Intratumoral Immune Cell Densities Are Associated with Lung Adenocarcinoma Gene Alterations

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Abstract

Rationale: Tumor-infiltrating immune cells affect lung cancer outcome. However, the factors that influence the composition and function of the tumor immune environment remain poorly defined and need investigation, particularly in the era of immunotherapy.

Objectives: To determine whether the tumoral immune environment is related to lung adenocarcinoma mutations.

Methods: This retrospective cohort included 316 consecutive patients with lung adenocarcinoma (225 men; 258 smokers) studied from 2001 to 2005 in a single center. We investigated the association of densities of intratumoral mature dendritic cells (mDCs), $CD8^+$ T cells, neutrophils, and macrophages with clinical and pathological variables and tumor cell mutation profiles obtained by next-generation sequencing.

Measurements and Main Results: In 282 tumors, we found 460 mutations, mainly in *TP53* (59%), *KRAS* (40%), *STK11* (24%), and *EGFR* (14%). Intratumoral $CD8^+$ T-cell density was high in smokers ($P = 0.02$) and *TP53*-mutated tumors ($P = 0.02$) and low in *BRAF*-mutated tumors ($P = 0.005$). Intratumoral mDC density was high with low pathological tumor stage ($P = 0.01$) and low with *STK11* mutation ($P = 0.004$). Intratumoral neutrophil density was high and low with *BRAF* mutation ($P = 0.04$) and *EGFR* mutation ($P = 0.02$), respectively. Intratumoral macrophage density was low with *EGFR* mutation ($P = 0.01$). Intratumoral $CD8^+$ T-cell and mDC densities remained strong independent markers of overall survival ($P = 0.001$ and $P = 0.02$, respectively).

Conclusions: Intratumoral immune cell densities (mDCs, $CD8^+$ T cells, neutrophils, macrophages) were significantly associated with molecular alterations in adenocarcinoma underlying the interactions between cancer cells and their microenvironment.

Keywords: lung adenocarcinoma; prognostic factors; mature dendritic cells; $CD8^+$ T cells; molecular alterations

At a Glance Commentary

Scientific Knowledge on the Subject: In lung adenocarcinoma, high density of intratumoral $CD8^+$ T cells and mature dendritic cells predicts increased survival, but the factors that could affect intratumoral immune infiltration remain poorly defined. Lung tumors show a large array of molecular alterations that may affect the tumor microenvironment, which provides the rationale for seeking a link between tumor molecular alterations and the immune environment.

What This Study Adds to the Field: We report a significant association between density of intratumoral $CD8^+$ T cells, mature dendritic cells, neutrophils, and macrophages and mutations in tumor cell genes. Furthermore, *STK11, KRAS*, and *TP53* mutations affected the strong prognostic value of immune cells.

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Non–small cell lung cancer (NSCLC) accounts for 85% of lung cancer cases, making it the leading cause of cancer-related death worldwide (1, 2). Lung adenocarcinoma is the most frequent NSCLC; its histological pattern is heterogeneous; it is significantly related to survival (3–9); and it features a high mutation rate (10, 11).

The prognostic value of *EGFR* and *KRAS* mutations in lung cancer is controversial; however, cases with *EGFR* mutation were recurrently found to be associated with better survival than those with *KRAS* mutation (12, 13). A high rate of *TP53* mutation has been reported in lung cancer, and its negative prognostic value is associated with nondisruptive mutation, inducing a gain of function, as compared with disruptive mutation, resulting in a loss of function (14). Similarly, we recently found *STK11* exons 1 and 2 mutations, resulting in a gain of oncogenic function (GOF) via the synthesis of a truncated STK11 isoform, associated with shorter survival as compared with the disruptive *STK11* exons 3–9 mutations, thereby resulting in a tumor-suppressive function (TSF) (15).

Beyond factors linked to tumor characteristics and staging, the tumor immune environment has a strong prognostic value in most solid cancers, including lung cancer (16). Intratumoral immune infiltration varies among patients, but the reason for the heterogeneity remains poorly understood. High density of mature dendritic cells (mDCs) and $CD8^+$ T cells was found to be associated with long-term survival in patients with NSCLC. Use of the combined quantification of mDC and $CD8^+$ T-cell density, called the *immunoscore*, could identify patients with the best outcome (17–20). Other immune cell populations belonging to the innate immune system, such as macrophages and neutrophils, may affect survival (21). Tumor-associated macrophages represent a major component of the immune microenvironment of NSCLC and have been reported as affecting prognosis (22, 23). High neutrophil count in NSCLC may be associated with increased risk of relapse, but not with overall survival (OS) (24).

The use of immune checkpoint inhibitors has emphasized the need to decipher how the tumor microenvironment develops and persists in the context of NSCLC. Some factors, such as cigarette smoking, affect the immune infiltrate density (25), but other factors, such as tumor antigenicity or individual susceptibility, are still under investigation. Cancer cells may orchestrate their own immune environment; indeed, we previously demonstrated that the immune environment was highly reproducible from the primary tumor to relapsing metastatic disease in colorectal and renal cell carcinoma (26). Therefore, tumor cell morphology and molecular alterations might reflect tumor biological heterogeneity and the capacity to recruit different immune cells.

In the present study, we retrospectively analyzed resected lung adenocarcinoma tissue to investigate the association of intratumoral immune cell densities with clinical and pathological variables and tumor cell mutation profiles. We found significant associations between intratumoral immune cell densities and tumor cell characteristics, which supports the hypothesis that cancer cells could orchestrate their immune environment.

