Loss of Heterozygosity in Epithelial Cells Obtained by Bronchial Brushing: Clinical Utility in Lung Cancer[1]

Charles A. Powell,[2] Scott Klares, George O'Connor, and Jerome S. Brody

The Pulmonary Center and the Department of Medicine, Boston Veterans Administration Medical Center, and Boston University School of Medicine, Boston, Massachusetts 02118

ABSTRACT

To determine whether loss of heterozygosity (LOH) could be a useful diagnostic test for lung cancer, we evaluated LOH in cells obtained from bronchial brushings. Cells from radiographically normal and abnormal lungs were obtained from 55 patients undergoing diagnostic bronchoscopy. Among 38 patients with lung cancer, LOH was present in at least one chromosomal locus in 79%, whereas cytology was positive for malignant cells in 37%. LOH was not restricted to the airway containing the tumor; fifty-three percent of the cancer patients had LOH in the contralateral lung, as did 59% of patients without lung cancer. There was an association between the extent of LOH and proximity to the cancer. The LOH score, which combined measures of fractional allelic loss and percentage of cells with allelic loss, was greater in subjects with positive cytology and on the side of the tumor. A LOH score >10 was positive in 58% of tumor-bearing lungs, in 13% of the contralateral lungs in cancer patients, and in no patients without cancer. Our results suggest that extensive and widespread allelic loss, as indicated by a high LOH score, may be diagnostic of lung cancer. Additional studies will be needed to clarify the clinical potential of using bronchial epithelial cell LOH as a biomarker and diagnostic test for lung cancer.

INTRODUCTION

Lung cancer is the leading cause of death from cancer in the United States with a 5-year mortality rate of ~85%, a rate that has been relatively unchanged over the past 3 decades (1). Previous attempts to improve survival have focused on the early detection of tumors by aggressive screening of high-risk individuals with sputum cytology and chest X-rays, but these methods have not improved mortality (2–4). Recent research has been directed to evaluating molecular diagnostic strategies to improve the early diagnosis of carcinoma. For example, nucleic acid-based methods, such as analysis of LOH[3] in DNA extracted from shed urothelial cells, show great promise for the early detection of tumors such as bladder carcinoma (5). These methods, when applied to cells microdissected from lung tumors and from preneoplastic bronchial epithelium, have demonstrated LOH at multiple chromosomal loci in tumors and have shown that LOH is an early event in the progression from normal to neoplastic cells (6, 7). These observations have encouraged the development of diagnostic tests based on the detection of LOH in readily obtainable clinical specimens (8). However, two recent studies of LOH in bronchial biopsies of smokers without apparent lung cancer have raised questions about the diagnostic significance of LOH (9, 10). Both studies found evidence of LOH at one or more chromosomal sites in histologically normal or minimally abnormal cells, casting doubt on the diagnostic potential of LOH as an early indicator of lung cancer.

Although the presence of LOH is nonspecific for lung cancer, there are data to suggest that LOH accumulates as the epithelium undergoes neoplastic transformation (10, 11). We undertook the present study to determine whether measuring the extent of LOH in the airway epithelial cells would be a useful diagnostic test for lung cancer. We first developed and validated a method for measuring and quantitating LOH in a mixed population of normal and abnormal cells obtained from bronchial brushings of airways at bronchoscopy. Using this method, we measured the presence and extent of LOH at 22 chromosomal loci in epithelial cells obtained from the lung bearing the suspect cancer and from the contralateral lung of a cohort of 55 adult patients undergoing diagnostic bronchoscopy for suspected lung cancer. The aims of this study were to assess the feasibility of measuring extent of allelic loss in a heterogeneous population of lung epithelial cells and to assess the sensitivity and specificity of LOH as a diagnostic marker for lung cancer.

MATERIALS AND METHODS

Subjects. Fifty-five patients undergoing diagnostic bronchoscopy at the Boston Veterans Administration Medical Center and Boston Medical Center were entered into the study after giving informed consent in accordance with Institutional Review Board procedures of each institution. All patients were scheduled for diagnostic bronchoscopy, usually to investigate a radiographic abnormality suspicious of lung carcinoma. Detailed clinical histories were recorded for each patient, and clinical records were periodically reviewed for at least 1 year after entry into the study to record final diagnoses, occurrence of new malignancies, and survival. Bronchoscopy resulted in a

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[2] To whom requests for reprints should be addressed, at Columbia University, 630 West 168th Street, Box 91, New York, NY 10032. Phone: (212) 305-0422; Fax: (212) 305-0089; E-mail: cap6@columbia.edu.

[3] The abbreviations used are: LOH, loss of heterozygosity; SCLC, small cell lung cancer; NSCLC, non-SCLC; FAL, fractional allelic loss.

diagnosis of cancer in 14 patients; the remaining diagnoses were made by needle aspiration, mediastinoscopy, thoracotomy, or by clinical progression (in 2 patients). Demographic and risk factor data, which included ethnicity, history of tobacco use, and occupation, were obtained by brief personal interviews. For smoking status, a former smoker was defined as one who had smoked cigarettes in the past but had stopped smoking at least 1 year before entry into the study. Cumulative cigarette pack-years was calculated by the formula: pack-years = (number of cigarette packs smoked/day) × (number of years smoked).

Bronchoscopic Collection of Cells for LOH Analysis. At bronchoscopy, the normal lung was evaluated first and brushings were obtained under direct vision from the lower lobe bronchus. Brushings were obtained with a cytology brush using two series of 10 strokes. The brushings were vortexed into normal saline and divided into aliquots for DNA analysis and for routine cytology analysis. Samples were then obtained with a new brush in the same fashion from the lung with the radiographic lesion. These brushings were directed toward the involved bronchial segment, either by direct vision if there was an endobronchial abnormality (12 subjects), or by brushing the bronchial segment that was abnormal on a chest radiograph or computerized tomography scan. Brushings were obtained first for routine diagnostic testing, and an additional brushing from the same area was then obtained for DNA analysis. Whole blood (2 ml) was collected in heparinized tubes from each patient.

