Patterns of *EGFR, HER2, TP53*, and *KRAS* Mutations of $p14^{arf}$ Expression in Non–Small Cell Lung Cancers in Relation to Smoking History

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Abstract

Mutations in the tyrosine kinase domain of the epidermal growth factor receptor *EGFR* are common in non–small cell lung cancer (NSCLC) of never smokers, whereas *HER2* mutations are rare. We have analyzed EGFR and HER2 mutations and the expression of the two products of the *CDKN2A* gene ($p14^{arf}$ and $p16^{INK4a}$) in 116 NSCLC that have been previously analyzed for TP53 and KRAS mutations in relation to smoking history of patients. *EGFR* mutations were detected in 20 of 116 (17%) tumors, whereas five (4.3%) tumors contained *HER2* mutations. No tumor contained both mutations. Of tumors with *EGFR* or *HER2* mutation, 72% were adenocarcinomas, 68% were from never smokers, and 32% were from former smokers. *EGFR* but not *HER2* mutations were mutually exclusive with *KRAS* mutation. Among never smokers, 11 of 16 tumors with *EGFR* mutation also had *TP53* mutation, in contrast with two of 17 tumors without *EGFR* mutation ($P = 0.0008$). Expression of $p14^{arf}$, but not $p16^{ink4a}$, was more frequently down-regulated in never smokers (62.5%) than ever smokers (35%; $P = 0.008$). All tumors with *EGFR* or *HER2* mutations and wild-type *TP53* showed down-regulation of $p14^{arf}$ expression. These observations suggest that functional inactivation of the $p14^{arf}$/p53 connection is required in tumors with *EGFR* or *HER2* mutations, consistent with the notion that these proteins are part of a fail-safe mechanism protecting cells against untimely or excessive mitotic signals. [Cancer Res 2007;67(12):5667–72]

Introduction

Lung cancer accounts for over a million annual deaths worldwide, ~90% attributable to smoking. Non–small cell lung cancer (NSCLC) represents 75% of primary lung cancers and includes squamous cell carcinomas (SCC), adenocarcinomas (ADC), and large-cell carcinomas (LCC). Irrespective of histologic type, common genetic alterations in NSCLC are mutations in *TP53*, defects in the *CDKN2a/RB* pathway, loss of alleles on chromosome 3p encompassing *FHIT, SEMA3B*, and *RASSF1A*, and aberrant promoter methylation in O^6*MGMT, CDKN2a, DAPK, TIMP-3*, or *RASSF1A*. Mutations at codon 12 in *KRAS* are found in 30% to 40% of ADC but are rare in other histologies (1). In NSCLC of smokers, there is a characteristic pattern of *TP53* mutations (2, 3), compatible with the effects of DNA damage by tobacco carcinogens, such as polycyclic aromatic hydrocarbons. Mutations in *EGFR*, encoding the epidermal growth factor receptor, have emerged as a frequent molecular alteration in NSCLC of never smokers. These mutations have attracted considerable clinical interest due to their association with tumor sensitivity to the antiproliferative effects of small-molecule tyrosine kinase (TK) inhibitors, erlotinib and gefinitib (4–6). Most of described *EGFR* mutations (85%) fall into two categories: point mutations in exon 21 (L858R; 40%) and in-frame deletions of two to nine residues in exon 19, encompassing residues of a conserved LREA motif (residues 748–751; 45%; ref. 7). Other mutations include rare insertions in exon 20, a minor hotspot at exon 18 (5%) and scattered missense mutations in exons 18 to 21. There is structural and biochemical evidence that the L858R mutation and the short deletions in exon 19 modify the geometry of the ATP binding cleft in the TK of *EGFR* (7), resulting in a hyperactive form of the receptor. *EGFR* mutations are inversely correlated with tobacco consumption and are reportedly frequent in ADC of women of Asian descent (8). R1-C1: one sentence mutations in *EGFR* may coexist with mutations in *TP53* (9, 10) but are mutually exclusive of mutations in *KRAS*, in agreement with the notion that *KRAS* is a downstream effector of *EGFR* signaling. Moreover, *HER2* (a member of the ERBB family to which *EGFR* also belongs) is dysregulated in many cancers. The most common alteration of *HER2* is overexpression with amplification, which is frequent in breast and ovarian cancers and is associated with poor prognosis (11). *HER2* can dimerize with other members of the ERBB family. The strongest and the most powerful heterodimer formed is *EGFR/HER2* (12). Recent studies have reported that mutations in the TK domain of *HER2* are detected in lung cancers (13).

