Limited role of Ki-67 proliferative index in predicting overall short-term survival in patients with typical and atypical pulmonary carcinoid tumors

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Pulmonary carcinoid tumors are currently classified as typical or atypical based on the mitotic index (2 per 10 hpf) and/or the presence of necrosis. Following incorporation of the Ki-67 index into the classification of GI carcinoid tumors, our oncologists have also been requesting this test as part of the work-up of pulmonary carcinoid tumors although there are currently no established criteria for interpreting Ki-67 index in these neoplasms. We utilized the Ariol[®] SL50 Image Analyzer system to measure the Ki-67 index in 101 pulmonary carcinoid tumors (78 typical and 23 atypical) and then correlated the Ki-67 index and the histological diagnoses in univariate and multivariable analysis with overall survival. The mean Ki-67 indices for the typical carcinoids (3.7 s.d. $\pm$ 4.0) and the atypical carcinoids (18.8 s.d. $\pm$ 17.1) were significantly different ($P < 0.001$) although the frequency distributions of Ki-67 indices in the two groups overlapped considerably. Receiver operating characteristic curve analysis showed that a Ki-67 index cutoff value of 5% provided the best fit for specificity and sensitivity in predicting overall survival. Histological diagnosis and the Ki-67 index cutoff of 5% were each independently strong predictors of survival ($P < 0.001$ and $P = 0.003$, respectively). When considered together in multivariable analysis, histological diagnosis was the stronger predictor of overall survival and a Ki-67 index cutoff of 5% did not provide additional significant predictive survival information within either the typical carcinoid or the atypical carcinoid patient group. A few typical carcinoid patients with Ki-67 indices of 5% appeared to have worse survival after 5 years than those with Ki-67 indices $< 5\%$, but the data set was insufficiently powered to further analyze this. These findings do not provide best evidence for the routine use of Ki-67 index to prognosticate overall short-term survival in patients with pulmonary carcinoid tumors.

Modern Pathology (2012) 25, 1258–1264; doi:10.1038/modpathol.2012.81; published online 11 May 2012

Keywords: atypical carcinoid; carcinoid; Ki-67; pulmonary

Neuroendocrine tumors (pulmonary carcinoid, small-cell carcinoma, and large-cell neuroendocrine carcinoma) comprise approximately 25% of invasive lung malignancies. Of these, typical carcinoids and atypical carcinoids are the least common, comprising only 0.9% and 0.1% of lung neoplasms, respectively.^{1,2} Currently, the World Health Organization (WHO) classification of lung tumors and the American Joint Commission on Cancer (AJCC)

staging manual define pulmonary carcinoids as typical and atypical based on the mitotic index and the presence or absence of necrosis. Typical carcinoids have < 2 mitotic figures per 2 mm² (or per 10 high power fields) and no necrosis. Atypical carcinoids have 2–10 mitotic figures per mm² (or per 10 high power fields) and focal tumor necrosis.^{3–5}

Patient cohorts with atypical carcinoids have a worse prognosis than those with typical carcinoids, but the clinical behavior of individual tumors remains unpredictable, with both tumors having the potential to metastasize and/or recur.^{6,7} As histological diagnosis provides somewhat limited prognostic information for individual patients with pulmonary carcinoid tumors,⁸ a variety of candidate prognosticators have been investigated, including tumor location within the lung, mutations, deletions,

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Presented in part at USCAP annual meeting San Antonio TX, March 2011.

Received 3 November 2011; revised 12 March 2012; accepted 13 March 2012; published online 11 May 2012

and/or instability in chromosomes, oncogenes, and/or tumor suppressor genes, cell/nuclear morphometry, Rb inactivation, p16, cyclin B1, cyclin D1, Bcl2, Bax expression, microsatellite instability, DNA ploidy, S-phase fraction, extent of apoptosis, and Ki-67 immunoreactivity.^{9–20}

