABLE 1. (*Continued*)

Study	Authors	Specimen Type	No. Cases	Antibody	Source	Dilution	Retrieval	Analysis System	Output Value	Referring Value	Cutoff Value %	Diagnostic Implications	Clinical Implications on Outcome
22	Laitinen, Cancer 2000	Resection	31	MIB-1	Immunotech	1:25	Yes	Manual count	% as labeling index	i.n.a.	No	Distinction between TC and AC; association with BCL-2 expression and apoptotic index	-
23	Granberg, J Clin Endocrinol Metab 2000	Resection and biopsy	43	MIB-1	Immunotech	1:100	Yes	Manual count	% as labeling index	i.n.a.	No	—	Worse prognosis
24	Al-Khafaji, Hum Pathol 1998	Resection	11	MIB-1	AMAC	1:50	Yes	Manual count	% as labeling index	Mean	<10% and ≤50%	No differences between metastatizing and nonmetastatizing tumors, independent of histology, but tumors with up to 50% Ki-67–labeled cells were atypical	—
25	Costes, Hum Path 1995	Resection	47	MIB-1	Immunotech	i.n.a.	Yes	Automated system	% as labeling index	Mean	4	Distinction between TC and AC	Prognostic stratification, independent of histology at multivariate analysis

Ventana Medical Systems Inc., Tucson, AZ; Immunotech, Westbrook, ME; AMAC, Westbrook, ME; Dako, Glostrup, Denmark; Dianova, Hamburg, Germany.
AC, atypical carcinoid; BCL-2, B-cell lymphoma 2; HG, high grade; i.n.a., information not available; LCNEC, large-cell neuroendocrine carcinoma; LG, low grade; LN, lymph node; NE, neuroendocrine; NEC, neuroendocrine carcinoma; SCLC, small-cell lung cancer; TC, typical carcinoid.

from the city of Kiel in Germany where the antibody was first raised and the number 67 from the clone position in the original 96-well plate generated immunizing mice with nuclei of the lymphoma cell line L428.[28,29] As the original monoclonal antibody to Ki-67 worked on frozen or fresh material only, subsequent antibodies have been developed to react with Ki-67 in formalin-fixed and paraffin-embedded material in a huge variety of malignancies, among which endocrine tumors in different anatomical sites.[30] These reagents included polyclonal Ki-67[31] and monoclonal MIB-1-3-5,[32–34] IND.64,[35] JG-67-2a,[34] Ki-S1,[36] Ki-S3,[37] Ki-S5,[36,38] and Ki-S11[39] antibodies, with most studies confirming the validity of results obtained with these reagents in the measurement of proliferative activity in routinely processed tissues and even cytological samples.[40–43] In more recent years, clone Mib-1 has been emerging as the most reliable and consistent reagent to recognize Ki-67 in paraffin sections[32,33] and its wide commercial availability allowed many investigative studies and meta-analyses to be performed on the clinical implications of Ki-67 to assess proliferative activity in different malignancies,[44,45] including lung.[46]

In NE tumor pathology, Ki-67 was first clinically investigated as prognostic factor in the pancreas,[47–49] then exported to many other types of intestinal NE tumors[50,51] until it was incorporated into the grading system of digestive tract NE neoplasms in the 2010 World Health Organization (WHO) classification.[52,53] However, Ki-67 has entered the clinical practice of other tumors, such as breast cancer, this outlining its role in the molecular classification[54] and clinical management of these oncologic patients.[55] In this evolving scenario of increasing clinical appraisal, it is not surprising that Ki-67 has been widely studied even in NE tumors of the lung.[56–72] However, clarifying its limits and defining practical applications can help clinicians and pathologists to better understand the potential lesson of Ki-67 in the management of NE lung tumor patients.