In this retrospective study, we have analyzed *EGFR* (exons 18–21) and *HER2* (exons 19 and 20) mutations in 116 NSCLC of well-characterized current, former, and never smokers, analyzed previously for *KRAS* and *TP53* mutations (2). In parallel, we have assessed by immunohistochemistry the expression of $p14^{arf}$ and $p16^{ink4a}$, the products of the *CDKN2a* locus (10, 14). $p16^{ink4a}$ is a cyclin kinase inhibitor that controls cyclin-dependent kinase 4/cyclin D1 complexes during progression into the G_1 phase of the cell cycle. $p14^{arf}$ exerts complex functions in cell cycle, apoptosis, and DNA repair (15). Its expression is regulated by *E2F*, a transcription factor activated as end product of the growth signaling cascade initiated by *EGFR* (16, 17). $p14^{arf}$ complexes with and neutralizes Mdm2, the main controller of wild-type (WT) p53 protein stability, thus allowing p53 to escape degradation and to

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Table 1. Gender, smoking status, and histology of patients and lung tumors

	All subjects (N = 116)	Never smokers (n = 33)	Former smokers (n = 38)	Current smokers (n = 45)
	n (%)	n (%)	n (%)	n (%)
Gender				
Female	31 (27)	29 (88)	2 (5)	0 (0)
Male	85 (73)	4 (12)	36 (95)	45 (100)
Tumor histology				
ADC	48 (41)	25 (76)	16 (42)	7 (16)
SCC	51 (44)	2 (6)	16 (42)	33 (73)
ADC-SCC	17 (15)	6 (18)	6 (16)	5 (11)

NOTE: Smoking status was defined as follows: never smokers, less than 100 cigarettes smoked in a lifetime; former smokers, smoking cessation for 2 yrs or more before diagnosis; and current smokers, over 35 pack-years.

exert antiproliferative effects. We reasoned that $p14^{arf}$ represents an important connection between the *EGFR* and/or *HER2* signaling pathways and the p53-dependent suppressive machinery. Here, we show that mutations in *EGFR* as well as *HER2* are associated with *TP53* mutation and/or with deficiency of $p14^{arf}$ expression in NSCLC of never and former smokers. This observation indicates that inactivation of the $p14^{arf}$/p53 connection is required for the development of tumors containing *EGFR* or *HER2* mutations.

Materials and Methods

Study subjects and tumors. The settings of the study have been described previously (2). The study is based on the INCO Central Europe Health Study, a hospital-based case-control study on lung cancer involving 600 cases and 600 controls. Each participant answered a questionnaire on lifestyle (residence, past health, direct and indirect exposure to tobacco, diet, alcohol consumption, sexual history, and occupational exposure to selected cancer risk factors). NSCLC from a total of 116 subjects from the Moscow area were selected into three groups: 33 never smokers (less than 100 cigarettes smoked in a lifetime; with or without exposure to involuntary smoking from the spouse or at workplace), 38 former smokers (smoking cessation for 2 years or more before diagnosis; average cumulative tobacco smoking, 27.6 pack-years), and 45 current smokers. The latter category was composed of heavy smokers (over 35 pack-years; average consumption, 48.8 pack-years). The characteristics of the patients and their cancers are summarized in Table 1.

Mutation analysis. DNA was extracted from areas of fresh formalin-fixed, paraffin-embedded tumor sections as selected by the pathologist and analyzed for *TP53* (exons 4–10) and *KRAS* mutations (codon 12) as described elsewhere (2). *EGFR* mutations were detected using PCR-based direct sequencing of the four exons of the TK domain (exons 18–21) using primers and annealing conditions as described by Pao et al. (6). *HER2* was amplified using the following sense and antisense primers for exons 19 and 20: 5′-GGATCCAGCCCACGCTCTT-3′ (19 forward) and 5′-CTGCAGC-CATGGGGTCCTT-3′ (19 reverse) and 5′-CCATACCCTCTCAGCGTA-3′ (20 forward) and 5′-GCTCCGGAGAGACCTGCAA-3′ (20 reverse). Amplifications were done by using a touchdown protocol from 65°C to 62°C for exon 19 and from 61°C to 58°C for exon 20.

