Tumor cellular proliferation is associated with enhanced immune checkpoint expression in stage I non–small cell lung cancer

Kyle G. Mitchell, MD,^a Edwin R. Parra, MD, PhD,^b David B. Nelson, MD,^a Jiexin Zhang, MS,^c Ignacio I. Wistuba, MD,^b Junya Fujimoto, MD, PhD,^b Jack A. Roth, MD,^a and Mara B. Antonoff, MD,^a the MD Anderson Lung Cancer Immune Microenvironment Working Group

ABSTRACT

Objectives: Ki67 is a marker for tumor proliferative activity and is known to have prognostic significance in multiple solid malignancies. We sought to characterize the relationships among Ki67 expression, immune cell infiltration, and immune checkpoint expression in patients with resected non–small cell lung cancer.

Methods: Specimens of patients undergoing resection of stage I to III non–small cell lung cancer (1997–2012) were analyzed using tissue microarrays. Proliferative index was quantified as the percentage of malignant cells expressing Ki67. Checkpoints expressed on malignant cells (programmed death ligand 1, B7H3, B7H4, indoleamine 2,3-dioxygenase 1) and lymphocytes (T-cell immunoglobulin and mucin-domain containing 3, V-domain suppressor of T-cell activation, tumor necrosis factor receptor superfamily member 4, lymphocyte activation gene 3, inducible T-cell co-stimulator) were analyzed in intratumoral and stromal compartments, respectively. Immune cell densities were quantified in intratumoral and peritumoral compartments in a representative subset.

Results: A total of 190 patients met inclusion criteria. Higher Ki67 expression was noted in squamous cell carcinoma (median 31.4% positive malignant cells vs 15.2% adenocarcinoma, $P < .001$), advanced-stage tumors (25.7% stages II/III vs 20.8% stage I, $P = .013$), and poorly differentiated tumors (28.8% vs 15.4% well/moderately, $P < .001$). Ki67 was positively correlated with intratumoral expression of programmed death ligand 1, B7-H3, and indoleamine 2,3-dioxygenase 1, and elevated stromal expression of lymphocyte activation gene 3 and inducible T-cell co-stimulator. Ki67 expression was inversely associated with intratumoral densities of CD57+ and CD4+ cells. The relationship between Ki67 and checkpoint expression was strongest in stage I tumors. Among patients with stage I, increased Ki67 was independently associated with worse overall survival.

Conclusions: Increased Ki67 expression is associated with biologically aggressive non–small cell lung cancer, enhanced immune checkpoint expression, and reduced intratumoral immune cell infiltration. These findings were strongest in early-stage disease and warrant further investigation in the context of novel therapeutic agents. (J Thorac Cardiovasc Surg 2019;158:911–9)

Intratumoral Ki67 expression is associated with poor OS in stage I NSCLC.

Central Message

Tumor proliferation (Ki67 expression) is positively associated with immune checkpoint expression in stage I NSCLC.

Perspective

Ki67 is a biomarker of tumor proliferative activity that has been shown to be associated with poor prognosis in NSCLC. By analyzing tumor expression of Ki67, we demonstrate associations between increased tumor proliferation and expression of immune checkpoints. These findings suggest a role for Ki67 as a marker of an immunosuppressive microenvironment in early-stage NSCLC.

See Commentaries on pages 920 and 922.

From the Departments of ^aThoracic and Cardiovascular Surgery, ^bTranslational Molecular Pathology, and ^cBioinformatics and Computational Biology, University of Texas MD Anderson Cancer Center, Houston, Tex.

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Address for reprints: Mara B. Antonoff, MD, Department of Thoracic and Cardiovascular Surgery, University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX 77030 (E-mail: MBAntonoff@mdanderson.org).

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Abbreviations and Acronyms

ACA	= adenocarcinoma
CD	= cluster of differentiation
CI	= confidence interval
DFS	= disease-free survival
FOXP3	= forkhead box protein 3
GZB	= granzyme B
ICOS	= inducible T-cell co-stimulator
IDO1	= indoleamine 2,3-dioxygenase 1
IQR	= interquartile range
LAG3	= lymphocyte activation gene 3
MC	= malignant cell
MST	= medial survival time
NSCLC	= non-small cell lung cancer
OS	= overall survival
PD1	= programmed cell death protein 1
PD-L1	= programmed cell death ligand 1
TME	= tumor microenvironment

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The success of immune checkpoint blockade in the management of non-small cell lung cancer (NSCLC) has brought the importance of the interactions between tumor and immune cells into sharp focus.¹⁻³ Development and application of these therapies have been permitted by a growing body of evidence demonstrating that innate and adaptive responses to tumor are characterized by a complex interplay of metabolic signaling and expression of immune checkpoints that can have immunosuppressive or immune-activating effects in the local tumor microenvironment (TME).⁴ As these novel therapies gain wider clinical use in the setting of early and advanced disease, there will be a need for predictors of therapeutic benefit, because not all patients will respond. An enhanced understanding of the interactions between tumor cells and the immune system may be useful for identification of biomarkers of response.

Ki67 is a nuclear protein that has roles in synthesis of ribosomal RNA and organization and maintenance of chromatin structure during mitosis.⁵⁻⁷ Tumorigenic properties of Ki67 have been proposed, and it has been further explored as a potential therapeutic target.⁷⁻⁹ Although it is detectable

throughout the cell cycle and is expressed constitutively at a low level in quiescent cells, its expression is highest during phases characterized by rapid cellular growth.^{7,10-12} In NSCLC, tumor proliferation has prognostic significance; a recent meta-analysis of 108 studies identified consistent associations between Ki67 expression and poor oncologic outcomes in several settings.¹³

Although the prognostic significance of Ki67 expression in NSCLC has been extensively studied, the relationship between tumor proliferative activity and characteristics of the local immune microenvironment are not well understood. In light of the known relationships among features of the immune microenvironment, post-therapeutic outcomes, and response to immune checkpoint inhibition, an understanding of the degree to which Ki67 reflects an immunologically favorable or unfavorable TME could help guide decisions regarding choice and timing of therapies.^{4,14,15} Moreover, given ongoing investigation into the development and clinical application of therapeutic agents targeting immune checkpoints expressed on both tumor and immune cells, delineation of the heretofore poorly understood relationships between tumor proliferative activity and immunosuppressive features is an incompletely developed and clinically salient line of inquiry.^{7,10} We hypothesized that tumor proliferative activity as quantified by Ki67 would reflect an immunosuppressive microenvironment and that it would, in turn, be associated with poor survival outcomes among chemotherapy-naïve patients undergoing definitive surgical therapy for NSCLC.

