Antonio Marchetti, Lara Felicioni, Sara Malatesta, Maria Grazia Sciarrotta, Luigi Guetti, Antonio Chella, Patrizia Viola, Carmela Pullara, Felice Mucilli, and Fiamma Buttitta

Clinical Features and Outcome of Patients With Non–Small-Cell Lung Cancer Harboring *BRAF* Mutations

Purpose. To investigate the prevalence, distribution, and prognostic role of *BRAF* mutations in a large cohorte of white patients with non–small-cell lung cancer (NSCLC).

Patients and methods. A retrospective series of 1,046 NSCLCs—comprising 739 adenocarcinomas (ADCs) and 307 squamous cell carcinomas (SCCs)—was investigated for *BRAF* mutations. High-resolution meeting analysis followed by sequencing and strip hybridization assay were used. All patients were also analyzed for *KRAS* and *EGFR* mutations.

Results. *BRAF* mutations were present in 36 ADCs (4.9%) and one SCC (0.3%; $P = 0.001$). Twenty-one of the mutations (56.8%) were V600E, and 16 (43.2%) were non-V600E. V600E mutations were significantly more prevalent in females (16 of 187 patients; 8.6%) than in males (five of 552 patients; 0.9%), as indicated by multivariate logistic regression analysis (hazard ratio [HR], 11.29; $P < 0.001$). V600E-mutated tumors showed an aggressive histotype characterized by micropapillary features in 80% of patients and were significantly associated with shorter disease-free and overall survival rates on both univariate (HR, 2.67; $P < 0.001$ and HR, 2.97; $P < 0.001$, respectively) and multivariate analyses (HR, 2.19; $P < 0.011$ and HR, 2.18; $P < 0.014$, respectively). All non-V600E mutations were found in smokers ($P < 0.015$) and were associated with neither clinicopathologic parameters nor prognosis. *BRAF* and *EGFR* were concomitantly mutated in two tumors.

Conclusion. We report for the first time to our knowledge that V600E and non-V600E *BRAF* mutations affect different patients with NSCLC. V600E mutations are significantly associated with female sex and represent a negative prognostic factor. In addition, we identified a number of other clinicopathologic parameters potentially useful for the selection of patients carrying *BRAF* mutations.

Introduction

In recent years, the introduction of new molecular targeted drugs, the effectiveness of which is closely dependent on the presence of specific genetic mutations in the tumor context,[1–5] has made a major contribution to the pharmacologic treatment of non–small-cell lung cancer (NSCLC). However, these mutations are present in subsets of patients; therefore, molecular target therapies, if indiscriminately administered to individuals with NSCLC, would induce low response rate and limited survival benefit. A large body of evidence shows that the presence of mutations in specific genes is closely related to particular clinicopathologic characteristics, including smoking habits, sex, and tumor histotype.[6,7] The finding that *EGFR* and *HER2* mutations preferably target never-smoker patients,[8–11] whereas *KRAS* mutations are more frequent in smokers,[12] suggests that adenocarcinomas (ADCs) in smokers and never smokers arise via different pathogenic pathways.[13] Mutations of *EGFR* and *HER2* are more frequent in females and almost exclusively present in lung ADC.[8–11,14] In addition, tumors with *EGFR* or *HER2* mutations often

Key points

- *A large body of evidence shows that the presence of mutations in specific genes is closely related to particular clinicopathologic characteristics, including smoking habits, sex, and tumor histotype.*

Key points

- *Somatic mutations of* BRAF *have been documented in different kinds of tumors, predominantly in malignant melanoma, thyroid papillary cancer, sporadic colorectal tumors, low-grade ovarian serous carcinoma, and lung tumors.*
- *A vast majority of these mutations correspond to the hotspot transversion mutation T1799A at exon 15, which causes the amino acidic substitution of V600E.*
- *The actual prevalence, distribution, and prognostic role of* BRAF *mutations in patients with NSCLC is still unclear.*
- *Treatment of patients with metastatic melanoma carrying the V600E* BRAF *mutation with PLX4032 has resulted in an overall response rate of 81%, with median progression-free survival of more than 7 months. These impressive results prompted us to evaluate a large number of lung tumors for* BRAF *mutations, with the aim of identifying potential common features in patients carrying* BRAF *mutations and assessing the impact of* BRAF *mutations on prognosis.*

share particular histopathologic subtypes, known as ADCs with lepidic and/or papillary features.[9,11,15]

BRAF codes for a nonreceptor serine/threonine kinase, activated downstream of the Ras protein, with a kinase domain structurally similar to other protein kinases, including epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2).[16,17] Somatic mutations of *BRAF* have been documented in different kinds of tumors, predominantly in malignant melanoma,[18] thyroid papillary cancer,[19] sporadic colorectal tumors,[20] low-grade ovarian serous carcinoma,[21] and lung tumors.[22,23] A vast majority of these mutations correspond to the hotspot transversion mutation T1799A at exon 15, which causes the amino acidic substitution of V600E.[16] A wide range of other missense mutations (non-V600E) have been detected in exons 11 and 15.[16] The actual prevalence, distribution, and prognostic role of *BRAF* mutations in patients with NSCLC is still unclear.