Methods

Patients

We analyzed primary lung adenocarcinoma tissue from 316 consecutive patients who underwent surgery between June 2001 and June 2005 in the Department of Thoracic Surgery of Cochin Hospital, Paris, France. We excluded patients who received neoadjuvant therapies and underwent sublobar resection, as well as those with noninterpretable results of next-generation sequencing (NGS). Approval by the institutional review board (CPP Ile-de-France II; 2008-133 and 2012 06-12) was obtained. Clinical and OS data were retrospectively collected, and pathological features were reviewed according to the latest World Health Organization 2015 and TNM 2009 classifications, as previously described (8).

Molecular Analysis

DNA was extracted from formalin-fixed, paraffin-embedded blocks containing the highest percentage of tumor cells (>30%) by using the illustra Nucleon BACC2 genomic DNA extraction kit (GE Healthcare Life Sciences, Little Chalfont, UK). Samples were characterized by using NGS and a custom AmpliSeq panel (AmpliSeq Ion Torrent; Life Technologies, Carlsbad, CA) that included *EGFR* (exons 18–21), *TP53* (exons 2–11), *KRAS* (exons 2–6), *BRAF* (exons 11–15), *NRAS* (exons 2–5), *HER2* (exons 18–21), and *STK11* (exons 1–9). The sequencing reads were processed by using Ion Torrent Suite V4.0 software (Life Technologies). *TP53* mutations were classified as "disruptive" and "nondisruptive" according to the method of Poeta and colleagues (27). Likewise, we classified *STK11* mutations as GOF when they were located in exons 1 and 2 and as TSF when they were located in exons 3–9 (15).

Quantification of the Intratumoral Immune Cell Densities

The intratumoral densities of $CD8^+$ T cells, mDCs, neutrophils, and macrophages were quantified after immunostaining with the antibodies for CD8 (SP16; Spring Bioscience, Pleasanton, CA), DC-LAMP (1010E1.01; Dendritics, Lyon, France), CD66b (G10F5; BD Biosciences, San Jose, CA), and CD68 (PG-M1; Dako, Carpinteria, CA), respectively. Images were acquired by using a NanoZoomer (Hamamatsu, Hamamatsu City, Japan) with NDPview software (Hamamatsu), and labeled cells in the entire section were counted as absolute numbers of positive cells per square millimeter with the use of CaloPix software (Tribvn, Châtillon, France) (18).

Statistical Analysis

Associations between categorical variables were analyzed by using the chi-square test or Fisher's exact test, and associations between continuous variables were evaluated by using Wilcoxon's signed-rank test or the *t* test. Factors that were significantly associated in univariate analysis were included in multivariate logistic regression analysis to identify factors independently associated with the biomarkers of interest. To study mDC, $CD8^+$ T-cell, neutrophil, and macrophage densities by gene mutations, we compared tumors harboring the studied mutation with wild-type tumors (for the same studied gene), regardless of the molecular status of the other genes. Median-based stratifications were used to dichotomize immune cell densities into high and low density. Survival was assessed by using the Kaplan-Meier method, and differences were analyzed by performing log-rank tests. Variables significantly

associated with OS ($P < 0.05$) in univariate analysis (age, histological grade, surgical resection, pathological TNM stage, vascular emboli, and $CD8^+$ T-cell and mDC densities) were included in multivariate analysis using a Cox proportional hazards regression model. Pleural invasion was excluded because it was included in TNM stage. Statistical analysis involved use of R (http://www.r-project.org/). $P < 0.05$ was considered statistically significant.

Results

Patient and Tumor Characteristics

We analyzed tissue from 316 patients with lung adenocarcinomas (297 [94%] lobectomies and 19 [6%] pneumonectomies), including 225 (71.2%) men (median age, 61 yr; range, 19–84 yr). Two hundred fifty-eight (81.7%) were smokers, and 58 (18.3%) were nonsmokers. Pathologic stages were I, II, III, and IV for 150 (48%), 83 (26%), 77 (24%), and 6 (2%) patients, respectively. According to the 2011 International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society classification, 223 patients had intermediate-grade (70.9%), 92 had high-grade (29.1%), and 1 had low-grade adenocarcinomas (Table 1).

Lung Adenocarcinoma Molecular Analysis

In 282 tumors, we found 460 mutations (88%): *TP53* in 186 of 316 patients (58.9%), *KRAS* in 128 of 316 (40%), *STK11* in 77 of 316 (24.3%), *EGFR* in 45 of 316 (14%), *BRAF* in 12 of 316 (4%), *HER2* in 7 of 316 (2%), and *NRAS* in 5 of 316 (1.5%) (Table 2). The distribution of the main gene mutations is reported in Figure E1 in the online supplement. We found 2 and 3 concomitant mutations in 120 and 17 tumors, respectively. In total, 40 tumors had a double mutation of *KRAS* and *STK11*, and 14 had *KRAS*, *STK11*, and *TP53* mutations; 20 *EGFR*-mutated tumors exhibited an additional *TP53* mutation.

STK11 mutation was associated positively with *KRAS* mutation ($P = 0.02$) and negatively with *EGFR* mutation ($P = 0.001$) (*see* Table E1 and Figure E1). Most *KRAS* mutations were in codons 12 and 13 (92%), and all were exclusive with *EGFR* and *NRAS* mutations. *HER2*, *NRAS*, and *BRAF* mutations were mutually exclusive, but two tumors had concomitant *KRAS* (both p.G12C) and *HER2* (p.G865R

Table 1. Correlations between Clinical, Pathological, and Intratumoral $CD8^+$ T Cells and Mature Dendritic Cell Densities