MATERIALS AND METHODS

Patient Population

Patients undergoing resection with definitive intent of primary stage I to III NSCLC between 1997 and 2012 and who had pathological Ki67 expression data available were eligible for analysis. Baseline clinicopathologic characteristics were retrospectively queried from a prospectively maintained departmental database. Because neoadjuvant therapy has been shown to be associated with changes in the TME, patients who received neoadjuvant chemotherapy or radiotherapy were excluded.¹⁶ Tumors were staged using the seventh edition of the American Joint Commission on Cancer staging system.¹⁷ This retrospective study was approved by the University of Texas MD Anderson Cancer Center's Institutional Review Board with a waiver of informed consent.

Immunohistochemical Staining and Image Analysis

For quantification of Ki67 and immune checkpoint expression, tissue microarrays were constructed using formalin-fixed, paraffin-embedded tumor blocks using methods that have been described.¹⁸ Briefly, tissue microarray sections were prepared using three 1.0-mm cores obtained from the center, periphery, and middle of the tumor. An automated staining system (BOND-MAX, Leica Microsystems, Vista, Calif) was used. After scanning at ×200 magnification using the Aperio AT Turbo system (Leica Microsystems), a trained pathologist reviewed scanned images (ImageScope, Leica Microsystems) and created a digital tissue microarray block with the Aperio eSlide Manager (Leica Microsystems). Tumor proliferation was quantified as the percentage of tumor cells expressing Ki67 (Ki-67/clone MIB-1 [M7240], dilution 1:100; Dako, Carpinteria, Calif). Percentage of cells expressing checkpoints that are predominantly

expressed on malignant cells (MCs) (programmed cell death ligand 1 [PD-L1], B7-H3, B7-H4, and indoleamine 2,3-dioxygenase 1 [IDO1]) were analyzed in the tumor compartment (tumor nests), and cell densities (cells/mm²) of those expressed on host tumor-associated immune cells (V-domain suppressor of T-cell activation, tumor necrosis factor receptor superfamily member 4, inducible T-cell co-stimulator [ICOS], lymphocyte activation gene 3 [LAG3], and T-cell immunoglobulin and mucin-domain containing 3) were examined in the stromal compartment (stroma tissue between tumor nests) in 184 of 190 cases (96.8%) as previously described.¹⁸ For a representative subset of tumors (171, 90.0%), whole tumor sections had been previously used for quantification of tumor-associated immune cell populations expressing cluster of differentiation (CD) 3, CD4, CD8, CD57, granzyme B (GZB), CD45RO, programmed cell death protein 1 (PD1), forkhead box P3 (FOXP3), and CD68 in five 1 mm² areas in the intratumoral (tumor nests and stroma of tumor) and peritumoral compartments using methods that have been previously published.¹⁹ Human tonsil tissue was used as a positive (stained with antibody) and negative (not stained) control. Stained slides were scanned and visualized using ScanScope Aperio AT Turbo scanner and ImageScope software (both Leica Microsystems), respectively, and the Aperio Image Toolbox (Leica Microsystems) was used to visualize and analyze imaging data.

Statistical Analysis

Associations among expression of Ki67, immune checkpoints, and immune cell densities were analyzed using Spearman's rank correlation test (range -1 [perfect negative correlation] to $+1$ [perfect positive correlation], with $r = 0$ indicating no correlation).²⁰ To control for multiple comparisons, all pairwise correlations and analyses of marker densities among samples dichotomized by Ki67 expression were evaluated using a false discovery rate-adjusted P value (q value).²¹ Because family-wise error rate methods are conservative and may lead to missed findings, we chose to use the false discovery rate method to identify as many significant relationships as possible (with the limitation of increased type I error rate) while controlling the rate of false-positive findings. Differences in Ki67 expression between groups were assessed using Mann-Whitney U and Kruskal-Wallis tests. Heat maps were generated to identify relative coexpression of immune checkpoints and patterns of immune cell infiltrates according to the degree of expression of Ki67 in MCs. For the immune checkpoints with which Ki67 was noted to have statistically significant pairwise correlations, the associations were further modeled by fitting a generalized linear model with Gaussian distribution and identity link to account for relevant clinicopathologic characteristics (age, sex, smoking status, histology, differentiation, pathologic stage; final model selection using Akaike information criterion). Overall survival (OS) was defined as the time from surgery to death; patients alive at the end of the study period were censored at the time of last contact. Disease-free survival (DFS) was defined as the time from surgery to disease recurrence or death; patients without a DFS event were censored at the date of last contact. Univariable Cox proportional hazard regressions were performed to analyze relationships between clinicopathologic parameters and Ki67 expression with OS. Variables with a P value less than .20 on univariable analysis were included in the multivariable model; stepwise backwards elimination was then performed until all variables in the final multivariable model met P less than .10 as the final selection criterion. Median follow-up duration was calculated as the median time from surgery to last contact or death. The Kaplan-Meier method was used to estimate survival, and the log-rank test was used to analyze differences in time-to-event outcomes between groups. All analyses were performed using SPSS (version 24.0.0; IBM, Armonk, NY) and R (version 3.3.0; <http://www.r-project.org>). Heat maps were generated using the native *Heatmap* function for R. Determination of the cutoff values of Ki67 that best discriminated postoperative OS was performed using the *FindCut* package for R, which determines the optimal number of cutoff points according to the Akaike information

TABLE 1. Baseline clinical, pathologic, and treatment characteristics of the examined cohort

Variable	N (%) or median (IQR)
Age, median (IQR) (y)	66.0 (60.0-74.0)
Sex	
Female	88 (46.3)
Male	102 (53.7)
Smoking	
Never	19 (10.0)
Former/current	171 (90.0)
Zubrod	
0	92 (48.4)
1	97 (51.1)
2	1 (0.5)
Histology	
ACA	114 (60.0)
SCC	72 (37.9)
NSCLC NOS	4 (2.1)
Differentiation	
Well/moderate	110 (57.9)
Poor	80 (42.1)
Extent of resection	
Sublobar	6 (3.2)
Lobectomy/bilobectomy	170 (89.5)
Pneumonectomy	14 (7.4)
Pathologic margin	
R0	182 (95.8)
R1	7 (3.7)
R2	1 (0.5)
Pathologic stage	
I	109 (57.4)
II	48 (25.3)
III	33 (17.4)
pT	
pT1	66 (34.7)
pT2	94 (49.5)
pT3	23 (12.1)
pT4	7 (3.7)
pN	
pN0	127 (66.8)
pN1	38 (20.0)
pN2	25 (13.2)
Adjuvant therapy	
Chemotherapy	36 (18.9)
Radiotherapy	17 (8.9)

IQR, Interquartile range; ACA, adenocarcinoma; SCC, squamous cell carcinoma; NSCLC NOS, non-small cell lung cancer, not otherwise specified.

criterion method and the optimal location of cutoff points according to the likelihood ratio test.²²

RESULTS

Patient and Tumor Characteristics

A total of 190 patients were eligible for analysis (Figure E1). Most patients were men (102, 53.7%), had

FIGURE 1. Tumor expression of Ki67 was noted to be higher in (A) squamous cell carcinoma than ACA, (B) poorly differentiated rather than moderately or well differentiated tumors, (C) ever smokers, and (D) patients with advanced pathological stage. ACA, Adenocarcinoma; SCC, squamous cell carcinoma.

early-stage disease (pathological stage I: 109, 57.4%), and underwent anatomic lobectomy (170, 89.5%) (Table 1). A majority of tumors studied were adenocarcinomas (ACAs) (114, 60.0%) and well or moderately differentiated (110, 57.9%).