MATERIALS AND METHODS

A general overview of articles thus far published on the issue of Ki-67 as an operational IHC marker of cell proliferation in lung NE tumors is shown in Table 1. The term "NE tumor" will be synonymously and interchangeably used with the more correct alternative "NE neoplasm" to encompass the whole spectrum of lung NE tumors.[73] Only articles dealing with the 1999 or 2004 WHO classifications[3,4] or equivalent systems[58,70] have been considered because they are more homogeneous for the definition of NE tumor categories.[1,2,4,74] A list of key questions was developed with regard to technical issues, diagnostic and prognostic implication, tumor grading, and relevance to therapy, and these formed the basis for the literature review. Our research was limited to the English literature available in PubMed by variably crossing different research terms, such as Ki-67 or Ki67 (either antigen or protein), MIB1, MIB-1, antibody, NE, tumor, neoplasm, pulmonary, lung, carcinoid, typical, atypical, LCNEC, SCLC, prognosis, survival, or therapy. As a whole, 2067 lung NE tumors were retrieved corresponding to 25 independent studies (Table 1). Our work did not intend to perform a quantitative meta-analysis but rather to provide a critical reappraisal

of the literature addressing frequently asked questions on NE lung tumor pathology and Ki-67 in daily clinical practice. Accordingly, a question-answer methodology has been pursued in the article, with relevant key points summing up major interpretation issues at the end of each answer. In the literature, the term hot spot has been used to indicate tumor areas with the highest concentration of nuclear decoration for Ki-67, whereas the term cold spot has been exploited by some studies to indicate the opposite phenomenon of tumor areas showing the minimal concentration of Ki-67 immunoreactive tumor cells[75] (Table 2).

Question 1. Are there relevant technical issues to Ki-67 IHC and evaluation of results?

Answer: Yes, there is no uniform methodology for Ki-67 IHC and evaluation of results, but most studies pinpointed monoclonal antibody MIB-1 on paraffin sections after antigen retrieval procedures and the assessment of a Ki-67 labeling index (LI) as the most widely agreed-upon methodologies, which have been optimized within each laboratory by longstanding experience on this marker.

Although there are no systematic investigations comparing different antibodies against Ki-67 in clinically worked up NE lung tumors, clone MIB-1 on paraffin sections has been used in all but three articles,[57,68,76] with different antibody dilutions (ranging from 1:25 to 1:1800) and antigen retrieval procedures being adopted within each laboratory usually by utilizing heat-induced unmasking systems in saline buffer and/or following specific manufacturer's instructions (Table 1). Although two articles used prediluted reagents,[57,77] five others did not provide details on antibody dilutions being applied to[58,59,61,62,68] and four lacked information about the antigen retrieval procedures in use.[59,61,67,78] Quantification of Ki-67 expression has been accomplished on surgical resection specimens by manual counting in all but three studies, in which automated systems of assessment were exploited.[58,68,79] A high overall agreement of manual Ki-67 LI evaluation and an automated evaluation method upon scanned slides have recently substantiated the value, reproducibility, and easiness of Ki-67 LI upon manual counting.[79] Small biopsy and cytology samples were used in six[57,64,66,76,80,81] and two studies,[62,77] respectively, to witness the applicability of these materials to accomplish Ki-67 evaluation. Although two works have imaginatively expressed Ki-67 results as either cumulative score[81] or staining index[78] by including the intensity of immunoreactivity or the density of stained cells in tumor tissue areas, respectively, all the remaining studies used the percentage of nuclear-stained tumor cells to substantiate a Ki-67 LI.[56–72,75–77,79,80,82] However, the way to select immunoreactive tumor cells differed somewhat among the diverse studies, with six of them even not providing useful contributory information for further evaluation.[57,70,72,77,80,81] Briefly, Ki-67 LI was assessed in nine studies pinpointing hot spot[63,64,68,69,71,75,78,79,82] or average labeling frequency fields[60] after scanning the entire tumor area at low magnification, whereas the quantification of positive tumor cells per 1 mm^2 [76] or 2 mm^2 [58] or randomly selected areas[61] was declared by others to better accomplish Ki-67 LI. In another study, both hot and cold tumor areas were screened, in the same tumor samples, at low magnification to count 1000 tumor nuclei and a separate evaluation was provided for comparison.[75] Evaluation of stained tumor cells on whole tissue sections of biopsy samples has also been used to maximize information obtainable from small material and avoid selection biases.[64] No particular details on the selection criteria of tumor cells but only the global number of tumor cells being assessed were included in other investigations, which did not thus contribute to unveiling this issue.[56,59,65–67] Interestingly, all studies dealing with LI determination were performed on biopsy or surgical specimens, except for two cytology investigations that expressed results either by quintiles[62] or by Ki-67 LI.[77] All tumor cells showing specific nuclear staining for Ki-67 were considered positive regardless of decoration patterns (diffuse, speckled, nucleolar, mitosis featuring), which are due to the differential expression of the protein during cell cycle progression.[24,25] Expectedly, a significant correlation of Ki-67 LI with mitotic count[59,65,69,75,76,79] or expression of cyclin B1[60] or histone H3 (a surrogate marker of mitoses)[75] has been described in NE tumors of both the lung and the pancreas[48] in virtue of the strong colinearity of the two indicators of cycling cells with variable correlation coefficients likely due to biological and technical reasons.[75,79]