		CD8			mDC			Immunoscore			
	Total %	Low	High	*P* Value	Low	High	*P* Value	0	1	2	*P* Value
Age ⩽70 yr	78.8	48.9	51.1	NS	50.2	49.8	NS	31.4	36.2	32.4	NS
Age >70 yr	21.2	54.4	45.6		49.2	50.8		36.8	29.2	33.0	
Men	71.2	46.6	53.4	0.06	51.9	48.1	NS	33.0	32.5	34.5	NS
Women	28.8	58.7	41.3		45.0	55.0		31.2	41.2	27.6	
L and I grade	70.9	54.0	46.0	0.07	48.3	51.7	NS	33.8	34.3	31.9	NS
H grade	29.1	40.5	59.5		53.6	46.4		28.6	36.9	34.5	
TTF-1^+	90.2	50.2	49.8	NS	48.9	51.1	NS	31.3	36.3	32.4	NS
TTF-1^-	9.8	48.2	51.8		60.7	39.3		44.5	22.2	33.3	
Lob	94	49.3	50.7	NS	49.8	50.2	NS	31.5	35.9	32.6	NS
Pneum	6	62.5	37.5		53.0	47.0		50.0	18.7	31.3	
Smokers	81.7	45.8	54.2	**0.02**	51.7	48.3	NS	32.6	32.6	34.8	NS
Nonsmokers	18.3	63.5	36.5		39.6	60.4		28.9	44.2	26.9	
COPD	38	44.1	55.9	NS	55.4	44.6	0.09	32.4	34.2	33.4	NS
No COPD	62	52.7	47.3		44.9	55.1		30.8	36.3	32.9	
Stages											
I	48	52.5	47.5	NS	46.8	53.2	NS	31.9	35.5	32.6	NS
II	26	42.3	57.7		56.2	43.8		32.4	33.8	33.8	
III–IV	26	52.7	47.3		50.0	50.0		33.8	35.1	31.1	
T ⩽3 cm	51.9	48.0	52.0	NS	44.3	55.7	**0.009**	28.4	35.1	36.5	**0.01**
T >3 cm	48.1	51.8	48.2		56.1	43.9		37.0	34.8	28.2	
pT1	24.4	48.5	51.5		36.8	63.2		23.5	38.2	38.3	
pT2	48.1	51.7	48.3	NS	50.3	49.7	**0.01**	34.3	33.6	32.1	NS
pT3–4	27.5	48.0	52.0		61.0	39.0		37.3	34.7	28	
pN0	67.4	49.8	50.2		50.2	49.8		32.5	35.0	32.5	
pN1	17.1	37.8	62.2	**0.049**	48.9	51.1	NS	26.7	33.3	40.0	NS
pN2	15.5	63.6	36.4		50.0	50.0		38.6	36.4	25.0	
Pleur inv	59	48.0	52.0	NS	56.1	43.9	**0.01**	34.3	35.5	30.2	NS
No pleur inv	41	53.0	47.0		41.0	59.0		29.9	34.2	35.9	
Vasc emb	55	47.0	53.0	NS	49.4	50.6	NS	30.8	35.2	34.0	NS
No vasc emb	45	53.4	46.6		48.7	51.3		32.8	36.2	31.0	

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; Lob = lobectomy; mDC = mature dendritic cell; NS = nonsignificant; Pleur inv = pleural invasion; Pneum = pneumonectomy; T = tumor size; TTF-1 = thyroid transcriptional factor 1; Vasc emb = vascular emboli.
Median-based stratifications were used to dichotomize continuous variables (mDC and $CD8^+$ T-cell densities) in high and low density. The immunoscore is the result of the combined analysis of mDC and $CD8^+$ T-cell densities, scored as follows: 0 = $mDC^{low}/CD8^{low}$; 1 = $mDC^{low}/CD8^{high}$ or $mDC^{high}/CD8^{low}$; 2 = $mDC^{high}/CD8^{high}$. Histological grade is defined by predominant architectural pattern and classified as low (L) and intermediate (I) or high (H) grade. The stage corresponds to pathological stage. *P* values are displayed when $P < 0.1$. Significant *P* values (univariate analysis) are shown in bold. The results of multivariate analyses are shown in Table E5.

Table 2. Correlations between Molecular Alterations of Tumoral Cell and Immune Cell Densities

		CD8			mDC			Immunoscore				Neutrophils			Macrophages		
	Total %	Low	High	*P* Value	Low	High	*P* Value	0	1	2	*P* Value	Low	High	*P* Value	Low	High	*P* Value
EGFR mut	14	58.5	41.5	NS	36.6	63.4	0.06	29.3	36.6	34.1	NS	66.7	33.3	**0.02**	66.7	33.3	**0.01**
EGFR wt	86	48.6	51.4		52.2	47.8		33.1	34.7	32.2		46.9	53.1		44	56	
KRAS mut	40	53.3	46.7	NS	51.6	48.4	NS	36.9	31.1	32.0	NS	51	49	NS	45.2	54.8	NS
KRAS wt	60	47.6	52.4		48.8	51.2		29.3	37.8	32.9		48.9	51.1		48.9	51.1	
TP53 nondisr	31.7	41.4	58.6		50.0	50.0		28.7	34.5	36.8		50	50		41.6	58.3	
TP53 disr	27.2	45.0	55.0	**0.02**	50.0	50.0	NS	31.2	32.5	36.3	NS	42	58	0.09	44.9	55.1	NS
TP53 wt	41.1	60.0	40.0		50.0	50.0		36.1	47.0	26.9		54.9	45.1		52.9	47.1	
STK11 gof	6.3	66.7	33.3		72.2	27.8		50.0	38.9	11.1		50	50		56.3	43.7	
STK11 tsf	18	60.0	40.0	0.07	65.4	34.6	**0.004**	48.1	28.8	23.1	**0.005**	43.6	56.4	NS	59	41	0.09
STK11 wt	75.7	46.3	53.7		44.5	55.5		27.3	36.1	36.6		51.1	48.9		44.2	55.8	
BRAF mut	4	91.0	9.0	**0.005**	72.7	27.3	NS	72.7	18.2	9.1	**0.01**	11.1	88.9	**0.04**	44.5	55.5	NS
BRAF wt	96	48.4	51.6		49.1	50.9		30.9	35.6	33.5		51.3	48.7		47.4	52.6	
Nb of mut																	
0	10.8	32.2	67.8		55.1	44.9		21.4	42.9	35.7		50	50		45.8	54.2	
1	45.9	55.6	44.4	NS	44.8	55.2	NS	30.8	39.1	30.1	NS	50	50	NS	46.6	53.4	**0.01**
2	38	45.4	54.6		50.9	49.1		34.3	27.8	37.9		48.3	51.7		43.8	56.2	
3	5.3	64.7	35.3		76.4	23.5		52.9	35.3	11.8		57.1	42.9		78.6	21.4	
$CD8^{low}$	50	—	—	NA	65.0	35.0	**0.0001**	—	—	—	NA	52.5	47.5	NS	55.7	44.3	**0.005**
$CD8^{high}$	50	—	—		35.0	65.0		—	—	—		47.9	52.1		37.6	62.4	
mDC^{low}	50	65.0	35.0	**0.0001**	—	—	NA	—	—	—	NA	54.7	45.3	NS	50.4	49.6	NS
mDC^{high}	50	35.0	65.0		—	—		—	—	—		46	54		44.4	55.6	