Aliquots of PCR products were examined by electrophoresis on 2% agarose gel containing ethidium bromide. PCR products were treated with 2 μL ExoSAP-IT (Amersham Biosciences) at 37°C for 15 min followed by inactivation at 80°C for 15 min and direct sequencing using Applied Biosystems Prism dye terminator cycle sequencing method (Perkin-Elmer) on ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Immunochemistry. Deparaffinized tissue sections were labeled with specific antibodies against $p16^{ink4a}$ and $p14^{arf}$. The monoclonal antibody $p16^{INK4a}$ (16P07) (Neomarkers) was used at 1:200 dilution after 60-min Tris-citrate (pH 6) retrieving treatment on a Ventana automated system. The secondary antibody was biotinylated antibody (goat anti-rabbit/goat anti-mouse) followed by streptavidin-peroxidase complex (View kit, Ventana Systems). Detection was done using 3,3′-diaminobenzidine with H_2O_2 (View kit). Results were expressed as expression scores combining the percentage of positive cells for the marker and the staining intensity (1–3 on an arbitrary, semiquantitative scale). Cases were considered as positive when the expression score was >60. This value corresponds to the minimal score of normal pulmonary cells (18). $p14^{arf}$ primary antibody was a polyclonal rabbit antibody (Neomarkers) used at 1:50 after 25-min treatment in citrate (pH 6). As described previously, the secondary antibody was a biotin retrieving labeled donkey anti-rabbit (1:1,250; Jackson ImmunoResearch) followed by detecting the streptavidin-biotin/horseradish peroxidase complex (DAKO). Normal lymphocytes, stromal cells, and normal epithelial cells (bronchial cells and alveolar cells) were considered as positive controls. $p14^{arf}$ was considered as down-regulated in tumors when the expression score was <60.

Statistical analysis. Relative risks and 95% confidence intervals (95% CI) were calculated after adjustment for sex, age, and education, based on unconditional multivariate logistic regression model using the SAS System

Table 2. Distribution of *EGFR* mutations in relation to smoking status, gender, and tumor histology

	Both genders (N = 116)			Female (n = 31)			Male (n = 85)		
	Mutation	No mutation	OR (95% CI)	Mutation	No mutation	OR (95% CI)	Mutation	No mutation	OR (95% CI)
Smoking status									
Never	16	17	1 (Ref)	14	15	1 (Ref)	2	2	1 (Ref)
Ever	4	79	0.05 (0.02–0.18)	1	1	1.07 (0.06–18.8)	3	78	0.04 (0.004–0.37)
Former	4	34	0.12 (0.04–0.43)	1	1	1.07 (0.06–18.8)	3	33	0.09 (0.01–0.90)
Current	0	45	—	0	0	—	0	45	—
Histology									
ADC	15	33	1 (Ref)	12	10	1 (Ref)	3	23	1 (Ref)
SCC	3	48	0.14 (0.04–0.51)	1	1	0.83 (0.05–15.1)	2	47	0.33 (0.05–2.09)
ADC-SCC	2	15	0.29 (0.06–1.45)	2	5	0.33 (0.05–2.10)	0	10	—

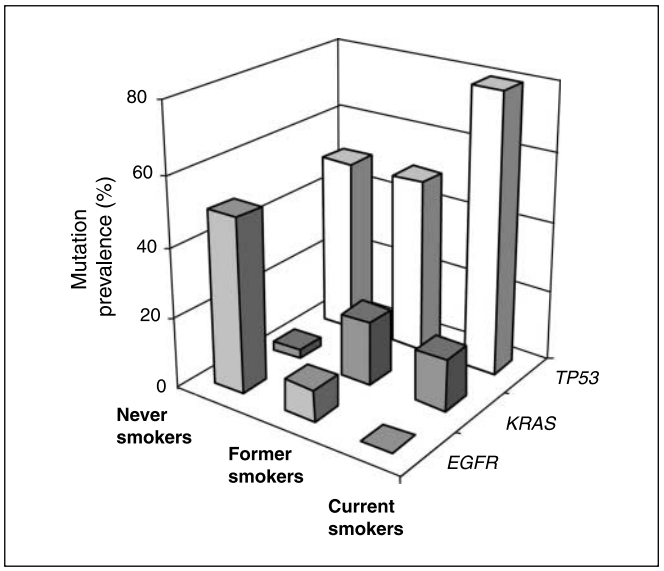

Figure 1. Prevalence of *TP53, KRAS*, and *EGFR* mutations in relation to smoking history. Percentage of tumors with mutation in *EGFR* (exons 18–21), TP53 (exons 4–9), and *KRAS* (codon 12), in relation to smoking status (never smokers, $n = 33$; former smokers, $n = 38$; and current smokers, $n = 45$). *TP53* and *KRAS* mutation data are from Le Calvez et al. (2).