More critical is the question of how Ki-67 LI should be calculated, because different methods have been provided to establish the optimal denominator, that is, the number of cells to be counted. Four to eight histological fields at ×20[68] or ×40[69,79] magnification, histological fields with average labeling incidence,[60] 2-mm^2 tumor areas taken at ×25 magnification,[58] 1-mm^2 tumor areas not otherwise specified,[76] or 400 to 2000 tumor cells being consecutively counted[56,59–61,63–67,71,75,78,82] have been used for assessing Ki-67 LI, which may account for some discrepant results and preclude a direct cross-study comparison.[68]

Another source of variability may derive from evaluating results of Ki-67 LI as mean[56,58–61,63–71,75,77,81,82] or median[57,64,79] thresholds, whereas other studies provided either poorly manageable categorical variables[76] or no useful information.[62,72,78,83] Reproducibility studies on Ki-67 LI evaluation by repeating the measurements in randomly selected carcinoid subsets[68] or comparing manual mitotic count with Ki-67 LI by different pulmonary pathologists in the same tumor samples[79] revealed encouraging results, with less than 1.5% of variability[68] and an outperformance of Ki-67 LI over mitotic count with regard to interobserver agreement.[79]

Relevant key points: At variance with the gastroenteropancreatic system,[84] there are no comparative studies evaluating different methods to perform and express Ki-67 results in lung NE tumors. However, most published investigations agreed on the opportunity of measuring Ki-67 LI in hot spot areas, taking into account all nuclear signals after visual scrutiny of the entire tumor. This would apply especially to TC or AC, whereas Ki-67 decoration is usually much more uniform in high-grade NE tumors. For practical purposes, Ki-67 LI should be calculated in surgical specimens by counting at least 2000 consecutive tumors cells in hot spot fields at ×40 magnification or 2 mm^2 for consistency with the histological classification, possibly in the same tumor area as that used for assessing mitotic count. In biopsy or cytology samples,

TABLE 2. Distribution of pKi-67 Labeling Index According to Histology and Selection Criteria of Lung Neuroendocrine Tumor Cells