New selective inhibitors of mutant *BRAF* have generated considerable interest. Treatment of patients with metastatic melanoma carrying the V600E *BRAF* mutation with PLX4032 has resulted in an overall response rate of 81%, with median progression-free survival of more than 7 months.[24] These impressive results prompted us to evaluate a large number of lung tumors for *BRAF* mutations, with the aim of identifying potential common features in patients carrying *BRAF* mutations and assessing the impact of *BRAF* mutations on prognosis.

Patients and methods

Patient selection

The study population was selected from a cohort of 1,370 patients with NSCLC who received radical resection of a primary NSCLC at the Department of Thoracic Surgery, University of Chieti (Chieti, Italy), or Department of Thoracic Surgery, University of Pisa (Pisa, Italy), during the period of 1996 to 2006. From this cohort, a series of 1,046 patients, including 739 consecutive patients affected by lung ADC and 307 consecutive patients with squamous cell carcinomas (SCC), was selected for the study. Follow-up data were obtained from 331 patients with histologic diagnosis of lung ADC. Patient stage at time of diagnosis was determined according to the TNM staging system.[25] On the basis of smoking history, deduced from anamnestic data, patients were classified as smokers, former smokers (stopped smoking at least 1 year before diagnosis of lung cancer), and never smokers (smoked fewer than 100 cigarettes).

For each patient, tumor and macroscopically normal lung tissue samples were snap frozen in liquid nitrogen within 10 minutes of excision and stored at –80°C. Adjacent pieces of tumor tissue were processed for histopathology and immunohistochemistry. All tumor specimens were subjected to macrodisssection before genomic analysis. In all cases, the amount of tumor cells equaled or exceeded 80% of the overall sample. Informed consent was obtained from all patients in the study. The study was conducted in accordance with the precepts of the Helsinki Declaration.

Genetic analysis

A highly sensitive detection procedure was used to detect *BRAF* mutations in tumor DNA. Genomic DNA was extracted and subjected to polymerase chain reaction amplification of exons 11 and 15, as reported previously.[22] The samples were then examined by high-resolution melting analysis (HRMA) on a Light Cycler 480 II (Roche Applied Science, Indianapolis, IN). Positive patient cases were subjected to independent polymerase chain reaction amplification and direct sequencing by capillary electrophoresis using the ABI Prism 3100 DNA analyzer (Applied Biosystems,

Foster City, CA). *BRAF* stripAssays (Vienna Lab Diagnostics, Vienna, Austria) were used to confirm the mutation in samples found to be positive by HRMA but with low mutated peaks in the electropherogram. Genetic analyses of *EGFR* in exons 18 to 21 and *KRAS* at codons 12 and 13 were performed as reported in previous studies.[9,26]

Statistical analysis

Variables measured in the study were investigated for association by using Fisher's exact test or χ^2 test, as appropriate. Associations of *BRAF* mutational status, as the dependent variable, with sex, smoking history, and stage of disease were also investigated by logistic regression analysis to account for the effect of the different variables. Disease-free survival (DFS) and overall survival (OS) were measured for each patient from the day of surgical treatment. Survival curves were estimated using the Kaplan-Meier method, and differences among them were evaluated by the log-rank test. Multivariate analysis was used to assess the effect of covariates on DFS and OS and was performed using Cox proportional hazards regression with a step-down method. $P < 0.05$ was considered significant. All statistical analyses were performed using SPSS version 15 (SPSS, Chicago, IL).

Results

Patient characteristics

A total of 1,046 patients with surgically resected NSCLC were included in the present analysis. All patients were white. Our study population consisted of a series of 739 patients with lung ADC, the histologic type of NSCLC in which the vast majority of *BRAF* mutations had been detected in previous studies,[23,27] and a series of 307 patients affected by SCC. Characteristics of patients with lung ADC and SCC are illustrated in Table 1. For 331 patients with lung ADC, follow-up data were obtained. In this latter series, no postoperative therapy was delivered to patients in stage I to II disease, and only a minority of patients with stage III to IV disease (27 patients) received adjuvant platinum-based chemotherapy. Patients with metastatic disease received surgery after or at the same time of single brain or

Key points

- *A total of 1,046 patients with surgically resected NSCLC were included in the present analysis.*
- *Our study population consisted of a series of 739 patients with lung ADC, the histologic type of NSCLC in which the vast majority of* BRAF *mutations had been detected in previous studies, and a series of 307 patients affected by SCC.*
- *For 331 patients with lung ADC, follow-up data were obtained. In this latter series, no postoperative therapy was delivered to patients in stage I to II disease, and only a minority of patients with stage III to IV disease (27 patients) received adjuvant platinum-based chemotherapy.*
- *Patients with metastatic disease received surgery after or at the same time of single brain or lung lesion removal.*

TABLE 1 - Clinicopathologic variables of patients with ADC and SCC

Characteristic	ADC (*N* = 739)		SCC (*N* = 307)	
	N	%	*N*	%
Age, years				
Mean	65		66.2	
SD	± 9.1		± 8.4	
Sex				
Male	552	74.7	289	94.1
Female	187	25.3	18	5.9
Smoking history				
Never	197	26.7	7	2.3
Former	272	36.8	90	29.3
Current	270	36.5	210	68.4
Pathologic stage				
I	412	55.8	189	61.6
II	109	14.7	55	17.9
III	194	26.3	58	18.9
IV	24	3.2	5	1.6

ADC = adenocarcinoma; SCC = squamous cell carcinoma; SD = standard deviation.