for Windows (release 9.1), as were pooled t tests for independent samples with equal variances, Satterthwaite t test for samples with unequal variances (when folded F tests $P < 0.05$), Pearson χ^2 analyses, Fisher's exact tests, and Î° statistics.

Results

***EGFR* and *HER2* mutations in relation to histology, gender, and smoking history.** A total of 23 *EGFR* mutations (exons 18–21) were found in 20 of 116 (17%) NSCLC cases (see detailed results in Supplementary Table). Of these mutated cases, 15 (75%) were ADC, 3 (15%) SCC, and 2 (10%) adeno-squamous (ADC-SCC) cases. Mutations were of the following types: deletions of variable size in exon 19, all encompassing codons 746 to 750 (14 mutations), missense mutations in exon 18 (2 mutations, I706T and S720P), exon 20 (2 mutations, S768I), and exon 21 (4 mutations, L858R, and 1 mutation L858Q). Three cases contained two mutations: in two cases, short deletions in exon 19 coexisted with a missense mutation (L858R or I706T); in the other case, two missense mutations were detected (L858R and S768I).

Mutations were inversely correlated with tobacco consumption, with no mutation in 45 current smokers, 4 in 38 (10%) former smokers, and 16 in 33 (48%) never smokers [odds ratio (OR), 0.05; 95% CI, 0.02–0.18]. In former smokers, 2 of the tumors with mutations were SCC and 2 were ADC. In never smokers, 13 of the tumors with mutations were ADC, 2 were ADC-SCC, and 1 was a SCC (Table 2).

Of patients with *EGFR* mutations, 15 were women and 5 were men. Thus, *EGFR* mutations were detected in 48% (15 of 31) of the women included in the study and in 6% (5 of 85) of men. *EGFR* mutations were found in 50% of ADC in never smokers, irrespective of gender (2 of 4 males and 11 of 21 females).

On the other hand, only 5 (4.3%) mutations were found in *HER2* [4 (10.5%) in former smokers and 1 (3%) in never smokers]. Of the 5 mutated cases, 3 were ADC, 1 was SCC, and 1 was ADC-SCC. Four of the five mutations were detected in former smokers, including 2 silent (G787G and L823L) and the 2 missense (E744G and R745D) mutations. The mutation T791I was found in a tumor of never smoker that harbored a *KRAS* mutation. All *HER2* mutations were detected in men and the three missense mutations were associated with mutant *TP53* and/or low expression of p14^{arf} (see details in Supplementary Table).

Patterns of *EGFR, HER2, KRAS,* and *TP53* mutations according to smoking history. Figure 1 shows the prevalence of *EGFR* mutations according to smoking history, combined with the prevalence of *TP53* and *KRAS* mutations as reported previously (2). None of the cases was found to contain mutations in all three genes (Table 3). In never smokers, *TP53* mutations were detected in 11 of 16 (69%) cases with *EGFR* mutation but in only 2 of 17 cases without *EGFR* mutation. Only one never smoker (N11) had a mutation in *KRAS*, without *TP53* or *EGFR* mutation but with *HER2* mutation. In former smokers, *EGFR* mutations were found in 10% (4 of 38) of the cases, *KRAS* mutations in 24%, and *TP53* in 59%. Of the four patients with *EGFR* mutations, none had a *TP53* or a *KRAS* mutation. In contrast, two of the eight cases with *KRAS* mutation also had a *TP53* mutation. In current smokers, *EGFR* mutations were absent, and *KRAS* and *TP53* mutations were found in, respectively, 16% and 84% of the cases. All but one case with *KRAS* mutation contained a *TP53* mutation. Overall, these results show that although *TP53* mutation load was particularly high in current smokers, *TP53* mutations were also detected in never smokers in relation to the presence of *EGFR* mutation. In contrast, mutations in *KRAS* were relatively rare in never smokers (6%) but more common in current (24%) and former smokers (21%). These differences were mostly due to differences in mutation patterns for ADC: *KRAS* mutations were detected twice as