Study	Authors	How to Get Results	pKi-67 Labeling Index			
			TC (n)	AC (n)	LCNEC (n)	SCLC (n)
1	Wart, Virchows Arch 2013	Hot spot fields at ×40 and whole tissue slide	Individual data not available	Individual data not available	—	—
2	Zheng, Acta Cytol 2013	i.n.a.[d]	3 (11)[b]	7 (8)[b]	60 (2)[b]	87 (34)[b]
3	Walts, Mod Pathol 2012	Eight hot spot fields at ×20[c]	3.7 (78)[a]	18.8 (31)[a]	—	—
4	Zahel, Virchows Arch 2012	Four hot spot fields at ×40[c]	1.8 mean to 2.5 hot (111)[a]	3.7 mean to 5.8 hot (82)[a]	—	—
5	Grimaldi, Front Endocrinol 2011	2000 cells[d]	2.9 (75)[a]	9.5 (31)[a]	—	—
6	Li, Appl Immunohistochem Mol Morphol 2011	i.n.a.	[0.06 (11)]	[0.41 (6)]	[1.29 (8)]	[1.83 (7)]
7	Tsuta et al., Hum Pathol 2011	1000 cells[d]	0.87 (6)[a]	9.9 (1)[a]	—	—
8	Tsuta et al., Am J Clin Pathol 2011	1000 cells in cold and hot spot fields[c]	0.1 cold to 2 hot (66)[a]	0.7 cold to 7.2 hot (12)[a]	36.8 cold to 55.9 hot (20)[a]	29 cold to 53.6 hot (15)[a]
9	Skov, J Thorac Oncol 2010	400 cells[d]	1.8 (48)[b]	4 (15)[b]	25.5 (27)[b]	41.8 (186)[b]
10	Das-Neves-Pereira, Eur J Cardiothorac Surg	1000 cells in hot spot fields	Individual data not available	—	—	—
11	Rugge, Clin Cancer Res 2008	2000 cells[d]	1.6 (58)[a]	8.8 (9)[a]	—	—
12	Aslan, Am J Clin Path 2005	i.n.a.[d]	1 (7)[b]	—	—	60 (13)
13	Pelosi, Am J Surg Pathol 2005	2000 cells in hot spot fields at ×400 magnification	2.3 (100)[a]	9 (36)[a]	47.5 (52)[a]	64.5 (32)[a]
	Pelosi, Am J Surg Pathol 2005	Whole tissue section for biopsy; hot spot fields at ×40 for surgical specimen counting 2000 tumor cells	1 (2) on biopsy; 5.8 (1) on surgical specimen[b]	6 (5) on biopsy; 11.8 (4) on surgical specimen[b]	—	81.8 (9) on biopsy[b]
14	Igarashi, Mod Path 2004	1000 cells in fields with average labeling incidence	1.3 (13)[a]	8.6 (5)[a]	52.2 (44)[a]	54.6 (49)[a]
15	Iyoda, Ann Thor Surg 2004	1000 cells in randomly selected fields[d]	—	—	41.9 (20)[a]	—
16	Lin, 2003	Quintiles (<5%; 6–25%; 26–50%; 51–75%; >75%)	<25 (low-grade NE tumors)		>50 (high-grade NE tumors)	
17	Pelosi, Cancer 2003	2000 cells in hot spot fields[c]	—	—	32.8 (11)[a]	—
18	Pelosi, Lung Cancer 2003	2000 cells in hot spot fields	<5% (29) and >5% (32)		<55% (31) and >55% (36)	
19	Van Eeden, Hum Pathol 2002	Positive cells per 1 mm^2	<25% (4)	<25% (4)	>25% (2)	
20	Arbiser, Mod Path 2001	1000 cells[d]	0.4 (5)[a]	0.8 (5)[a]	25 (5)[a]	42 (5)[a]
21	Helpap, Virchows Arch 2001	2000 cells in hot spot fields	0.9 (i.n.a.)	5.6 (i.n.a.)	66.3 (i.n.a.)	
22	Laitinen, Cancer 2000	i.n.a.[d]	<1 (21)[a]	10–20 (10)[a]	—	—
23	Granberg, J Clin Endocrinol Metab 2000	i.n.a.[d]	0.9 (43)[b]	—	—	—

24	Al-Khafaji, Hum Pathol 1998	i.n.a.	<10 (6)	≤50% (5)	—	—	
25	Costes, Hum Path 1995	2 mm^2 at ×25 with randomly selected fields[d]	0.5 (31)[a]	2.4 (16)[a]	—	—	
Surgical specimens only			1.72[e] /2.58[f] (n = 209[e]/355[f])	8.04[e]/9.13[f] (n = 77[e]/161[f])	47.57[e]/47.24[f] (n = 69[e]/83[f])	53.43[e]/61.02[f] (n = 54[e]/47[f])	
Surgical specimens and biopsies			1.65[e]/2.60[f] (n = 318[e]/358[f])	7.41[e]/11.04[f] (n = 100[e]/170[f])	41.51[e]/47.57[f] (n = 91[e]/83[f])	50.16[e]/64.36[f] (n =287[e]/56[f])	