Associations Between Tumor Proliferation and Clinicopathologic Characteristics

No differences in Ki67 expression were observed between men (median 23.1% positive tumor cells; interquartile range [IQR], 12.5-34.8) and women (median 21.0% positive tumor cells [10.6-35.3], $P = .659$) (distribution of Ki67 expression for the study cohort provided in Figure E2). Former or current smokers had higher intratumoral expression of Ki67 (median 24.6% positive tumor cells [13.2-35.9]) than never-smokers (median 11.4% positive tumor cells [7.7-16.5], $P < .001$). Ki67 expression was higher in squamous cell carcinoma (median 31.4% positive tumor cells [22.8-40.2]) than in ACA (median 15.2% positive tumor cells [9.0-26.4], $P < .001$) (Figure 1). Measured expression of Ki67 was highest in stage III tumors (median 28.5% positive tumor cells [18.7-36.7] vs stages I (median 20.8% [9.6-30.5]) and II (median 19.2% [13.1-38.2], $P = .025$). Likewise, pathological nodal involvement was associated with higher Ki67 (pN0 median 20.8% positive tumor cells [9.7-30.5] vs pN+ median 28.3% [16.5-38.5], $P = .003$). Poorly

differentiated tumors (median 28.8% positive tumor cells [20.6-38.0]) were noted to have higher expression of Ki67 than those that were moderately or well differentiated (median 15.4% positive tumor cells [9.0-28.4], $P < .001$). Ki67 expression was positively correlated with pathologic tumor size ($r = 0.191$, $P = .018$).

Tumor Proliferation Is Associated With Enhanced Immune Checkpoint Expression on Tumor and Immune Cells in Early-Stage Non-Small Cell Lung Cancer

Tumor proliferative activity was observed to be strongly correlated with expression of several immune checkpoints in the tumor compartment by MCs, including PD-L1 ($r = 0.287$, $P < .001$), B7H3 ($r = 0.235$, $P = .006$), and IDO1 ($r = 0.225$, $P = .008$) (Figure 2, A). Increased Ki67 expression was also associated with enhanced expression of LAG3 by tumor-associated immune cells in the stromal compartment ($r = 0.245$, $P = .004$). Ki67 was weakly associated with increased expression of the costimulatory molecule ICOS ($r = 0.175$, $P = .036$). Tumors characterized by Ki67 expression greater than the observed median were noted to have increased densities of cells expressing the inhibitory checkpoint PD1 in the intratumoral (median 625.8 cells/mm² [IQR, 365.8-931.2] vs 464.6 [289.1-687.1], $P = .032$) and peritumoral (1066.7 [714.9-1519.5] vs 875.2 [603.6-1124.1], $P = .043$) compartments.

FIGURE 2. Associations between tumor expression of Ki67 and features of the immune microenvironment. A, Elevated Ki67 expression was associated with statistically significant increases in expression of the checkpoint molecules PD-L1, B7H3, and IDO1; however, no clustering of immune checkpoint expression was observed. B, Tumors with high expression of Ki67 were associated with increased peritumoral infiltration by several immune cell populations. In contrast, increased Ki67 expression was associated with a statistically significant decrease in densities of immune cells expressing CD4 and CD57 in the tumor compartment. The cohort was ordered along the x axis according to decreasing Ki67 expression; the dendrograms on the left y axis indicate relative similarity or dissimilarity between rows (expression of each individual marker). *SCC*, Squamous cell carcinoma; *ACA*, adenocarcinoma; *IDO1*, indoleamine 2,3-dioxygenase 1; *PD-L1*, programmed death ligand 1; *LAG3*, lymphocyte activation gene 3; *ICOS*, inducible T-cell co-stimulator; *TIM3*, T-cell immunoglobulin and mucin-domain containing 3; *OX40*, tumor necrosis factor receptor superfamily, member 4; *VISTA*, V-domain suppressor of T cell activation; *NSCLC NOS*, non-small cell lung cancer, not otherwise specified; *PD1*, programmed cell death protein 1.

Although Ki67 expression was not independently associated with expression of B7H3 or IDO1 after controlling for relevant clinicopathologic characteristics, its expression remained associated with increased tumor expression of PD-L1 ($P = .043$).

Of note, expression of Ki67 by MCs was inversely correlated with infiltration of the tumor compartment by cells expressing CD4 ($r = -0.208$, $P = .015$) and CD57 ($r = -0.220$, $P = .010$). No association was observed between Ki67 expression and densities of CD8⁺ cells in the tumor compartment ($r = 0.125$, $P = .170$). In the peritumoral compartment, increased infiltration by tumor-associated immune cells expressing CD3 ($r = 0.390$, $P < .001$), CD4 ($r = 0.242$, $P = .008$), CD8 ($r = 0.384$, $P < .001$), CD45RO ($r = 0.321$, $P < .001$), FOXP3 ($r = 0.347$, $P < .001$), and GZB ($r = 0.285$, $P = .002$) was associated with Ki67 expression (Figure 2, B). Upon subgroup analysis after stratification by histology, relationships between Ki67 and checkpoint expression were preserved in ACAs, whereas these associations did not meet statistical significance in squamous cell carcinomas (Tables E1 and E2). Upon subgroup analysis after stratification by pathologic stage, the associations between Ki67 expression and checkpoint expression were present in pathologic stage I tumors but absent in stages II and III

(Table E3), suggesting that the relationship between Ki67 expression and an immunosuppressive microenvironment may be absent in more advanced stages of disease.