DNA Analysis. The yield of the bronchoscopy brushings was determined by hemocytometer to be 100,000- 500,000 cells of which 90% were histologically classified as epithelial cells. The cells were washed twice with PBS, and 0.2–1 μg of genomic DNA was extracted with proteinase K (0.1 mg/ml) at 55°C for 12 h using standard procedures (12). Normal, nonlung (control) DNA was obtained from peripheral leukocytes and prepared in the same manner as the bronchial epithelial cells. DNA samples were examined for LOH by PCR-based microsatellite analysis using four-color fluorescent primers, robotics, and capillary electrophoresis to facilitate precise quantitation and automation. All DNA templates were amplified with a panel of fluorescent PCR primers from chromosomal regions 3p, 9p, 11q, and 17p that were obtained from Research Genetics (Huntsville, AL) and PE Applied Biosystems (Foster City, CA). The primers were selected to amplify chromosomal regions that had been reported to frequently demonstrate LOH in lung tumors (6, 7, 13, 14). The microsatellite markers and cytogenetic locations were as follows: D3S2387 (3p26.3), D3S2409 (3p21.31), D3S1300 (3p14.3), D3S1285 (3p14.1), D3S1233 (3p13), D3S2406 (3p12.2); GATA62FO3 (9p24), D9S925 (9p22.3), D9S169 (9p21.3), D9S741 (9p21), D9S1118 (9p13); D11S2371 (11q13.3), D11S2002 (11q21), D11S2000 (11q22.3), D11S1986 (11q22.3), D11S1998 (11q23.2), D11S925 (11q23.3–24); D17S513 (17p12), D17S969 (17p12), D17S1303 (17p12), D17S520 (17p12), and D17S122 (17p11.2). All PCR reactions were performed on the automated 877 Integrated Thermal Cycler from PE Applied Biosystems. Each 5-μl reaction consisted of 10 ng of DNA, 1.5 mM $MgCl_2$, 0.5 μM of each primer, 0.125 units of AmpliTaq Gold Taq polymerase from PE Applied Biosystems with GeneAmp PCR Buffer II. Amplification was done at 95°C for 10 min, followed by 10 cycles of 95°C for 15 s, 55°C for 30 s, 72°C for 60 s, and 20 cycles of 89°C for 15 s, 55°C for 30 s, 72°C for 60 s, with a final extension at 72°C for 10 min. Several water blank controls were included in each PCR. From each of the six different reaction products, 1 μl was pooled. All products in the pooled sample were amplified from the same template and distinguished by allele size and emission wavelength. Reactions were then analyzed by capillary electrophoresis on the PE Applied Biosystems 310 Genetic Analyzer with Genescan 2.1 and Genotyper 2.0 software. All DNA templates were coded such that investigators were unaware of the cytological and clinical data from patients until the PCR data analysis was complete. Only primers that demonstrated heterozygosity in leukocyte DNA were considered informative. Most DNA templates were amplified with a panel of 22 primers, with the mean number of informative primers being 14 ± 4 per patient. There was no difference in the mean number of informative primers between the patients with or without cancer nor between patients with or without LOH.

Determination of Allelic Loss. Allelic loss was calculated using a normalized allele ratio (LOH ratio) equation in which R = (A1)(N2)/(A2)(N1) where A1 and A2 are the heights of the alleles from lung and N1 and N2 are the heights of the alleles from the DNA from blood (Refs. 15 and 16; Fig. 1). For informative primers, based on our mixing experiments (see below), LOH was scored as a 20% decrease in the height ratio of an allele in the lung compared with height ratio of the same allele in leukocyte DNA. Therefore, cases in which the LOH ratio was ≤0.8 or ≥1.25 were scored as LOH. For the purposes of subsequent data analyses, the LOH ratio was represented by R or by 1/R if R was >1. All instances of LOH were verified by repetition, and the mean LOH ratio was used for data analysis.

Mixing Studies. Because cells obtained from bronchial brushings will include mixed cell populations of normal and abnormal epithelial cells, we sought to determine the sensitivity and precision of our LOH assay. Experiments were performed mixing DNA from brushings of an endobronchial small cell tumor that showed complete loss of one allele at primer D11S2002 with varying amounts of DNA from the contralateral lung that was normal with a LOH ratio = 1. As the proportion of DNA from the lung bearing the tumor was increased, there was a proportional decrease in the LOH ratio (Fig. 2). These studies demonstrate that the proportion of DNA with allelic loss and, therefore, the percentage of cells with allelic loss corresponded to the LOH ratio. Repetition of this mixing experiment revealed the SE of the LOH ratio to be 9**%,** validating the precision of the method and confirming that the LOH ratio cutoff value of 20% was outside the 95% confidence interval of the method. The mixing experiment was duplicated using DNA templates from two lung cancer cell lines (NCIH446 and NCI441) amplified with primer D9S1118 (data not shown).

Data Analysis. The extent of allelic loss incorporates measurements of the fraction of informative primers with allelic loss (FAL) and the percentage of cells with LOH at each microsatellite (severity). The FAL was calculated by dividing the number of primers with LOH by the total number of informative primers for each template. The severity of allelic loss for each microsatellite was a measure of the proportion of cells in a bronchial brushing sample displaying complete loss of one allele. The severity was determined by the LOH ratio for each DNA template with each microsatellite. For example, a LOH ratio of 0.5 meant that 50% of cells in the sample were homozygous for that allele.