[a]Surgical specimens.
[b]Biopsy samples.
[c]Declared hot spot areas.
[d]Not declared hot spot areas.
[e]Not declared hot spot areas (in parenthesis the corresponding number of tumors).
[f]Declared hot spot areas (in parenthesis the corresponding number of tumors).
TC, typical carcinoid; AC, atypical carcinoid; LCNEC, large-cell neuroendocrine carcinoma; SCLC, small-cell lung cancer; i.n.a., information not available; NE, neuroendocrine.

in which the number of tumor cells may be lower than 2000 and the 2-mm^2 criterion unsuitable, it could be reasonable to calculate Ki-67 LI on all tumor cells. For experienced pathologists, manual counting of Ki-67 LI upon visual inspection or eyeball estimation differs little from more sophisticated, time-consuming, or cumbersome methods.[84] Additional work and reproducibility studies are needed to address the optimal procedure for evaluating Ki-67 in lung NE tumors.

Question 2. Is there a diagnostic role for Ki-67 LI in lung NE tumors?

Answer: No, the classification on NE lung tumors is currently guided by morphology alone, but a practical utility for this marker has been emerging for separating TC/AC from high-grade NE tumors in limited diagnostic material.

As outlined in Table 2, the weighted average of Ki-67 LI values across different studies in which this evaluation could be done differed between TC and AC but not between LCNEC and SCLC when considering both surgical specimens only or biopsy/cytology and surgical specimens as a whole, with minor differences if hot spot values of Ki-67 LI were taken into account. The distribution of Ki-67 LI values across the different categories of lung NE tumors according to the type of specimens (only excised specimens or excised and biopsy samples) and the way to select tumor cells (declared hot spot areas versus not declared hot spot areas) is shown in Table 2. In three large studies[8,64,85] accounting for 628 surgically excised NE tumors of the lung (one of which in abstract form only),[85] the value of Ki-67 LI ranged from 2.3% to 4.15% in 211 TC, 9% to 17.8% in 131 AC, 47.5% to 70.0% in 153 LCNEC, and 64.5% to 77.5% in 133 SCLC, in substantial agreement with the expected proliferation rates of these tumors.

Significant differences in the Ki-67 LI distribution have been described in several studies between TC and AC,[58–60,64–66,68,69,71,72,79] between LCNEC and SCLC,[64] or across the entire spectrum of lung NE tumors,[81] whereas other authors did not support this correlation at all[56,70,75] or limited the failure to poorly differentiated NE tumors only.[60] Proposed cutoff thresholds of Ki-67 LI ranged from 2.5 to approximately 30% for carcinoids,[58,59,62,64–66,68,69,75,76,79,82] with two studies detecting 50% or more in few AC,[68,70] whereas the separation of SCLC and LCNEC, if any,[64] is of more limited clinical impact.[1,86] In another study on 190 lung NE tumors published in an abstract form only, although there were differences in Ki-67 LI among diverse tumor categories, the incorporation of this marker as a primary criterion in the classification scheme of lung NE tumors was not further supported.[85] A possible explanation why Ki-67 LI could not effectively split biologically adjacent tumor variants could be the imperfect correlation with mitotic count. This causes the frequency distributions of Ki-67 LI to consistently overlap between these adjacent tumor variants,[68,69,75] also taking into account the high interobserver variability existing, for example, in high-grade NE tumor subclassification.[87]

One of the most agreed-upon uses of Ki-67 LI with important clinical implications deals with the distinction of low to intermediate grade from poorly differentiated NE tumors (especially SCLC) in small biopsy or cytology samples,[57,62,64,71,77] especially in the presence of crush artifacts or