Key points

- *The mutational status of the* BRAF *gene was investigated in 1,046 resected primary lung tumors, comprising 739 lung ADCs and 307 SCCs.*
- BRAF *mutations were detected in 37 tumors (3.5%). None of the matching normal samples showed evidence of mutation, indicating the somatic nature of all mutational events.*
- *Twenty-one mutations (56.7%) were V600E hotspot mutations, and 16 (43.3%) were non-V600E mutations distributed in narrow areas between codons 594 and 606 (exon 15) and 446 and 449 (exon 11).*
- *Thirty-six (97.3%) of the 37 mutations of the* BRAF *gene were found in lung ADCs. The frequency of* BRAF *mutations in ADCs was 4.9%.*

■ **TABLE 2 - Genomic mutations in *BRAF* gene**

***BRAF* mutation**	**Change**		**Histologic type**		***P***
	Nucleotide	**Aminoacid**	**ADC**	**SCC**	
Exon					0.001
15	T1799A	V600E	21	—	
	A1781G	D594G	2	—	
	T1790G	L597R	2	—	
	C1789G	L597V	1	—	
	T1790A	L597Q	1	—	
	G1798T	V600L	1	—	
	A1801G	K601E	1	—	
	A1803T	K601N	1	—	
	T1810A	W604R	—	1	
	G1817C	G606A	1	—	
	G1817T	G606V	1	—	
11	G1397T	G466V	2	—	
	G1406C	G469A	1	—	
	G1406T	G469V	1	—	
Total					0.001
N			36	1	
%			4.9	0.3	

ADC = adenocarcinoma; SCC = squamous cell carcinoma.

lung lesion removal. No patient indicated a history of thyroid, breast, melanocytic, or ovarian cancer. Median follow-up for this population was 45 months after surgical treatment, and median DFS and OS were 43 (95% CI, 26.2 to 59.8) and 65 months (95% CI, 52.1 to 77.8), respectively. At the time of analysis, 139 deaths (42%) and 161 recurrences (48.6%) had occurred in this series. As expected, median DFS and OS were longer in patients with stage I to II than in those with stage III to IV disease (70.5 vs. 23.1 months; $P < 0.001$ and not reached vs. 39.0 months; $P < 0.001$, respectively). No significant differences in DFS or OS were observed according to sex or smoking history.

■ *BRAF* mutations

The mutational status of the *BRAF* gene was investigated in 1,046 resected primary lung tumors, comprising 739 lung ADCs and 307 SCCs. Tumor DNA was subjected to HRMA, and the alterations detected were confirmed by sequencing. In three patient cases with low mutated peaks in the electropherogram, the mutation was further confirmed by *BRAF* stripAssays, a sensitive diagnostic detection method. *BRAF* mutations were detected in 37 tumors (3.5%). None of the matching normal samples showed evidence of mutation, indicating the somatic nature of all mutational events. As illustrated in Table 2, 21 mutations (56.7%) were V600E hotspot mutations, and 16 (43.3%) were non-V600E mutations distributed in narrow areas between codons 594 and 606 (exon 15) and 446 and 449 (exon 11).

■ Correlations with clinicopathologic data and mutations of *EGFR* and *KRAS*

Thirty-six (97.3%) of the 37 mutations of the *BRAF* gene were found in lung ADCs (Table 2). The frequency of *BRAF* mutations in ADCs was 4.9%. In the series of 307 SCCs, only one non-V600E mutation (0.3%) was detected in a tumor from a male smoker. The histology of this latter tumor was confirmed by immunohistochemistry with anti-p63 and anti–thyroid transcription factor 1 monoclonal antibodies to exclude the possibility of an adenosquamous

nature. As shown in Table 3, all 15 non-V600E mutations (2%) detected in ADCs were found in smokers ($P = 0.015$). On the other hand, the 21 V600E *BRAF* mutations (2.8%) were significantly more frequent in females (16 of 187 patients; 8.6%) than in males (five of 552 patients; 0.9%; $P < 0.001$) and in never smokers (10 of 197 patients; 5.1%) than in smokers or former smokers (11 of 542 patients; 2%; $P = 0.04$). Other clinicopathologic parameters, including age, tumor size, nodal status, and tumor stage, were not significantly associated with V600E *BRAF* mutations (Table 3).

Association of V600E *BRAF* mutations with clinicopathologic parameters was also evaluated by logistic regression analysis to take into consideration the reciprocal effects of the covariates investigated. As shown in Table 4, *BRAF* mutations were found to be independently associated only with female sex (hazard ratio [HR], 11.29; $P < 0.001$).