Definition of abbreviations: disr = disrupted; gof = gain of function; mDC = mature dendritic cell; mut = mutated; NA = not applicable; nondisr = nondisruptive; NS = nonsignificant; Nb of mut = number of mutations (represents the association of several recurrent mutations); tsf = tumor-suppressive function; wt = wild type.

The intratumoral densities of adaptive immune cells ($CD8^+$ T cells and mDCs), and innate immune cells (neutrophils and macrophages) were quantified after immunostaining with the antibodies for CD8 (SP16; Spring Bioscience), DC-LAMP (1010E1.01; Dendritics), CD66b (G10F5; BD Biosciences), or CD68 (PG-M1; Dako), respectively. Median-based stratifications were used to dichotomize continuous variables of immune cell densities in high and low density. The immunoscore is the result of the combined analysis of mDC and $CD8^+$ T-cell densities, scored as follows: 0 = $mDC^{low}/CD8^{low}$; 1 = $mDC^{low}/CD8^{high}$; or $mDC^{high}/CD8^{low}$; and 2 = $mDC^{high}/CD8^{high}$. *P* values in univariate analyses are displayed when $P < 0.1$. Significant *P* values are shown in bold. *TP53* mutations were classified as nondisruptive, inducing a gain of function, and disruptive mutations resulting in a loss of function. *STK11* mutations were separated as gain of oncogenic function mutations and tumor-suppressive function mutations.

and p.N850Y) mutations, and two other ones had concomitant *KRAS* (p.G12D and p.Q61H) and *BRAF* (p.G469R and p.G469V, respectively) mutations. Lung tumors from smokers more frequently harbored *KRAS* ($P = 0.0007$), *STK11* ($P = 0.009$), and/or *TP53* ($P = 0.0002$) mutations, whereas tumors from nonsmokers more frequently had *EGFR* mutations ($P = 0.0000001$) (*see* Table E2).

TP53 mutations were subclassified as disruptive (n = 86) or nondisruptive (n = 100). *TP53* mutations, especially the nondisruptive subtype, were associated with young age ($P = 0.005$), large tumors ($P = 0.006$), vascular emboli ($P = 0.001$), and high tumor stage ($P = 0.007$). We found 20 GOF and 57 TSF mutations for *STK11* mutations. *STK11* mutations ($P = 0.009$), especially the GOF subtype ($P = 0.04$), were significantly associated with thyroid transcriptional factor 1–negative tumors (*see* Table E2).

Intratumoral Immune Cell Analysis

First, we examined the association of the density of two immune cell subtypes belonging to the adaptive immune environment—$CD8^+$ T cells and mDCs—with clinical, pathological, and molecular variables. As we previously reported (18), intratumoral $CD8^+$ T-cell and mDC densities were heterogeneously distributed among patients. The median densities of mDCs and $CD8^+$ T cells in all studied tumors were 2.16 and 409 cells per square millimeter, respectively, and the densities of these two cell subtypes were significantly related ($P = 0.0001$) (Table 2). High density of $CD8^+$ T cells was associated with smoking ($P = 0.02$) and *TP53* mutation ($P = 0.02$), as well as, although not significantly, with *STK11* mutation ($P = 0.07$) and high histological grade ($P = 0.07$). Conversely, low density of intratumoral $CD8^+$ T cells was associated with *BRAF* mutation ($P = 0.005$). High density of mDCs was associated with small tumors ($P = 0.009$), low pathological tumor stage ($P = 0.01$), and tumors without pleural invasion ($P = 0.01$), as well as, although not significantly, with *EGFR* mutation ($P = 0.06$). Low density of mDCs was significantly associated with *STK11* mutation ($P = 0.004$) (Tables 1, 2, and E3). We found no association between mDC or $CD8^+$ T-cell densities and *KRAS*, *HER2*, or *NRAS* mutation. Since we sometimes found concomitant *KRAS*, *STK11*, and *TP53* mutations, we analyzed whether there was an association of densities of mDCs and $CD8^+$ T cells with number of mutations, but we found no association (Table 2).