MKI67, a nuclear protein that is present during mitosis and the G1, S, and G2 phases of the cell cycle but not during G0, has been shown to be a good marker for cellular proliferation. Ki-67 index, the percentage of tumor nuclei that stain positive using commercially available monoclonal antibody Ki-67, correlates with mitotic figures and provides an objective method that can partially compensate for some of the difficulties associated with mitotic counts.^{21–23} As a group, atypical carcinoids are associated with significantly higher Ki-67 indices than are typical carcinoids, but there is currently no consensus in the literature regarding the optimal cutoff value of Ki-67 index that helps distinguish typical from atypical carcinoids and/or prognosticate survival in patients with pulmonary carcinoid tumors.^{9,17,24–28}

Following incorporation of the Ki-67 index into the WHO classification and the AJCC staging of gastroenteropancreatic neuroendocrine tumors,^{4,29,30} oncologists at our hospital have been requesting Ki-67 index as part of the pathological evaluation of pulmonary carcinoids although there are currently no established consensus or evidence-based guidelines for interpretation of the Ki-67 index in these tumors. This retrospective study was designed to (a) determine whether the mean Ki-67 indices were significantly different in typical and atypical pulmonary carcinoid tumors, (b) select the optimal cutoff Ki-67 index value that would help predict overall survival in pulmonary carcinoid patients, (c) determine whether histology, optimal cutoff Ki-67 index, or both correlate with overall survival, and (d) determine which of the three data (histological diagnosis, optimal cutoff Ki-67 index, or both) would be the best predictor of overall survival in patients with pulmonary carcinoid tumors.

Materials and methods

With IRB approval, slides from 101 patients with pulmonary carcinoid tumors (78 typical and 23 atypical) (accrued between 1 January 1997 and 30 July 2010) were retrieved from the surgical pathology files at Cedars-Sinai Medical Center. The cases were diagnosed as typical and atypical carcinoid tumors utilizing standard WHO criteria (mitotic index and/or presence of tumor necrosis) and had been established as pulmonary primaries by clinical and imaging information. Patients ranged from 16 to 88 years of age at surgery. The typical carcinoids ranged in size from 0.6 to 5.7 cm and the atypical carcinoids ranged from 0.6 to 5.5 cm. Staging information is shown in Table 1.

Table 1 Staging of pulmonary carcinoid tumors

Stage	Typical carcinoids (N = 78)	Atypical carcinoids (N = 23)
IA	45	4
IB	8	0
IIA	7	2
IIB	2	0
IIIA	4	6
IIIB	0	1
IV	0	7
Not available	12	3

The patients were treated with lobectomy (61), wedge biopsy (21), bilobectomy (5), segmentectomy (3), and pneumonectomy (1). The remaining 10 patients underwent only a biopsy of the lung lesion or of a metastasis at our hospital. The following specimens were studied from the 101 patients: 93 lung specimens (76 typical carcinoids and 17 atypical carcinoids), 4 lymph nodes (2 with typical carcinoid and 2 with atypical carcinoid), 2 liver biopsies with atypical carcinoid, and 2 chest-wall biopsies with atypical carcinoid.

Pathology slides were reviewed and histological diagnoses were confirmed by one of the authors (AM). Carcinoid tumorlets,³¹ defined as carcinoid tumors <0.5 cm, were excluded from the study.

Overall survival information was obtained from pathology and thoracic surgery departmental databases, computerized patient medical records, and/or hospital tumor registry data. Disease-specific survival information was not available. Available follow-up ranged from 2 to 142 months (median 26 months). There was no significant difference ($P > 0.05$) in the median length of follow-up for the typical carcinoid (28 months; range 0.1–142 months) and the atypical carcinoid (17 months; range 0.1–111 months) groups.

Immunohistochemical Staining

A serial section cut from a representative formalin-fixed paraffin-embedded block of each tumor and appropriate positive and negative controls were immunostained utilizing standard laboratory protocols. Ki-67 immunostaining was performed using predilute rabbit monoclonal Ki-67 antibody (30-9) (Ventana Medical Systems Inc., Tucson, AZ, USA) with ultraview DAB detection accomplished on the Ventana Ultra instrument.