Tumors affected by *BRAF* mutations were revised histologically according to the new international multidisciplinary classification of lung ADC,[15] and detailed morphologic data will be published separately (submitted for publication). According to this classification, 80% of the tumors with V600E mutations were histopathologically classified as infiltrating lung ADCs with a predominant (50% of patients) or secondary (30%) micropapillary component, an uncommon histologic pattern associated with tumor aggressiveness. Tumors with non-V600E mutations were histologically more heterogeneous and showed micropapillary features in 12% of patients.

This series of lung ADCs was also investigated for *EGFR* and *KRAS* mutations. *KRAS* mutations were observed in 203 patients (27%) and *EGFR* mutations in 86 (12%). All tumors with *BRAF* mutations were found to be negative for *KRAS* mutations, whereas two tumors with V600E *BRAF* mutations showed concomitant *EGFR* mutations (in both, deletion in exon 19).

Survival analyses

Follow-up data were obtained for 331 patients with lung ADC. This series comprised all 36 patients with *BRAF* mutations who emerged from the analysis of the 739 patients with lung ADC and a consecutive series of patients with ADC from the period of 2001 to 2004. The 2001-to-2004 series contained 17 (47%) of the 36 patients with *BRAF* mutations (10 V600E and seven non-V600E) and 295 wild-type patients, for a total of 312 patients.

In the series of 331 lung ADCs, the postoperative survival curves (Figure 1), estimated using the Kaplan-Meier method, revealed that patients with V600E *BRAF* mutations had shorter median DFS and OS than patients without V600E mutations (15.2 vs. 52.1 months; $P < 0.001$ and 29.3 vs. 72.4 months; $P < 0.001$, respectively). No differences in DFS or OS were observed between patients with and without non-V600E mutations (42.8 vs. 43.2 months; $P = 0.84$ and 56.4 vs. 65.1 months; $P = 0.42$, respectively). No significant differences in DFS or OS were seen when *BRAF* mutations were not separated into the two main types (V600E and non-V600E; data not shown). The joint effect of sex, smoking history, and pathologic stage was examined using stepwise Cox regression analysis. Table 5 shows the results of the univariate and multivariate survival analyses. In the univariate analysis, only stage and *BRAF* V600E mutation status were significantly associated with shorter DFS (HR, 2.82; $P < 0.001$ and HR, 2.67; $P < 0.001$, respectively) and OS (HR, 3.26; $P < 0.001$ and HR, 2.97; $P < 0.001$, respectively). Multivariate analysis confirmed that pathologic stage and V600E mutations were the only independent and significant factors to predict DFS (HR, 2.54; $P < 0.001$ and HR, 2.19; $P < 0.011$,

Key points

- *All 15 non-V600E mutations (2%) detected in ADCs were found in smokers.*
- *The 21 V600E* BRAF *mutations (2.8%) were significantly more frequent in females (16 of 187 patients; 8.6%) than in males (five of 552 patients; 0.9%) and in never smokers (10 of 197 patients; 5.1%) than in smokers or former smokers (11 of 542 patients; 2%).*
- *Other clinicopathologic parameters, including age, tumor size, nodal status, and tumor stage, were not significantly associated with V600E* BRAF *mutations.*
- *According to the new international multidisciplinary classification of lung ADC, 80% of the tumors with V600E mutations were histopathologically classified as infiltrating lung ADCs with a predominant (50% of patients) or secondary (30%) micropapillary component, an uncommon histologic pattern associated with tumor aggressiveness.*
- *In the series of 331 lung ADCs, the postoperative survival curves, estimated using the Kaplan-Meier method, revealed that patients with V600E* BRAF *mutations had shorter median DFS and OS than patients without V600E mutations (15.2 vs. 52.1 months; 29.3 vs. 72.4 months, respectively).*

■ **TABLE 3 - Comparison of clinicopathologic variables with *BRAF* mutations in series of 739 patients with lung ADC**

Variable	Total No. of patients (*N* = 739)	V600E				
		Mutated (*N* = 21)		Wild type (*N* = 718)		*P*
		N	%	*N*	%	
Age, years						0.19; NS
Mean		67.7		64.7		
SD		± 6.5		± 9.1		
Sex						< 0.001
Male	552	5	0.9	547	99.1	
Female	187	16	8.6	171	91.4	
Smoking history						0.042
Smoker/former smoker	542	11	2	531	98	
Never smoker	197	10	5.1	187	94.9	
Tumor size						0.1; NS
T1	322	6	1.9	316	98.1	
T2	330	9	2.7	321	97.3	
T3	58	4	6.9	54	93.1	
T4	29	2	6.9	27	93.1	
N status						0.13; NS
N0	457	8	1.8	449	98.2	
N1	111	5	4.5	106	95.5	
N2	166	8	4.8	158	95.2	
N3	5	0	0	5	100	
Stage						0.11; NS
I	412	7	1.7	405	98.3	
II	109	4	3.7	105	96.3	
III	194	8	4.1	186	95.9	
IV	24	2	8.3	22	91.7	

ADC = adenocarcinoma; NS = not significant.