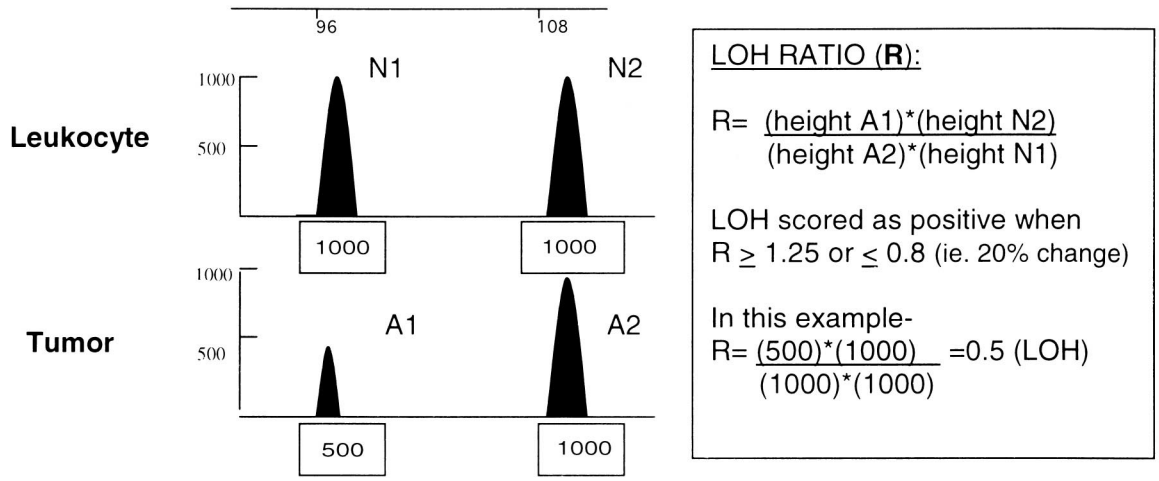

Fig. 1 Example of LOH analysis. The panels are the electropherograms resulting from the amplification of the DNA templates from leukocytes (normal DNA) and tumor. The horizontal axis is allele size in bp. The vertical axis is allele height in arbitrary fluorescent units. *N1* and *N2* are the alleles amplified from leukocyte DNA, and *A1* and *A2* are the alleles amplified from tumor DNA.

To define a value of the extent of LOH that had the greatest sensitivity while retaining specificity, we incorporated measurements of FAL and severity [the percentage of DNA (~cells) with LOH] into a LOH score. The total LOH score was obtained by assigning a value of 1 for all LOH sites with ratios between and a value of 2 for all LOH ratios of 45% or more (ratios of <0.55). The total LOH score was calculated as the sum of the above LOH values divided by the maximum potential LOH score [2 × (number of informative primers) × 100]. This total LOH score may range from 0–100%.

$$\text{Total LOH score} = \frac{(\text{number of loci with LOH ratio of } 0.55\text{–}0.80) + (2 \times \text{number of loci with LOH ratio} > 0.55)}{(2 \times \text{total number of informative loci})} \times 100$$

Comparisons between groups were performed using either the Mann-Whitney U Test for independent samples or the Wilcoxon test for matched samples. The *t* test was used to compare the FAL between airways containing lung cancer and the contralateral airways of the patients with lung cancer. The reported *P*s are two-sided. The 95% confidence limit for the proportion of alleles demonstrating allele-specific loss was computed as described by Zar (17).

RESULTS

Patient Information. The demographic characteristics of the patient population are shown in Table 1. Thirty-eight of the 55 patients were diagnosed with lung cancer of the following cell types: 12 adenocarcinomas, 10 squamous cell carcinomas, 9 large cell carcinomas, 5 SCLCs, and 2 NSCLCs of unspecified cell type. The remaining 17 patients were diagnosed with other aerodigestive tract tumors (primary esophageal and head and neck cancers), other malignancies (lymphoma and mesothelioma), chronic interstitial and granulomatous disease, and acute infections (bronchitis, pneumonia, tuberculosis). None of these 17 patients were determined to have pulmonary neoplasms, primary nor metastatic. Endobronchial abnormalities were visible in 12 (32%) of the 38 patients with lung cancer. Bronchial brushing cytology was positive in 14 (37%) of the patients with lung cancer, including 10 of 12 patients with visible endobronchial abnormalities.

Examples of LOH. The variation in the proportion of cells with allele loss is demonstrated in Fig. 3. Complete loss of one allele is demonstrated in Fig. 3*a*. Cells from this patient came directly from a visible small cell carcinoma; microsatellite analysis revealed complete allelic loss at D3S2387 on chromosome 3p26 [LOH ratio (R) = 0]. More frequently, analysis revealed partial allelic loss as is seen in Fig. 3b. This example demonstrates 37% allelic loss of the shorter allele in cells obtained from the same patient using primer D9S925. Complete allelic loss occurred in three patients, all at chromosome 3p. However, each patient with complete allelic loss also demonstrated partial allele loss (r = 0.30-0.60) at a different chromosomal region, such as 9p or 11q. This suggests that the cells within the tumor were genetically heterogeneous in terms of allelic loss, rather than being genetically clonal.