Pre-treatment was performed on the Ventana Ultra instrument using CC1 buffer. The slides were counterstained with hematoxylin and a bluing reagent. MKI67 was entirely localized to the nucleus, where it was detected as a homogeneous darkly stained reaction product (Figure 1). The areas of tumor on each Ki-67-stained slide were circled before scanning by the image analyzer.

Figure 1 Atypical pulmonary carcinoid tumor: (a) H&E; (b) Ki-67 (brown).

Ki-67 Index

The percentage of Ki-67-positive tumor nuclei (Ki-67 index) was quantitated for each case using the Ariol[®] SL-50 Image Analyzer and imaging software (Genetix Corp, Boston, MA, USA) (Figure 2) in accordance with the manufacturer's recommendations and protocols previously validated in our laboratory. The Ariol[®] system utilizes an automated bright-field microscope and software to detect, classify, and count the Ki-67 immunostained nuclei based upon predetermined specific classifiers that include color, saturation, intensity, size, and shape. Briefly, after the Ki-67-stained slide had been scanned at $5\times$, a trained clinical laboratory scientist (DI), who was blinded to the histological diagnoses and patient survival data, selected at least eight fields representative of the range of Ki-67 immunostaining in the previously encircled tumor for evaluation by the instrument at $20\times$. Necrotic tumor cells, inflammatory cells, and other non-tumor cells were excluded when possible. The software calculates the percentage of positive nuclei in each field and then determines the overall percent positive value.

Figure 2 Atypical pulmonary carcinoid tumor with Ki-67-positive nuclear images identified by the Ariol[®] System.

Assessment of the Reproducibility of Ki-67 Measurements

Five repeated measurements of Ki-67 index were performed in each of the four pulmonary carcinoid tumors chosen at random from our cases. The variability in Ki-67 index measurements was obtained for each case.

Statistical Analysis

The mean Ki-67 indices for the typical and the atypical carcinoids were compared using Fisher's exact test and the median follow-up times for the typical and the atypical carcinoid groups were compared using the Wilcoxon two-sample test. The P values <0.05 were considered significant. The sensitivity and specificity of various Ki-67 index values for prediction of overall survival in patients with typical and atypical carcinoids were calculated using standard formulas. Receiver-operating characteristic curves were used to establish cut-points of various Ki-67 indices on overall survival and to identify the optimal cut-off Ki-67 index that selects the best fit for sensitivity and specificity. Kaplan-Meier methodology was used to determine the rates of overall survival by histological diagnosis, by optimal cut-off Ki-67 index, and by both parameters. Cox proportional hazards models were used to determine the prognostic value of histology-based diagnoses and optimal cut-off Ki-67 index. Power analysis using a Cox regression of the log hazards ratio was performed to estimate whether negative results are significant to 80% power at a 0.05 significance level. All statistical analyses were

Figure 3 Distribution of Ki-67 index in pulmonary carcinoid tumors.

performed using SAS v9.1 software (SAS Institute, Inc., Cary, NC, USA).

Results

As shown in Table 1, there were too few cases to analyze the data by pathological stage. All repeat Ki-67 index measurements per case showed <1.5% variability in results. The mean Ki-67 indices for typical carcinoids (mean 3.7, s.d. $\pm$ 4.0) and atypical carcinoids (mean 18.8, s.d. $\pm$ 17.1) were significantly different ($P < 0.001$) although the frequency distributions of Ki-67 indices in the two groups showed considerable overlap (Figure 3). There were no significant differences between the distribution of Ki-67 index values in the lung and other specimens when data were analyzed by typical carcinoid, atypical carcinoid, and both diagnoses combined.

The Ariol[®] system evaluated the Ki-67 index in 2132–17082 (mean 6651) tumor cells per case. Receiver-operating characteristic curve analysis indicated that Ki-67 cut-offs of 5%, 4%, and 3% yielded specificities of 69%, 61%, and 44% and sensitivities of 71%, 76%, and 88%, respectively, in predicting overall survival in the pulmonary carcinoid patients. A Ki-67 cut-off of 5% provided the best fit for specificity and sensitivity and was identified as the optimal cut-off Ki-67 index (Figure 4).