Key points

- *Multivariate analysis confirmed that pathologic stage and V600E mutations were the only independent and significant factors to predict and OS. These results were confirmed in the consecutive series of 312 patients with lung ADC.*

■ **TABLE 4 - Association between V600E *BRAF* mutations and independent covariates computed by multivariate logistic regression analysis**

Variable	Category	Logistic regression analysis		
		HR	95% CI	*P*
Sex	Female/male	11.29	3.65 to 34.87	< 0.001
Smoking	Never smoker/smoker	1.19	0.45 to 3.21	0.7; NS
Stage	III + IV/I + II	2.25	0.92 to 5.51	0.08; NS

HR = hazard ratio; NS = not significant.

respectively) and OS (HR, 2.92; $P < 0.001$ and HR, 2.18; $P = 0.014$, respectively). These results were confirmed in the consecutive series of 312 patients with lung ADC.

Discussion

The present study was devised to investigate the prevalence, distribution, and prognostic role of *BRAF* mutations in a

Non-V600E				
Mutated (*N* = 15)		Wild type (*N* = 724)		*P*
N	%	*N*	%	
65.9 ± 7.6		64.8 ± 9.2		0.72; NS
14 1	2.5 0.5	538 186	97.5 99.5	0.13; NS
15 0	2.8 0	527 197	97.2 100	0.015
5 9 1 0	1.6 2.7 1.7 0	317 321 57 29	98.4 97.3 98.3 100	0.61; NS
10 2 3 0	2.2 1.8 1.8 0	447 109 163 5	97.8 98.2 98.2 100	0.97; NS
9 3 3 0	2.2 2.8 1.5 0	403 106 191 24	97.8 97.2 98.5 100	0.79; NS

large cohort of white patients with NSCLC. The first aim was to define potential clinicopathologic parameters that could help in the selection of patients to undergo mutational screening. In this respect, our results indicate that *BRAF* mutations are almost completely confined to the ADC histotype; the prevalence of *BRAF* mutations in lung ADC is close to 5%; and the two main types of *BRAF* mutations, V600E and non-V600E, affect different patients and are associated with different pathologic features of lung ADCs. In addition, we evaluated the prognostic role of this important biomolecular marker and report for the first time to our knowledge that the V600E hotspot mutation represents a negative prognostic factor in patients with radically resected NSCLC.

BRAF mutations belong to a series of clinically relevant genomic alterations that affect a minority of patients with NSCLC. Most previous studies of *BRAF* mutations in NSCLC have been performed in relatively small cohorts.[17,22,23,28] A large but heterogeneous series of 916 patients from Japan, Taiwan, Australia, and the United States, predominantly of Eastern origin, was recently investigated by Pratilas et al.[27] The authors reported *BRAF* mutations in 2% of the tumors in the entire series, with differences among groups ranging from 1.3% in Japan and 3% in Australia. Sasaki et al.[28] reported that the frequency of *BRAF*

Key points

- *The present study was devised to investigate the prevalence, distribution, and prognostic role of* BRAF *mutations in a large cohort of white patients with NSCLC. The first aim was to define potential clinicopathologic parameters that could help in the selection of patients to undergo mutational screening.*
- *Our results indicate that* BRAF *mutations are almost completely confined to the ADC histotype; the prevalence of* BRAF *mutations in lung ADC is close to 5%; and the two main types of* BRAF *mutations, V600E and non-V600E, affect different patients and are associated with different pathologic features of lung ADCs.*
- *In addition, we evaluated the prognostic role of this important biomolecular marker and report for the first time to our knowledge that the V600E hotspot mutation represents a negative prognostic factor in patients with radically resected NSCLC.*