Incidence and Severity of LOH. LOH at any of the 22 chromosomal loci in bronchial epithelial DNA from the tumor-bearing airway occurred in at least one chromosomal locus in 30 (79%) of 38 patients (Tables 2 and 3). However, LOH was present in brushings from the contralateral lung in 20 (53%) of 38 patients. In 75% (P <0.05 *versus* random distribution of allele loss, *i.e.*, 50%) of these instances, the same allele was affected, a finding that is consistent with the concept of allele-specific loss (Fig. 3*c*). There were five instances of allele-specific loss in four patients without lung cancer. Although the number of patients was small, there was no difference in the frequency of allele-specific loss among patients with different lung cancer cell types, nor was there a predilection for a specific chromosomal region to be affected. Thus, LOH at one or more

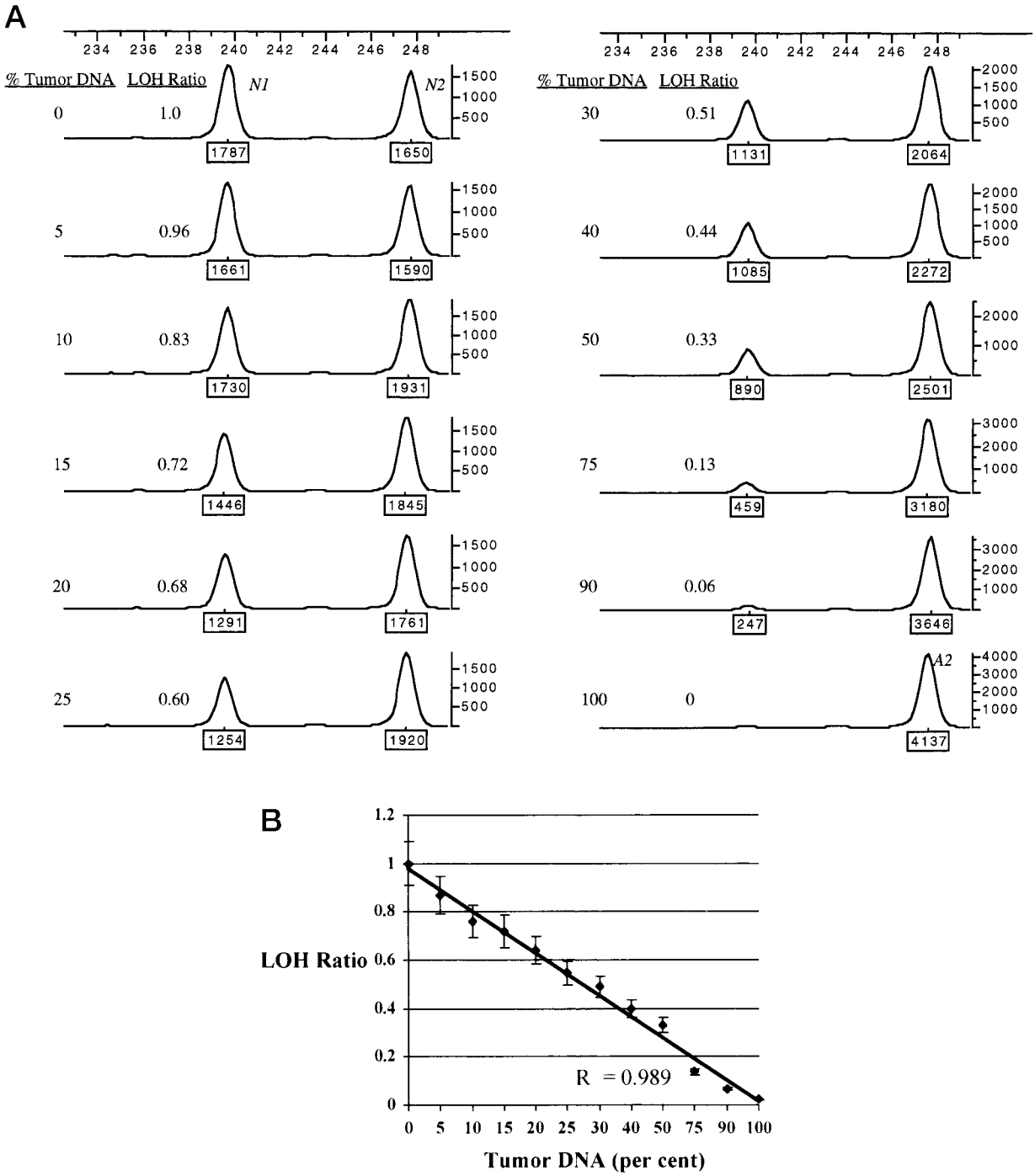

Fig. 2 Mixing experiment. Epithelial cell DNA obtained from a visible tumor was serially mixed with autologous leukocyte DNA from a patient with small cell carcinoma. As the amount of DNA from the tumor containing only A2 was increased in the template, there was a gradual decrease in the height of *N1* (*A*). The LOH ratio (normalized allele ratio) correlated in a linear fashion with the amount of DNA from the tumor (*B*).

sites was frequent and bilateral in the airways of smokers who have cancer. We did not observe microsatellite instability in our samples.

Comparison of tumor-bearing and contralateral lung (Fig. 4) showed that both the proportion of informative primer sites that demonstrated LOH (FAL) and the percentage of cells demonstrating allelic loss, expressed by the LOH score, was greater on the side of the tumor. The same was true for tumors with positive cytology *versus* negative cytology or when an airway abnormality was brushed. Thus, the extent of allelic loss

Table 1 Demographics

	Total (n = 55)	Lung cancer (n = 38)	No lung cancer (n = 17)
Age (mean ± SD)	66 ± 10	67 ± 8	61 ± 12
Sex-no. (%)			
Male	51 (93)	37 (97)	14 (82)
Female	4 (7)	1 (3)	3 (18)
Race-no. (%)			
Black	8 (15)	5 (13)	3 (18)
White	47 (85)	33 (87)	19 (82)
Smoking history			
Smokers-no. (%)	51 (93)	38 (100)	13 (76)
Pack-years (median)	70	75	50
Current[a]-no. (%)	31 (61)	23 (61)	13 (100)
Indication for bronchoscopy-no. (%)			
Mass/nodule	41 (74)	36 (94)	5 (29)
Infiltrate	3 (6)	0	3 (18)
Hemoptysis	5 (9)	1 (3)	4 (24)
Effusion	2 (4)	0	3 (12)
Other[b]	4 (7)	1 (3)	3 (18)

[a] Current smokers used tobacco within the past 12 months.

