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Tobacco Smoke-induced DNA Damage and an Early Age of Smoking Initiation Induce Chromosome Loss at 3p21 in Lung Cancer[1]

Tomoko Hirao, Heather H. Nelson, Tara Devi S. Ashok, John C. Wain, Eugene J. Mark, David C. Christiani, John K. Wiencke, and Karl T. Kelsey[2]

Department of Cancer Cell Biology [T. H., H. H. N., T. D. S. A., K. T. K.] and Occupational Health Program [D. C. C.], Harvard School of Public Health, Boston, Massachusetts 02115; Thoracic Surgery Unit, Department of Surgery [J. C. W.], Department of Pathology [E. J. M.], and Pulmonary and Critical Care Unit, Department of Medicine [D. C. C.], Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114; and Laboratory for Molecular Epidemiology, Department of Epidemiology and Biostatistics, University of California at San Francisco, San Francisco, California 94143 [J. K. W.]

ABSTRACT

The short arm of chromosome 3 is thought to harbor a novel oncogenic locus that is important in the genesis of lung cancer. The region at 3p21 is believed to contain a distinct locus that is sensitive to loss from the action of tobacco smoke carcinogens and has been reported to be specifically targeted for deletion in lung cancer. To investigate whether 3p21 alteration in lung cancer is associated with carcinogen exposure, PCR-based analysis was performed to detect loss of heterozygosity (LOH) on chromosome 3 at 3p21 in non-small cell lung carcinoma (NSCLC). We also measured instability at the *BAT-26* locus, because the mismatch DNA repair gene, *hMLH1*, is found at 3p21. LOH at 3p21 was analyzed for association with the clinical features of NSCLC, p53 mutation status, polynuclear aromatic hydrocarbon-DNA adduct levels (measured using ^{32}P-postlabeling) and carcinogen exposure information including cigarette smoking and asbestos exposure. Of 219 lung cancers, 150 cases (68.5%) were informative at the *D3S1478* locus, and 44.2% of squamous cell carcinoma cases and 30.2% of adenocarcinoma cases showed 3p21 LOH. None of the cancers showed *BAT-26* instability. The prevalence of 3p21 LOH was higher in both current and former smokers compared with never smokers and was higher in p53 mutated cases. Among squamous cell carcinoma cases, there was a strong association of increased 3p21 LOH with increasing polynuclear aromatic hydrocarbon-DNA adducts levels ($P = 0.03$), as well as an increased prevalence LOH with earlier age of smoking initiation ($P = 0.02$). Our results confirm that 3p21 LOH is strongly associated with measures of biologically effective dose of exposure to tobacco carcinogens. Our results also suggest that alterations of *hMLH1* are not related to any of the reported associations, because there was no evidence of microsatellite instability. Finally, LOH in 3p21 may be an early molecular event in NSCLC, because it is significantly associated with a tendency to start smoking at a young age.

INTRODUCTION

Lung cancer is the leading cause of cancer death in both women and men in the United States (1) and is increasing in incidence worldwide. In the United States, 80% of lung cancer deaths in men and 75% in women are estimated to be attributable to cigarette smoking (1), and smoking is accepted as a major cause of lung cancer (2–4). Recent studies have suggested that specific genetic alterations in lung cancer occur in premalignant clones long before the appearance of overt malignancies. Furthermore, these changes may persist for many years after smoking cessation (5). Wiencke *et al.* (6) reported recently that, in former smokers, age at smoking initiation was inversely associated with increasing PAH[3]-DNA adduct levels in normal lung tissue. This suggests that smoking during adolescence may produce physiological alterations that lead to increased DNA adduct persistence (6). Little attention has been paid to the relationship between early smoking initiation and somatic mutation.

The loss of wild-type alleles at 3p, 9p, and 17p is widely described in various human malignancies, and LOH at 3p has been reported to be a relatively early event in lung carcinogenesis (7–11). 3p deletion is a common finding in lung cancer that was first detected in small cell lung carcinoma by cytogenetic analysis (12). Recent studies have shown that 3p deletion can occur in preneoplastic epithelial lesions as well as in invasive cancers (11, 13). LOH at 3p occurs considerably more frequently in patients who smoke than in those who have never smoked (14).

