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Biology of Human Tumors

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Nondisruptive p53 Mutations Are Associated with Shorter Survival in Patients with Advanced Non–Small Cell Lung Cancer

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Abstract

Purpose: *TP53* mutations in early-stage non–small cell lung cancer (NSCLC) may be associated with worse survival but their prognostic role in advanced NSCLC is controversial. In addition, it remains unclear whether mutated patients represent a clinically homogeneous group.

Experimental Design: We retrospectively examined *TP53* mutations and outcome in a training cohort of 318 patients with stage IIIB–IV NSCLC: 125 epidermal growth factor receptor (*EGFR*) wild-type (wt) and 193 *EGFR* mutated (mut). An independent validation cohort of 64 *EGFR*-mut patients was subsequently analyzed. Mutations were classified as "disruptive" and "nondisruptive" according to their predicted degree of disturbance of the p53 protein structure and function.

Results: In the training cohort, *TP53* mutations were found in 43 of the 125 *EGFR*-wt patients (34.4%). Of these, 28 had nondisruptive *TP53* mutations and a median overall survival (OS) of 8.5 months, compared with 15.6 months for the remaining 97 patients ($P = 0.003$). In the *EGFR*-mut group, *TP53* mutations were found in 50 of the 193 patients (25.9%). The OS for the 26 patients with *TP53* nondisruptive mutations was 17.8 months versus 28.4 months for the remaining 167 patients ($P = 0.04$). In the validation cohort, the 11 patients with nondisruptive *TP53* mutations had a median OS of 18.1 months compared with 37.8 months for the 53 remaining patients ($P = 0.006$). In multivariate analyses, nondisruptive *TP53* mutations had an independent, significant association with a shorter OS.

Conclusions: Nondisruptive mutations in the *TP53* gene are an independent prognostic factor of shorter survival in advanced NSCLC. *Clin Cancer Res; 20(17); 4647–59. ©2014 AACR.*

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Introduction

The majority of patients diagnosed with non–small cell lung cancer (NSCLC) present with advanced disease stage and have extremely poor prognosis (1). The tumor suppressor gene *TP53* is the most frequently mutated in NSCLC (2). It encodes a 393-aa protein with three distinct domains (Supplementary Fig. S1). The transactivation domain is the target of posttranscriptional modification by proteins such as the serine-protein kinase ATM or the E3 ubiquitin-protein ligase Mdm2 (3). The DNA-binding domain (DBD), encoded by exons 5 to 8 of the *TP53* gene, comprises residues 102–292 and recognizes a consensus sequence in the promoter of several genes involved in DNA repair, cell-cycle arrest, or apoptosis (*CDKN1A*, *SFN*, *FAS*, *BAX*, *DDB2*, and others; refs. 4–6). The sequence-specific transcriptional activity mediated by the DBD is the primary mechanism accounting for the tumor suppressor activities of p53. Within the DBD, loops L2 (residues 163–195) and L3 (residues 236–251) bind to a Zn atom and play a key role in the interaction with DNA (7). Finally, the C-terminal

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Translational Relevance

The majority of patients diagnosed with non–small cell lung cancer (NSCLC) present with advanced disease stage and have extremely poor prognosis. The tumor suppressor gene *TP53* is the most frequently mutated in NSCLC, but its prognostic role in advanced NSCLC is controversial, and it remains unclear whether mutated patients represent a clinically homogeneous group. This study shows that a specific group of mutations in the *TP53* gene, namely nondisruptive mutations, are an independent prognostic factor of shorter survival in advanced NSCLC. Our results pave the way for more widespread testing of *TP53* mutations in unresectable NSCLC and indicate that the disruptive/nondisruptive categorization is clinically relevant and should be applied in all studies of *TP53* in lung cancer. Clinical trials are warranted to determine whether patients with advanced NSCLC with nondisruptive mutations can benefit from drugs that reactivate p53.

domains are responsible for oligomerization and negative regulation of the protein. Under normal conditions, the p53 protein binds to the ubiquitine ligase Mdm2 and is quickly degraded. Some cellular stresses (DNA damage, cell-cycle abnormalities, and hypoxia) block Mdm2 binding to p53 which then oligomerizes to an active tetramer (2).

Mutations in the *TP53* gene are present in 32.5% of NSCLC tumors (8). Many of these are mutations in the DBD that lead to a stable protein, with significant loss of activity. The mutated protein accumulates in the nucleus of cells and exerts a dominant-negative effect by heterodimerizing with the (wild-type) wt p53 expressed from the remaining allele (9). Recent evidence also indicates that, in addition to abrogating tumor-suppressor properties, some mutations confer new "gain of function" (GOF) activities to the mutated p53 protein that contribute to tumor progression (10, 11). Mechanisms of mutated p53 GOF include interference with p53-related proteins (p63, p73, etc; ref. 12) and aberrant upregulation of genes that promote cancer progression or drug resistance (13).

A variety of criteria have been used to categorize *TP53* mutations. Poeta and colleagues (14) proposed a classification in "disruptive" and "nondisruptive" according to their functional effects on the p53 protein. Disruptive mutations include (i) any mutation that originates a stop codon (nonsense, frameshift, and intronic), (ii) missense mutations located inside the L2 or L3 loops replacing one residue by another of a different polarity or charge and, (iii), in-frame deletions within the L2 or L3 loops. Nondisruptive mutations are all those not classified as disruptive and include (i) missense mutations and in-frame deletions located outside the L2-L3 loops and (ii) missense mutations within the L2-L3 loops but replacing one residue with another of the same polarity or charge. Disruptive mutations likely lead to a complete, or almost complete, loss of activity of the p53 protein. In contrast, nondisruptive mutations can retain some of the functional properties of wt-p53, and more importantly, experimental evidence shows that they often associate with GOF activities (15, 16).

Determination of *TP53* mutations by standard techniques requires an amount of material that is often only available in resected tumors. For this reason, most studies of *TP53* status in NSCLC have been performed in stages I to III. The small numbers of patients included in some of these studies, the differences in follow-up, and the various criteria used to classify *TP53* mutations have led to contradictory results. This last aspect is particularly important as the heterogeneity of *TP53* mutations has been demonstrated to correlate with similarly heterogeneous clinical outcomes in other types of tumor (14, 17, 18).

We have analyzed *TP53* mutations in a training cohort of 318 patients with advanced NSCLC [125 *EGFR*-wt and 193 *EGFR*-mutated (mut)] and correlated *TP53* mutations with clinical parameters. We validated our findings in an independent cohort of 64 *EGFR*-mut patients.