[b] Other indications for bronchoscopy included chronic cough, esophageal carcinoma, routine evaluation of lung cancer resection margin, and vasculitis.

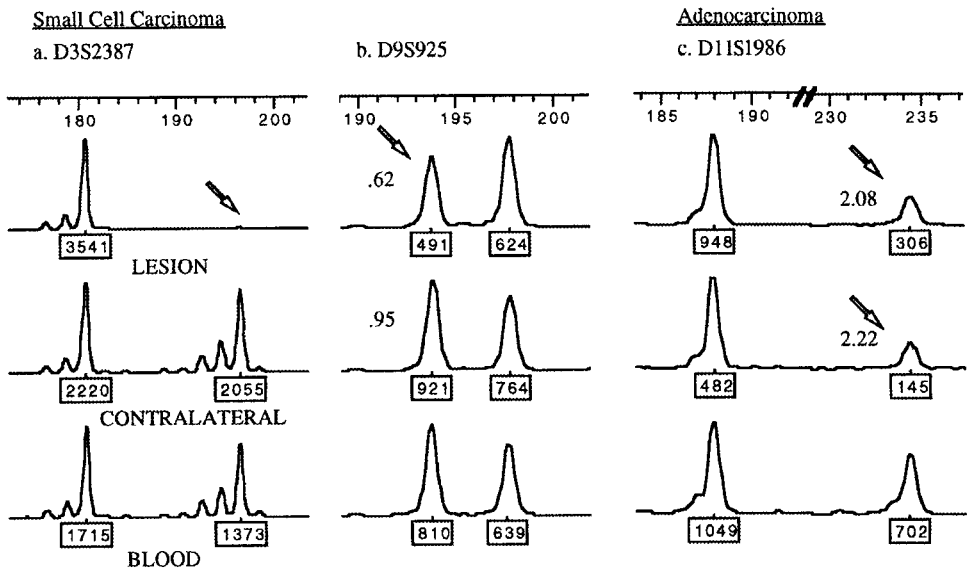

Fig. 3 Examples of microsatellite LOH. The *bottom row* of each panel is the electropherogram of the PCR products resulting from amplification of leukocyte DNA. The *middle* and *top* panels are electropherograms of products from amplification of DNA from the contralateral lung (*middle*) and from the airway containing the radiographic abnormality (*top*). Each column (*a-c*) of three panels contains the products amplified from DNA from one individual by a single primer. *Arrows* point to the alleles demonstrating LOH. The LOH ratios are provided adjacent to the *arrows*. *a*, complete loss of larger allele (196 bp) in tumor from a patient with small cell carcinoma using primer D3S2387 (3p21). *b*, same patient demonstrating partial allele loss in the tumor at microsatellite D9S925 (9p22). *c*, LOH in DNA from both the lesion and from the contralateral airway in a patient with adenocarcinoma at microsatellite D11S1986 (11q22). Note the same allele is lost in both airways; this is consistent with the concept of allele-specific loss.

increased as one moved closer to the tumor, as has been seen in studies of cells dissected from pathological specimens (10, 11).

We examined the sensitivity, specificity, and predictive values of the LOH score as a diagnostic test for lung cancer in the 38 patients who were determined to have lung cancer and in the 17 patients with another diagnosis (Table 4). Using a LOH score >10, the sensitivity was 58%, which was greater than the sensitivity of cytology (37%). The LOH score was >10 in the contralateral lung in five (13%) of 38 patients. This LOH score of >10 was 100% specific in the 17 patients without lung cancer. Despite often having at least one LOH site in lesion or contralateral lung, none of these 17 patients had a positive LOH score (>10) in cells from either lung. The positive and negative predictive values of the LOH score were 100% and 52%, re-

Table 2 Patient data

					LOH score		FAL	
Pathology	Age	Cigarette pack-years	Cytology	Stage[a]	Lesion	Contralateral	Lesion	Contralateral
Adenocarcinoma	68	100	Positive	1B	10	0	20	0
Adenocarcinoma	73	55	Positive	3B	18	0	35	0
Adenocarcinoma	84	45	Positive	2B	26	0	47	0
Adenocarcinoma	62	45	Positive	1A	50	4	67	8
Adenocarcinoma	67	100	Negative	3B	35	7	46	14
Adenocarcinoma	74	75	Negative	4	21	6	41	12
Adenocarcinoma	63	60	Negative	2A	0	0	0	0
Adenocarcinoma	73	55	Negative	4	4	0	7	0
Adenocarcinoma	64	50	Negative	3A	0	0	0	0
Adenocarcinoma	56	50	Negative	1A	0	0	0	0
Adenocarcinoma	80	30	Negative	2B	12	3	18	7
Adenocarcinoma	53	20	Negative	3B	20	21	30	33
Large cell	72	80	Positive	2A	17	21	33	43
Large cell	60	75	Positive	4	7	0	14	0
Large cell	62	75	Positive	3B	50	0	50	0
Large cell	75	250	Negative	3A	11	6	22	11
Large cell	74	98	Negative	4	60	3	67	6
Large cell	50	95	Negative	3B	18	18	36	36
Large cell	60	85	Negative	3B	17	9	33	18
Large cell	46	70	Negative	1A	0	0	0	0
Large cell	75	60	Negative	3A	0	5	0	10
Squamous	77	80	Positive	3A	3	7	6	13
Squamous	71	80	Positive	1B	65	0	90	0
Squamous	72	75	Positive	4	16	16	25	3
Squamous	61	60	Positive	4	64	5	91	9
Squamous	67	180	Negative	1A	3	3	5	5
Squamous	77	120	Negative	3A	9	19	12	25
Squamous	75	60	Negative	3A	0	0	0	0
Squamous	62	60	Negative	1B	5	0	9	0
Squamous	61	40	Negative	3B	14	0	29	0
Squamous	66	28	Negative	1B	0	4	0	7
Non-small cell	74	150	Negative	3B	18	4	36	7
Non-small cell	71	75	Negative	1A	3	9	7	19
Small cell	61	90	Positive	L	38	3	38	6
Small cell	64	50	Positive	L	89	0	100	0
Small cell	66	20	Positive	L	50	0	50	0
Small cell	69	125	Negative	L	0	0	0	0
Small cell	64	110	Negative	E	50	0	75	0
Mesothelioma	77	150			10	NA[b]	20	NA
Lymphoma	71	50			0	0	0	0
Esophageal cancer	66	40			0	4	0	8
Esophageal cancer	49	22			0	7	0	14
Head and neck cancer	54	60			0	8	0	15
Ependymoma	65	40			0	3	0	6
UIP	47	45			0	9	0	18
Granulomatous disease	61	80			6	7	12	14
Granulomatous disease	71	75			3	NA	7	NA
Granulomatous disease	63	0			4	8	8	16
Sarcoidosis	35	0			8	3	15	7
Vasculitis	72	0			0	0	0	0
Pneumonia	75	0			0	0	0	0
Pulmonary edema	46	40			0	0	0	0
Active tuberculosis	67	25			0	0	0	0
Bronchitis	71	100			0	0	0	0
Bronchitis	67	80			0	0	0	0