Many genetic alterations have been described in lung cancer, but their association with individual patterns of exposure to tobacco and other lung carcinogens has been less well studied. Because previous work has suggested that loss of 3p21 is tobacco associated and possibly an early event in lung carcinogenesis (15), we assessed LOH using a well defined polymorphic marker in this region (*D3S1478*) and examined whether this genetic alteration was associated with self-reported patterns of tobacco use, asbestos exposure, and other life-style factors in our prospective surgical case series of NSCLC. In an effort to more specifically investigate whether detectable DNA damage was also associated with LOH at 3p21, we further compared the mean DNA adduct burden, derived from ^{32}P-postlabeling of normal lung DNA, in patients with and without LOH. Finally, because the *hMLH1* DNA repair gene, also located in the 3p21 region, has been hypothesized to be targeted for deletion, we tested the association of LOH and *BAT-26* instability. *BAT-26* is a reliable marker of *hMLH1* related microsatellite instability.

MATERIALS AND METHODS

Study Population. Eligible cases consisted of all newly diagnosed patients with resectable lung cancer who received treatment at the Massachusetts General Hospital Thoracic Surgery, Oncology, and Pulmonary Services from November 1992 through December 1996 (16). Patients with recurrent disease or with nonoperable tumors were excluded. Of the 461 case patients enrolled consecutively in the parent study, a random subset of 219 was analyzed for somatic loss at 3p21. Tumor-derived DNA was obtained from archived pathology specimens as described previously (17), and comparative constitutive DNA was derived from circulating blood lymphocytes (QIAamp DNA Blood Mini kit; Qiagen). Demographic and epidemiological data, including all of the data on tobacco use, were gathered by interviewer review of a self-administered questionnaire completed by patients and reviewed by a single reviewer during the hospitalization for thoracic surgery.

LOH Analysis. To evaluate LOH at chromosome 3p21, the microsatellite marker *D3S1478* was amplified by PCR containing [α-^{32}P]dCTP (DuPont NEN Life Science Products). Primer sequences can be obtained from the Genome Database. PCR was performed for 30 cycles with the annealing temperature of 62°C. Two μl of PCR product were mixed with 4 μl of loading buffer, denatured, and separated by electrophoresis on a 6% polyacrylamide-7 M urea gel at 60W at room temperature. PCR products were detected by autoradiography (Biomax film; Eastman Kodak). LOH was visually scored by >50% reduction in allele intensity.

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[2] To whom requests for reprints should be addressed, at Department of Cancer Cell Biology, Harvard School of Public Health, 665 Huntington Avenue, Boston, MA 02115-6021.

[3] The abbreviations used are: PAH, polynuclear aromatic hydrocarbon; LOH, loss of heterozygosity; NSCLC, non-small cell lung cancer.

***BAT-26* Instability.** For *BAT-26* instability detection, PCR containing [α-^{32}P]dCTP (DuPont NEN Life Science Products) was performed for 30 cycles with the annealing temperature of 58°C. Primer sequences can be obtained from the Genome Database. Four μl of PCR product were mixed with 4 μl of loading buffer, denatured, and separated by electrophoresis on a 6% polyacrylamide-7 M urea gel. The temperature of the gel was maintained at 55°C. The gel was then dried, and autoradiography was performed.

p53 Analysis. p53 mutations were detected using PCR-SSCP of exons 5–10 as described previously by Nelson *et al.* (17). Briefly, the primer sequences of Toguchida *et al.* (18) were used for amplification of DNA derived from adjacent 10-μm paraffin sections that were used to generate DNA using standard protocols (19). PCR amplification of primary tumor DNA was used for p53 mutation sequencing to avoid polymerase artifact.

DNA Adducts. PAH-DNA adduct levels were assessed in normal, nontumorous lung tissue that was obtained as part of the surgical protocol. Adducts were measured as described by Wiencke *et al.* (6).

Statistical Analysis. Statistical analyses of 3p21 LOH, patient demographics, and tumor traits included χ^2, Wilcoxon rank-sum of means, trend test, and unconditional logistic regression. All *P*s represent two-sided statistical tests and are considered statistically significant for $P < 0.05$.

Results

Of the 219 cases studied, 150 (68.5%) were informative at the *D3S1478* locus. The prevalence of 3p21 LOH was 34% (51 of 150). It was 33.6% when we restricted the data to the NSCLC cases (48 of 143). All further analyses of 3p21 LOH and patient traits were restricted to the NSCLC cases ($n = 143$).

