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ORIGINAL REPORT

Clinical Impact of Immune Microenvironment in Stage I Lung Adenocarcinoma: Tumor Interleukin-12 Receptor β2 (IL-12Rβ2), IL-7R, and Stromal FoxP3/CD3 Ratio Are Independent Predictors of Recurrence

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ABSTRACT

Purpose
Mounting evidence suggests that tumor-infiltrating immune cells have prognostic value for patients with solid organ malignancies. Our aim was to investigate the prognostic significance of the immune microenvironment in patients with stage I lung adenocarcinoma (ADC).

Patients and Methods
Using tissue microarray and immunohistochemistry, we investigated eight types of tumor-infiltrating immune cells in the tumor nest and tumor-associated stroma as well as tumor expression of five cytokines in a uniform cohort of 956 patients with stage I lung ADC (478 each in training and validation cohorts).

Results
Although a high density of stromal forkhead box P3 (FoxP3) –positive cells was associated with shorter recurrence-free probability (RFP; $P = .043$), the relative proportion of stromal FoxP3 to CD3 was a stronger predictor of recurrence (5-year RFP, 85% for high *v* 77% for low ratio; $P = .004$). High expression of tumor interleukin-12 receptor β2 (IL-12Rβ2) was associated with better outcome (5-year RFP, 90% for high *v* 80% for low expression; $P = .026$), whereas high expression of tumor IL-7R was associated with worse outcome (5-year RFP, 76% for high *v* 86% for low expression; $P = .001$). In multivariate analysis, these immune markers were independently associated with recurrence. Although IL-7R remained significant for poor overall survival, all the markers remained prognostic for recurrence in patients with stages IA and IB disease as well as for patients with tumors ≤ 2 cm.

Conclusion
Our investigation confirms the biologic and prognostic significance of the tumor immune microenvironment for patients with stage I lung ADC and provides support for its use to stratify clinical outcome and immunotherapeutic interventions.

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INTRODUCTION

Therapeutic decisions in solid malignancies are dictated by the TNM staging system, which is based on anatomic factors. The need to advance beyond the TNM staging system has been addressed by examining the tumor immune microenvironment, which is influenced by the type, density, and location of tumor-infiltrating immune cells.[1-6] For colorectal cancer, an immune score based on the density of cytotoxic CD8+ and memory CD45R0+ lymphocytes in the tumor center and the invasive margin has proven to be a stronger predictor of clinical outcome than the conventional staging system.[7] These results have led to discussions about incorporating immunologic parameters into the routine diagnostic and prognostic assessment of tumors.[8]

More than 160,000 deaths resulting from lung cancer are projected to occur during 2012 in the United States alone, making it the most lethal malignancy.[9] In the recent National Lung Screening Trial, computed tomography (CT) screening was shown to decrease mortality related to lung cancer.[10] On the basis of this trial, the National Comprehensive Cancer Network recently issued guidelines for lung cancer screening that recommend helical low-dose CT for high-risk patients.[11] With the earlier detection afforded by CT screening, it is anticipated that increasing numbers of patients will be diagnosed with early-stage lung cancer. Surgery alone remains

the standard of care for patients with stage I lung cancer, yet as many as 27% will experience disease recurrence within 5 years.[12]

The prognostic utility of tumor-infiltrating immune cells has been investigated for lung cancer[13-17]; however, these studies investigated heterogeneous stages and histologies and used survival as the main end point, rendering their application to early-stage patients difficult. In our study, we investigated the prognostic utility of tumor-infiltrating immune cells as well as cytokines in a uniform cohort of patients with stage I lung adenocarcinoma (ADC), the most common histologic type of lung cancer. To elucidate the biologic significance of the tumor immune microenvironment, we investigated immune cells that have shown prognostic significance in both the tumor nest and tumor-associated stroma for other solid malignancies—CD3 (pan T cell), CD4 (helper T cell), CD8 (cytotoxic T cell), CD20 (B cell), CD45R0 (memory T cell), forkhead box P3 (FoxP3; regulatory T cell), CD56 (natural-killer cell), and CD68 (macrophage)—as well as tumor expression of cytokines CCR7, CXCL12, CXCR4, interleukin-7 receptor (IL-7R), and IL-12Rβ2.[18] Most importantly, we chose recurrence, rather than survival, as the study end point, because recurrence is more clinically relevant to patients with stage I disease and is not confounded by factors that might influence survival.

PATIENTS AND METHODS

Patients

Our training cohort comprised patients diagnosed with pathologic stage I lung ADC at Memorial Sloan-Kettering Cancer Center between 1995 and 2005 and has been well characterized[19]; patients in the validation cohort received the same diagnosis between 2002 and 2009. Institutional review board approval was obtained. Data were obtained from the prospectively maintained thoracic surgery database. No patients received neoadjuvant chemotherapy or radiation therapy.