[a] Clinical stage: L, limited stage small cell carcinoma; E, extensive stage small cell carcinoma.
[b] NA, not available; UIP, usual interstitial pneumonitis.

spectively. These values compare favorably with those of cytology, which demonstrated positive and negative predictive values of 100% and 41%, respectively. Combining positive cytology and LOH score >10 increased the sensitivity in all subjects with cancer to 66%. The sensitivity of cytology combined with the LOH score was 80% in SCLC *versus* 55% in NSCLC, but this difference was not statistically significant.

Chromosomal Locations of LOH. Cumulative analysis of all chromosomal regions did not demonstrate significant differences in the incidence of LOH or extent of allelic loss

Table 3 LOH score and FAL

	Cancer		No cancer	
	Lesion	Contralateral	Lesion	Contralateral
Any LOH %	79	53	29	47
FAL	30 ± 4.5	7.7 ± 1.8	3.6 ± 1.5	6.5 ± 1.8
LOH score	21 ± 3.7	4.5 ± 1	1.8 ± 0.8	3.3 ± 0.9
LOH score > 10%	58	13	0	0

between SCLC and NSCLC patients or between NSCLC cell types. However, the incidence of allelic loss and of a LOH score >10 on chromosome 3p was higher in small cell carcinoma tumors compared with NSCLCs (Fig. 5). The microsatellites affected most frequently were in the regions of 3p21 and 3p14, which is consistent with data derived from microdissection of lung tumors (18). The chromosomal distribution of microsatellites with LOH was not useful in distinguishing small cell carcinoma from NSCLC.

Cigarette Smoking and LOH. To determine the relation between cigarette smoking and allelic loss, we plotted cumulative cigarette exposure *versus* FAL and the LOH score. To reduce confounding by the presence of tumors, we limited our analysis to cells obtained from the nontumor lung from patients with cancer and from the lesion-bearing lung in patients without lung cancer. Cumulative cigarette exposure did not correlate with the FAL or the LOH score in the contralateral nonlesion-bearing lung in either cancer or noncancer patients (data not shown).

DISCUSSION

We have devised and validated a sensitive, semiquantitative, and reproducible PCR-based method for assessment of LOH in a genetically heterogeneous population of cells using fluorescent microsatellite primers and capillary electrophoresis of epithelial cell DNA obtained at bronchoscopy. Mixing tumor cell lines and patient samples that were either heterozygous and or homozygous at a specific allele established the sensitivity and precision of the LOH assay. LOH was detected with 95% confidence if the change in the allele height ratio was ≥20%. This value is identical to that reported by Wang *et al.* (19) using fluorescent primers and capillary electrophoresis to analyze LOH in bladder cells. Our cutoff value, which was determined by the data presented in Fig. 2, is also in the same general range as those chosen by other investigators using radio-isotope labeling to evaluate LOH. (20, 21). In our study, the procedures following DNA extraction (PCR reaction preparation, data collection, and data analysis) are automated, making the method suitable for screening large patient populations.

We found, as have others, that LOH at any of the examined chromosomal loci was frequent in the airway epithelial cells of smokers. It was present in the contralateral lung of subjects with cancer and in both lungs of patients who had lesions on chest radiograph that proved not to be cancer. Thus, the presence of LOH is a nonspecific indicator of chromosomal DNA damage and it does not in and of itself indicate the presence of cancer. Brushings with positive cytology tended to have LOH at several microsatellites, often with an allele ratio <0.45. Brushings with negative cytology often demonstrated LOH, but the number of microsatellites involved was less, and the incidence of allele ratio <0.45 was less than in brushings with positive cytology. We, therefore, constructed a measure of the extent of LOH (LOH score) that incorporated the proportion of microsatellites with LOH or FAL and the percentage of cells with allelic loss or the LOH ratio. A positive LOH score >10 was determined by analysis of sensitivity and specificity of the score. The LOH score was positive (>10) in 55% of brushings from the tumor side and was negative in brushings from both lungs in the 17 patients who did not have lung cancer. The LOH score was >10 in brushings from four (11%) contralateral lungs in patients with cancer. It is unclear if a positive LOH score from the contralateral lung represented an early malignant lesion in the contralateral lung. Although none of the patients developed clinically evident cancer over the year of follow-up, second cancers do appear after treatment at a rate of 1–2% per year for NSCLC and 6% per year for SCLC (22).