Materials and Methods

Patients

In the training cohort, we retrospectively analyzed a total of 318 patients with advanced NSCLC, divided into *EGFR*-wt and *EGFR*-mut groups. All patients were unresectable, stage IIIB, or IV NSCLC (sixth TNM staging system) with histologic tissue and clinical data available. Samples were obtained at the time of diagnosis of metastatic disease in all cases. Written informed consent was provided by all patients. Approval was obtained from each hospital's institutional review board and ethics committee. The *EGFR*-wt group consisted of 125 tumor samples from patients diagnosed between 2006 and 2012 in six different European hospitals. We selected all those patients with enough tumor tissue available and complete follow-up who also fulfilled the following criteria (i) stage IIIB–IV NSCLC, (ii) no surgery, (iii) first-line platinum-based chemotherapy, (iv) *EGFR*-wt. The *EGFR*-mut group was composed of 193 patients from the Spanish Lung Adenocarcinoma Database (19) and the EURTAC clinical trial (20). Since several molecular biology studies had already been performed on the biopsies from these patients (21), many of them had insufficient tumor sample remaining. We therefore selected all the patients from these trials who had sufficient tumor tissue.

The validation cohort consisted of 64 tumor samples from *EGFR*-mut patients diagnosed between 2006 and 2012 in two different hospitals in Spain and Colombia. All were stage IIIB–IV, nonsurgical patients with complete follow-up.

Processing and dissection of tumor samples

All samples were formalin-fixed, paraffin-embedded pretreatment tumor tissues, stained with haematoxilin/eosin, and evaluated by an expert pathologist. The small percentage of specimens with more than 60% tumor infiltration in

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an area greater than 2.2 mm^2 were macrodissected; the rest of the samples were microdissected as previously described (21). In both cases, cells were dissected directly into 30 μL of PCR buffer (Ecogen) plus proteinase K (40 μg/mL) and incubated overnight at 60°C. Proteinase was inactivated by incubation at 100°C for 10 minutes and the resulting cell extract used for mutational analyses. Previous validation experiments had demonstrated that our genetic techniques can detect a mutation in *TP53*, *KRAS*, *BRAF*, and *PIK3CA* in samples containing as little as 10% tumor cells.

Analysis of *TP53* mutations

PCR followed by sequencing to determine mutations when they are distributed throughout a gene is an expensive, time- and sample-consuming procedure. Therefore, we set up and validated a high-resolution melting (HRM) technique to quickly and efficiently screen for mutations in exons 5 to 8 of *TP53*.

Cell extracts (1–2 μL) were mixed with 10 μL of MeltDoctor HRM Master Mix (Applied Biosystems), primers (0.3 μmol/L final, Supplementary Table S1) and, only in the case of exon 5b, MgCl2 (1 mmol/L additional). DNAs from cell lines with known *TP53* status were used as controls (Supplementary Table S2). All cell lines were purchased from the American Type Culture Collection (ATCC) and passaged for fewer than 6 months after resuscitation, except for the PC9 cell line, which was kindly provided by F. Hoffman-La Roche Ltd. ATCC performs cell line authentication by short tandem repeat profiling. In addition, all the cell lines were validated in our laboratory by repeatedly genotyping them for *EGFR*, *KRAS*, *PIK3CA*, *BRAF*, *TP53*, and *CTNNB1* genes. The genotyping was also performed in the same purified DNA samples that were used in our experiments. In all cases, the genotypes of the cells exactly matched those described in the COSMIC database.

HRM amplification reactions were carried out for 50 cycles (Supplementary Table S3). The melt analysis was performed with the 7900HT Fast Real-Time PCR System (Applied Biosystems). Conditions were 95°C for 1 second, 72°C for 90 seconds, and an HRM step rising from 72°C to 95°C at 0.1°C per second. The dissociation curves were analyzed using Applied Biosystems HRM Software v2.0 and the HRM products of all samples that seemed mutated or unclear were sequenced. Finally, all mutated samples were reconfirmed by standard PCR followed by Sanger sequencing (Supplementary Tables S1 and S3).

To address the issue of heterogeneity, we analyzed at least two separate tumor areas in a total of 74 of our samples (42 in the training and 32 in the validation cohort). The genotype of the different regions of the same sample was identical in all cases, indicating that the distribution of *TP53* mutations within the tumor was homogeneous.

Description of the mutations was based on the Universal Mutation Database for *TP53* (http://p53.fr/; ref. 13) and obtained using the MUT-*TP53* 2.0 Excel spreadsheet tool (22), which was also used to perform comparison of our dataset with previous publications using the mean and 95% confidence interval (CI) of p53 activity as measured by transactivation with the WAF1 promoter. In the validation cohort, the mean of our study was −1.27 for *EGFR*-wt and −1.34 for *EGFR*-mut, compared with −1.26 and −1.29 in the two comprehensive NSCLC studies reported in the tool.

Classification of *TP53* mutations

Mutations were classified as "disruptive" and "nondisruptive" according to Poeta and colleagues (14), with the only modification being that, for our analyses, glycine was considered to be a nonpolar side chain residue.

Detection of hotspot *KRAS*, *BRAF*, and *PIK3CA* mutations

Controls used for detection of *KRAS*, *BRAF*, and *PIK3CA* hotspot mutations are presented in Supplementary Table S2, primers and probes in Supplementary Table S4, and amplification conditions in Supplementary Table S5. Mutations in codons 12 and 13 of *KRAS* were examined by HRM analysis and reconfirmed by standard sequencing, as described for *TP53* mutations. Mutations V600E in *BRAF* and E542K, E545K and H1047R in *PIK3CA* were screened by an allelic discrimination assay. Amplification was carried out in 12.5-μL volumes using from 1 μL of DNA sample, 6.25 μL of Taqman Genotyping Master Mix (Applied Biosystems), 0.6 pmol of each primer, and 0.2 pmol of probes. All mutated samples were confirmed by standard PCR plus sequencing.