All hematoxylin and eosin–stained slides were re-reviewed by a pathologist (K.K.), and problem cases were reviewed by two pathologists (K.K., W.D.T.). All slides were evaluated for lymphatic, vascular, and visceral pleural invasion[19] as well as predominant histologic subtype according to the new IASLC/ATS/ERS (International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society) classification of lung ADC.[20] Staging was based on the seventh edition of the TNM Cancer Staging Manual.[21] All patients were observed until death or last follow-up (assessed April 2011). Slides for the validation cohort were reviewed by the same pathologists.

Immunohistochemical Analysis

Formalin-fixed, paraffin-embedded tumor specimens were used for tissue microarray construction. For each tumor, the slide with the most severe inflammatory reaction was chosen. From each tumor in the training cohort, four representative cores with the most abundant inflammatory reaction, 0.6 mm in size, were marked—two each from the tumor nest and tumor-associated stroma. For the validation cohort, the number of cores was increased—six from the tumor nest and three from the tumor-associated stroma. Lung tissues from eight normal samples were included as controls. The standard avidin-biotin-peroxidase complex technique was used for immunohistochemical staining for the antihuman antibodies (Appendix Table A1, online only).

Scoring of Immunohistochemistry

Representative images and scoring of immunohistochemistry are shown in the Data Supplement. Under a high-power field (magnification, $\times$400), each core was scored for degree of immune-cell infiltration in the tumor nest and tumor-associated stroma semiquantitatively. Normal controls showed sparse staining for all stains. The scores for each core were averaged to give one score per patient. For each patient, immune-cell infiltration was defined by a score of 1 (average, 1 to 1.67), 2 (average, 1.67 to 2.33), or 3 (average, $>$ 2.33). For statistical analysis, a score of 1 was considered to be low, and 2 and 3 were considered to be high.

For cytokines, we scored the tumor stain on the basis of intensity and distribution, as previously described.[22] For CCR7, CXCL12, CXCR4, and IL-7R, a score of <1 was regarded as negative and ≥ 1 as positive. For IL12-Rβ2, the score was based on intensity only, because the distribution was diffuse. IL12-Rβ2 intensity of <1 was regarded as negative and ≥ 1 as positive. Normal controls demonstrated staining for IL12-Rβ2, as previously described,[23] but not for the other four cytokines.

Statistical Analysis

Associations between clinicopathologic variables and each marker were analyzed using Pearson's χ^2 test. Recurrence-free probability (RFP) was estimated using the Kaplan-Meier method, with follow-up starting at the time of surgery. Patients whose disease did not recur during study follow-up were censored at the time of last contact or death without documented recurrence. Differences in RFP between subgroups of patients were compared using the nonparametric log-rank test. Multivariate analyses were performed using the Cox proportional hazards regression model to estimate the effect of the immune markers of interest on RFP, with adjustment for clinicopathologic factors. All significance tests were two sided, and all used a 5% level of significance. Statistical analyses were conducted using SAS statistical software (version 8.02; SAS Institute, Cary, NC).

RESULTS

Clinicopathologic Variables

A total of 956 patients were included in the study, 478 each in training and validation cohorts. Clinicopathologic variables are listed in Table 1. The validation cohort had a higher percentage of patients with stage IA disease, likely as an effect of this cohort being more recent, resulting in a higher percentage of wedge resections, tumors without lymphatic and pleural invasions, and lepidic-predominant morphology.

Of the available clinicopathologic variables, sex ($P = .002$), stage (IA *v* IB; $P < .001$), lymphatic invasion ($P = .013$), vascular invasion ($P = .01$), and tumor morphology ($P < .001$) were significantly associated with recurrence (Table 2), in concordance with published results. The presence of visceral pleural invasion reflected a tendency for higher rates of recurrence ($P = .075$).

Tumor-Infiltrating Immune Cells

Each immune cell in the tumor nest and tumor-associated stroma was independently assessed for its ability to predict recurrence (Table 2). A high density of FoxP3-positive cells in the stroma was significantly associated with recurrence ($P = .043$; Fig 1A). Because FoxP3-positive regulatory T cells are a subset of the entire T-cell population, we next investigated the relative proportion of FoxP3-positive to CD3+ cells. Interestingly, we observed that among patients with high stromal FoxP3, those with high stromal CD3 had better RFP compared with those with low stromal CD3. In fact, the cohort with high stromal FoxP3 and concurrent high-level stromal CD3 infiltration demonstrated a recurrence rate similar to that of the group with low stromal FoxP3 (data not shown). On the basis of this observation, we devised a stromal FoxP3 risk index in which tumors containing high stromal FoxP3 and low stromal CD3 are considered high risk and tumors with low stromal FoxP3 are considered low risk, as are tumors with high stromal FoxP3 and concurrent high-level stromal CD3