Histological diagnosis and an optimal cut-off Ki-67 index of 5% were each independently strong predictors of overall survival ($P < 0.001$ and $P = 0.003$, respectively) (Figures 5 and 6). As shown in the Kaplan–Meier curves, patients with atypical carcinoid had a statistically shorter overall survival time compared with patients with typical carcinoid after the first year.

When considered together in multivariable analysis, histological diagnosis was a stronger predictor of overall survival ($P < 0.001$) than optimal cut-off Ki-67 index ($P = 0.678$). Several other cut-points of Ki-67 index were examined and none was stronger than histological diagnosis.

Survival curves for patients stratified by both histological diagnosis and Ki-67 index are shown in Figure 7. Patients with atypical carcinoid had

Figure 4 Ki-67 index and overall survival in patients with pulmonary carcinoid tumors (ROC: receiver operating characteristic).

Figure 5 Overall survival and histological diagnosis.

Figure 6 Overall survival at Ki-67 index cutoff of 5%.

significantly shorter overall survival than those with typical carcinoid whether the typical carcinoid Ki-67 index was <5% ($P = 0.0004$) or equal to 5% ($P = 0.0272$). Survival rates and 95% confidence intervals at 1 year and 5 years are shown in Table 2. Although a few typical carcinoid patients with Ki-67 index of 5% appear to have worse survival after approximately 5 years, the difference in overall survival among typical carcinoid patients with Ki-67 index <5% and equal to 5% was not significant ($P = 0.1572$).

Given that there were more metastatic lesions in the atypical carcinoid than in the typical carcinoid group of patients and the metastatic lesions had

Figure 7 Overall survival stratified by histological diagnosis and Ki-67 index.

Table 2 Survival rates and 95% confidence intervals

	Survival rates	
	1-year	5-year
Typical <5%	98.1 (87.4–99.7)	95.2 (81.7–98.8)
≥5%	94.1 (65.1–99.1)	87.4 (58.1–96.7)
Atypical	78.4 (52.1–91.3)	21.9 (5.5–45.3)

significantly higher Ki-67 indices than the primary lesions in the corresponding group, the data stratified by typical carcinoid and atypical carcinoid diagnoses were also analyzed as described above by specimen type, including pulmonary specimens, metastases, and all cases. There were no significant differences in Ki-67 indices or overall survival in this analysis.

Power analysis using a Cox regression of the log hazards ratio on the Ki-67 index covariate with an anticipated death rate of 6%, an estimated regression coefficient of 1.292, and a standard deviation of 0.9135 estimated that a future study with a total sample of 94 typical carcinoid patients would achieve 80% power at a 0.05 significance level.

Discussion

Our data confirm that mean Ki-67 indices are significantly different in typical and atypical pulmonary carcinoid tumors but show a considerable overlap in the distribution of values in both groups of tumors. Receiver-operating characteristic curve analysis identified a Ki-67 index value of 5% as the best cut-off value that significantly correlates with overall short-term survival in patients with pulmonary carcinoid tumors. Analysis of our overall survival data by histology, optimal cut-off Ki-67 index, and both shows that histological diagnosis still provides the strongest prognostic value and that the addition of Ki-67 index does not provide further significant prognostic information. The presence of a few patients with typical carcinoids and Ki-67 index

of 5% who appear to have a slightly worse prognosis suggests the need for future studies with larger cohorts of typical carcinoid patients and longer follow-up to clarify whether or not Ki-67 index could provide additional significant prognostic information for these patients. Our current study is limited by the use of a relatively small retrospective cohort of patients with pulmonary carcinoid tumors, lack of sufficient number of cases to analyze data stratified by stage, age at initial diagnosis, comorbidities, or treatment/therapy received, use of overall rather than disease-free survival information, limited follow-up information to evaluate the prognosis of tumors that are known to recur and/or metastasize many years after initial diagnosis,^{31,32} and use of a quantitative method to assess immunohistochemical staining that awaits technical improvements.³³

Although the Ariol[®] image analyzer is dependent on the sampling method used for quantitation and other operator-dependent variables, it has several advantages over ‘eyeball’ estimations of Ki-67 immunostaining that favor obtaining more reproducible Ki-67 indices. Within the tumor fields selected by the operator, the instrument (a) evaluates all nuclei other than those intentionally excluded by the operator, (b) provides a permanent record of the fields/cells evaluated, and (c) applies consistent/identical criteria (set of classifiers) in the evaluation of each cell.