Increased Proliferative Index Is Negatively Associated With Survival After Resection of Pathological Stage I Non-Small Cell Lung Cancer

After a median follow-up time of 70.0 months, there were 121 deaths and 123 DFS events; median survival time (MST) and DFS time for the entire cohort were 78.7 (95% confidence interval [CI], 50.5-106.9) months and 62.1 (95% CI, 37.5-86.7) months, respectively. Because an interaction was observed between Ki67 expression and pathological stage (OS interaction term $P = .004$; DFS interaction term $P = .002$) and Ki67 was not associated with survival in advanced stages, the cohort was stratified and further analysis was restricted to patients with pathological stage I disease (109/190; 57 deaths and 58 DFS events; MST 138.3 months, 95% CI, 104.2-172.4; median DFS time 125.0 months, 95% CI, 84.0-166.1). The optimal cutoffs to define low-, intermediate-, and high-risk stage I tumors according to postoperative OS were 7.17% or less (Ki67_{Low}; 12/109; MST not reached), 7.17% to 25.73% (Ki67_{Intermediate}; 55/109; MST 125.0 months, 95% CI, not reached), and greater than 25.73% (Ki67_{High}; 42/109;

FIGURE 3. Intratumoral expression of Ki67 (Ki67 low: $\leq 7.17\%$; Ki67 intermediate: $7.17\% - 25.73\%$; Ki67 high: $> 25.73\%$) was associated with worse OS among patients with pathological stage I tumors. CI, Confidence interval.

MST 51.7 months, 95% CI, 14.9-88.5 months), respectively (log-rank $P < .001$) (Figure 3). Post hoc analysis identified expression of PD-L1 (Ki67_{High} median 36.7% of tumor cells PD-L1⁺ [IQR, 17.1-88.1] vs Ki67_{Low} 16.3% [8.8-19.1] and Ki67_{Intermediate} 22.8% [12.4-54.3], $P = .002$) and IDO1 (Ki67_{High} 6.6 [IQR, 3.0-21.0], Ki67_{Low} 2.1 [1.0-4.6], Ki67_{Intermediate} 4.5 [1.8-13.5], $P = .027$) to be highest among Ki67_{High} tumors. Likewise, densities of tumor-associated immune cells expressing the inhibitory checkpoint PD1 were highest in Ki67_{High} tumors in both intratumoral (691.5 [IQR, 386.8-1100.1] cells/mm² vs Ki67_{Low} 300.2 [202.6-455.1] and Ki67_{Intermediate} 521.4 [356.9-764.2], $P = .005$) and peritumoral (Ki67_{High} 1118.4 [746.6-1715.4], Ki67_{Low} 910.4 [724.9-1103.3], Ki67_{Intermediate} 815.6 [627.3-1185.8], $P = .034$) compartments. On multivariable analysis, Ki67 expression remained independently associated with an increased hazard of death (Ki67_{Intermediate} HR 4.33, 95% CI, 1.00-18.67; Ki67_{High} HR 8.23, 95% CI, 1.91-35.41) (Tables 2 and Table E4) and DFS events (Tables E5 and E6) (Video 1).

DISCUSSION

In this analysis of chemotherapy-naïve patients undergoing definitive surgical therapy for NSCLC, we identify associations between high tumor proliferative activity as quantified by Ki67 and enhanced immune checkpoint expression on both tumor cells and immune cells within the local TME, particularly among stage I tumors. Tumors characterized by high proliferative activity were associated with reduced intratumoral infiltration by cells expressing

markers for helper T lymphocytes (CD4) and natural killer cells (CD57), although they were also marked by increased peritumoral inflammatory infiltrates. Finally, high Ki67 expression identified a subgroup of patients with pathological stage I disease who were at a markedly increased hazard of death after resection. To the authors' knowledge, the present study represents the first characterization of the relationship between tumor proliferation and an immunosuppressive TME in NSCLC.

The ability of MCs to evade immunosurveillance plays a crucial role in the development and progression of tumors. Critical to this process is the suppression of immune responses by expression of immune checkpoints that can diminish host immune cell activity and induce lymphocyte death. These checkpoints may act via independent pathways, and their degree of coexpression may be a function of molecular subtype.^{18,23,24} Although the PD-L1/PD1 axis currently tends to be the ligand-receptor pair of greatest current scrutiny, expression of IDO1 (an enzyme involved in tryptophan catabolism), the transmembrane immunoregulatory protein B7H3, and the lymphocyte-inactivating LAG3 have been shown to have prognostic significance in NSCLC.²⁴⁻²⁷ Exploration of therapies targeting these checkpoints is ongoing.^{28,29} In the present study, by analyzing tumor expression of Ki67 and several immune checkpoints, we highlight associations between an immunosuppressive local microenvironment and rapid tumor proliferation. Because the efficacy of cytotoxic therapy and immunotherapy are enhanced by tumor growth and checkpoint expression, respectively, we propose that pretreatment expression of Ki67 be further explored as a predictive biomarker among patients with NSCLC receiving checkpoint inhibitors, cytotoxic agents, or novel combinations thereof.^{30,31}

In the present study, our observations of an association between tumor proliferation and an immunosuppressive microenvironment are supported by the inverse associations between Ki67 and intratumoral infiltration by cells expressing CD4, a marker for helper T lymphocytes, and CD57, a marker of natural killer cells. In contrast, tumors characterized by high expression of Ki67 were observed to have a highly inflamed peritumoral compartment marked by increased densities of a variety of immune cell populations expressing CD3, CD4, CD8, CD45RO, FOXP3, and GZB. Likewise, tumor-associated immune cell expression of the T-cell stimulatory factor ICOS was enhanced in highly proliferative tumors. Taken together, these findings suggest an appropriate lymphocytic response to tumor growth activity in the peritumoral compartment with a contradictory inability of immune cells to infiltrate the intratumoral compartment and tumor nests. Given the proposed importance of spatial interactions between malignant and immune cells, this compartment-specific difference in relative immune cell infiltration warrants further investigation.³²

TABLE 2. Univariable and multivariable analysis of factors associated with overall survival among patients with pathological stage I tumors (n = 109)

Variable	N (%)	Univariable		Multivariable	
		HR (95% CI)	P	HR (95% CI)	P
Age					
<65 y	45 (41.3)	Reference		Reference	
≥65 y	64 (58.7)	2.32 (1.30-4.12)	.004	2.99 (1.55-5.75)	.001
Sex					
Female	63 (57.8)	Reference			
Male	46 (42.2)	1.45 (0.86-2.45)	.164		
Smoker					
Never	14 (12.8)	Reference			
Ever	95 (87.2)	1.53 (0.65-3.59)	.328		
Zubrod					
0	61 (56.0)	Reference			
1	48 (44.0)	0.73 (0.43-1.23)	.237		
FEV1					
>70% Predicted	82 (75.2)	Reference			
≤70% Predicted	27 (24.8)	1.72 (0.97-3.04)	.065		
Tumor size (cm)	n/a	1.15 (0.94-1.42)	.184	1.26 (1.00-1.60)	.051
Differentiation					
Well/moderate	69 (63.3)	Reference			
Poor	40 (36.7)	1.80 (1.06-3.06)	.029		
Histology					
ACA	73 (67.0)	Reference			
SCC	33 (30.3)	2.15 (1.26-3.68)	.005		
NSCLC NOS	3 (2.8)	1.60 (0.38-6.69)	.522		
Extent of resection					
Lobectomy/bilobectomy	105 (96.3)	Reference		Reference	
Sublobar	4 (3.7)	7.58 (2.57-22.36)	<.001	5.00 (1.66-15.06)	.004
Margin					
R0	107 (98.2)	Reference			
R1	2 (1.8)	2.71 (0.66-11.18)	.169		
Ki67 expression*					
Low	12 (11.0)	Reference		Reference	
Intermediate	55 (50.5)	4.33 (1.02-18.41)	.047	4.33 (1.00-18.67)	.050
High	42 (38.5)	8.22 (1.96-34.52)	.004	8.23 (1.91-35.41)	.005
Adjuvant chemotherapy					
None	91 (83.5)	Reference		Reference	
Chemotherapy	18 (16.5)	0.39 (0.15-0.98)	.044	0.42 (0.16-1.07)	.070