Table 1. Patient Demographic and Clinical Characteristics for the Two Study Cohorts

Variable	Training Cohort No.	Training Cohort %	Validation Cohort No.	Validation Cohort %	*P*
All patients	478		478		
Age, years					.12
Median	68		69		
Range	33-89		23-96		
Mean	67.7		68.6		
SD	9.8		10.1		
Sex					.55
Female	302	64	292	61	
Male	176	37	186	39	
Smoking status					.19
Never	73	15	86	18	
Former	329	69	335	70	
Current	74	16	57	12	
Laterality					.65
Left	203	42	195	41	
Right	275	58	283	59	
Surgical procedure					< .001*
Wedge resection	48	10	92	19	
Segmentectomy/lobectomy/ pneumonectomy	430	90	386	81	
Stage					< .001*
IA	283	59	346	72	
IB	195	41	132	28	
Lymphatic invasion					< .001*
Absent	371	78	303	63	
Present	107	22	175	37	
Vascular invasion					.88
Absent	364	76	367	77	
Present	114	24	111	23	
Pleural invasion					.036*
Absent	387	81	412	86	
Present	91	19	66	14	
Morphology					< .001*
Lepidic	35	7	83	17	
Acinar	220	46	167	35	
Papillary	132	28	93	20	
Micropapillary	12	3	46	10	
Solid	60	13	63	13	
Variants	19	4	26	5	
Mutation					
Wild type	NA		280	59	
EGFR	NA		119	25	
KRAS	NA		79	17	

Abbreviations: NA, not applicable; SD, standard deviation.
*Indicates significant *P* value.

infiltration. We found stromal FoxP3 risk index to be a strong predictor of recurrence; low-risk patients had a 5-year RFP of 85%, compared with 77% for high-risk patients ($P = .004$; Fig 1B). These results were replicated in the validation cohort (Figs 1E, 1F). None of the other immune cells had significant prognostic value.

Tumor Expression of Cytokines

Although the five investigated cytokines are known to be expressed on both tumor and immune cells, our immunohistochemical analysis was optimized for staining on tumor cells. Of the five cytokines, IL-12Rβ2 and IL-7R were found to be prognostic (Table 2). Higher-level expression of IL-12Rβ2 was associated with reduced risk of recurrence (5-year RFP, 90% for high *v* 80% for low level; $P = .026$; Fig 1C), whereas higher-level expression of IL-7R was associated with increased risk of recurrence (5-year RFP, 76% for high *v* 86% for low level; $P = .001$; Fig 1D). These results were replicated in our validation cohort (Figs 1G, 1H). The associations between these two cytokine expressions and the densities of immune cells are shown in the Data Supplement. IL-12Rβ2 expression was associated with low tumor density of CD68 ($P = .006$), whereas IL-7R expression was associated with high density of stromal CD3 ($P = .02$), tumor CD68 ($P < .001$), and stromal CD68 ($P = .002$; CD68 is the marker for tumor-associated macrophages). We did not find significant associations between expression of the other three cytokines (CCR7, CXCL12, and CXCR4) and recurrence.

Multivariate Analysis

After identifying three prognostic immune markers—stromal FoxP3 risk index, tumor IL-12Rβ2, and tumor IL-7R—we next performed a multivariate analysis, with adjustment for other currently known prognostic clinicopathologic factors, including sex, disease stage (IA *v* IB), and lymphatic invasion. The multivariate analysis confirmed that all three immune markers remained independently associated with recurrence (Table 3).

Correlation of Immune Parameters With Clinicopathologic Factors

To gain additional biologic insights, we next investigated the association of the three immune markers with clinicopathologic factors (Fig 2). Combining results from the two cohorts, we found a significant association between stromal FoxP3 risk index score and lymphatic invasion ($P = .038$), vascular invasion ($P < .001$), and high-grade morphology ($P < .001$). IL-12Rβ2 expression had a significant association with low-grade morphology ($P = .019$) and presence of *EGFR* mutation and lack of *KRAS* mutation ($P = .0075$). IL-7R expression had a significant association with higher stage ($P < .001$), larger tumor size ($P < .0013$), lymphatic invasion ($P < .001$), vascular invasion ($P < .001$), high-grade morphology ($P < .001$), and presence of *KRAS* mutation and lack of *EGFR* mutation ($P < .001$).