poor tissue preservation,[55,60,62] in which nuclear markers are more suitable for the diagnostic interpretation than cytoplasmic markers. In fact, nuclear markers are easier to scrutiny because there is no passive diffusion of cytoplasmic proteins into adjacent cells, but chromatin-related molecules, such as Ki-67, are likely to remain tightly associated to nuclear remnants even when filamentous changes occur due to tumor cell fragmentation. Thresholds up to 25% to 30% of Ki-67 LI have been quoted as a useful diagnostic adjunct to exclude poorly differentiated NE tumors, which are associated with an exceedingly high proliferation index,[57,62,64,71] whereas thresholds of less than 3% would support a diagnosis of low-grade NE tumor and thresholds between 3% and 30% would indicate indeterminate tumors that most often consisted of AC with very few poorly differentiated tumors.[75] A comparative assessment of Ki-67 LI in biopsy[64] or cytology samples[77] and paired surgical specimens was available from two studies, with similar but not perfectly overlapping results in the setting of low to intermediate malignant lung NE tumors in keeping with those obtained in pancreatic NE tumors,[88] likely owing to either sampling or methodological issues.[64,77] Although it is mandatory to avoid major pitfall in the management of lung cancer patients and Ki-67 LI assessment is effective to assist this task,[64] worth noting, however, is that Ki-67 LI on small biopsy sample may represent the only available data of cell proliferation for clinical decisions in inoperable patients.[89]

Relevant key points: Ki-67 LI is not part of the current WHO diagnostic criteria for classifying lung NE tumors and should not be used to differentiate TC and AC owing to considerable overlapping in the distribution of Ki-67 indices between biologically adjacent NE tumor categories. The assessment of Ki-67 LI, however, is useful as a diagnostic adjunct in small biopsy or cytology specimens with poor preservation or crush artifact, to avoid misdiagnosing low- to intermediate-grade NE tumors as poorly differentiated NE carcinoma.[57,62,64,71,77] Ki-67 LI does not serve to make specific diagnoses of lung NE subtypes, rather it very sensitively parallels the inherent proliferative properties of the tumors under evaluation.

Question 3: Is there a prognostic role for Ki-67 LI?

Answer: Possibly, Ki-67 LI has been emerging as a promising prognostic factor in excised specimens especially of low- to intermediate-grade lung NE tumors, although more data are needed to establish its ideal role.

As indicated in Table 1, at least 12 articles have investigated the prognostic inference of Ki-67 LI in diverse categories of lung NE tumors, especially TC and AC,[58–60,63,65,66,68,69,76,78,82,83] but results are sometimes conflicting and not conclusive yet to authorize a well-recognized role as a prognostic factor for Ki-67 LI in lung NE tumors. Some authors denied any relevance for this marker to pinpoint differences in patients' life expectancy inside individual tumors categories,[60,66,76] whereas others indicated a worse prognosis in TC[80] or purported a role as metastasis predictor alone[78] or upon fascin overexpression (a protein involved in cell migration).[82] Only six studies have indeed supported a prognostic role of Ki-67 LI in surgically excised TC and/or AC,[58,59,65,68,69,80] but results are far from being conclusive. In fact, Ki-67 LI seemed to accurately segregate TC and AC into two distinct prognostic categories by cutoff values between 2.5% and 5.8%,[58,59,65,68,69] which turned out independent of morphology in three studies totaling 220 carcinoids.[58,59,65] In another study dealing with 43 TC, patients with increased Ki-67 expression had significantly shorter survival time.[80] A note of caution, however, has been advanced on the limited role of Ki-67 LI in predicting survival of low to intermediate malignant lung NE tumors when lumping TC and AC, inasmuch as a threshold of 5% did not offer substantial better survival information over morphology, also within individual tumor categories.[68] Similar conclusions on the lack of an independent prognostic efficacy of Ki-67 LI in lung NE tumors have recently been published in an abstract form only.[85] Interestingly, however, several studies have revealed that a Ki-67 LI cutoff of 4% to 5% could differentiate between lower and higher malignant NE tumors in the setting of TC and AC,[58,59,65,68,69,82] similar to what already demonstrated in analogous NE tumors of the pancreas.[48,49] Although conceptually reasonable, no studies have so far addressed a role of Ki-67 LI in the prognostic stratification of poorly differentiated NE tumors, at variance with what has been proposed in other endocrine organs, such as the pancreas.[90]