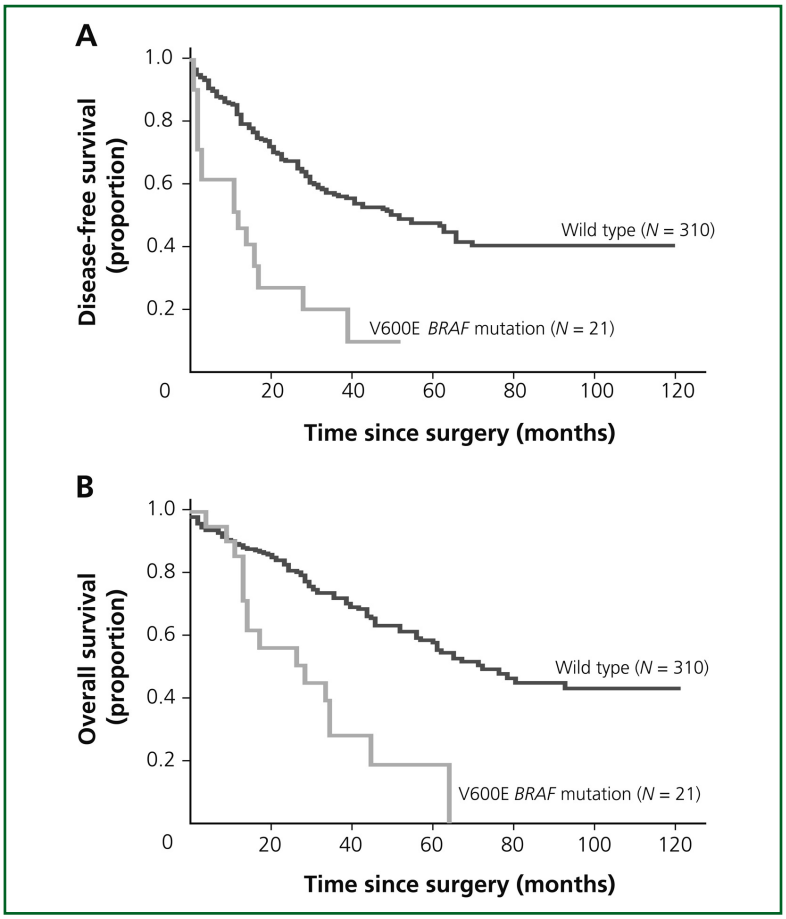

FIGURE 1 ■ *A)* Disease-free and *(B)* overall survival curves in 331 patients with lung adenocarcinoma based on presence or absence of V600E *BRAF* mutation.
Curve differences are statistically significant.

mutations in Japanese patients was low (0.8%), whereas Yousem et al.,[29] who restricted their analysis to V600E mutations, reported a frequency of 2.9% in ADCs of US patients. To our knowledge, our study represents the largest survey of *BRAF* mutations in patients with NSCLC conducted to date. In a homogeneous cohort of white patients, we report *BRAF* mutations in 3.5% of the tumors and in 4.9% of lung ADCs. The incidence of V600E mutations in ADCs was 2.8%, in agreement with data reported by Yousem et al.[29] The number of mutational events in our panel allowed us to compare them statistically with clinicopathologic parameters. Data analysis revealed that hotspot V600E and non-V600E mutations were distinct events. V600E mutations were significantly more prevalent in females (approximately 9% of females with ADCs had V600E mutations) and were independent of smoking history. In addition, V600E mutations were frequently associated with a more aggressive tumor histotype, characterized by micropapillary features, and were independently associated with poor prognosis. On the other hand, non-V600E mutations were only seen in smokers and were not associated with any of the clinicopatologic parameters investigated or

■ TABLE 5 - Univariate and multivariate survival analyses of 331 patients with lung ADC*

Variable	Category	DFS analysis					
		Univariate			Multivariate		
		HR	95% CI	*P*	HR	95% CI	*P*
Sex	Female/male	1.37	0.93 to 2.03	0.11; NS	1.42	0.8 to 2.52	0.23; NS
Smoking	Never smoker/ smoker	1.01	0.65 to 1.54	0.99; NS	1.37	0.76 to 2.49	0.29; NS
Stage	III + IV/I + II	2.82	1.96 to 4.07	< 0.001	2.54	1.74 to 3.71	< 0.001
V600E	Mutated/wild type	2.67	1.89 to 5.12	< 0.001	2.19	1.2 to 4.01	0.011
Non-V600E	Mutated/wild type	1.1	0.45 to 2.69	0.84; NS	1.15	0.47 to 2.84	0.76; NS

Sex, smoking, and non-V600E variables were removed from model at last step of multivariate analysis.

*Series included all 36 patients with *BRAF* mutations found in entire series of 739 lung ADCs.

ADC = adenocarcinoma; DFS = disease-free survival; HR = hazard ratio; NS = not significant; OS = overall survival.

with prognosis. To the best of our knowledge, this is the first time that different somatic mutations affecting the same gene have been associated with different type of patients, pathologic tumor features, and clinical behavior.

In previous studies, because of the relatively low number of patient cases investigated or the particular cohorts of patients examined, the number of V600E and non-V600E mutations detected was too low to allow separate analysis. Considering these two different types of mutations together results in an extremely difficult interpretation of data. On the basis of our observations, we reanalyzed the rough supplemental data published by Pratilas et al.[27] and found that in their series as well, V600E mutations were much more frequent in females (eight of 11 patients; 72% vs. 16 of 21; 76% in our series) and non-V600E mutations in smokers (five of six patients; 83% vs. 16 of 16; 100% in our series). Another interesting observation that emerged from our data (strengthened by comparison with data previously published by Pratilas et al.) was that the rare *BRAF* mutations detected in SCCs (notably associated with smoking history) were non-V600E (one in our series and two in series of Pratilas et al.). In both series, V600E mutations were restricted to lung ADCs.

The presence of V600E *BRAF* mutations in approximately 9% of females affected by lung ADC is an important finding that emerged from our statistical analysis. This information could be useful in the selection of patients for treatment with specific *BRAF* inhibitors. The high frequency of *BRAF* mutations in females is intriguing and could be reminiscent of the presence of *EGFR* mutations in NSCLC, for which, it has been postulated, sex hormones or environmental factors may be responsible.[13,30–32] An association between *BRAF* mutations and female sex has also been reported in patients with colorrectal cancer.[33] During the review process, an article appeared in which Paik et al.[34] reported 18 *BRAF* mutations (3%) in a multiethnic series of 697 patients affected by lung ADC. Because of the small number of patients with *BRAF* mutations in their series, the authors could not perform a comparison of the clinical characteristics and outcomes among *BRAF* mutation subtypes. However, analysis of their raw data revealed a high frequency of V600E mutations in females, in agreement with our results.