DISCUSSION

The current TNM staging system relies solely on anatomic factors and is limited in its ability to discriminate a subset of patients with stage I disease with poor clinical outcome. In fact, for stage I lung ADC, tumor size is the only standard prognosticator available. In our study, we have demonstrated the prognostic power of immunologic parameters for stage I lung ADC, identifying three immune markers that are predictive of recurrence. This immunologic observation has both prognostic and therapeutic implications.

Tumor-infiltrating immune cells have shown prognostic value for several solid malignancies, including colorectal,[1,2,7] ovarian,[3] and breast cancers[4] (Appendix Table A2, online only). Galon et al[1] have advocated, through their work in colorectal cancer, the use of three important parameters of tumor-infiltrating lymphocytes (TILs)—type, density, and location—to predict clinical outcome. In our study of a uniform cohort of patients with stage I lung

Table 2. Univariate Analysis of 5-Year RFP in Patients With Stage I Lung Adenocarcinoma in Training Set According to Clinical, Pathologic, and Immune Parameters

Variable	No. of Patients	5-Year RFP (%)	95% CI (%)	*P*
Sex				.002*
Male	176	74	66 to 81	
Female	302	87	83 to 91	
Stage				< .001*
IA	283	89	85 to 93	
IB	195	73	67 to 81	
Lymphatic invasion				.013*
Absent	371	84	80 to 88	
Present	107	76	68 to 85	
Vascular invasion				.01*
Absent	364	84	80 to 88	
Present	114	76	68 to 85	
Visceral pleural invasion				.075
Absent	387	84	79 to 88	
Present	91	77	68 to 87	
Morphology grade				< .001*
Low	35	92	82 to 100	
Intermediate	352	84	80 to 89	
High	72	71	60 to 83	

	Tumor				Stroma			
Marker	No. of Patients	5-Year RFP (%)	95% CI (%)	*P*	No. of Patients	5-Year RFP (%)	95% CI (%)	*P*
CD3				.660				.600
Low	264	82	77 to 97		309	82	78 to 87	
High	193	83	77 to 89		151	83	77 to 89	
CD4				.554				.105
Low	221	83	77 to 89		215	84	79 to 90	
High	236	82	77 to 87		236	80	74 to 85	
CD8				.136				.794
Low	227	84	78 to 89		266	82	77 to 87	
High	227	81	76 to 87		194	83	77 to 89	
CD20				.417				.389
Low	315	83	78 to 88		306	83	78 to 88	
High	140	81	74 to 88		144	80	73 to 87	
CD45RO				.880				.583
Low	182	84	78 to 89		221	83	77 to 88	
High	270	81	76 to 87		220	83	78 to 89	
FoxP3				.846				.043*
Low	245	83	77 to 88		287	85	80 to 89	
High	205	83	78 to 89		160	80	74 to 87	
CD56				.318				.215
Low	435	83	79 to 87		408	83	79 to 87	
High	7	67	38 to 100		9	100	100 to 100	
CD68				.064				.094
Low	310	85	80 to 89		302	85	81 to 90	
High	133	78	71 to 86		115	79	72 to 87	
FoxP3/CD3 ratio				.972				.004*
Low	268	83	77 to 89		318	85	81 to 90	
High	168	83	78 to 89		120	77	69 to 85	
IL-12Rβ2				.026*	†			
Low	365	80	76 to 85					
High	92	90	84 to 97					
IL-7R				.001*	†			
Low	282	86	81 to 90					
High	172	76	69 to 83					
CCR7				.564	†			
Low	321	81	77 to 86					
High	130	84	77 to 91					
CXCL12				.422	†			
Low	225	82	77 to 88					
High	236	83	77 to 88					
CXCR4				.819	†			
Low	248	82	77 to 88					
High	203	83	78 to 89					

Abbreviations: FoxP3, forkhead box P3; IL-7R, interleukin-7 receptor; IL-12Rβ2, interleukin-12 receptor β2; RFP, recurrence-free probability.
*Indicates significant *P* value.
†Stroma not applicable for this marker.