Statistical analyses

For patients from the EURTAC clinical trial, progression-free survival (PFS) and overall survival (OS) were calculated from time of randomization; maximum time between randomization and start of treatment was 15 days. For the remaining patients, PFS and OS were calculated from the start of treatment. Median PFS and OS were estimated using the Kaplan–Meier method and distribution curves were compared with a two-sided log-rank test. Cox regression univariate analysis was used to generate survival HRs. The effect of *TP53* nondisruptive mutations on OS was assessed by correlating all the studied covariates with interaction terms. χ^2 test or Fisher exact test were used when response was compared with prognostic factors. In the multivariate analysis of the training cohort, we included all standard covariates, although *KRAS* mutations and squamous histology were almost exclusively present in the *EGFR*-wt group. All statistical analyses were performed using the IBM Statistical Package for Social Science (SPSS) for Windows version 19. Level of significance was set bilaterally at 0.05.

Results

Patient characteristics and *TP53* status (training cohort)

Table 1 summarizes the characteristics of the 318 patients analyzed in the training cohort. Median follow-up was 14.36 months; 20.00 months for those still alive at the conclusion of the study. One hundred and twenty-five patients were *EGFR*-wt, and 193 were *EGFR*-mut. Mutations in the *EGFR* gene were significantly associated with female

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Table 1. Characteristics of the patients, according to *EGFR* status

	Training cohort			Validation cohort
Characteristic	***EGFR* wt ($n = 125$) (%)**	***EGFR* mut ($n = 193$) (%)**	***P* value[a]**	***EGFR* mut ($n = 64$) (%)**
Median age (range)	62 (29–82)	66 (29–86)	0.003	61 (21–87)
Gender			<0.001	
Male	93 (74.4)	47 (24.4)		22 (34.4)
Female	32 (25.6)	146 (75.6)		42 (65.6)
Smoking status			<0.001	
Never smokers	17 (14.3)	141 (73.1)		29 (72.5)
Current and former smokers	102 (85.7)	52 (26.9)		11 (27.5)
Unknown	6	0		24
Histology				
Squamous	34 (27.2)	1 (0.5)	<0.001[b]	2 (3.1)
Nonsquamous	91 (72.8)	192 (99.5)		62 (96.9)
Large cell	16 (12.8)	3 (1.6)		0 (0)
Adenocarcinoma	68 (54.4)	177 (91.7)		59 (92.2)
Undifferentiated	6 (4.8)	6 (3.1)		1 (1.6)
Others	1 (0.8)	6 (3.1)		2 (3.1)
Stage			0.67	
IIIB	11 (8.8)	14 (7.3)		5 (7.8)
IV	114 (91.2)	179 (92.7)		59 (92.2)
ECOG PS			0.004	
0	18 (15.3)	57 (29.8)		9 (15.8)
≥1	100 (84.7)	134 (70.2)		48 (84.2)
Unknown	7	2		7
Brain metastases			0.47	
Yes	16 (13.8)	21 (10.9)		21 (35.0)
No	100 (86.2)	172 (89.1)		39 (65.0)
Unknown	9	0		4
Bone metastases			0.09	
Yes	40 (34.5)	48 (24.9)		26 (41.3)
No	76 (65.5)	145 (75.1)		37 (58.7)
Unknown	9	0		1
Response			0.17	
CR	6 (5.4)	10 (6.0)		8 (13.1)
PR	45 (40.2)	90 (53.9)		36 (59.0)
SD	44 (39.3)	50 (29.9)		16 (26.2)
PD	17 (15.2)	17 (10.2)		1 (1.6)
NE	13	26		3
Responders (CR+PR)	51 (45.5)	100 (59.9)	0.02	44 (72.1)
Non responders (SD+PD)	61 (54.5)	67 (40.1)		17 (27.9)
TP53 mutated vs. wt			0.13	
P53 mutated (D+NoD)	43 (34.4)	50 (25.9)		17 (26.6)
wt	82 (56.6)	143 (74.1)		47 (73.4)
KRAS status (wt vs. mutated)			<0.001	
wt	94 (75.2)	144 (99.3)		NA
Mutated	31 (24.8)	1 (0.7)		
Not determined	0	48		
KRAS mutation			<0.001	
wt	94 (75.2)	144 (99.3)		NA
G12C	15 (12.0)	1 (0.7)		
Other mutations	16 (12.8)	0 (0.0)		

NOTE: KRAS mutations were not determined in the validation cohort, composed entirely of *EGFR*-mut patients.

Abbreviations: CR, complete response; D, disruptive; ECOG PS, Eastern Cooperative Oncology Group Performance Status; NA, not analyzed; NE, not evaluable; NoD, nondisruptive; PD, progressive disease; PR, partial response; SD, stable disease.

[a]*P* values calculated excluding the "unknown" or "not determined" samples.

[b]*P* value calculated for squamous versus nonsquamous.

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gender, never smoking status, older age, histology, and Eastern Cooperative Oncology Group Performance Status (ECOG PS) $\geq$1 (Table 1). More than 90% of *EGFR*-mut tumors were adenocarcinomas, whereas 27% of the *EGFR*-wt patients had squamous cell carcinomas. The percentage of samples carrying *TP53* mutations was higher in the *EGFR*-wt than in the *EGFR*-mut group (34.4% vs. 25.9%) but the difference was not significant ($P = 0.13$). All *EGFR*-wt patients received platinum-based chemotherapy, whereas 146 *EGFR*-mut patients received erlotinib and 47 chemotherapy (Supplementary Table S6). Response to first-line therapy was significantly better in the *EGFR*-mut group than in the *EGFR*-wt group ($P = 0.02$). Finally, 72 *EGFR*-wt and at least 90 *EGFR*-mut patients received two or more lines of therapy (Supplementary Table S6).

Mutations in the *TP53* gene were not significantly associated with gender, histology, stage (IIIB or IV), PS or presence of brain, and bone metastases in either the *EGFR*-wt or the *EGFR*-mut group (Supplementary Table S7). About smoking history, the frequency of *TP53* mutations was significantly higher in former or current smokers only in the *EGFR*-wt group. The different types of *TP53* mutations detected in our study and their distribution are shown in Table 2, Supplementary Table S8, and Supplementary Figs. S1 and S2. Remarkably, there were differences in the types of *TP53* mutations between the two groups of patients. Frameshift and in-frame deletions were exclusively found in *EGFR*-mut patients, where they accounted for 18% of the total number of mutations. Transversions (replacing a purine by a pyrimidine or vice versa) were predominant in *EGFR*-wt patients, whereas transitions (that change a purine by a purine or a pyrimidine by a pyrimidine) made up the majority of point mutations in *EGFR*-mut patients. Tobacco smoking is known to induce transversions (23), and the percentage of former and current smokers was significantly higher in the *EGFR*-wt group than in the *EGFR*-mut group (85.7% vs. 26.9%, $P < 0.001$).