Next, we analyzed two immune cell subtypes belonging to innate immunity: neutrophils and macrophages. The median densities of neutrophils and macrophages in the whole studied population were 28.2 and 405 cells per square millimeter, respectively. The densities of neutrophils and macrophages were not significantly associated with major clinical features, except that high neutrophil density was associated with tumor size greater than 3 cm ($P = 0.02$) and high macrophage density was associated with older age ($P = 0.04$) (*see* Table E4). High neutrophil density was associated with *BRAF* mutation ($P = 0.02$) and *EGFR* wild-type tumors ($P = 0.04$), and high macrophage density was associated with *EGFR* wild-type tumors ($P = 0.01$) (Table 2).

Prognostic Value of Intratumoral Immune Cell Density, Molecular Alterations, and Their Combined Analysis

Among our patients with resected lung adenocarcinomas, the OS rates at 2, 3, 5, 7, and 8 years were 70.3% (95% confidence interval, 64.9–75.2) 62.3% (56.7–67.6), 52.8% (47.1–58.4), 45% (39.2–51.0), and 43.4% (37.4–49.6), respectively. In univariate analysis, OS was related to age ($P = 0.01$), histological grade ($P = 0.009$), pathological stage ($P = 0.0000001$), extent of resection ($P = 0.004$), tumor size ($P = 0.03$), pleural invasion ($P = 0.001$), vascular emboli ($P = 0.00004$), $CD8^+$ T-cell density ($P = 0.002$), and mDC density ($P = 0.0001$) (*see* Table E5). Combined analysis of mDC and $CD8^+$ T cells for the immunoscore (17, 23) allowed us to identify patients with a good ($mDC^{high}/CD8^{high}$), intermediate ($mDC^{low}/CD8^{high}$ or $mDC^{high}/CD8^{low}$), or poor prognosis ($mDC^{low}/CD8^{low}$) ($P = 0.00008$) (Table 3).

We found no significant association between tumor cell molecular alterations and OS. However, nondisruptive *TP53* and GOF *STK11* mutations were associated with worse prognosis, albeit not significantly ($P = 0.07$ and $P = 0.08$, respectively). The median OS was lower for patients with nondisruptive *TP53* mutation than for those with wild-type *TP53* tumors or disruptive *TP53* mutations (44 vs. 79 mo). Similarly, the median OS was lower for patients with GOF *STK11* mutation than for those with wild-type *STK11* tumors or TSF *STK11* mutations (38 vs. 68 mo) (Figure 1). The presence of more than one mutation was not associated with prognosis, but it concerned only a few patients for each combination (data not shown). Multivariate analysis revealed that OS was associated with pathologic stage ($P = 0.00000001$), vascular emboli ($P = 0.01$), histological grade ($P = 0.02$), mDC density ($P = 0.02$), and $CD8^+$ T-cell density ($P = 0.001$) in resected lung adenocarcinomas (Tables 3 and E5).

We performed a subgroup analysis to evaluate the prognostic value of mutations in terms of $CD8^+$ T-cell or mDC density. For patients with low $CD8^+$ T-cell density, survival was shorter among those with GOF *STK11* mutations than among those with TSF mutations or wild-type *STK11* tumors ($P = 0.029$) (*see* Table E6). Similarly, for patients with high mDC density, survival was shorter for those with GOF *STK11* mutations than for those with TSF mutations or wild-type *STK11* tumors (70 vs. 111 mo), and it was shorter among those with nondisruptive *TP53* mutations than among those with wild-type *TP53* tumors or disruptive *TP53* mutations (61 vs. 111 mo) (Figure 1). *KRAS* mutation was not significantly associated with OS, but survival was shorter with *KRAS* mutations in $CD8^{high}$ and mDC^{high} lung adenocarcinomas than with wild-type *KRAS* tumors (84 vs. >140 mo and 85 vs. 111 mo, respectively) (Figure 2).

Table 3. Prognostic Impact of the Intratumoral Immune Environment

	Univariate Analysis			Multivariate Analysis		
	5-yr Survival (%)	95% CI	*P* Value	OR	95% CI	*P* Value
$CD8^{low}$	45.4	37.4–53.5	**0.002**	1.69	1.23–2.34	**0.001**
$CD8^{high}$	62.7	54.5–70.2				
mDC^{low}	44.2	36.3–52.4	**0.0001**	1.46	1.06–2.01	**0.02**
mDC^{high}	63.8	55.6–71.2				
Immunoscore						
0	36.6	27.5–46.7	**0.00008**			
1	60.8	51–69.8				
2	64.4	54.2–73.4				
Neutrophils low	52.4	43.4–61.1	NS			
Neutrophils high	58.2	51–68.1				
Macrophages low	56.2	47.1–65	NS			
Macrophages high	54.4	45.8–62.8				

Definition of abbreviations: CI = confidence interval; mDC = mature dendritic cell; NS = nonsignificant; OR = odds ratio; TNM = tumor, lymph node, metastasis.

Median-based stratifications were used to dichotomize continuous variables (immune cell densities) in high and low density. The immunoscore is the result of the combined analysis of mDC and $CD8^+$ T-cell densities, scored as follows: 0 = $mDC^{low}/CD8^{low}$; 1 = $mDC^{low}/CD8^{high}$ or $mDC^{high}/CD8^{low}$; and 2 = $mDC^{high}/CD8^{high}$. Significant *P* values are shown in bold. Variables significantly associated ($P < 0.05$) with survival in univariate analysis (age, histological grade, surgical resection, pathological TNM stage, vascular emboli, $CD8c^+$ T-cell density, and mDC density) were included in multivariate analysis with a Cox proportional hazards regression model. Pleural invasion was excluded because it is included in TNM stage. The prognostic impact of the other clinical and histological parameters is described in Table E5.