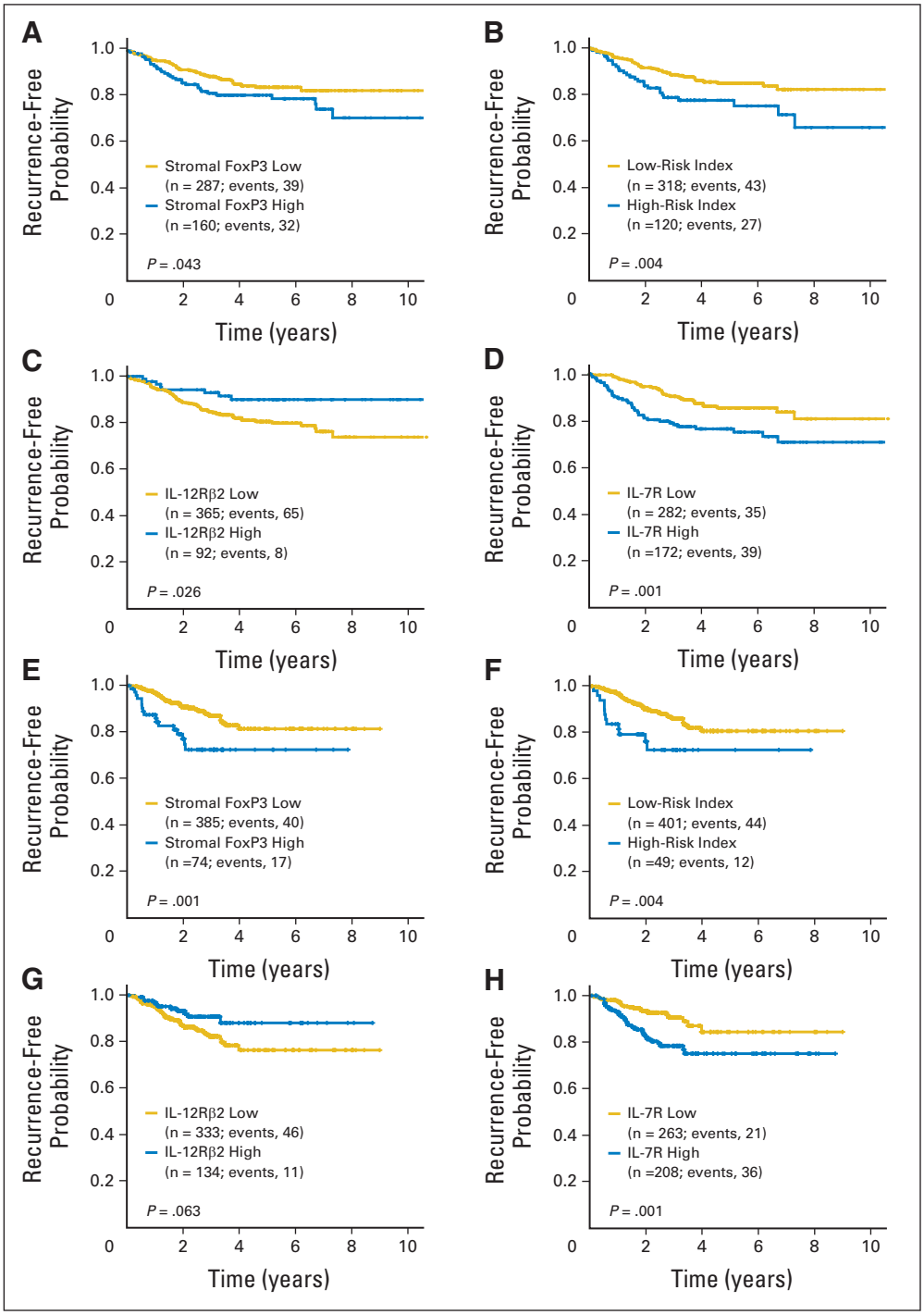

Fig 1. Recurrence-free probability (RFP) in (A–D) training and (E-H) validation cohorts by (A, E) stromal forkhead box P3 (FoxP3) density, (B, F) stromal FoxP3 risk index, (C, G) interleukin-12 receptor $\beta 2$ (IL-12R$\beta 2$), and (D, H) IL-7R. (A) RFP was lower in the group with high density of stromal FoxP3 (n = 160; 5-year RFP, 80%) than in the group with low density (n = 287; 5-year RFP, 85%; P = .043). (B) Patients with high stromal FoxP3 risk index score (defined as those with high density of stromal FoxP3 and low density of stromal CD3) had lower RFP (n = 120; 5-year RFP, 77%) compared with those with low score (n = 318; 5-year RFP, 85%; P = .004). (C) RFP for patients with high-level expression of IL-12R$\beta 2$ was higher (n = 92; 5-year RFP, 90%) than that for patients with low-level expression (n = 365; 5-year RFP, 80%; P = .026). (D) RFP for patients with high-level expression of IL-7R was lower (n = 172; 5-year RFP, 76%) than that for patients with low-level expression (n = 282; 5-year RFP, 86%; P = .001). (E–H) Results were replicated in validation cohort.