Currently there is no consensus on how to best quantitate immunohistochemical staining. For Ki-67 index the seventh edition of the AJCC Cancer Staging Manual recommends evaluating ‘2000 tumor cells in areas of highest nuclear labeling’.⁴ In our experience with quantitating immunohistochemical staining, repeated attempts at selecting ‘the hot spots’ resulted in considerable variation in Ki-67 index measurements. Measurements from multiple areas generally provide more consistent results as reflected in the low variability (<1.5%) in repeat Ki-67 index measurements per case as stated in the results section. In our study, the Ariol[®] system evaluated Ki-67 immunostaining in 2132–17082 (mean 6651) tumor cells per case. Others emphasize the importance of ‘random counting of sufficient numbers of tumor cells’ to provide the reproducibility required for a reliable classification parameter.³⁴ Indeed, in a recent paper, Yang *et al*³⁵ state ‘the extent of intratumoral heterogeneity is not well established, and few data exist to support the use of the highest-labeling foci vs randomly selected regions’ to determine the Ki-67 index. In an effort to take tumor sampling and intratumoral heterogeneity in Ki-67 immunoreactivity into account, we analyzed microscopic fields representative of the range in Ki-67 staining observed within each of the tumor sections studied rather than analyze only the field(s) containing the largest number of immunostained tumor cells (so called ‘hot spots’), as has been discussed or recommended for gastroenteropancreatic carcinoids by some groups.^{4,36}

Our review of the literature identified several studies that address prognosis in patients with pulmonary carcinoid tumors using histological criteria, Ki-67 immunoreactivity, and a variety of other covariates. Ki-67 index cut-off values that were used in these studies vary from 3.4²⁸ to 16%.⁹ Some workers concluded that Ki-67 index did or might provide additional prognostic information for pulmonary carcinoid patients beyond that obtained using histological diagnosis alone or in combination with one or more of the other covariates they studied,^{9,12,15,16,25,27,28,37} whereas others found that Ki-67 index *per se* provided no substantial additional prognostic information for their pulmonary carcinoid patients.^{14,18,38} Differences in case selection, histological criteria, Ki-67 antibody, immunostaining protocol, Ki-67 indices measured in typical and atypical carcinoids, Ki-67 index cut-off value used in analysis, and immunostain assessment protocol (field and cell selection, visual estimate vs image analysis), length of follow-up, and/or parameter studied (disease-free survival, progression-free survival, overall survival, response to platinum-based therapy) preclude direct cross-study comparisons.

The results of this retrospective study underscore the limitations in automatically assuming that 'new data' are better or more informative than older data obtained with conventional histopathology. Ki-67 indices were 'quantitated' using current instrumentation, methodology similar to that used in other laboratories, and in accordance with manufacturers' protocols. Although our results show that the Ki-67 index correlates with survival in patients with pulmonary carcinoid tumors, multivariable analysis clearly shows that the 'new data' are less powerful than the older. Moreover, the results underscore the need to critically evaluate the clinical applicability of 'new' tests using an evidence-based approach. A review of our results using an evidence-based approach can conclude that the study provides only level III evidence and that there is no best evidence to resolve that Ki-67 index should be routinely evaluated in pulmonary carcinoid tumors. A larger prospective study that provides better levels of evidence is needed before we definitely conclude that Ki-67 index is not a useful test in the evaluation of pulmonary carcinoids.

Acknowledgement

We thank Catherine Bresee, MS for providing the statistical analyses and Alison Kinney, CTR and Donato Kusuanco, BS for their assistance in obtaining patient survival information.

Disclosure/conflict of interest

The author declares no conflict of interest.

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