Table 1 summarizes the relationship between 3p21 LOH, clinical features, and characteristics of the tumors. Nineteen of 43 squamous cell carcinomas (44.2%) exhibited LOH at 3p21, as did 26 of 86 adenocarcinomas (30.2%; $P = 0.12$ for squamous cell carcinoma *versus* adenocarcinoma). There was no significant association of 3p LOH with age, gender, or stage (Table 1).

We next examined 3p LOH status and carcinogen exposures. LOH was more frequent in current smokers (32.3%) and ex-smokers (37.3%), than in never-smokers (18.2%), but this difference was not statistically significant. Evaluating all cases, as shown in Table 1, the mean value for pack-years and number of years smoked tended to be higher in LOH-positive cases. However, this did not reach statistical significance. There was a borderline significant association in the unadjusted data of a history of asbestos exposure with LOH at 3p ($P = 0.1$).

Table 1 *Demographics, histology, and carcinogen exposure history by LOH at 3p21*[a]

	LOH present	LOH absent
Age	66.9 ± 9.8	66.5 ± 10.0
Gender		
Male	26	46
Female	22	49
Histology		
Adenocarcinoma	26	60
Large cell	3	7
Squamous cell	19	24
Other[b]	0	4
Stage		
I	25	59
II	10	10
III	11	21
IV	2	5
Smoking status		
Never	2	9
Former	25	42
Current	21	44
Pack-years smoked	63.0 ± 42.3	55.4 ± 40.9
Years smoked	39.6 ± 15.1	37.7 ± 17.2
Age started smoking	17.8 ± 5.5	18.8 ± 7.0
Asbestos exposure[c]		
No	39	87
Yes	8	8

[a] Values are counts, or mean ± SD.
[b] Two bronchioalveolar carcinomas and two adenosquamous cell carcinomas.
[c] Asbestos exposure information missing for one squamous cell carcinoma case, $P = 0.1$.

We also examined the association between 3p21 LOH and smoking as a categorical variable, defined by tertiles (Fig. 1). LOH prevalence appeared to increase with increasing pack-years in all cases; however, this trend was not statistically significant. A younger age of smoking initiation was associated with a significantly higher LOH prevalence among the squamous cell carcinoma cases ($P < 0.02$; Fig. 1). Interestingly, neither pack-years ($P = 0.83$) nor years of smoking ($P = 0.34$) were significantly associated with LOH at 3p21 in patients with squamous cell carcinomas. When all cases were examined using a logistic model, the association of LOH and youngest age of onset of smoking was borderline significant ($P = 0.10$), controlling for the effect of histology ($P = 0.07$).

To further study the relationship between 3p21 loss and smoking carcinogen dose, we investigated the relationship between prevalence of LOH and PAH-DNA adduct levels in normal lung tissue from 70 cases. DNA adducts were evaluated by stratifying adducts into tertiles. The highest adduct tertile had the highest LOH prevalence when looking at all cases with both measurements, and there was a dose-related increase in 3p21 LOH with increasing adduct tertiles overall that was highly significant in the squamous cell carcinoma cases ($P < 0.05$; Fig. 2).

Finally, 3p21 LOH was analyzed stratified by p53 and K-*ras* mutation status. There was no apparent association between LOH and induction of mutation at the K-*ras* locus (Table 2). The prevalence of 3p21 LOH was borderline significantly higher in those tumors with mutated p53. As shown in Table 2, 47.6% of p53 mutant adenocarcinoma cases had 3p21 LOH compared with 26.7% of the wild-type cases ($P < 0.08$). In squamous cell carcinoma, 53.9% of p53 mutated case showed LOH, compared with 37% in wild-type cases.

When logistic models were used to examine these data, the association of p53 mutation and LOH was significant after adjustment for pack-years of smoking and histology (squamous *versus* adenocarcinoma; $P < 0.03$; odds ratio, 2.6). Additional modeling of the association of age at smoking initiation, DNA adduct level, and LOH was not done, because these variables are colinear and the number of subjects studied was limited.

For 73 of the 219 cases, we also examined tumor tissue for instability at the *BAT-26* locus and found no tumors with any evidence of changes in allele length (Table 1).