ADC, we investigated eight markers of TILs and found the FoxP3/CD3 ratio in tumor-associated stroma to be significantly associated with recurrence. FoxP3 is a marker of regulatory T cells, a subset of lymphocytes known to suppress the host immune response. In patients with lung cancer, regulatory T cells are thought to play protumor roles,[24] and their association with worse prognosis has been shown for all histologic types, including ADC.[17,25] Interestingly, FoxP3 in the stroma only—and not in the tumor nest—was associated with recurrence, emphasizing the importance of assessing the location of TILs within the tumor microenvironment. In fact, the significance of immune cells in the tumor stroma has been shown in non–small-cell lung cancer. In patients with stages I to IIA disease, Dieu-Nosjean et al[26] demonstrated the presence of tertiary de novo lymphoid structure in the tumor microenvironment, a structure they termed the tumor-induced bronchus-associated lymphoid tissue (Ti-BALT). The presence of mature dendritic cells in Ti-BALT correlated with prolonged overall and disease-free survival.

Table 3. Multivariate Analysis

Variable	HR	95% CI	*P*
Sex (male *v* female)	1.74	1.07 to 2.83	.025
Stage (IB *v* IA)	2.23	1.34 to 3.70	.002
Lymphatic invasion	1.52	0.91 to 2.53	.108
Stromal FoxP3 risk index	2.00	1.22 to 3.27	.006
IL-12Rβ2	2.24	1.02 to 4.95	.045
IL-7R	1.65	1.01 to 2.68	.045

Abbreviations: FoxP3, forkhead box P3; HR, hazard ratio; IL-7R, interleukin-7 receptor; IL-12Rβ2, interleukin-12 receptor β2.

Furthermore, we demonstrate that in addition to type, density, and location, a fourth characteristic of TILs—the relative proportion of pro- and antitumor immune cells—is an important parameter. Our close examination of patients with high densities of stromal FoxP3 revealed that among these patients, a concurrent high density of stromal CD3 predicted better outcome. This suggests that even in the presence of high stromal FoxP3, a high density of CD3 may overcome the protumor effects of FoxP3-positive regulatory T cells. In addition to revealing prognostic value, this finding has significant implications for devising potential immunomodulatory therapy for patients with lung ADC; an intervention that decreases FoxP3 and increases CD3 would likely be beneficial. Of interest, cyclophosphamide has been shown to modulate the tumor immune microenvironment by depleting regulatory T cells.[27] Also, in a murine melanoma model, activation of a T-cell costimulatory receptor, 4-1BB, has been shown to result in decreased tumor infiltration of regulatory T cells.[28] Because FoxP3-positive regulatory T cells are thought to be a subset of CD4+ T cells, we also investigated stromal FoxP3 density and its relation to CD4 density. Although stromal densities of CD4 and FoxP3 showed significant correlation, combining CD4 and FoxP3 densities did not result in significant prognostic findings (Data Supplement).

In our analysis of chemokine expression on tumor cells, we found two to be of prognostic significance, one with antitumor associations (IL-12Rβ2) and one with protumor associations (IL-7R). IL-12Rβ2 is one of the two subunits that form the receptor for IL-12. Tumors expressing IL-12Rβ2 were associated with low-grade morphology, *EGFR* mutation, and less recurrence. Given that IL-12Rβ2 expression is observed on normal lung epithelium,[23] it is plausible that lung ADC progression is accompanied by loss of IL-12Rβ2 expression. In fact, in preclinical models, mice deficient in IL-12Rβ2 have been shown to spontaneously develop lung ADC.[29]

After observing that IL-12Rβ2 expression is associated with less-aggressive tumors, we investigated how tumor expression of IL-12Rβ2 is associated with the protumor stromal environment. In a group of patients identified as high risk by the stromal FoxP3 risk

Fig 2. Association of stromal forkhead box P3 (FoxP3) risk index score and interleukin-12 receptor β2 (IL-12Rβ2) and IL-7R expression with stage, tumor size, lymphatic (Ly) and vascular invasion (V), morphologic grade, and mutation status. Histograms show percentage of patients for each immune marker. Stromal FoxP3 risk index score was significantly associated with lymphatic invasion ($P = .038$), vascular invasion ($P < .001$), and high-grade morphology ($P < .001$). IL-12Rβ2 expression was significantly associated with low-grade morphology ($P = .019$) and presence of *EGFR* mutation and lack of *KRAS* mutation ($P = .0075$). IL-7R expression was significantly associated with higher stage ($P < .001$), larger tumor size ($P < .001$), lymphatic invasion ($P < .001$), vascular invasion ($P < .001$), high-grade morphology ($P < .001$), and presence of *KRAS* mutation and lack of *EGFR* mutation ($P < .001$). Int, intermediate.