Discussion

The objective of our study was to decipher the link between the heterogeneity of intratumoral immune cell density and tumor cell features. We postulated that tumor cell characteristics could govern, in part, the immune environment within lung tumors. An effect of the tumor cell on its immune environment was suggested by our previous study demonstrating a similar immune pattern observed from primary to metastatic tumors (26). Therefore, we examined the associations of intratumoral mDC and $CD8^+$ T-cell densities for adaptive immunity and neutrophil and macrophage densities for innate immunity with pathological and molecular variables in a series of resected lung adenocarcinomas. Intratumoral immune cell density (mDCs, $CD8^+$ T cells, neutrophils, macrophages) was significantly associated with tumor characteristics, particularly the molecular alterations underlying the interactions between cancer cells and their microenvironment.

The clinical and morphological characteristics of our series were consistent with those in a series of white subjects, and we confirmed the prognostic value of histological grade based on predominant architectural pattern (8). Interestingly, we found a high rate of mutations among the 316 patients we studied: 280 patients (88%) had tumors with at least one mutation in the *EGFR*, *KRAS*, *TP53*, *STK11*, *BRAF*, *HER2*, or *NRAS* gene. In accordance with the literature, the most common mutations were in *TP53* (58.9%), *KRAS* (40%), *STK11* (24.3%), and *EGFR* (14%), and some were mutually exclusive (10, 28). *TP53* and *STK11* mutations are frequently associated with another mutation (10, 14, 29). We found that *STK11* mutation was positively associated with *KRAS* mutation, as previously reported (30), and that it was negatively associated with *EGFR* mutation. The prognostic value of gene alterations is still controversial in lung

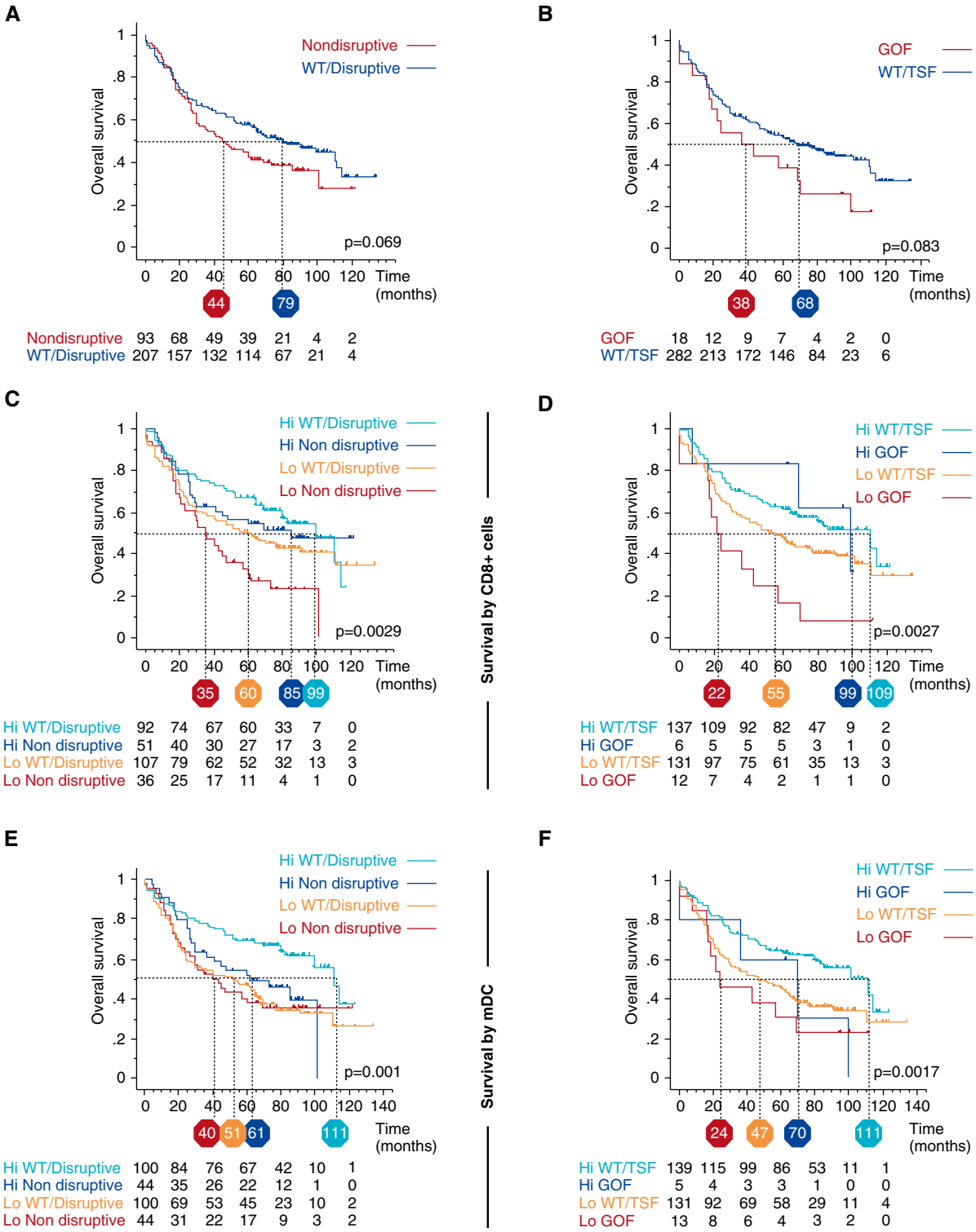

Figure 1. Overall survival (OS) of patients with lung adenocarcinoma by presence of (*A*) *TP53* or (*B*) *STK11* mutation and prognostic value of density of (*C* and *D*) $CD8^+$ T cells and (*E* and *F*) mature dendritic cells (mDCs) by mutation status of (*C* and *E*) *TP53* and (*D* and *F*) *STK11* in tumor cells. *TP53* mutations were classified as nondisruptive or disruptive; we grouped disruptive mutations and wild-type (WT) tumors for survival analysis. Nondisruptive mutations induced a gain of function, as compared with disruptive mutations, which induced a loss of function. Likewise, for *STK11*, we differentiated tumor-suppressive function (TSF) mutations from gain of oncogenic function (GOF) mutations and grouped TSF mutation tumors with WT tumors for survival analysis. Median-based stratifications were used to dichotomize mDC and $CD8^+$ T-cell density as high or low. OS curves were estimated by using the Kaplan-Meier method, and differences between patient groups were evaluated by using the log-rank test. The *horizontal dashed lines* represent median survival.