HR, Hazard ratio; CI, confidence interval; FEV1, forced expiratory volume in 1 second; ACA, adenocarcinoma; SCC, squamous cell carcinoma; NSCLC NOS, non-small cell lung cancer, not otherwise specified. *Low expression: Ki67 ≤7.17%; Intermediate expression: Ki67 7.17%-25.73%; High expression: Ki67 >25.73%.

In this cohort of chemotherapy-naïve patients with resected NSCLC, the prognostic effect of Ki67 expression was moderated by pathological stage such that it retained prognostic significance only in patients with stage I disease. The observation that Ki67 was more closely related to an immunosuppressive microenvironment in stage I disease than in more advanced stages provides a possible mechanistic explanation for the differential prognostic significance that similarly varied according to pathologic stage. In concert with this observation, several previous analyses have identified associations between

tumor proliferation and postoperative survival in patients with stage I NSCLC.¹³ Further, by trichotomizing our institutional cohort of patients with stage I disease, we identified a subgroup defined by an easily applicable cutoff (Ki67 expression in ~25% of tumor cells) with postoperative survival approaching that historically observed in stage II disease.³³ Whether intratumoral Ki67 expression defines a subset of stage I tumors at sufficiently high risk of treatment failure that the use of adjuvant therapy is justified is a subject that warrants further investigation.

Tumor Cellular Proliferation Is Associated with Enhanced Immune Checkpoint Expression in Resected Non-Small Cell Lung Cancer

Kyle G. Mitchell, Edwin R. Parra, David B. Nelson, Jiejin Zhang, Ignacio I. Wistuba, Junya Fujimoto, Jack A. Roth, Mara B. Antonoff, MD Anderson Lung Cancer Immune Microenvironment Working Group

VIDEO 1. A summary of the key findings of the study and their relevance. Video available at: [https://www.jtcvs.org/article/S0022-5223\(19\)31015-3/fulltext](https://www.jtcvs.org/article/S0022-5223(19)31015-3/fulltext).

Study Limitations

Despite the limitations imposed on this study by its retrospective nature, the long follow-up period allowed for discrimination of patients with relatively low-risk disease into 3 distinct risk strata. Although our findings regarding the associations between tumor proliferation and checkpoint expression are intriguing in the possibility of their application to the context of immune checkpoint inhibitors, we restricted our analysis to patients who had not received neoadjuvant therapy. It is unclear what impact the exposure to previous therapies and potential clonal selection might have on these relationships. Further study is needed to clarify whether these findings are preserved in the context of alterations in tumor biology that are associated with disease progression from the locoregional to the advanced setting. Further, because the cohort was defined by the availability of Ki67 expression data, a small subset lacked other immunohistochemistry data, and the study cohort reflects only a small subgroup of all patients with NSCLC undergoing resection at our institution during the study period. Finally, an exploratory analysis of tumors stratified by histology demonstrated that these associations were not present in squamous cell carcinomas; this observation warrants further investigation. As biomarkers are developed for the purposes of prediction and prognostication in NSCLC, it may well be the case that a signature of several markers tailored to histopathologic subtypes may be the optimal approach. No association was observed between expression of Ki67 and immune checkpoints in stage II and III tumors after stratification according to pathologic stage. Further analyses of trials using novel therapies in the neoadjuvant and adjuvant settings are needed to define the clinical significance of this apparent stage-specific relationship.

CONCLUSIONS

We identified associations between tumor proliferative activity as quantified by tumor expression of Ki67 and features associated with an aggressive tumor biology.

Additionally, rapidly proliferating tumors were associated with a high degree of stromal inflammatory response but reduced infiltration of several cell populations in the tumor compartment. These results highlight the need for further investigation in the prospective setting to identify whether pretreatment quantification of Ki67 can be used as a predictive marker for response to novel therapies.

Webcast

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Conflict of Interest Statement

Roth discloses unrelated relationships with Genprex (equity, consulting) and Varian (grant support). All other authors have nothing to disclose with regard to commercial support.

The MD Anderson Lung Cancer Immune Microenvironment Working Group coauthors include Erin M. Corsini, MD, Ara A. Vaporciyan, MD, Wayne L. Hofstetter, MD, Reza J. Mehran, MD, Stephen G. Swisher, MD, David C. Rice, MD, Boris Sepesi, MD, Garrett L. Walsh, Carmen Behrens, MD, Neda Kalhor, MD, Annikka Weissferdt, MD, DrMed, Cesar A. Moran, MD, and J. Jack Lee, PhD, MS, DDS.

References

- Borghaei H, Paz-Ares L, Horn L, Spigel DR, Steins M, Ready NE, et al. Nivolumab versus docetaxel in advanced nonsquamous non-small-cell lung cancer. *N Engl J Med*. 2015;373:1627-39.
- Brahmer J, Reckamp KL, Baas P, Crino L, Eberhardt WE, Poddubskaya E, et al. Nivolumab versus docetaxel in advanced squamous-cell non-small-cell lung cancer. *N Engl J Med*. 2015;373:123-35.
- Forde PM, Chaft JE, Smith KN, Anagnostou V, Cottrell TR, Hellmann MD, et al. Neoadjuvant PD-1 blockade in resectable lung cancer. *N Engl J Med*. 2018;378:1976-86.
- Sharma P, Hu-Lieskova S, Wargo JA, Ribas A. Primary, adaptive, and acquired resistance to cancer immunotherapy. *Cell*. 2017;168:707-23.
- Cuylens S, Blaukopf C, Politi AZ, Muller-Reichert T, Neumann B, Poser I, et al. Ki-67 acts as a biological surfactant to disperse mitotic chromosomes. *Nature*. 2016;535:308.
- Rahmanzadeh R, Hüttmann G, Gerdes J, Scholzen T. Chromophore-assisted light inactivation of pKi-67 leads to inhibition of ribosomal RNA synthesis. *Cell Prolif*. 2007;40:422-30.
- Sobecki M, Mrouj K, Colinge J, Gerbe F, Hay P, Krasinska L, et al. Cell-cycle regulation accounts for variability in Ki-67 expression levels. *Cancer Res*. 2017;77:2722-34.
- Zheng JN, Ma TX, Cao JY, Sun XQ, Chen JC, Li W, et al. Knockdown of Ki-67 by small interfering RNA leads to inhibition of proliferation and induction of apoptosis in human renal carcinoma cells. *Life Sci*. 2006;78:724-9.
- Lee JJ, Liu D, Lee JS, Kurie JM, Khuri FR, Ibarguen H, et al. Long-term impact of smoking on lung epithelial proliferation in current and former smokers. *J Natl Cancer Inst*. 2001;93:1081-8.