Our results are consistent with prior studies in lung cancer that have measured LOH in cells dissected from tumors and from adjacent normal lung and in bronchial biopsies taken from smokers and former smokers who did not have lung cancer. These studies have shown that LOH occurs in current and former smokers without lung cancer, both in atypical and cytologically normal cells (9, 10) and that LOH accumulates as carcinogenesis progresses. Analysis of premalignant lesions in the airways of healthy smokers has demonstrated that the extent of LOH, as measured by fractional regional loss (fraction of chromosomal arms examined with LOH), accumulates as the histological stage progressed from dysplasia to carcinoma *in situ* (10). Analysis of squamous cell carcinomas has demonstrated that the extent of LOH, as measured by FAL, was higher in clinically advanced stage tumors than in small tumors that were radiographically occult (11). This concept of step-wise accumulation of DNA damage from "normal" to tumor tissue serves as the basis of the tumor progression models first put forth by Fearon and Vogelstein (23) and validated in lung cancer by Gazdar and coworkers (6, 7, 24). In these models, LOH, subsequent inactivating mutations at the sites of growth suppression genes, and activating mutations in oncogenes all accumulate as cells progress from premalignant to transformed phenotypes.

Allelic loss in the contralateral lung of 53% of the cancer patients is consistent with the concept of field cancerization and is confirmed by two recent studies of bronchial biopsies in smokers without cancer (9, 10). In our study, 80% of the microsatellites that demonstrated LOH in the contralateral lung were also involved in the lung containing the tumor, involving the same allele 75% of the time. Furthermore, each instance of contralateral LOH in the patients without lung cancer involved the same allele. This allele-specific loss suggests the presence of genetically determined allelic susceptibility to LOH. Allele-specific loss has been found in several previous studies, and several explanations have been proposed (7, 25). Clonal proliferation of a progenitor cell with a somatic mutation that confers growth advantage may explain the widespread appearance of the same mutation in lung epithelial cells. Alternately, allele-specific mutations may result from inherited differences that predispose specific alleles to DNA damage. It is known that allelic methylation of genes results in genetic imprinting during em-

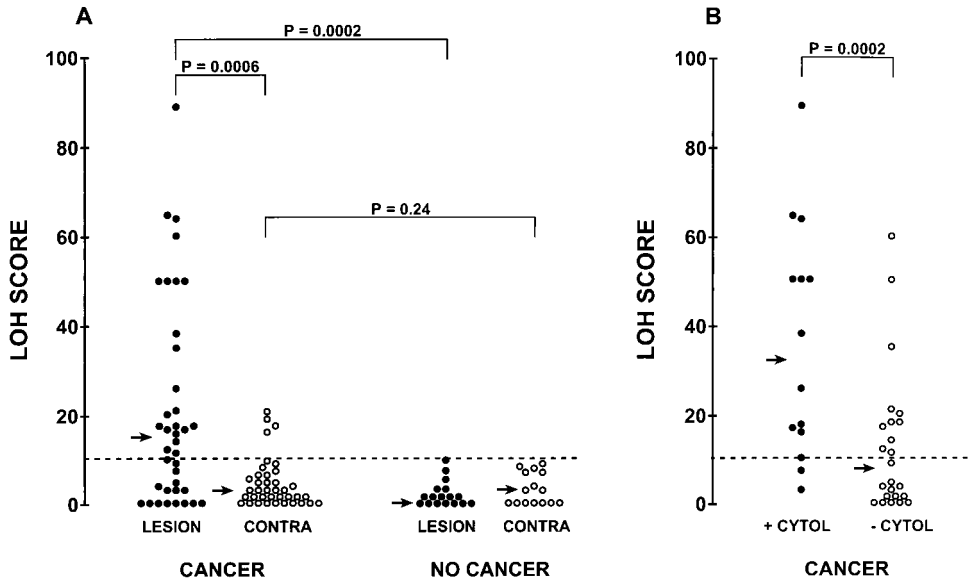

Fig. 4 Individual LOH scores in subjects. *A*, comparison of LOH score in epithelial cells obtained from the side of the lesion and contralateral lung in patients with cancer and with no cancer. ●, samples from the side of the lesion; ○, samples from the contralateral lung; *arrows*, the median values for the groups. The LOH score was higher on the lesion *versus* contralateral side ($P = 0.0006$) in cancer patients and higher on the lesion side ($P = 0.0002$) cancer *versus* noncancer patients. However, there was no difference in LOH score ($P = 0.24$) between the contralateral lung of cancer *versus* noncancer patients. *Dashed line*, a LOH score of 10. None of the noncancer patients had a LOH score >10, whereas 58% of the cancer patients had a LOH score >10. *b*, LOH score from the side of the lesion in cancer patients with positive (●) and negative (○) cytology. Those with positive cytology had scores that were greater ($P = 0.0002$) than those with negative cytology.

Table 4 Comparison of diagnostic tests

	Sensitivity	Specificity	Positive predictive value	Negative predictive value
LOH present	79	42	75	47
Cytology positive	37	100	100	41
LOH score >10	58	100	100	52
Cytology positive or LOH score >10	66	100	100	57

bryonic development (26). These specific methylated alleles may be targets of carcinogen-induced mutations, as demonstrated by benzo(*a*)pyrene adducts formation clustering preferentially at specific methylated codons on p53 (27) and for loss of imprinting of insulin-like growth factor II in lung cancer (28). Although the mechanisms for allele-specific loss remains to be determined, its presence in clinical samples may provide insights into genetic factors that determine susceptibility to genetic damage and lung cancer.

There was no statistically significant correlation between cumulative cigarette dose (pack years) and extent of LOH in the contralateral lung of cancer patients. These results are similar to the biopsy studies of smokers without cancer that demonstrated no correlation between extent of LOH and amount of cigarette consumption. The absence of this correlation suggests that other genetic and environmental factors are important for the progression of DNA damage. The ultimate biological significance of allelic loss in bronchial epithelial cells remains to be determined. In a few instances, the detection of LOH clustering in chromosomal regions has led to the cloning or a tumor suppressor gene such as *p53* or *FHIT*. However, given the frequency of allelic loss in nonmalignant epithelial cells, it is most unlikely that LOH always indicates deletion of a tumor suppressor gene. Alternately, LOH may be a biomarker of chromosomal instability that occurs as tumor suppressor genes, such as *p53*, are inactivated during carcinogenesis.