Two recent reports have shown that *KRAS* mutations are associated with shorter survival in chemotherapy-treated advanced NSCLC (24, 25). Therefore, samples were analyzed for *KRAS* mutations (exons 12–13). Where material was still available, *BRAF* and *PIK3CA* hotspots were also tested (Supplementary Fig. S3). As expected, only one of the 32 *KRAS*-mutated patients had a concomitant *EGFR*-mut (Supplementary Table S7). Finally, mutations in the *PIK3CA* gene were infrequent and only one patient had a *BRAF* mutation (Supplementary Table S6).

Patient characteristics and *TP53* status (validation cohort)

The characteristics of the 64 patients analyzed in the validation cohort are presented in Table 1. Median follow-up was 27.32 months for all patients and 29.32 months for those still alive at the conclusion of the study. All patients were *EGFR*-mut, and 92.2% had adenocarcinomas. The percentage of samples carrying *TP53* mutations was similar to that observed among the *EGFR*-mut patients of the training cohort (26.6% vs. 25.9%). The profile of *TP53* mutations was also comparable; 12% were deletions (Table 2) and 60% of point mutations were transitions (Supplementary Fig. S2). First-line treatment was erlotinib or gefitinib in 89% of the patients and 59.5% received at least two lines of therapy (Supplementary Table S6).

Survival and clinical characteristics, *EGFR*, and *KRAS* mutational status

For all 318 patients in the training cohort, survival was significantly associated with conventional prognostic factors (Table 3). OS was 26.5 months in the *EGFR*-mut group versus 12.8 months in the *EGFR*-wt group ($P < 0.001$, Supplementary Fig. S4). Within the *EGFR*-wt group, OS was significantly associated with gender, smoking history, histology, and presence of bone metastases. The G12C mutation correlated with a significantly worse outcome in this group, whereas the rest of the mutations in the *KRAS* gene did not

Table 2. Summary of *TP53* mutations identified in our study, classified according to their functional effects

	Training cohort		Validation cohort
Type of mutation	*EGFR* wt ($n = 43$)	*EGFR* mutated ($n = 50$)	*EGFR* mutated ($n = 17$)
Disruptive	15 (35%)	24 (48%)	6 (35%)
Frameshift deletions	0	5 (10%)	2 (12%)
In-frame deletions within L2-L3	0	3 (6%)	0
Nonsense	8 (19%)	1 (2%)	3 (17%)
Missense within L2-L3[a]	7 (16%)	13 (26%)	1 (6%)
Intronic	0	2 (4%)	0
Nondisruptive	28 (65%)	26 (52%)	11 (65%)
In-frame deletions outside L2-L3	0	1 (2%)	0
Missense[b]	28 (65%)	25 (50%)	11 (65%)

[a]Replacing a residue with another of a different polarity or charge.
[b]Outside the L2-L3 loops or, if within L2-L3, replacing a residue with another of the same polarity or charge.

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Table 3. Results of the univariate and multivariate analyses of selected factors for OS in the training cohort

		Univariate analysis		Multivariate analysis	
Variable	***N***	**HR for death (95% CI)**	***P* value**	**HR for death (95% CI)**	***P* value**
EGFR status					
Mutated	193	1		1	
wt	125	1.96 (1.46–2.62)	<0.001	1.22 (0.77–1.93)	0.41
Age	315	0.99 (0.98–1.01)	0.69	1.01 (0.98–1.02)	0.84
Gender					
Female	178	1		1	
Male	140	1.77 (1.32–2.36)	<0.001	1.29 (0.85–1.94)	0.24
Smoking status					
Never smokers	17	1		1	
Current + former smokers	102	1.78 (1.33–2.40)	<0.001	1.18 (0.74–1.88)	0.48
Histology					
Nonsquamous	283	1		1	
Squamous	35	2.49 (1.67–3.72)	<0.001	2.32 (1.32–4.08)	0.003
Stage					
IIIB	25	1		1	
IV	293	1.70 (0.95–3.05)	0.08	2.08 (0.75–5.78)	0.16
ECOG PS					
0	75	1		1	
≥1	234	1.83 (1.26–2.65)	0.001	2.03 (1.24–3.34)	0.005
Brain metastases					
No	272	1		1	
Yes	37	1.46 (0.95–2.24)	0.09	1.56 (0.94–2.58)	0.09
Bone metastases					
No	221	1		1	
Yes	88	1.80 (1.32–2.46)	<0.001	2.25 (1.53–3.29)	<0.001
TP53					
wt+ D	264	1		1	
NoD	54	2.08 (1.46–2.97)	<0.001	1.89 (1.09–3.26)	0.02
KRAS status					
wt+ others	254	1		1	
G12C	16	2.19 (1.42–3.36)	<0.001	2.10 (1.19–3.71)	0.01

Abbreviations: D, disruptive; ECOG PS, Eastern Cooperative Oncology Group Performance Status; NoD, nondisruptive.

(Supplementary Table S9). OS for the 15 patients with a G12C mutation was 7.6 months, compared with 14.4 months for patients with other genotypes ($P = 0.03$; Supplementary Fig. S5). The number of patients harboring *PIK3CA* or *BRAF* mutations was too low for reliable statistical analyses. Finally, in the *EGFR*-mut group, only an ECOG PS of 0 was significantly associated with a better OS (Supplementary Table S9).

Survival and *TP53* mutations

In the training cohort, the 93 patients with *TP53* mutations had a median OS of 17.5 months versus 22.9 months for the 225 *TP53*-wt patients (Supplementary Fig. S6), but the difference was not statistically significant (HR, 1.45; CI 95%, 0.95–2.22; $P = 0.09$). However, because different types of mutations in the *TP53* gene are known to have different effects on the functionality of the protein, we classified *TP53* mutations into disruptive and nondisruptive and found that patients with advanced NSCLC with these two types of *TP53* mutation constitute distinct prognostic groups. In the training cohort, the median OS in patients with nondisruptive mutations was 13.3 months compared with 24.6 months in patients *TP53*-wt or carrying disruptive mutations (HR, 2.08; 95% CI, 1.46–2.97; $P < 0.001$; Fig. 1, Table 3). The association of nondisruptive mutations with shorter survival was maintained when the patients were divided according to *EGFR* status. In the *EGFR*-wt group, median OS was 8.5 months for patients with nondisruptive *TP53* mutations versus 15.6 months for other patients (HR, 2.04; 95% CI, 1.27–3.26; $P = 0.003$). In the *EGFR*-mut group, OS was 17.8 months for patients with *TP53* nondisruptive mutations compared with 28.4 months for the remaining patients (HR, 1.79; 95% CI, 1.02–3.13; $P = 0.04$; Fig. 2, Supplementary Table S9).