Relevant key points: Ki-67 LI has been proposed as a prognostic factor in excised specimens of TC and AC, with cutoff values ranging from 2.5% to 5.8%, sometimes but not always independent of morphology. The existence of conflicting results and the lack of widely agreed-upon cutoff thresholds to stratify these tumors preclude making a recommendation at this time. Additional information is needed to establish the ideal role of Ki-67 LI in the prognostic assessment of lung NE tumors, ideally helping to predict prognosis within individual tumor categories. Because there is not much variability in survival for TC, LCNEC, and SCLC, the tumor category where there would be the greatest potential to predict prognosis is within AC. When lumping TC and AC together, it is not surprising that Ki-67 may help with prognosis, but this is not really adding anything to existing diagnostic capabilities.

Question 4: Is there an established role for Ki-67 LI in tumor grading?

Answer: No, in lung NE tumors, the concept of tumor grading as a biological continuum paralleling increasing malignancy is tautologically included into the current WHO classification, according to which TC are considered low malignant, AC intermediate malignant, and LCNEC and SCLC high malignant tumors.

In this setting, tumor grade of lung NE tumors refers to the degree of biologic aggressiveness and is related to, but different from, differentiation that is in turn defined by morphology.[73] The main reason why Ki-67 LI assessment cannot currently claim any primacy in the grading system of lung NE tumors over morphology to realize the clinical three-tier spectrum regards its suboptimal correlation with histological features used for classification, especially mitotic count[75,79] and necrosis, which causes adjacent categories of traditionally assessed tumors to partially imbricate with each other. This also reflects the fact that morphology itself is insufficient in separating borderline/overlapping lesions for either low to intermediate malignant or high malignant tumors.[3,7,91] To try to overcome the drawback of the largely expected close but

not perfect colinearity of Ki-67 and mitotic count, the grading system devised for digestive NE tumors (G1: <2 mitoses per 2 mm^2 and/or Ki-67 LI ≤2%; G2: 2–20 mitoses per 2 mm^2 and/or Ki-67 LI >2% but ≤20%; G3: >20 mitoses per 2 mm^2 and Ki-67 LI >20%)[52,92] has been tested on 111 TC and 83 AC to identify tumor subpopulations from lower to higher aggressive biological behavior.[69] All TC corresponded to G1 tumors using the mean (1.8%) but not hot spot (2.5%) values of Ki-67 LI, whereas all AC resulted in G2 tumors by either threshold (3.7% and 5.8%, respectively). Distribution of tumors across this scheme indicated that 72.3% more patients with G2 tumors (no tumor fit with G3 criteria) developed lymph node metastases, 18.2% more distant metastases, and 20% more died, when compared with traditionally assessed AC, thereby concluding that Ki-67 LI in addition to mitotic count could improve the prediction of clinical behavior of lung carcinoids.[69] This work, however, was not supported by multivariate analysis to validate superiority of this grading system in lung NE tumors in comparison with the traditional criteria adopted in WHO 2004 classification, and in this data set, the method used to apply the 2004 WHO criteria resulted in an extraordinary finding of significantly greater lymph node metastases in TC (25.2%) compared with AC (13.4%).[1] Other works have correlated the distribution of Ki-67 LI with tumor grade according to the usual diagnostic categories by light microscope,[75,77] but they reflected variations in tumor cell differentiation along the clinicopathologic spectrum of lung NE tumors rather than introducing a grading system based on Ki-67 LI.

Relevant key points: Establishing a lung-specific grading system based on a widely agreed-upon marker, such as Ki-67 LI, alone or better in combination with other morphologic parameters, in analogy with other NE tumors elsewhere in the body, is a desirable and clinically warranted goal, also because this marker is familiar to most oncologists and pathologists. However, to date, the existing data do not support a recommendation to apply to the lung the grading systems devised for NE tumors in other anatomical sites, particularly the gastrointestinal tract. Nonetheless, the behavioral heterogeneities within individual lung NE tumor subtypes (especially AC and LCNEC) may be opportunities in future research to develop a specifically devised grading procedure for lung NE tumors where Ki-67 LI as defined by widely agreed-upon criteria according to a grading system could play a role even within individual tumor categories.