The LOH score calculation we devised is based, in part, on arbitray cutoff values that may theoretically introduce bias. However, the application of this method to our data illustrates the important principle that LOH is frequently detected in the epithelium of cigarette smokers, but extensive LOH is rare in the absence of cancer. Although the use of our measure of LOH in the diagnosis of lung cancer remains to be validated, the association of LOH score with lung cancer validates it as a potential biomarker (29). Future studies measuring the extent of LOH may be directed to the genetic and epidemiological analysis of cohorts of cigarette smokers who demonstrate inter-individual differences in the extent of allelic loss, relative to cigarette exposure. These studies may provide insights into the genetic factors such as race, gender, polymorphism in metabolic enzymes, and the environmental factors such as ambient air pollution and diet that may be responsible for the accumulation of allelic loss within cells of the bronchial epithelium. Prospective studies of cigarette smokers may also determine whether the LOH score will be a useful biomarker to identify smokers who are at risk for developing lung cancer and to assess epithelial cell responses to chemoprevention and to smoking cessation.

Our study and the work of others indicate that LOH analysis may have clinical use in the diagnosis of lung cancer. The

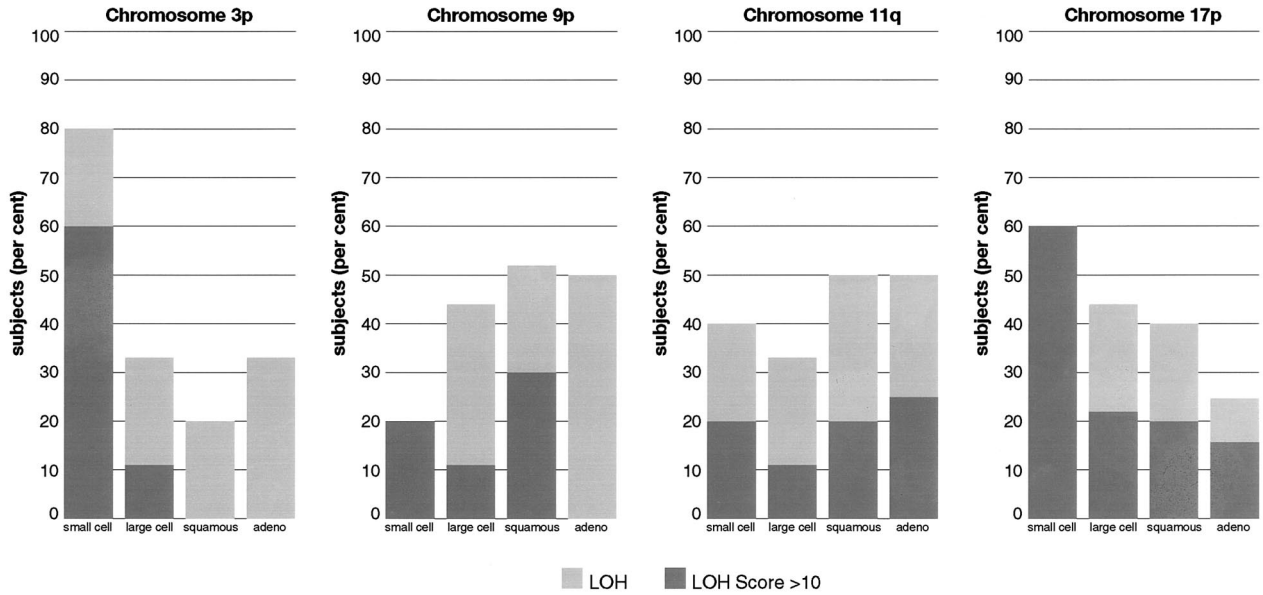

Fig. 5 Distribution of LOH on chromosomes 3p, 9p, 11q, and 17p for various histological cell types. The percentage of subjects with LOH at any microsatellite and a LOH score >10 are shown. Subjects with small cell carcinoma showed an increased incidence of LOH and LOH score >10 compared with subjects with other histological cell types ($P < 0.05$).

presence of LOH is nonspecific, but sensitive for the presence of cancer. These results indicate that molecular alterations associated with lung tumors are commonly detected in the epithelial cells of smokers without cancer. This paradigm similarly holds true for analyses of other genetic alterations including trisomy 7 (30), trisomy 20 (31), and p16 methylation (32). The analysis of the extent of LOH demonstrates that a LOH score >10 is specific and sensitive for the diagnosis of lung cancer. Compared with cytology, the LOH score is more sensitive while retaining a positive predictive value of 100%. The LOH score and cytology were complementary, resulting in a combined test sensitivity of 66%. This suggests that the simultaneous analysis of bronchoscopy brushings for extent of LOH and for cytological malignant cells may increase the diagnostic yield of the procedure.

With the addition of new microsatellite primer pairs at other chromosomal loci shown to be involved in lung cancer, the sensitivity and the negative predictive value of the LOH assay should increase. The method and the panel of primers need to be validated in a prospective study of a larger population of patients that will have greater statistical power before this test can be considered to have clinical use. It is clear that the specific criteria used in defining a positive LOH score in this study may not be applicable to all studies of LOH. Presently, most allelic loss analysis is performed using microdissected tissues as the source of DNA templates that are amplified by P32-labeled PCR primers. The values of a positive LOH score will likely vary depending on the methods used in the study. Whether the LOH score will correlate with the presence of lung cancer, regardless of the specific methods, remains to be determined.

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