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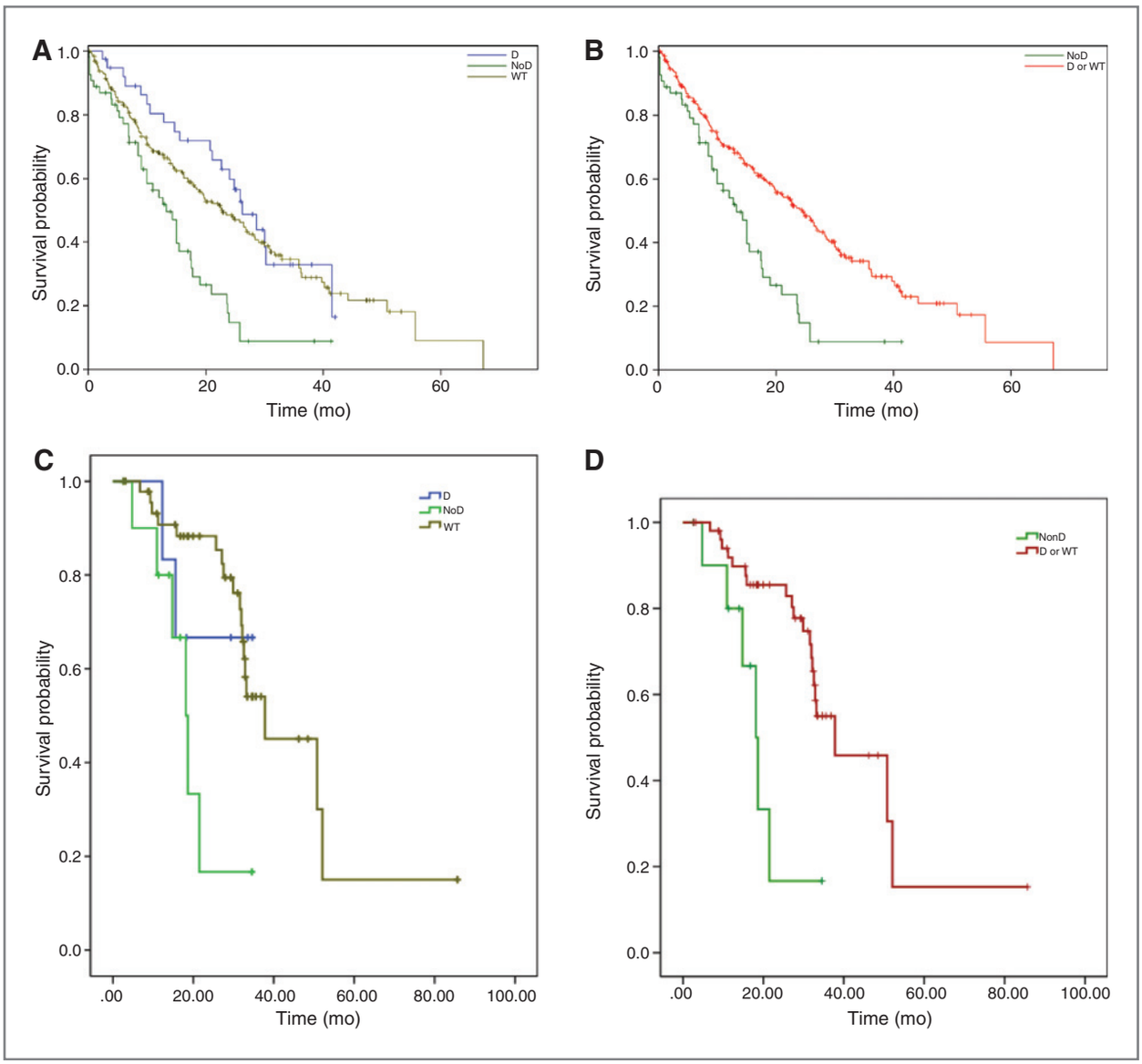

Figure 1. Kaplan–Meier plots of OS among patients in the training and validation cohorts, according to *TP53* mutation status. A, median survival for patients in the training cohort with nondisruptive mutations (green; $N = 54$, of whom 40 died) was 13.3 months, compared with 26.2 months for patients with disruptive mutations (blue; $N = 39$, of whom 21 died) and 22.9 months for patients with wt *TP53* (brown; $N = 225$, of whom 126 died; $P < 0.001$). B, median survival for patients in the training cohort with nondisruptive mutations was 13.3 months (green), whereas for the remaining patients it was 24.6 months (red; $P < 0.001$). C, median survival for patients in the validation cohort (all of them *EGFR*-mut) with nondisruptive mutations ($n = 11$, of whom 6 died) was 18.1 months, compared with 37.8 months for *TP53*-wt patients ($n = 47$, of whom 18 died) and not reached among patients with disruptive mutations ($n = 6$, of whom 2 died; $P = 0.01$). D, median survival for *EGFR*-mut patients in the validation cohort with nondisruptive mutations was 18.1 months, whereas it was 37.8 months for the remaining patients ($P = 0.006$).

Because we had found that the *KRAS* G12C mutation was associated with worse outcome in *EGFR*-wt patients, we excluded the 15 patients carrying this mutation from the analysis of the *EGFR*-wt population. For the remaining patients, OS was 9.0 months for those with nondisruptive mutations, compared with 22.6 months for those with disruptive mutations and 16.3 months for those *TP53*-wt ($P = 0.005$; Supplementary Fig. S7).