10. Sun X, Kaufman PD. Ki-67: more than a proliferation marker. *Chromosoma*. 2018;127:175-86.
11. Bullwinkel J, Baron-Luhr B, Ludemann A, Wohlenberg C, Gerdes J, Scholzen T. Ki-67 protein is associated with ribosomal RNA transcription in quiescent and proliferating cells. *J Cell Physiol*. 2006;206:624-35.
12. Miller I, Min M, Yang C, Tian C, Gookin S, Carter D, et al. Ki67 is a graded rather than a binary marker of proliferation versus quiescence. *Cell Rep*. 2018;24:1105-12.e1105.
13. Wei DM, Chen WJ, Meng RM, Zhao N, Zhang XY, Liao DY, et al. Augmented expression of Ki-67 is correlated with clinicopathological characteristics and prognosis for lung cancer patients: an up-dated systematic review and meta-analysis with 108 studies and 14,732 patients. *Respir Res*. 2018;19:150.
14. Chen L, Diao L, Yang Y, Yi X, Rodriguez BL, Li Y, et al. CD38-mediated immunosuppression as a mechanism of tumor cell escape from PD-1/PD-L1 blockade. *Cancer Discov*. 2018;8:1156-75.
15. Soo RA, Chen Z, Yan Teng RS, Tan HL, Iacopetta B, Tai BC, et al. Prognostic significance of immune cells in non-small cell lung cancer: meta-analysis. *Oncotarget*. 2018;9:24801-20.
16. Parra ER, Villalobos P, Behrens C, Jiang M, Pataer A, Swisher SG, et al. Effect of neoadjuvant chemotherapy on the immune microenvironment in non-small cell lung carcinomas as determined by multiplex immunofluorescence and image analysis approaches. *J Immunother Cancer*. 2018;6:48.
17. Edge S, Byrd DR, Compton CC, Fritz AG, Greene F, Trotti A III, eds. *AJCC Cancer Staging Handbook*. 7th ed. New York: Springer; 2010.
18. Parra ER, Villalobos P, Zhang J, Behrens C, Mino B, Swisher SG, et al. Immunohistochemical and image analysis-based study shows that several immune checkpoints are co-expressed in non-small cell lung carcinoma tumors. *J Thorac Oncol*. 2018;13:779-91.
19. Parra ER, Behrens C, Rodriguez-Canales J, Lin H, Mino B, Blando J, et al. Image analysis-based assessment of PD-L1 and tumor-associated immune cells density supports distinct intratumoral microenvironment groups in non-small cell lung carcinoma patients. *Clin Cancer Res*. 2016;22:6278-89.
20. Spearman C. The proof and measurement of association between two things. *Am J Psychol*. 1904;15:72-101.
21. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Ser B Stat Methodol*. 1995; 57:289-300.
22. Chang C, Hsieh M-K, Chang W-Y, Chiang AJ, Chen J. Determining the optimal number and location of cutoff points with application to data of cervical cancer. *PLoS One*. 2017;12:e0176231.
23. Kadara H, Choi M, Zhang J, Parra ER, Rodriguez-Canales J, Gaffney SG, et al. Whole-exome sequencing and immune profiling of early-stage lung adenocarcinoma with fully annotated clinical follow-up. *Ann Oncol*. 2017;28:75-82.
24. Schalper KA, Carvajal-Hausdorf D, McLaughlin J, Altan M, Velcheti V, Gaule P, et al. Differential expression and significance of PD-L1, IDO-1, and B7-H4 in human lung cancer. *Clin Cancer Res*. 2017;23:370-8.
25. Altan M, Pelekanou V, Schalper KA, Toki M, Gaule P, Syrigos K, et al. B7-H3 Expression in NSCLC and its association with B7-H4, PD-L1 and tumor-infiltrating lymphocytes. *Clin Cancer Res*. 2017;23:5202-9.
26. He Y, Yu H, Rozeboom L, Rivard CJ, Ellison K, Dziadziusko R, et al. LAG-3 protein expression in non-small cell lung cancer and its relationship with PD-1/PD-L1 and tumor-infiltrating lymphocytes. *J Thorac Oncol*. 2017;12:814-23.
27. Zou W, Chen L. Inhibitory B7-family molecules in the tumour microenvironment. *Nat Rev Immunol*. 2008;8:467.
28. Loo D, Alderson RF, Chen FZ, Huang L, Zhang W, Gorlatov S, et al. Development of an Fc-enhanced anti-B7-H3 monoclonal antibody with potent antitumor activity. *Clin Cancer Res*. 2012;18:3834-45.
29. Spranger S, Koblisch HK, Horton B, Scherle PA, Newton R, Gajewski TF. Mechanism of tumor rejection with doublets of CTLA-4, PD-1/PD-L1, or IDO blockade involves restored IL-2 production and proliferation of CD8(+) T cells directly within the tumor microenvironment. *J Immunother Cancer*. 2014;2:3.
30. Rizvi H, Sanchez-Vega F, La K, Chatila W, Jonsson P, Halpenny D, et al. Molecular determinants of response to anti-programmed cell death (PD)-1 and anti-programmed death-ligand 1 (PD-L1) blockade in patients with non-small-cell lung cancer profiled with targeted next-generation sequencing. *J Clin Oncol*. 2018;36:633-41.
31. Denkert C, Loibl S, Müller BM, Eidtmann H, Schmitt WD, Eiermann W, et al. Ki67 levels as predictive and prognostic parameters in pretherapeutic breast cancer core biopsies: a translational investigation in the neoadjuvant GeparTrio trial. *Ann Oncol*. 2013;24:2786-93.
32. Barua S, Fang P, Sharma A, Fujimoto J, Wistuba I, Rao AUK, et al. Spatial interaction of tumor cells and regulatory T cells correlates with survival in non-small cell lung cancer. *Lung Cancer*. 2018;117:73-9.
33. Yang L, Wang S, Zhou Y, Lai S, Xiao G, Gazdar A, et al. Evaluation of the 7(th) and 8(th) editions of the AJCC/UICC TNM staging systems for lung cancer in a large North American cohort. *Oncotarget*. 2017;8:66784-95.