index, we observed that patients with high-level expression of IL-12Rβ2 (n = 91) experienced less-frequent recurrence than those with low-level expression (n = 25; 5-year RFP, 88% for high-level *v* 73% for low-level expression; $P = .086$). This suggests that even in the presence of unfavorable stromal immune-cell infiltrates, high-level expression of IL-12Rβ2 on tumor cells may play a protective role. Thus, therapies targeted to maintain IL-12Rβ2 expression on tumor cells are of interest. In patients with stages I to IV lung ADC, methylation of the *IL-12Rβ2* gene was shown to be associated with less mRNA expression in vivo and shorter survival.[30] The recent publication of a phase I/II study of DNA methyltransferase inhibitor and a histone deacetylase inhibitor in patients with recurrent metastatic lung cancer is promising.[31] Furthermore, in preclinical models, T cells genetically modified to secrete IL-12 have shown intrinsic resistance to regulatory T cells.[32] IL-12–secreting T cells are of special interest, because this resistance could potentially overcome the protumor associations of regulatory T cells. In addition, the association of IL-12 and IL-12Rβ2 expression on tumor cells warrants further investigation.

In contrast to IL-12Rβ2 expression, IL-7R expression was associated with aggressive tumor features: high-grade morphology, lymphovascular invasion, larger tumor size, *KRAS* mutation, and more-frequent recurrence. IL-7 and IL-7R are implicated in lung cancer lymphangiogenesis via c-Fos/C-Jun–dependent vascular endothelial growth factor D (VEGF-D) upregulation.[33] In breast cancer,