In the multivariate Cox proportional-hazard model (Table 3), the presence of a nondisruptive *TP53* mutation was significantly associated with decreased OS (HR, 1.89; 95% CI, 1.09–3.26; $P = 0.02$) after adjustment for all covariates. The interaction terms of nondisruptive *TP53* mutations with the rest of the covariates were not statistically significant. The presence of a nondisruptive *TP53* mutation remained an independent prognostic factor for shorter OS in the *EGFR*-wt group (HR, 1.78; 95% CI, 1.03–3.07; $P = 0.04$). Squamous histology also emerged as an independent prognostic factor in this group. In the *EGFR*-mut patients, only ECOG PS ≥ 1

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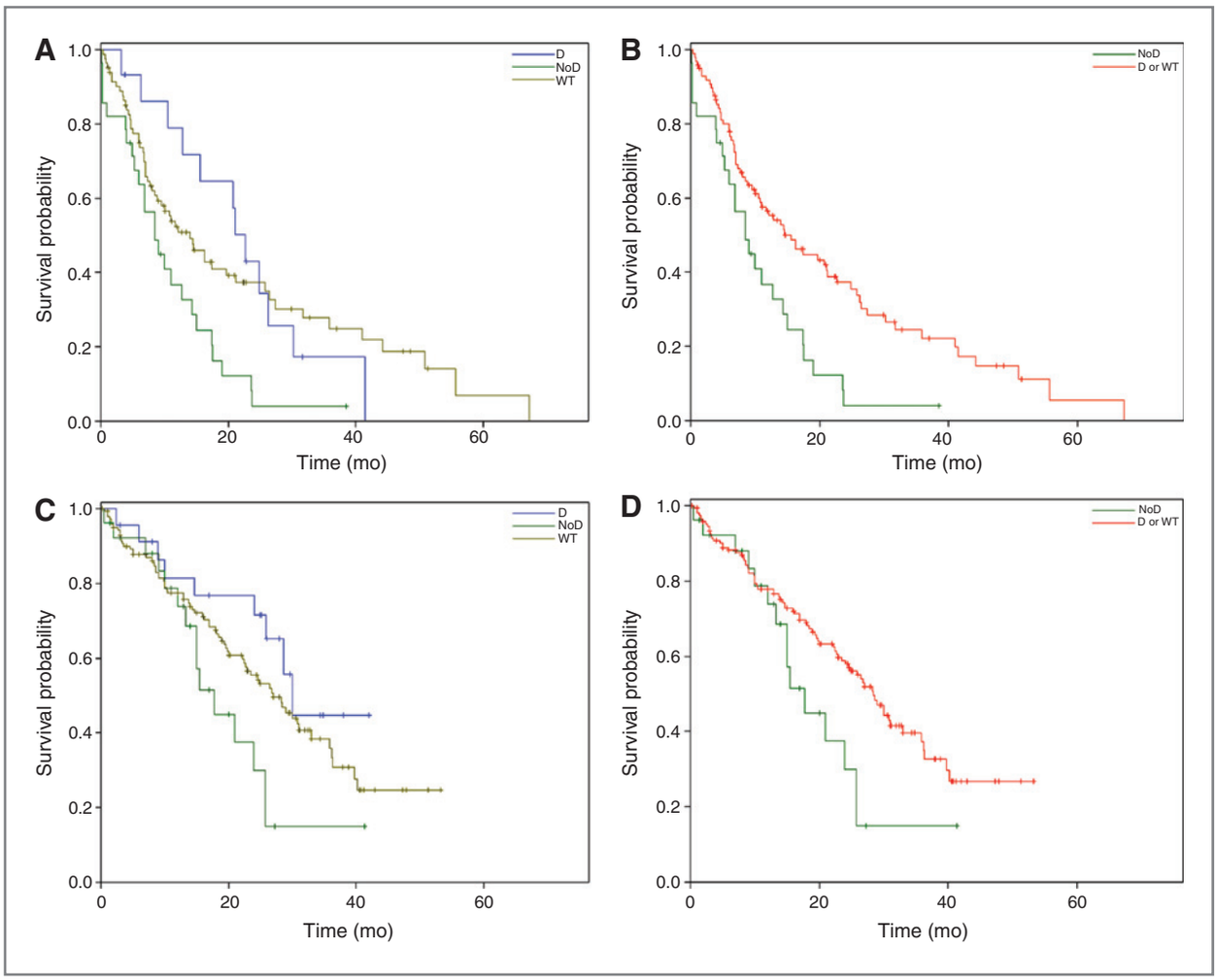

Figure 2. Kaplan–Meier plots of OS for the *EGFR*-wt and *EGFR*-mut populations in the training cohort, according to *TP53* mutation status. A, median survival for *EGFR*-wt patients in the training cohort with nondisruptive mutations (green; $N = 28$, of whom 25 died) was 8.5 months, compared with 22.6 months for patients with disruptive mutations (blue; $N = 15$, of whom 12 died) and 14.0 months for the patients with wt *TP53* (brown; $N = 82$, of whom 56 died; $P = 0.01$). B, median survival for *EGFR*-wt patients in the training cohort with nondisruptive mutations was 8.5 months (green), compared with 15.6 months (red) for the rest of the patients ($P = 0.003$). C, median survival for *EGFR*-mut patients in the training cohort with nondisruptive mutations ($n = 26$, of whom 15 died) was 17.8 months, compared with 27.0 months for *TP53*-wt patients ($n = 143$, of whom 70 died) and 30.0 months among patients with disruptive mutations ($n = 24$, of whom 9 died; $P = 0.061$). D, median survival for *EGFR*-mut patients in the training cohort with nondisruptive mutations was 17.8 months, whereas it was 28.4 months for the rest of the patients ($P = 0.04$).

was associated with survival, but the presence of a nondisruptive *TP53* mutation almost reached statistical significance (HR, 1.71; 95% CI, 0.97–3.02; $P = 0.06$; Supplementary Table S10).

The association of nondisruptive mutations with shorter survival was maintained in the validation cohort of stage IIIB–IV *EGFR*-mut patients, that had a median follow-up considerably longer than the training cohort (27.32 vs. 14.36 months). The OS for the 11 patients with *TP53* nondisruptive mutations was 18.1 months versus 37.8 months for the 53 patients *TP53*-wt or carrying disruptive mutations (HR, 3.84; 95% CI, 1.46–10.11; $P = 0.006$; Fig. 1, Table 4). In the multivariate analysis, the presence of a nondisruptive *TP53* mutation emerged as the only independent prognostic factor for OS (HR, 6.11; 95% CI, 1.43–26.09; $P = 0.01$) when either a backward of a forward stepwise selection variable procedure was carried out (Table 4).