Table 3. Alterations of *TP53* and *KRAS* in relation to *EGFR* mutations

EGFR mutation		Total			Never smokers			Former smokers			Current smokers		
		With (%)	Without (%)	OR (95%CI)	With (%)	Without (%)	OR (95%CI)	With (%)	Without (%)	OR (95%CI)	With	Without (%)	OR (95%CI)
Total		20	94		16	17		4	34		0	45	
TP53	No mutation	9 (45)	36 (38)	1 (Ref)	5 (31)	15 (88)	1 (Ref)	4 (100)	14 (41)	1 (Ref)	0	7 (16)	1 (Ref)
	With mutation	11 (55)	58 (62)	0.76 (0.29–2.01)	11 (69)	2 (12)	16.5 (2.69–101)	0	20 (59)	—	0	36 (84)	—
KRAS	No mutation	19 (100)	80 (83)	1 (Ref)	15 (100)	16 (96)	1 (Ref)	4 (100)	26 (76)	1 (Ref)	0	38 (84)	1 (Ref)
	With mutation	0	16 (17)	—	0	1 (6)	—	0	8 (24)	—	0	7 (16)	—

NOTE: Results of TP53 mutation analysis were not available for two tumors of current smokers.

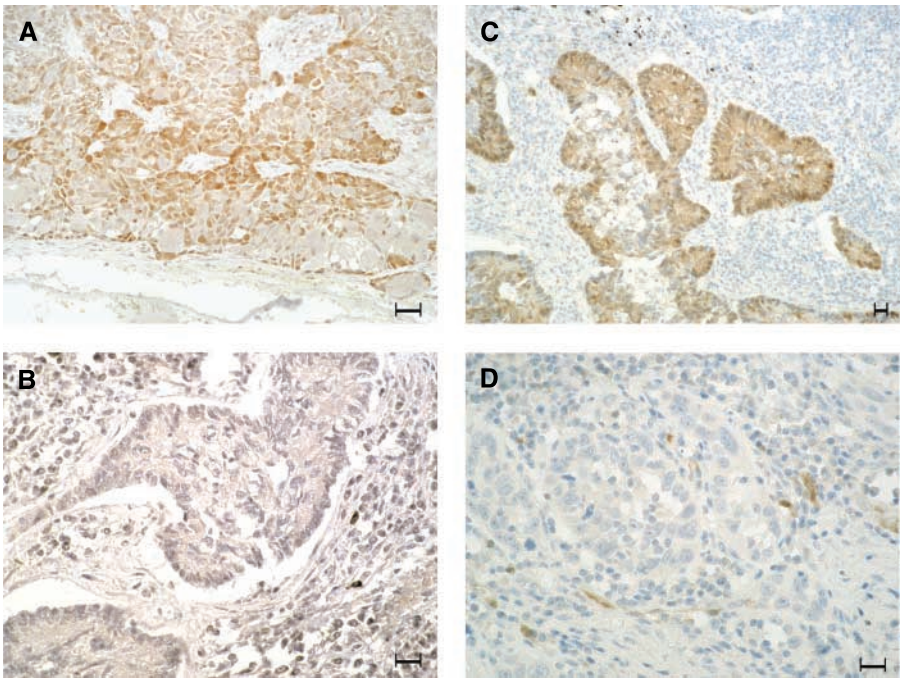

Figure 2. Immunohistochemical detection of $p14^{arf}$ an $p16^{INK4a}$ in lung tumors. Results were expressed as expression scores combining the percentage of cells positive for each marker and the staining intensity (1–3 on an arbitrary, semiquantitative scale). Representative examples of staining with different scores in SCC (*D*) and ADC (*A–C*). $p14^{arf}$ staining in ADC (*A* and *B*). Expression scores, 120 (*A*) and 20 (*B*). $p16^{ink4a}$ staining in ADC (*C*) and SCC (*D*). Expression scores, 80 (*C*) and 0 (*D*). Note the presence of positive nontumor cells in the stroma of the specimen in (*D*). *Bars*, 10 μm.

frequently in ADC of former smokers (38%) than in ADC of never or current smokers (19% in each group).