Discussion

Deletion of one copy of the short arm of chromosome 3 is observed frequently in lung cancer. 3p LOH has been reported to be a relatively early event in lung carcinogenesis (7, 8, 13) and has been detected in preneoplastic epithelial lesions (13). The frequency of 3p LOH in lung cancer has been reported to be 49–86% (13). The prevalence of LOH at 3p has also been observed to be higher in squamous cell carcinoma than in adenocarcinoma: 50–83% in squamous cell carcinoma *versus* 36–61% in adenocarcinoma (20). We have detected LOH at 3p in 33.6% of 143 informative NSCLC cases, and the presence of LOH was more frequent in squamous cell carcinoma cases (44.2%) than in adenocarcinoma cases (30.2%). The somewhat lower prevalence of LOH observed in our study may be attributable to our use of only one marker, or it might also be attributable to differences in study design. We used a prospective enrollment strategy, whereas previous investigations used primarily retrospective and convenience designs. In addition, the enrollment criterion for our study was strictly surgical, and our use of a conservative method for detecting LOH may also account for the lower prevalence of LOH at 3p.

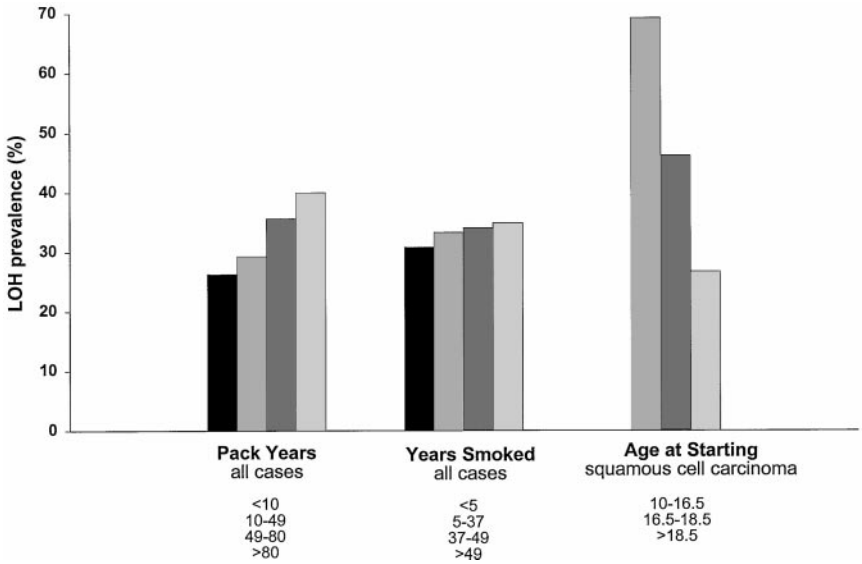

Fig. 1. The prevalence of LOH at 3p21 in NSCLC by quartiles of pack-years smoked, quartiles of years smoked, and tertiles of age at starting smoking.

In our study, although the prevalence of 3p21 LOH increased with cumulative smoking dose, there was a strong significant association between 3p21 LOH and increasing PAH-DNA adducts levels. In addition, there was a higher prevalence of 3p21 LOH in individuals who started smoking at younger ages. Our result, showing that 3p LOH is associated with measurable PAH-DNA adducts implies that 3p21 LOH is clearly induced by tobacco carcinogens. These observations are consistent with previous work of other investigators (14, 15) and indicate that deletion of 3p is an important and early event in lung carcinogenesis.

Although different measures of tobacco smoke exposure are related, we found the strongest association of LOH at 3p21 to be with the age of onset of smoking. One novel interpretation of this data is that LOH induced in lungs that are still developing can result in propagation of this lesion and yield large fields of cells with LOH. Field effects are well described for the squamous cell histology and less well documented for adenocarcinomas. This mirrors our finding that early smoking is more strongly associated with LOH at 3p21 in squamous cell carcinoma. Hence, we believe that developmental factors may account for the higher prevalence of LOH at 3p21 among patients who begin smoking early in life.

The association of an early age of initiation of smoking, DNA adduct persistence, and LOH at 3p could further indicate that a gene in this region is important in facilitating DNA damage repair, either directly or indirectly. The *hMLH1* gene is located at 3p21, but this protein is not likely to be responsible for bulky DNA adduct repair. In addition, the absence of *BAT-26* instability in these tumors suggests that *hMLH1* is not the gene responsible for the associations we observed with tobacco carcinogen exposure and DNA adducts. However, because DNA repair genes cluster on some chromosomes (21) and there is a recent report of a novel DNA repair gene in the precise locus (3p21) that we examined (22), it is possible that an important gene of this sort is located in this region. Further speculation on this possibility awaits cloning of the gene(s) at this locus that is important in the genesis of lung cancer.