Response, PFS, and *TP53* mutations

Among all 318 patients in the training cohort, median PFS was 7.0 months in the *TP53* nondisruptive group versus 8.3 months for the remaining patients ($P = 0.05$; Supplementary Fig. S8). However, no difference of PFS was observed when the patients were divided in the *EGFR*-wt and *EGFR*-mut groups (Supplementary Fig. S9). When patients were stratified by treatment type rather than *EGFR* mutation status, among the 172 patients that received erlotinib, PFS was 11.0

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Table 4. Results of the univariate and multivariate analyses of selected factors for OS in the in the validation cohort

		Univariate analysis		Multivariate analysis	
Variable	***N***	**HR for death (95% CI)**	***P* value**	**HR for death (95% CI)**	***P* value**
Age	63	0.98 (0.95–1.01)	0.24	0.99 (0.95–1.03)	0.60
Gender					
Female	42	1		1	
Male	22	1.27 (0.56–2.89)	0.57	1.60 (0.57–4.46)	0.37
Stage					
IIIB	5	1		1	
IV	59	2.14 (0.45–10.14)	0.34	0.66 (0.04–10.38)	0.76
ECOG					
0	9	1		1	
≥1	48	1.52 (0.44–5.30)	0.51	3.15 (0.60–16.73)	0.18
Brain metastases					
No	39	1		1	
Yes	21	0.86 (0.35–2.11)	0.75	0.99 (0.35–2.81)	0.99
Bone metastases					
No	37	1		1	
Yes	26	1.70 (0.74–3.88)	0.21	1.44 (0.46–4.53)	0.53
TP53					
wt+ D	53	1		1	
NoD	11	3.84 (1.46–10.11)	0.006	6.11 (1.43–26.09)	0.01

NOTE: All patients in the validation cohort were *EGFR*-mut. *EGFR* and *KRAS* status were not included in the analyses. Smoking history was also excluded due to the high number of patients with no data available.
Abbreviations: D, disruptive; NoD, nondisruptive.

months for those harboring *TP53* nondisruptive mutations versus 15.0 months for the remaining patients ($P = 0.14$). No difference in PFS was observed according to the type of *TP53* mutation among the 146 patients treated with chemotherapy (Supplementary Fig. S10).

Finally, no association between nondisruptive *TP53* mutations and response to therapy was observed. In the training cohort, 23 of the 54 patients (50%) with nondisruptive mutations had complete or partial responses to therapy, compared with 128 among the remaining 264 patients (55%; $P = 0.63$).

Discussion

In the present study, we have analyzed *TP53* mutations in a large cohort of patients with advanced, nonresectable NSCLC and we provide evidence of an association of *TP53* status and OS. Our results demonstrate that nondisruptive *TP53* mutations define a distinct prognostic group of patients with significantly shorter survival. In contrast, those patients with disruptive mutations showed a nonsignificant trend toward better OS, compared with *TP53*-wt patients. In the multivariate analysis, the HRs for nondisruptive *TP53* mutations were similar to those obtained for other widely used markers of poor prognosis, such as the PS ≥ 1. These findings, which have been validated in an independent cohort, indicate that *TP53* nondisruptive mutations could be a clinically useful prognostic marker in advanced NSCLC.

It is now universally accepted that *EGFR* mutations define two types of NSCLC with different biology, therapeutic options, and outcome (19). In the present study, we have found that patients with the G12C *KRAS* mutation also have significantly worse outcome. Although the issue of *KRAS* mutations in lung cancer is controversial, a recent study of 484 patients found that they are significantly associated with shorter survival in advanced NSCLC (OS of 7.7 for patients with the G12C mutation vs. 15.0 months for those *KRAS* wt; ref. 24). In the present study, the prognostic value of *TP53* nondisruptive mutations was not dependent on *EGFR* or *KRAS* status, and was observed both in chemotherapy-treated, *EGFR*-wt patients as well as in *EGFR*-mut patients treated in first line with erlotinib or chemotherapy.

We found *TP53* mutations in 34% (43/125) of the *EGFR*-wt patients, in accordance with the frequency described in the COSMIC database for NSCLC (8). The frequency of *TP53* mutations dropped to 26% in the *EGFR*-mut patients. Patients with tobacco-associated lung cancer have a higher frequency of *TP53* mutations than patients who never smoked (26). Tobacco-associated lung cancer is also characterized by a higher number of transversions in the *TP53* gene, whereas a high percentage of transition mutations are found in never smokers. In our study, 63% of the *TP53* point mutations in the *EGFR*-wt group were transversions,

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compared with 53% (training cohort) or 60% (validation cohort) of transitions in the *EGFR*-mut group. In addition, frameshift or in-frame deletions were found exclusively in *EGFR*-mut patients.

Although most studies on the prognostic role of p53 in human cancers have only dealt with wt versus mutated patients, recent reports have demonstrated the usefulness of categorizing *TP53* mutations since different mutant proteins can have widely disparate biologic effects (14, 17, 18). Our study strongly supports this new approach: we found that only nondisruptive mutations were significantly associated with worse OS, whereas *TP53* mutations as a whole did not significantly correlate with outcome.

A weak prognostic role for *TP53* mutations in NSCLC has been suggested by two meta-analyses (27, 28), but the issue is still controversial. Reports are contradictory probably due to the fact that some investigators consider *TP53* mutations as a whole, whereas others do categorize them, but use a wide range of criteria. Most authors have analyzed early-stage, surgically resected tumors where sufficient tissue is available for mutational testing by standard techniques. When *TP53* mutations were uncategorized, some studies found no association with outcome (29) or only identified a trend, which was lost in the multivariate analysis (30). Other studies observed an association between *TP53* mutations and response to adjuvant therapy (31) or with shorter OS, either alone (32; only in stage I disease) or in combination with other molecular markers (33). In studies of early-stage NSCLC, a prognostic value for particular types of *TP53* mutations is usually found, although results are contradictory. Truncated, but not missense, mutations have been associated with shorter PFS or OS in some studies (34, 35), whereas others have reached the opposite conclusion (36). Finally, some authors have reported an association between shorter OS and the presence of particular types of *TP53* mutations, such as "severe flexible and contact" mutants (37), "truncated, structural, and DNA contact mutations" (38), or mutations in particular exons and codons (39, 40). In the case of advanced NSCLC, a significant percentage of patients cannot be biopsied and, in those where a biopsy is feasible, the amount of tumor tissue obtained is often scarce and can be easily consumed by routine testing. To the best of our knowledge, only two reports in stage IIIB–IV NSCLC have analyzed the clinical relevance of *TP53* mutations, but both studies included a limited number of patients and did not classify the mutations. One of these studies found an association of *TP53* mutations with shorter median OS in 70 cases of advanced NSCLC (4 vs. 9 months; ref. 41), whereas the other reported no association in a population of 88 patients (42). Taken together, all these discordant reports highlight the importance of establishing a widely accepted, clinically relevant classification of *TP53* mutations that can allow the comparison of different studies. Our results indicate that the disruptive/nondisruptive categorization can fulfill this criterion.