Key points

- *V600E mutations were significantly more prevalent in females (approximately 9% of females with ADCs had V600E mutations) and were independent of smoking history.*
- *V600E mutations were frequently associated with a more aggressive tumor histotype, characterized by micropapillary features, and were independently associated with poor prognosis.*
- *Non-V600E mutations were only seen in smokers and were not associated with any of the clinicopatologic parameters investigated or with prognosis.*
- *The presence of V600E* BRAF *mutations in approximately 9% of females affected by lung ADC is an important finding that emerged from our statistical analysis. This information could be useful in the selection of patients for treatment with specific* BRAF *inhibitors.*

	OS analysis					
	Univariate			Multivariate		
	HR	95% CI	*P*	HR	95% CI	*P*
	1.39	0.91 to 2.13	0.13; NS	1.2	0.63 to 2.27	0.58; NS
	1.1	0.69 to 1.76	0.68; NS	1.09	0.56 to 2.09	0.81; NS
	3.26	2.21 to 4.80	< 0.001	2.92	1.95 to 4.37	< 0.001
	2.97	1.96 to 5.81	< 0.001	2.18	1.17 to 4.04	0.014
	1.56	0.51 to 5.04	0.42; NS	1.46	0.46 to 4.64	0.52; NS

Key points

- *The most striking result obtained in the present study was the association of* BRAF *mutations with poor prognosis.*
- *In conclusion, in this study, we identified a number of clinicopathologic parameters potentially useful for the selection of patients with NSCLC carrying* BRAF *mutations in their tumors and demonstrated a prognostic role for V600E* BRAF *mutations in patients with NSCLC.*

It has previously been reported that *BRAF*, *EGFR*, and *KRAS* mutations are mutually exclusive events.[9,13] In our series, we confirmed these data for *EGFR-KRAS* and *BRAF-KRAS*, whereas we found two tumors with concomitant V600E *BRAF* and *EGFR* mutations. Because we used a sensitive detection technique, it is possible that *BRAF* and *EGFR* mutations in these two tumors affected subpopulations of neoplastic cells.

The most striking result obtained in the present study was the association of *BRAF* mutations with poor prognosis. Data from univariate and multivariate logistic regression analyses indicated a trend for an association between V600E mutations and T status, N status, and pathologic stage. These results, together with the observation that tumors harboring V600E mutations frequently showed micropapillary features, suggest an association between V600E mutations and tumor aggressiveness, in agreement with previously published data.[29] When V600E *BRAF* mutations were analyzed comparatively with follow-up data, they clearly stratified patients into markedly different survival groups; patients with V600E mutations in the tumor had significantly shorter DFS and OS times than those without mutations. Multivariate Cox regression analysis indicated an independent association of V600E mutations with poor DFS and OS. A similar association has been reported in patients with colorectal cancer carrying *BRAF* mutations.[20,33,35,36]

In conclusion, in this study, we identified a number of clinicopathologic parameters potentially useful for the selection of patients with NSCLC carrying *BRAF* mutations in their tumors and demonstrated a prognostic role for V600E *BRAF* mutations in patients with NSCLC. Additional large multicentric studies are needed to extend and confirm our results.

References

1. Sun S, Schiller JH, Spinola M, et al: New molecularly targeted therapies for lung cancer. J Clin Invest 117:2740-2750, 2007.
2. Rosell R, Moran T, Queralt C, et al: Screening for epidermal growth factor receptor mutations in lung cancer. N Engl J Med 361:958-967, 2007.
3. Horn L, Pao W: EML4-ALK: Honing in on a new target in non–small-cell lung cancer. J Clin Oncol 27:4232-4235, 2009.
4. Choi YL, Soda M, Yamashita Y, et al: EML4-ALK mutations in lung cancer that confer resistance to ALK inhibitors. N Engl J Med 363:1734-1739, 2010.
5. Pao W, Girard N: New driver mutations in non-small-cell lung cancer. Lancet Oncol 12:175-180, 2011.
6. Dacic S, Shuai Y, Yousem S, et al: Clinicopathological predictors of EGFR/KRAS mutational status in primary lung adenocarcinomas. Mod Pathol 23:159-168, 2010.
7. Marchetti A, Felicioni L, Pelosi G, et al: Frequent mutations in the neurotrophic tyrosine receptor kinase gene family in large cell neuroendocrine carcinoma of the lung. Hum Mutat 29:609-616, 2008.
8. Shigematsu H, Lin L, Takahashi T, et al: Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. J Natl Cancer Inst 97:339-346, 2005.
9. Marchetti A, Martella C, Felicioni L, et al: EGFR mutations in non–small-cell lung cancer: Analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment. J Clin Oncol 23:857-865, 2005.
10. Shigematsu H, Takahashi T, Nomura M, et al: Somatic mutations of the HER2 kinase domain in lung adenocarcinomas. Cancer Res 65:1642-1646, 2005.
11. Buttitta F, Barassi F, Fresu G, et al: Mutational analysis of the HER2 gene in lung tumors from Caucasian patients: Mutations are mainly present in adenocarcinomas with bronchioloalveolar features. Int J Cancer 119:2586-2591, 2006.
12. Marchetti A, Buttitta F, Pellegrini S, et al: Bronchioloalveolar lung carcinomas: K-ras mutations are constant events in the mucinous subtype. J Pathol 179:254-259, 1996.
13. Shigematsu H, Gazdar AF: Somatic mutations of epidermal growth factor receptor signaling pathway in lung cancers. Int J Cancer 118:257-262, 2006.
14. Stahel RA: Adenocarcinoma, a molecular perspective. Ann Oncol 18:ix147-ix149, 2007 (suppl 9).
15. Travis WD, Brambilla E, Noguchi M, et al: International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society international multidisciplinary classification of lung adenocarcinoma. J Thorac Oncol 6:244-285, 2011.
16. Garnett MJ, Marais R: Guilty as charged: B-RAF is a human oncogene. Cancer Cell 6:313-319, 2004.
17. Davies H, Bignell GR, Cox C, et al: Mutations of the BRAF gene in human cancer. Nature 417:949-954, 2002.