Expression of $p14^{arf}$ and $p16^{ink4}$ in relation to *TP53, KRAS,* and *EGFR/HER2* mutation patterns. The observation that *TP53* mutation is more common in never smokers with than without *EGFR* mutation led us to formulate the hypothesis that inactivation of p53 function may be required for carcinogenesis driven by mutant *EGFR*. *In vitro* studies in human diploid fibroblasts have shown that activation of growth signaling cascades resulted in increased expression of $p14^{arf}$, leading to accumulation of p53 and growth suppression (16). This model is consistent with the fact that *TP53* mutation is uncommon in knockout mice lacking $p14^{arf}$ expression (19). We thus analyzed by immunohistochemistry the expression of $p14^{arf}$ and of $p16^{ink4a}$, the two product of *CDKN2a*, a locus often altered by deletion, mutation, or hypermethylation in lung cancer (see Introduction). Results were expressed as an expression score combining staining intensity and distribution in tumor cells (see methods). Figure 2 shows an example of staining patterns in tumors with low (expression score < 60) and normal (expression score ≥ 60) $p14^{arf}$ or $p16^{ink4a}$ expression. Results are presented in Table 4. Loss of $p14^{arf}$ expression seemed to be more common in never smokers (63%) than in former smokers (47%) or

Table 4. Expression of $p14^{arf}$ and $p16^{Ink4a}$ in lung cancers according to smoking status

		p14 negative, ES ≤ 60 (%)	p14 positive, ES > 60 (%)	OR (95%CI)	p16 negative, ES ≤ 60 (%)	p16 positive, ES > 60 (%)	OR (95%CI)
Smoking*	Never	20 (62.5)	12 (37.5)	1 (Ref)	17 (53)	15 (47)	1 (Ref)
	Ever	28 (35)	52 (65)	0.94 (0.15–5.87)	53 (65)	29 (35)	0.34 (0.04–3.37)
	Former	17 (47)	19 (53)	1.24 (0.19–8.27)	23 (61)	15 (39)	0.31 (0.03–2.99)
	Current	11 (25)	33 (75)	0.48 (0.07–3.62)	30 (68)	14 (32)	0.52 (0.05–0.57)
	P_{trend}			0.15			0.73
EGFR †	No mutation	31 (34)	61 (66)	1 (Ref)	57 (61)	37 (39)	1 (Ref)
	Mutation	17 (85)	3 (15)	5.22 (1.08–25.30)	13 (65)	7 (35)	2.14 (0.56–8.18)
TP53 †	Mutation	21 (48)	23 (52)	1 (Ref)	23 (51)	22 (49)	1 (Ref)
	No mutation	27 (41)	39 (59)	1.321 (0.49–3.54)	45 (79)	22 (33)	2.10 (0.85–5.22)
KRAS †	Mutation	42 (44)	53 (56)	1 (Ref)	61 (63)	36 (37)	1 (Ref)
	No mutation	5 (31)	11 (69)	0.55 (0.15–1.97)	8 (50)	8 (50)	0.44 (0.14–1.38)

Abbreviation: ES, expression score (see Materials and Methods).
*OR, adjusted for age, sex, and education.
†OR, adjusted for age, sex, smoking, and education.