It remains to be explained why nondisruptive mutations, that likely result in a p53 protein which can retain some functionality, are associated with shorter OS in advanced NSCLC, whereas disruptive mutations are not. Nondisruptive mutations could be more frequent in smokers whose tumors develop a high genetic instability (29) and other, as yet unknown, genetic alterations may be responsible for the worse outcomes. This would make our findings spurious, positioning nondisruptive *TP53* mutations as a bystander of those alterations. However, several lines of evidence suggest that this is not the case and that nondisruptive *TP53* mutations are responsible for the poor outcome of the patients.

First, nondisruptive mutations were predictive of shorter survival in the *EGFR*-mut patients, both in the training and in the validation cohorts. More than 70% of these patients were never smokers, whose tumors have a lower genetic instability and fewer mutations than tobacco-associated lung cancer. In addition, in contrast with the *EGFR*-wt group, the frequency of *TP53* mutations among the *EGFR*-mut patients did not depend on the smoking status (Supplementary Table S7). Furthermore, nondisruptive mutations represented 65% of the *TP53* mutations in the validation cohort, composed entirely of *EGFR*-mut patients, a majority of whom are never smokers.

Second, p53 is a "master protein" that regulates multiple cell processes. It plays a key role in tumorigenesis and *TP53* mutations are well-known drivers in many types of human cancer. Experimental evidence shows that many nondisruptive mutations, rather than causing simple loss of function of wt p53, induce GOF activities that can be exerted through direct transcriptional regulation or through inactivation of p63/p73 (12). These GOF activities are dominant over the *TP53*-wt allele and lead to increased tumorigenicity, growth rate, motility, metastasis and invasiveness, and decreased chemosensitivity in cell models (43). However, these phenotypes do not always appear together and different p53 mutants have been demonstrated to have different, complex patterns of GOF. At least 11 of the nondisruptive mutations found in our study (Supplementary Table S8) have been shown to induce GOF activities in cell models (Supplementary Table S11), including hotspot mutations such as R175H or R273H. Examples of these GOF activities are the downregulation of apoptotic (*FAS*) and cell-arrest (*CDKN1A*) genes and the upregulation of immortalizing (*TERT*), mitogenic (*EGR1*, *MYC*), stress-protective (*HSPA1A*), angiogenic (*ANGPT1*), or drug resistance (*ABCB1*, *AXL*) genes. Overexpression of the Axl tyrosine-kinase receptor (43), associated with the epithelial-to-mesenchimal transition, leads to resistance to tyrosine-kinase inhibitors.

The tumorigenic GOF activities can explain the worse survival observed in patients carrying nondisruptive *TP53* mutations. In contrast, disruptive mutations are less likely to acquire GOF activity, which can explain why these mutations do not affect survival in advanced NSCLC. In addition, many of them are missense or frameshift mutations that lead to a truncated p53 protein that might be unable to form tetramers and thus to exert a dominant-negative effect (44). A wide-range analysis of mRNA and

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miRNA can determine what particular sets of genes show an altered expression in patients with different types of *TP53* mutations and can further refine the prognostic significance of such mutations.

The number of published studies that have used the disruptive/nondisruptive categorization of *TP53* mutations is very limited and all of them have been performed in surgically resected head and neck squamous cell carcinoma (HNSCC). In the first report proposing this classification, Poeta and colleagues (14) found that nondisruptive mutations were associated with shorter OS (3.9 vs. 5.4 years for the *TP53*-wt patients), although the difference did not reach statistical significance. In contrast, patients with disruptive mutations showed a significantly shorter OS (2.0 years). These results were confirmed in an independent cohort of patients (45). *TP53* disruptive mutations also led to treatment failure through locoregional recurrence in HNSCC (46). In contrast with these studies, we found no association of *TP53* disruptive mutations with a shorter OS in advanced NSCLC. Several reasons might help to explain this discrepancy. First, *TP53* mutations can function differently in different cell contexts. For instance, the R175H nondisruptive mutant upregulated the transcription of the human telomerase reverse transcriptase gene in osteosarcoma cells, whereas it had no effect on a lung adenocarcinoma cell line (13). Second, we analyzed stage IIIB–IV, nonresectable lung cancer, while the patients in the HNSCC studies were all surgically resected. Several biomarkers are known to have opposing prognostic values in early and advanced tumors, such as the DNA repair gene *ERCC1*. Overexpression of ERCC1 was associated with adverse prognosis in advanced-stage NSCLC (47) but correlated with longer OS in early-stage, surgically resected patients (48). High levels of ERCC1 expression indicate an efficient DNA repair system that can prevent the appearance of new genetic alterations that promote invasiveness and metastases, thus explaining its association with better outcome in early-stage, resected tumors. In contrast, in advanced, nonresectable NSCLC, where the standard treatment is platinum-based chemotherapy, a proficient DNA repair capacity within the tumor can eliminate the adducts generated by platinum and therefore correlate with a poor prognosis. Similar mechanisms may be operating in disruptive mutations in *TP53*, which can drastically reduce the DNA repair capacity of tumor cells (49).

Although our study shows a prognostic impact of nondisruptive *TP53* mutations in advanced NSCLC, it also indicates that they have no predictive value. We found no significant association of any type of *TP53* mutations with duration or type of response, although there was a trend toward shorter PFS in erlotinib-treated patients carrying nondisruptive mutations. We lack an adequate explanation for this finding. However, we can hypothesize that most GOF activities of the nondisruptive *TP53* mutations in our study could promote an aggressive behavior of the tumor after progression rather than induce a quicker resistance to drugs. In transformed cell models, resistance to DNA-damaging chemotherapeutic agents is not necessarily associated with increased proliferating or metastatic capacity, at least in some GOF p53 mutants (50).

In conclusion, we have demonstrated that nondisruptive mutations in *TP53* are an independent prognostic factor of shorter survival in advanced NSCLC. Clinical trials are warranted to determine whether patients with this type of mutation would benefit from drugs that reactivate mutant p53.

Disclosure of Potential Conflicts of Interest

B. Massuti reports receiving speakers bureau honoraria from and is a consultant/advisory board member for Roche. No potential conflicts of interest were disclosed by the other authors.

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