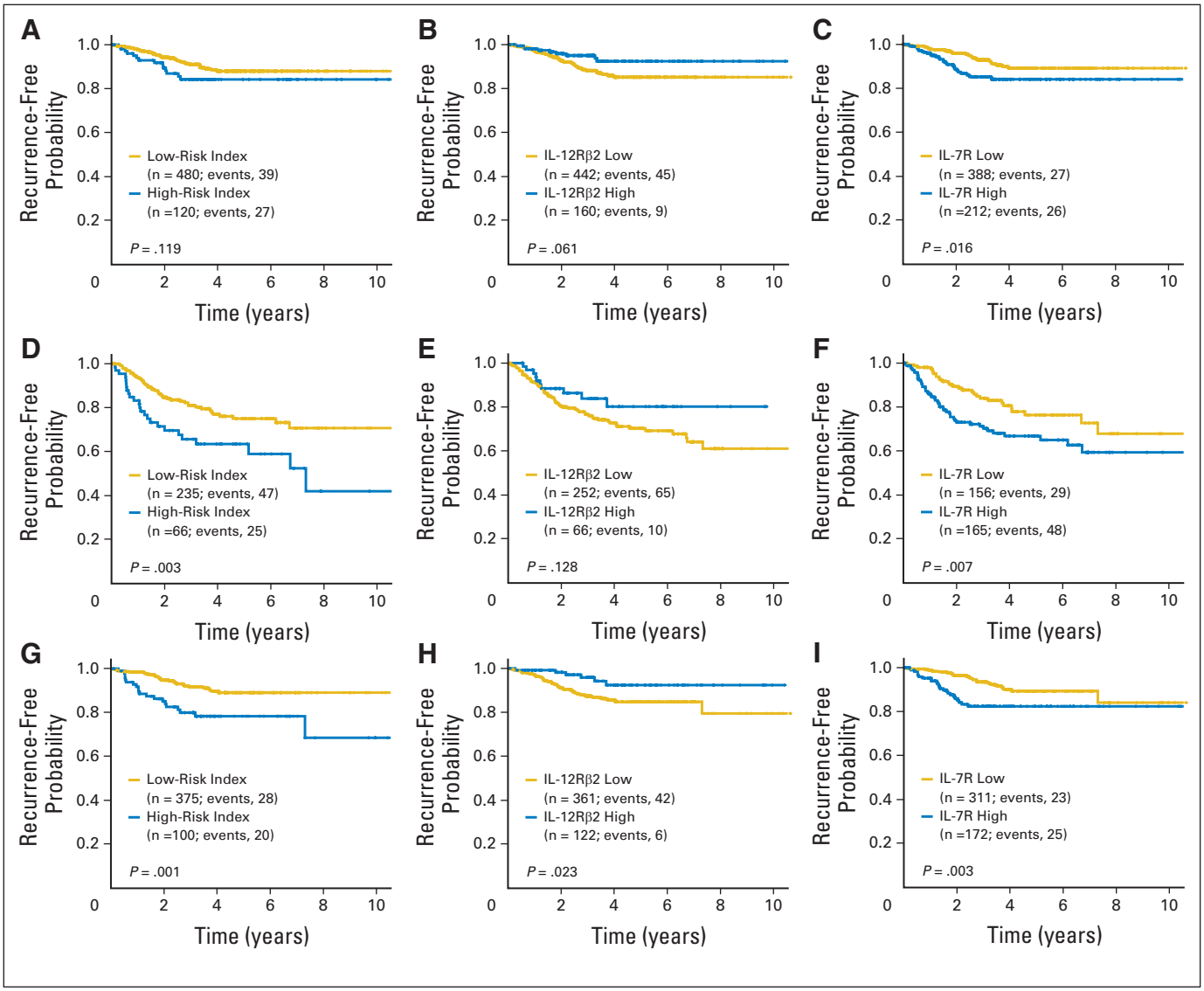

Fig 3. Recurrence-free probability (RFP) by three prognostic immune markers for patients with (A-C) stages IA and (D-F) IB disease and for (G-I) tumors ≤ 2 cm. (A) Among patients with stage IA disease, stromal forkhead box P3 (FoxP3) risk index score was not prognostic. (B) There was tendency for patients with high-level expression of interleukin-12 receptor $\beta 2$ (IL-12Rβ2) to have lower RFP compared with patients with low-level expression ($P = .061$). (C) IL-7R expression further stratified prognosis among patients with stage IA disease ($P = .016$). (D) Among those with stage IB disease, patients with low stromal FoxP3 risk index score had 5-year RFP of 75%, compared with 63% for those with high score ($P = .003$). (E) IL-12Rβ2 expression did not stratify clinical outcome among patients with stage IB disease. (F) Patients with low-level expression of IL-7R had 5-year RFP of 76%, compared with 67% for those with high-level expression ($P = .007$). (G) For tumors ≤ 2 cm, patients with low stromal FoxP3 risk index score had 5-year RFP of 89%, compared with 79% for those with high score ($P = .001$). (H) Patients with low-level expression of IL-12Rβ2 had 5-year RFP of 85%, compared with 92% for those with high-level expression ($P = .023$). (I) Patients with low-level expression of IL-7R had 5-year RFP of 89%, compared with 82% for those with high-level expression ($P = .003$).

IL-7R has been shown to induce tumor growth and lymphangiogenesis through upregulation of VEGF-D.[34,35] Its ligand, IL-7, is produced by stromal and epithelial cells and plays a central role in T-cell development, in addition to providing a potent lymphocyte-survival factor through the JAK-STAT pathway.[36] The role of the IL-7/IL-7R axis in the tumor immune microenvironment in lung ADC warrants more investigation.

The prognostic significance of the immune markers was further strengthened by their ability to stratify within currently known prognosticators—stage (IA and IB; Figs 3A to 3F), tumors ≤ 2 cm (Figs 3G to 3I), and morphologic grade (Data Supplement). Patients with stage IB disease with a high stromal FoxP3 risk index or high-level expression of IL-7R experienced outcomes similar to those of patients with stage II disease, for whom adjuvant chemotherapy is recommended. The ability to prognosticate within tumors ≤ 2 cm is especially important, because this represents a population in which incidence is expected to increase with the adoption of more-widespread CT screening, but in which a standardized prognostic factor is lacking. When we assessed overall survival as an end point, IL-7R remained a significant prognosticator in both the training ($P = .007$) and validation cohorts ($P = .02$), whereas findings were not significant for FoxP3/CD3 risk index ($P = .21$) or IL-12Rβ2 ($P = .51$). The ability of IL-7R to prognosticate both recurrence-free and overall survival merits further investigation of its biologic role (Data Supplement).

One limitation of our study is the semiquantitative nature of immunohistochemical scoring. Although a digital analysis was attempted using the Aperio ScanScope XT (Aperio, Vista, CA), the results were confounded by anthracotic pigments picked up as positive stains, a unique problem encountered in lung specimens. Accurate analysis and discrimination of the tumor from the tumor-associated stroma were best achieved by direct visualization under the microscope.

Our findings shed light on the complex tumor immune microenvironment in stage I lung ADC. First, we demonstrated that in the tumor-associated stroma, immune infiltrates rich in FoxP3-positive regulatory T cells create a protumor environment, and this environment may be overcome when there is a concurrently high density of CD3+ lymphocytes. Second, IL-12Rβ2 is expressed on normal lung epithelium and on less-aggressive tumors but to a lesser extent on more-aggressive tumors. Furthermore, tumors expressing IL-12Rβ2 tend to do well despite an unfavorable immune environment. Third, IL-7R expression on tumors is associated with aggressive features.

In conclusion, we have identified prognostic immune factors in this first, to our knowledge, large-scale study of the tumor immune microenvironment in patients with stage I lung ADC, a population anticipated to increase with widespread CT screening. For a population in which tumor size is currently the main prognostic factor, our results provide important prognostic tools and demonstrate the feasibility of using a multidisciplinary approach to advance beyond the limitations of the current staging system. More importantly, these findings provide a crucial foundation for future investigations into immunomodulatory therapies for lung ADC.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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