Question 5: Is there a predictive role for Ki-67 LI in therapeutic decisions?

Answer: No, the therapy of NE lung tumors is basically guided by morphology and tumor staging by tumor, node, metastasis (TNM) system, with TC being usually treated by surgery, AC, and LCNEC by multimodality approach especially in advanced stage and SCLC by almost exclusive chemoradiotherapy.[5]

The role of Ki-67 LI may be directed to improve diagnosis for better chance of cure, especially in challenging cases of small biopsy or cytology specimens,[64] but its direct implications in establishing the type, timing, and results of therapy have not been evaluated by randomized trials, but at the moment, a role as dynamic biomarker of treatment efficacy has not been provided. A correlation between excision repair cross-complementation 1 expression, a resistance factor against platinum-based chemotherapy in lung cancer, and Ki-67 LI has been described in diverse lung NE tumors considered as a whole, although the correlation was weak and the significance disappeared within different tumor types.[66]

Another study investigated the relationship between Ki-67 LI and thymidylate synthase expression, an enzyme involved in DNA synthesis whose expression acts as a resistance factor to fluoropyrimidine therapy, but results showed that these two markers were independently regulated.[93] Likewise, the expression of mammalian target of rapamycin signaling activation pathways, an attractive target for mammalian target of rapamycin inhibitors such as everolimus in NE tumors, did not correlate with Ki-67 LI.[8] In conventional NSCLC, the predictive impact of Ki-67 to treatment has remained unclear.[94]

Relevant key points: There are no randomized trials documenting that establishing Ki-67 LI in lung NE tumors may guide therapy, beyond refining better diagnosis in difficult cases, usually with small crushed biopsy specimens. Future work will determine the role of evaluating Ki-67 in lung NE tumors other than SCLC (most often are AC and LCNEC, the less familiar categories of these tumors), for deciding chemotherapy intervention especially in symptomatic patients with clinically aggressive tumors.

CONCLUSIONS AND PERSPECTIVES

Three decades after its introduction in the medicine practice and 20 years after its proven prognostic relevance for pancreatic and digestive NE tumors, Ki-67 continues to be a protagonist marker also in lung NE tumors. Conflicting results may be stemming from several factors, including selection of patients, number and type of tumors under evaluation, histological criteria used for classification, variability in the choice of antibodies and immunostaining protocols, Ki-67 staining cutoff thresholds, assessment criteria (automated analysis, manual counting, eyeball estimation, field and cell selection, number of analyzed cells), length and accuracy of follow-up, and/or clinical parameters under evaluation, which may have prevented direct cross-study comparisons. Establishing a lung-specific and clinically meaningful grading system based on Ki-67 LI, alone or in combination with other parameters, with defined cutoff thresholds and uniform procedures for assessing Ki-67 LI is clinically warranted.

KEY REMARKS

- The most agreed-upon procedure to express Ki-67 is to calculate the percentage of stained tumor cells on at least 2000 cells in hot spot areas (Ki-67 labeling index).
- Lung NE tumors are classified by morphology, and Ki-67 does not provide relevant information because of overlapping values in biologically adjacent tumor categories.
- Ki-67 LI is useful to avoid misdiagnosing TC and AC as SCLC.

- Avoid untested and untrusting grading systems devised for NE tumors of other anatomical sites because of the expected different biology.
- Ki-67 LI correlates closely but not perfectly with mitotic count introducing more ample information on NE tumor cell population: a more sensitive grading system should hopefully include both parameters.

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This work is dedicated to the memory of Carlotta, an extraordinarily lively girl who untimely died of cancer in the prime of life.

After this paper was accepted for publication, an innovative evidence-based proposal of a three-tier, morphology-independent grading system of NE lung tumors was reported on, which combined managerially Ki-67 LI, mitotic counting and necrosis assessment in a large series of surgically excised tumors.[95] In particular, lung-specific cut-off thresholds were generated for these tumors, which provided an effective tool for accurately predicting prognosis and biological aggressiveness.

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