adenocarcinoma. In this study, we found a trend for a survival disadvantage with nondisruptive *TP53* or GOF *STK11* mutation ($P = 0.07$ and $P = 0.08$, respectively), as previously reported by another team (14, 15).

The strong independent prognostic value of mDC and $CD8^+$ T-cell densities that was previously reported (18–20, 31, 32) was confirmed in our cohort with lung adenocarcinomas. We found significant associations of mDC and $CD8^+$ T-cell densities with patient clinical characteristics and tumor morphological and molecular features. $CD8^+$ T-cell density was associated with smoking, and mDC density was associated with tumor size, pleural invasion, and pathological tumor stage. $CD8^+$ T-cell density was also associated with *TP53* and *BRAF* mutations, and mDC density was associated with *STK11* mutation. We found no significant association between immune infiltration and histological grade, although we found some association of increased $CD8^+$ T-cell density in high-grade tumors ($P = 0.07$). $CD8^+$ T cells may be attracted by inflammation before tumor emergence in smoking patients (25), when an increased mutational load was reported (33), or by a high rate of tumor cell death, as in high-grade adenocarcinomas. High $CD8^+$ T-cell density may reflect an efficient immune response setting, and the threshold density of such cells could be critical for cytotoxic function, as was suggested by the intermediate prognosis we previously observed in patients with $mDC^{high}CD8^{low}$ tumors (18). This finding contrasts with mDC density, which is likely associated more with a specific antitumor immune response.

We found neutrophil and macrophage densities to be associated with clinical characteristics (high density of macrophages with older age and high density of neutrophils with large tumor size) and oncogenic driver genes (high density of both cell subtypes with *EGFR* wild-type tumor and high density of neutrophils with *BRAF* mutation). Our results agree with data reported in the literature suggesting that macrophages and neutrophils may be implicated in the pathogenesis of lung cancer and are probably recruited within a proinflammatory context (23–34).

Overall, we found *TP53*, *STK11*, *EGFR*, and *BRAF* mutations in tumor cells to be significantly associated with specific intratumoral immune characteristics. Similar findings have been reported in colorectal carcinoma, with an abundance of tumor-infiltrating T cells associated with microsatellite instability and a favorable prognosis (35, 36). Similarly, in glioblastomas, tumor-infiltrating T-cell density was associated with molecular alterations (37). Immune cell infiltration was suggested to differ by *EGFR* and *KRAS* mutational status in lung adenocarcinoma tumors (38, 39). However, in these previous studies, individual quantifications of each subtype of immune cells were missing, and all immune cell subtypes were analyzed, without distinguishing $CD4^+$ from $CD8^+$ T cells and without quantifying mDCs, suggested to be key cells for priming a specific antitumoral immune response. In our present study, we found a link between increased density of mDCs and *EGFR* mutation and no association between immune cell density and *KRAS* mutation. Interestingly, *STK11* and *TP53* were the gene mutations most linked to the immune environment and survival. Indeed, these genes are not considered driver oncogenes, but their frequency is high in lung adenocarcinoma. Molecular analyses of both these genes are relevant, particularly when they are associated with other gene mutations, such as *EGFR* mutation. Indeed, the presence of *EGFR* and *TP53* mutations in combination was previously linked to poor survival (27). GOF *STK11* mutation and nondisruptive *TP53* mutation may confer aggressiveness to tumors for cancer cell survival and spread, possibly through modification of immune cells, particularly adaptive cells. Additional experiments should be performed to study in depth the link between differentiation and activation status of $CD8^+$ T cells and tumor mutational status.

A whole-exome sequencing analysis of a lung adenocarcinoma series suggested an association between the number of gene mutations (high mutation burden) and tumor immunogenicity that was indicative of sensitivity to PD-1 pathway–targeted therapies (40). In our study, the mutational load evaluated with the seven genes tested by NGS was not associated with immune infiltration level (data not shown), but it would probably have differed had we explored the true mutational load determined by whole-genome sequencing.

Molecular alterations may affect the tumor immune environment by the production of immunogenic peptides from somatic gene mutations, thereby promoting a protective immune response mediated by immunodominant T cells against cancer-mutated epitopes (41). We previously reported the presence of specific anti-*TP53* IgG and IgA in the supernatant of intratumoral B-cell cultures from lung cancer, suspected of participating in protective antitumor immune responses (42). Mutated *EGFR*, *KRAS*, and *STK11* antigens were not included in this set of experiments, and further analysis of the related specific immunoglobulins is needed. The mutational status of tumor cells might also affect the pattern of cytokines and chemokines secreted by tumor cells, which may affect the chemoattractant properties of the tumor microenvironment and the shaping of its immune microenvironment. Indeed, in a previous study, disease-free survival and OS were found to be shorter among patients with high IL-8 expression than in those with low IL-8 expression. In that study, disease-free survival and OS were significantly shorter in patients with mutant *KRAS*/high IL-8 level than in those with wild-type *KRAS*/low IL-8 level (43). In our present study, we quantified neutrophils, which are recruited by IL-8, and found both increased neutrophil and macrophage densities in *EGFR* wild-type tumors. *EGFR*-mutated tumors are probably less inflammatory in patients without a smoking history, but this may also be due to less cumulative mutation. We also found increased density of neutrophils in *BRAF*-mutated tumors, these tumors also being enriched in $CD8^+$ T cells, for a mixed inflammatory environment.