18. Curtin JA, Fridlyand J, Kageshita T, et al: Distinct sets of genetic alterations in melanoma. N Engl J Med 353: 2135-2147, 2005.
19. Kimura ET, Nikiforova MN, Zhu Z, et al: High prevalence of BRAF mutations in thyroid cancer: Genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma. Cancer Res 63:1454-1457, 2003.
20. Samowitz WS, Sweeney C, Herrick J, et al: Poor survival associated with the BRAF V600E mutation in microsatellite-stable colon cancers. Cancer Res 65:6063-6069, 2005.
21. Singer G, Oldt R 3rd, Cohen Y, et al: Mutations in BRAF and KRAS characterize the development of low-grade ovarian serous carcinoma. J Natl Cancer Inst 95:484-486, 2003.
22. Brose MS, Volpe P, Feldman M, et al: BRAF and RAS mutations in human lung cancer and melanoma. Cancer Res 62:6997-7000, 2002.
23. Naoki K, Chen TH, Richards WG, et al: Missense mutations of the BRAF gene in human lung adenocarcinoma. Cancer Res 62:7001-7003, 2002.
24. Flaherty KT, Puzanov I, Kim KB, et al: Inhibition of mutated, activated BRAF in metastatic melanoma. N Engl J Med 363:809-819, 2010.
25. Sobin LH, Witteking CH (eds): TNM Classification of Malignant Tumours (ed 6). New York, NY, John Wiley & Sons, 2002.
26. Marchetti A, Milella M, Felicioni L, et al: Clinical implications of KRAS mutations in lung cancer patients treated with tyrosine kinase inhibitors: An important role for mutations in minor clones. Neoplasia 11:1084-1092, 2009.
27. Pratilas CA, Hanrahan AJ, Halilovic E, et al: Genetic predictors of MEK dependence in non-small cell lung cancer. Cancer Res 68:9375-9383, 2008.
28. Sasaki H, Kawano O, Endo K, et al: Uncommon V599E BRAF mutations in Japanese patients with lung cancer. J Surg Res 133:203-206, 2006.
29. Yousem SA, Nikiforova M, Nikiforov Y: The histopathology of BRAF-V600E-mutated lung adenocarcinoma. Am J Surg Pathol 32:1317-1321, 2008.
30. Yamamoto H, Toyooka S, Mitsudomi T: Impact of EGFR mutation analysis in non-small cell lung cancer. Lung Cancer 63:315-321, 2009.
31. Raso MG, Behrens C, Herynk MH, et al: Immunohistochemical expression of estrogen and progesterone receptors identifies a subset of NSCLCs and correlates with EGFR mutation. Clin Cancer Res 15:5359-5368, 2009.
32. Nose N, Uramoto H, Iwata T, et al: Expression of estrogen receptor beta predicts a clinical response and longer progression-free survival after treatment with EGFR-TKI for adenocarcinoma of the lung. Lung Cancer 71:350-355, 2011.
33. Roth AD, Tejpar S, Delorenzi M, et al: Prognostic role of KRAS and BRAF in stage II and III resected colon cancer: Results of the translational study on the PETACC-3, EORTC 40993, SAKK 60-00 trial. J Clin Oncol 28:466-474, 2010.
34. Paik PK, Arcila ME, Fara M, et al: Clinical characteristics of patients with lung adenocarcinomas harboring *BRAF* mutations. J Clin Oncol 29:2046-2051, 2011.
35. Yokota T, Ura T, Shibata N, et al: BRAF mutation is a powerful prognostic factor in advanced and recurrent colorectal cancer. Br J Cancer 104:856-862, 2011.
36. Richman SD, Seymour MT, Chambers P, et al: *KRAS* and *BRAF* mutations in advanced colorectal cancer are associated with poor prognosis but do not preclude benefit from oxaliplatin or irinotecan: Results from the MRC FOCUS trial. J Clin Oncol 27:5931-5937, 2009.