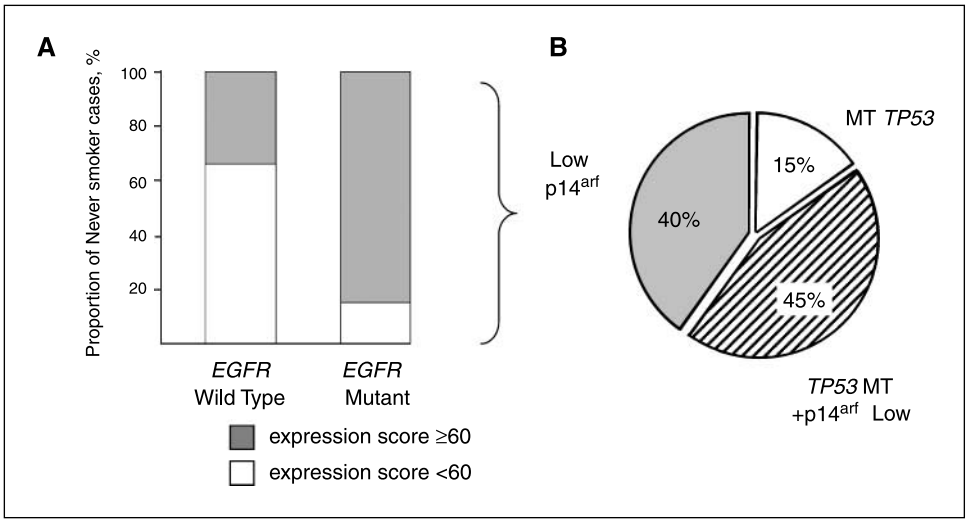

Figure 3. Concordance *TP53, EGFR* mutations and p14^{arf} expression in never smokers. *A,* percentage of tumors with down-regulated (*gray*) or normal (*white*) p14^{arf} expression among tumors with WT or mutant *EGFR*. *B,* among tumors with mutant *EGFR*, proportion of cases with down-regulated p14^{arf} expression (*gray*), *TP53* mutation (*white*), or both (*shaded*).

current smokers (25%). This trend was not observed with p16^{ink4a}, the loss of expression of which was marginally more common in current smokers (68%) and former smokers (61%) than in never smokers (53%). Among the 20 tumors containing *EGFR* mutations, 17 (85%) showed low p14^{arf} expression. Figure 3 shows the interrelation between *TP53* status and p14^{arf} expression in tumors containing *EGFR* mutations. All the tumors with WT *TP53* showed deficiency of p14^{arf} expression. Thus, overall, the p14^{arf}/p53 pathway was altered in all tumors containing mutant *EGFR*, either by mutation of *TP53* (3 cases), by loss of expression of p14^{arf} (9 cases), or by both (8 cases). Despite the low number of *HER2* alterations, a similar concordance between mutation and p14^{arf} expression was observed with *HER2*. All cases with missense *HER2* mutation were deficient for p14^{arf} expression, two of them also harboring a *TP53* mutation (see Supplementary Table).

Discussion

Somatic mutations in the TK domain of *EGFR* have emerged recently as common alterations in ADC of never smokers (8), whereas mutations in *HER2* are not frequent (13, 20, 21). *EGFR* mutation prevalence is inversely correlated with tobacco consumption (22, 23). Furthermore, *EGFR* and *KRAS* mutations exhibit a mutually exclusive distribution in ADC, suggesting that they belong to redundant oncogenic pathways. *EGFR/HER2* and *KRAS* mutations have been reported to be mutually exclusive (24); however, thus far, little is known on whether these mutations may coexist with *TP53* mutations. *TP53* has been reported as mutated in ADC of never smokers with prevalences between 15% and 50% according to different studies (2). In a study by Kosaka et al. (10), *TP53* mutations were found at similar prevalences in tumors with or without *EGFR* or *KRAS* mutations, suggesting that mutation in *TP53* occur independently of mutation in *EGFR* or *KRAS*.

In the present study, conducted on a series of 116 patients with well-defined smoking history, we found that *HER2* mutations are detectable but rare in lung cancer (4.3%), whereas *EGFR* mutations are more common in (*a*) tumors of never smokers (16 of 33, 48%) as opposed to former smokers (4 of 38, 10%) or current smokers (0%) and (*b*) ADC (15 of 20 tumors, 75%) as opposed to ADC-SCC (2 of 20, 10%) and to SCC (3 of 20, 15%). *EGFR* mutations are mutually exclusive with *KRAS* but not *TP53* mutations. On the other hand, *HER2* mutations are mutually exclusive with *EGFR* mutations but can coexist with *KRAS* or *TP53* mutations. The overall type and distribution of *EGFR* mutations is compatible with previous reports: mutation in exon 19 represented 61% of the mutations and mutations in exons 20 and 21 represented, respectively, 9% and 22%.

It has been suggested that the prevalence of *EGFR* mutations was particularly high in ADC of never smoking women (25). In the present study, cases harboring *EGFR* mutations were equally distributed among male and female never smokers (50% in each group). This observation suggests that the high prevalence of *EGFR* mutations in females is mostly an effect of the overrepresentation of women in the "never smoker" category, compatible with patterns of tobacco consumption among genders.