Tumor mutation in driver genes is the hallmark of cellular pathways. Because molecular alterations and immune infiltrates are related, these interactions need further exploration, as does the use of targeted therapies themselves, which might affect the tumor environment. These findings may affect disease management, as was reported for BRAF inhibition in metastatic melanoma, which induces a favorable tumor immune microenvironment, thus providing support for a potential synergy between BRAF-targeted therapy and immunotherapy (44).

In summary, our data demonstrate that clinical, morphological, and molecular characteristics of cancer cells explain in part

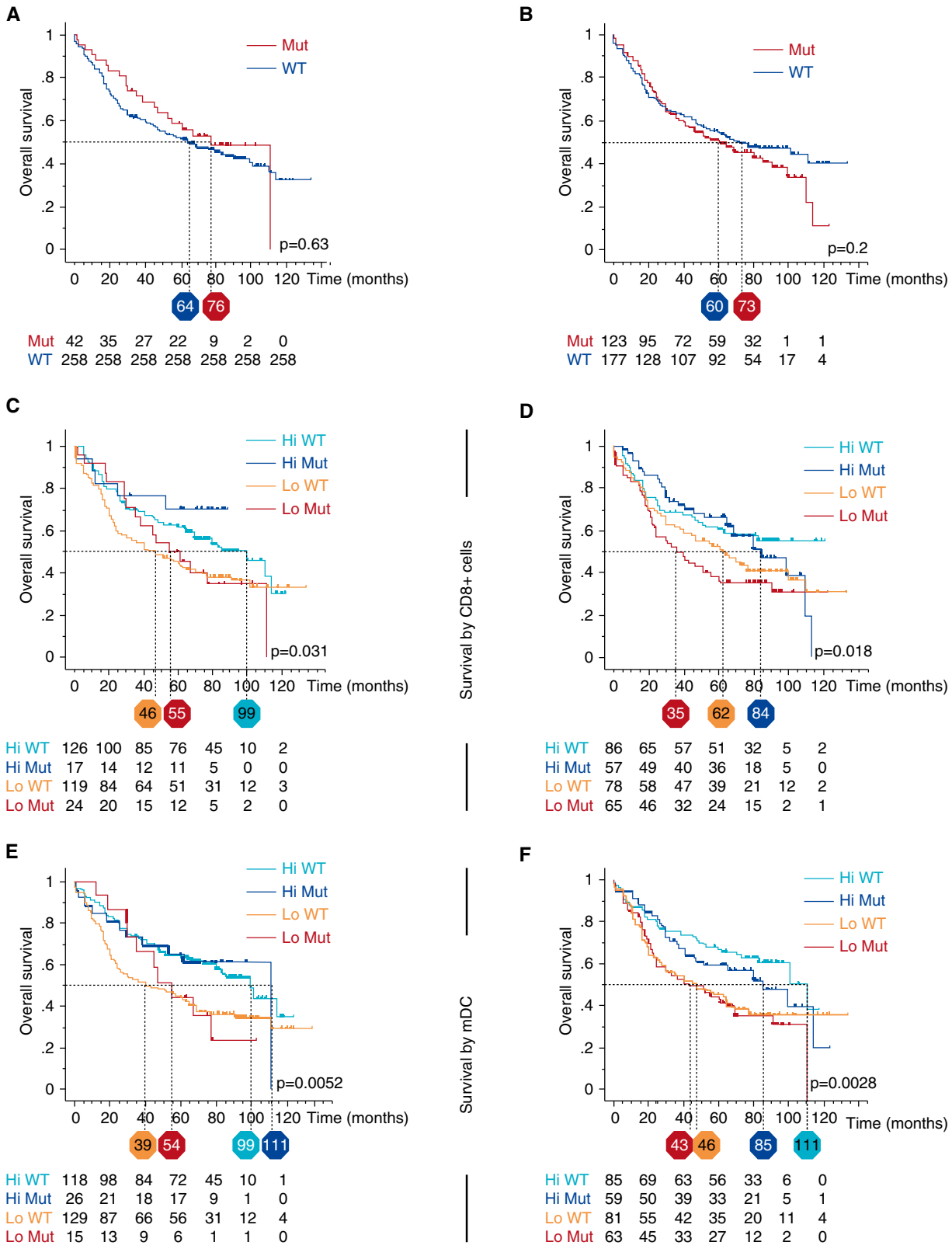

Figure 2. Overall survival (OS) of patients with lung adenocarcinoma by mutation status of (*A*) *EGFR* or (*B*) *KRAS* in tumors and prognostic value of density of (*C* and *D*) $CD8^+$ T cells and (*E* and *F*) mature dendritic cells (mDCs) by mutation status of (*C* and *E*) *EGFR* and (*D* and *F*) *KRAS* in tumors. Median-based stratifications were used to dichotomize mDC and $CD8^+$ T-cell density as high or low. OS curves were estimated by using the Kaplan-Meier method, and differences between patient groups were evaluated by using the log-rank test. The *horizontal dashed lines* represent median survival. Mut = mutation; WT = wild-type.

the heterogeneity of intratumoral immune cell infiltration among tumors, which supports the idea of dynamic interactions between tumor cells and their microenvironment. Further studies are needed to decipher the precise effect of distinct tumor cell mutations on the functions of tumor-infiltrating immune cells, which may affect patient outcomes and the responsiveness of the tumors to targeted therapies combined with immunotherapies. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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