Key Words: non-small cell lung cancer, immune microenvironment, immune checkpoints, Ki67

FIGURE E1. Consolidated Standards of Reporting Trials diagram depicting selection of eligible patients. *MDACC*, MD Anderson Cancer Center; *NSCLC*, non–small cell lung cancer.

FIGURE E2. Distribution of Ki67 among the study cohort depicted by (A) histogram and density function and (B) quantile plot.

TABLE E1. Associations between tumor expression of Ki67 and expression of immune checkpoints among patients with adenocarcinoma and squamous cell carcinoma

Adenocarcinoma		
Variable	r	FDR-corrected P
PD1 _{Intratumoral}	0.218	.060
PD1 _{Peritumoral}	0.149	.225
PD-L1	0.410	<.001
B7H3	0.237	.045
B7H4	−0.115	.324
IDO1	0.272	.028
VISTA	0.100	.384
LAG3	0.292	.016
TIM3	0.190	.089
ICOS	0.195	.085
OX40	0.218	.059
Squamous cell carcinoma		
Variable	r	FDR-corrected P
PD1 _{Intratumoral}	−0.124	.705
PD1 _{Peritumoral}	0.041	.927
PD-L1	0.080	.802
B7H3	−0.103	.749
B7H4	−0.055	.885
IDO1	0.251	.395
VISTA	−0.138	.655
LAG3	0.142	.655
TIM3	−0.102	.749
ICOS	−0.139	.655
OX40	−0.006	.962

FDR, False discovery rate; *PD1*, programmed cell death protein 1; *PD-L1*, programmed cell death ligand 1; *IDO1*, indoleamine 2,3-dioxygenase 1; *VISTA*, V-domain suppressor of T-cell activation; *LAG3*, lymphocyte activation gene 3; *TIM3*, T-cell immunoglobulin and mucin-domain containing 3; *ICOS*, inducible T-cell co-stimulator; *OX40*, tumor necrosis factor receptor superfamily, member 4.

TABLE E2. Associations between tumor expression of Ki67 and expression of immune checkpoints among patients with pathologic stage I adenocarcinoma and squamous cell carcinoma

Adenocarcinoma		
Variable	r	FDR-corrected <i>P</i>
PD1 _{Intratumoral}	0.266	.043
PD1 _{Peritumoral}	0.130	.422
PD-L1	0.493	<.001
B7H3	0.338	.019
B7H4	−0.189	.179
IDO1	0.298	.041
VISTA	0.156	.2700
LAG3	0.345	.016
TIM3	0.282	.043
ICOS	0.254	.054
OX40	0.276	.043
Squamous cell carcinoma		
Variable	r	FDR-corrected <i>P</i>
PD1 _{Intratumoral}	0.326	.499
PD1 _{Peritumoral}	0.065	.879
PD-L1	0.046	.879
B7H3	−0.064	.879
B7H4	−0.055	.879
IDO1	0.401	.499
VISTA	−0.276	.499
LAG3	0.042	.879
TIM3	−0.081	.879
ICOS	−0.276	.499
OX40	−0.079	.879

FDR, False discovery rate; *PD1*, programmed cell death protein 1; *PD-L1*, programmed cell death ligand 1; *IDO1*, indoleamine 2,3-dioxygenase 1; *VISTA*, V-domain suppressor of T-cell activation; *LAG3*, lymphocyte activation gene 3; *TIM3*, T-cell immunoglobulin and mucin-domain containing 3; *ICOS*, inducible T-cell co-stimulator; *OX40*, tumor necrosis factor receptor superfamily, member 4.

TABLE E3. Associations between tumor expression of Ki67 and expression of immune checkpoints among patients with pathologic stage I (n = 109) and stages II/III (n = 81) non-small cell lung cancer

Stage I		
Variable	r	FDR-corrected <i>P</i>
PD1 _{Intratumoral}	0.151	.226
PD1 _{Peritumoral}	0.162	.225
PD-L1	0.349	.002
B7H3	0.320	.004
B7H4	0.025	.868
IDO1	0.276	.016
VISTA	−0.001	.993
LAG3	0.253	.025
TIM3	0.117	.337
ICOS	0.150	.225
OX40	0.056	.687
Stage II/III		
Variable	r	FDR-corrected <i>P</i>
PD1 _{Intratumoral}	0.067	.643
PD1 _{Peritumoral}	0.145	.468
PD-L1	0.212	.272
B7H3	0.092	.553
B7H4	−0.010	.935
IDO1	0.234	.272
VISTA	0.018	.909
LAG3	0.224	.272
TIM3	0.079	.603
ICOS	0.262	.272
OX40	0.178	.325

FDR, False discovery rate; *PD1*, programmed cell death protein 1; *PD-L1*, programmed cell death ligand 1; *IDO1*, indoleamine 2,3-dioxygenase 1; *VISTA*, V-domain suppressor of T-cell activation; *LAG3*, lymphocyte activation gene 3; *TIM3*, T-cell immunoglobulin and mucin-domain containing 3; *ICOS*, inducible T-cell co-stimulator; *OX40*, tumor necrosis factor receptor superfamily, member 4.

TABLE E4. Univariable and multivariable analysis of factors associated with overall survival among patients with pathological stage I tumors, with inclusion of all clinically relevant variables without elimination (n = 109)

Variable	N (%)	Univariable		Multivariable	
		HR (95% CI)	P	HR (95% CI)	P
Age					
<65 y	45 (41.3)	Reference			
≥65 y	64 (58.7)	2.32 (1.30-4.12)	.004	3.12 (1.61-6.04)	.001
Sex					
Female	63 (57.8)	Reference		Reference	
Male	46 (42.2)	1.45 (0.86-2.45)	.164	1.15 (0.67-1.97)	.613
Smoker					
Never	14 (12.8)	Reference		Reference	
Ever	95 (87.2)	1.53 (0.65-3.59)	.328	1.29 (0.52-3.19)	.589
Zubrod					
0	61 (56.0)	Reference		Reference	
1	48 (44.0)	0.73 (0.43-1.23)	.237	0.92 (0.50-1.70)	.798
FEV1					
>70% Predicted	82 (75.2)	Reference		Reference	
≤70% Predicted	27 (24.8)	1.72 (0.97-3.04)	.065	1.09 (0.58-2.07)	.793
Tumor size (cm)	n/a	1.15 (0.94-1.42)	.184	1.23 (0.96-1.59)	.105
Differentiation					
Well/moderate	69 (63.3)	Reference		Reference	
Poor	40 (36.7)	1.80 (1.06-3.06)	.029	1.46 (0.80-2.67)	.213
Histology					
ACA	73 (67.0)	Reference		Reference	
SCC	33 (30.3)	2.15 (1.26-3.68)	.005	1.12 (0.57-2.22)	.741
NSCLC NOS	3 (2.8)	1.60 (0.38-6.69)	.522	1.23 (0.26-5.68)	.795
Extent of resection					
Lobectomy/bilobectomy	105 (96.3)	Reference			
Sublobar	4 (3.7)	7.58 (2.57-22.36)	<.001		
Margin					
R0	107 (98.2)	Reference			
R1	2 (1.8)	2.71 (0.66-11.18)	.169		
Ki67 expression*					
Low	12 (11.0)	Reference		Reference	
Intermediate	55 (50.5)	4.33 (1.02-18.41)	.047	3.91 (0.89-17.18)	.071
High	42 (38.5)	8.22 (1.96-34.52)	.004	6.12 (1.21-30.89)	.028
Adjuvant chemotherapy					
None	91 (83.5)	Reference		Reference	
Chemotherapy	18 (16.5)	0.39 (0.15-0.98)	.044	0.39 (0.15-1.02)	.055