One candidate gene located on the short arm of chromosome 3, at 3p25, the *von Hipple-Lindau* tumor suppressor gene, appeared to have association with renal cell carcinoma but has been found to be involved only infrequently in lung cancer. Several studies have examined associations of another 3p region, including the *FHIT* locus and p53 status. Horio *et al.* (23) reported that there was a significant association between the presence of p53 mutation and 3p deletions in 71 NSCLCs. Burke *et al.* (24) studied 106 NSCLCs and found that 3p14 LOH was associated with p53 missense mutations, whereas

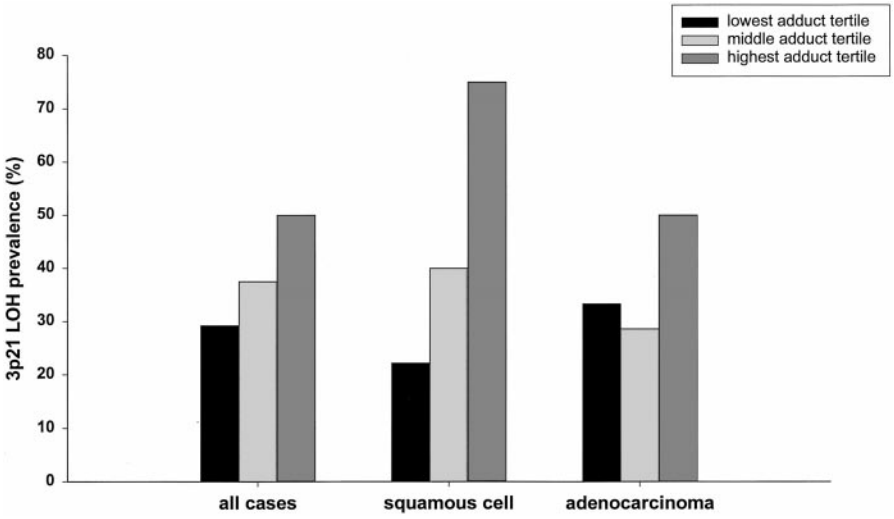

Fig. 2. The prevalence of LOH at 3p21 in NSCLC stratified by lung polynuclear aromatic adduct tertiles for all cases ($n = 70$) and restricted to histologies ($n = 43$ for adenocarcinoma and $n = 22$ for squamous cell carcinoma).

Table 2 *LOH at 3p21, p53 mutation, K-ras mutation, and carcinogen exposure stratified by histology*

	Adenocarcinoma		Squamous cell	
	LOH present	LOH absent	LOH present	LOH absent
Asbestos exposure				
No	23	57	15	20
Yes	3	3	3	4
Age	68.4 ± 11.0	66.0 ± 10.3	65.7 ± 8.3	68.3 ± 8.8
Pack-years smoked	50.1 ± 38.3	48.7 ± 39.8	76.4 ± 44.6	67.0 ± 37.2
Years smoked	36.5 ± 15.9	36.5 ± 18.6	43.8 ± 14.4	39.8 ± 14.9
Age started smoking[a]	19.3 ± 6.8	18.3 ± 6.4	16.2 ± 3.2	20.1 ± 9.5
p53[b]				
Wild-type	16	44	10	17
Mutant	10	11	7	6
K-*ras* codon 12				
Wild-type	19	44	NA[c]	NA
Mutant	7	16	NA	NA
BAT-26[d]				
Negative	10	23	9	11
Positive	0	0	0	0

[a] $P < 0.08$, squamous cell carcinoma.
[b] p53 data not available for 8 cases; $P < 0.08$, adenocarcinoma.
[c] NA, not analyzed.
[d] Restricted to cases where both LOH at 3p21 and *BAT-26* instability were examined.

Geradts *et al.* (25), in 103 resected NSCLCs, found no correlation of 3p LOH with abnormality in p53 immunohistochemical staining. In our study, there was an increasing prevalence of 3p21 LOH among cases with p53 mutations, which is consistent with a role of p53 in the maintenance of genetic stability.

Of interest, the histological differences in various genetic alterations have been observed within NCSLC; p53 mutations are more prevalent in squamous cell carcinomas, *ras* mutations are more common in adenocarcinomas, and 3p LOH is more frequently seen in squamous cell carcinomas (20, 26). The induction of mutations at these loci in specific tissues is likely to occur in a specific order but possibly at varying intervals of time. The order may differ in different histological tissue types and may thereby represent a differing pathogenesis and progression toward frank malignancy. However, large-scale chromosome loss does not seem likely to be associated with genomic instability in NSCLC.

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