The *CDKN2a* locus encodes two distinct suppressor proteins, p16^{Ink4a} and p14^{arf}, that are often down-regulated in lung cancers by several mechanisms, including promoter methylation, loss of heterozygosity, point mutations, and loss of expression (26). How alterations in these genes correlate with *EGFR* mutation in lung cancer is not clearly understood. In a recent study, Toyooka et al. (27) have shown that the probability of having *EGFR* mutation was lower among tumors with methylated *CDKN2a* gene. The patterns of alterations of p14^{arf} in lung cancers are much less well described as those of p16^{ink4a}. Eymin et al. (28) have shown that loss of p14^{arf} expression tended to correlate with high expression of Mdm2 in primary lung cancers, suggesting a role for Mdm2/p14^{arf} in modulating the activity of p53. Nicholson et al. (29) have detected homozygous deletions, but not intragenic mutations of p14^{arf} in 19% of NSCLC, equally distributed among *TP53* mutated and WT tumors. *CDKN2a* promoter methylation has been described in lung cancer cell lines (30), in DNA from bronchial lavages (31), and in primary SCC (32), but how methylation correlates with expression is poorly documented. To determine whether down-regulation of p16^{ink4a} and p14^{arf} correlates with the mutation status of *EGFR, HER2, KRAS*, and *TP53* in lung cancers, we have analyzed their expression by immunohistochemistry, a method that captures inactivation of these products by several mechanisms. In agreement with previous reports, p16^{ink4a} was frequently down-regulated in lung cancers, with a nonsignificant tendency for more frequent loss of expression in current smokers. In contrast, p14^{arf} was significantly more frequently down-regulated in never smokers than in current smokers. Among patients with *EGFR* mutations, 85% showed down-regulation of p14^{arf} expression, whereas the 15% of the cases that retained p14^{arf} expression harbored a mutation in *TP53*.

Furthermore, the three tumors carrying missense mutation of *HER2* were down-regulated for $p14^{arf}$, and two of them were mutant for *TP53*. These results suggest that mutation in *EGFR/HER2* requires the functional inactivation of the $p14^{arf}$/p53 connection, either by down-regulation of $p14^{arf}$, by mutation of *TP53*, or by both mechanisms. This interpretation is consistent with a model in which hyperproliferative signals generated by mutant *EGFR* may induce $p14^{arf}$, resulting in the activation of p53 as part of a fail-safe mechanism to counter mitotic signaling. The fact that some cancers show both *TP53* mutation and low $p14^{arf}$ expression could reflect a selection for the loss of one or both the *INK4* genes that flank the *ARF* locus. Alternatively, mutation of *TP53* could reflect the fact that loss of $p14^{arf}$ might not completely abrogate p53 function, perhaps due to its participation to other signaling pathways (33–35). Further dissection of these mechanisms will require the analysis of the status of other partners in the p53/$p14^{arf}$ connection, in particular mdm2.

According to this model, only cells with a functional deficiency in the $p14^{arf}$/p53 connection may bypass growth suppression and clonally expand to cause cancer. This hypothesis requires that *EGFR* mutation is an early event in lung carcinogenesis, which may occur in normal lung, and that mutation in *TP53* and/or inactivation of $p14^{arf}$ is the rate-limiting event that permits clonal expansion. It is intriguing to note that a concordance with $p14^{arf}$/p53 inactivation was not observed for *KRAS* mutations. Indeed, constitutive activation of *KRAS* is expected to induce an oncogenic stress that activates the $p14^{arf}$/p53 pathway, leading to the same type of selection pressure for inactivating this pathway as with *EGFR* mutation. One possible explanation is that these mutations trend to arise in distinct etiologic context, perhaps as the consequence of distinct mechanisms of carcinogenesis. Of the 16 cases with *KRAS* mutation in the present series, only 1 was a never smoker, whereas 8 and 7 were former or current smokers, respectively. In line with this hypothesis, Toyooka et al. (27) have shown that there were differences in the evolvement of epigenetic alterations between *EGFR*- and *KRAS*-mediated tumorigenesis. Further studies are needed to determine whether such differences in genetic and epigenetic alterations may predict different tumor behavior and response to therapy.

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Patterns of *EGFR, HER2, TP53*, and *KRAS* Mutations of p14^{arf} Expression in Non–Small Cell Lung Cancers in Relation to Smoking History

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