HR, Hazard ratio; CI, confidence interval; FEV1, forced expiratory volume in 1 second; ACA, adenocarcinoma; SCC, squamous cell carcinoma; NSCLC NOS, non–small cell lung cancer, not otherwise specified. *Low expression: Ki67 ≤7.17%; Intermediate expression: Ki67 7.17%-25.73%; High expression: Ki67 >25.73%.

TABLE E5. Univariable and multivariable analysis of factors associated with disease-free survival among patients with pathological stage I tumors (n = 109)

Variable	N (%)	Univariable		Multivariable	
		HR (95% CI)	P	HR (95% CI)	P
Age					
<65 y	45 (41.3)	Reference		Reference	
≥65 y	64 (58.7)	2.06 (1.17-3.62)	.012	2.29 (1.27-4.12)	.006
Sex					
Female	63 (57.8)	Reference			
Male	46 (42.2)	1.44 (0.86-2.43)	.167		
Smoker					
Never	14 (12.8)	Reference			
Ever	95 (87.2)	1.58 (0.67-3.70)	.294		
Zubrod					
0	61 (56.0)	Reference			
1	48 (44.0)	1.32 (0.79-2.22)	.292		
FEV1					
>70% Predicted	82 (75.2)	Reference			
≤70% Predicted	27 (24.8)	1.60 (0.91-2.84)	.106		
Tumor size (cm)	n/a	1.14 (0.93-1.40)	.212		
Differentiation					
Well/moderate	69 (63.3)	Reference			
Poor	40 (36.7)	1.69 (1.00-2.86)	.049		
Histology					
ACA	73 (67.0)	Reference			
SCC	33 (30.3)	1.91 (1.12-3.25)	.017		
NSCLC NOS	3 (2.8)	1.41 (0.34-5.90)	.639		
Extent of resection					
Lobectomy/bilobectomy	105 (96.3)	Reference			
Sublobar	4 (3.7)	5.62 (1.95-16.23)	.001	3.70 (1.27-10.84)	.017
Margin					
R0	107 (98.2)	Reference			
R1	2 (1.8)	2.62 (0.64-10.83)	.182		
Ki67 expression*					
Low	12 (11.0)	Reference		Reference	
Intermediate	55 (50.5)	4.29 (1.01-18.23)	.048	4.48 (1.04-19.33)	.044
High	42 (38.5)	7.56 (1.80-31.73)	.006	8.71 (2.04-37.19)	.003
Adjuvant chemotherapy					
None	91 (83.5)	Reference			
Chemotherapy	18 (16.5)	0.42 (0.17-1.05)	.063		

HR, Hazard ratio; CI, confidence interval; FEV1, forced expiratory volume in 1 second; ACA, adenocarcinoma; SCC, squamous cell carcinoma; NSCLC NOS, non-small cell lung cancer, not otherwise specified. *Low expression: Ki67 ≤7.17%; Intermediate expression: Ki67 7.17%-25.73%; High expression: Ki67 >25.73%.

TABLE E6. Univariable and multivariable analyses of factors associated with disease-free survival among patients with pathological stage I tumors, with inclusion of all clinically relevant variables without elimination (n = 109)

Variable	N (%)	Univariable		Multivariable	
		HR (95% CI)	P	HR (95% CI)	P
Age					
<65 y	45 (41.3)	Reference		Reference	
≥65 y	64 (58.7)	2.06 (1.17-3.62)	.012	2.64 (1.40-5.00)	.003
Sex					
Female	63 (57.8)	Reference		Reference	
Male	46 (42.2)	1.44 (0.86-2.43)	.167	1.20 (0.71-2.05)	.494
Smoker					
Never	14 (12.8)	Reference		Reference	
Ever	95 (87.2)	1.58 (0.67-3.70)	.294	1.34 (0.54-3.31)	.528
Zubrod					
0	61 (56.0)	Reference		Reference	
1	48 (44.0)	1.32 (0.79-2.22)	.292	0.94 (0.52-1.70)	.826
FEV1					
>70% Predicted	82 (75.2)	Reference		Reference	
≤70% Predicted	27 (24.8)	1.60 (0.91-2.84)	.106	1.15 (0.62-2.12)	.667
Tumor size (cm)	n/a	1.14 (0.93-1.40)	.212	1.19 (0.93-1.53)	.162
Differentiation					
Well/moderate	69 (63.3)	Reference		Reference	
Poor	40 (36.7)	1.69 (1.00-2.86)	.049	1.35 (0.75-2.44)	.321
Histology					
ACA	73 (67.0)	Reference		Reference	
SCC	33 (30.3)	1.91 (1.12-3.25)	.017	1.05 (0.54-2.04)	.892
NSCLC NOS	3 (2.8)	1.41 (0.34-5.90)	.639	1.09 (0.24-5.01)	.911
Extent of resection					
Lobectomy/bilobectomy	105 (96.3)	Reference			
Sublobar	4 (3.7)	5.62 (1.95-16.23)	.001		
Margin					
R0	107 (98.2)	Reference			
R1	2 (1.8)	2.62 (0.64-10.83)	.182		
Ki67 expression*					
Low	12 (11.0)	Reference		Reference	
Intermediate	55 (50.5)	4.29 (1.01-18.23)	.048	3.96 (0.91-17.28)	.068
High	42 (38.5)	7.56 (1.80-31.73)	.006	6.00 (1.21-29.81)	.028
Adjuvant chemotherapy					
None	91 (83.5)	Reference		Reference	
Chemotherapy	18 (16.5)	0.42 (0.17-1.05)	.063	0.43 (0.17-1.12)	.084

HR, Hazard ratio; CI, confidence interval; FEV1, forced expiratory volume in 1 second; ACA, adenocarcinoma; SCC, squamous cell carcinoma; NSCLC NOS, non-small cell lung cancer, not otherwise specified. *Low expression: Ki67 ≤7.17%; Intermediate expression: Ki67 7.17%-25.73%; High expression: Ki67 >25.73%.