Spectral Karyotyping Detects Chromosome Damage in Bronchial Cells of Smokers and Patients with Cancer

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Rationale: Lung cancer is a multistep process that is preceded and often accompanied by molecular cytogenetic lesions in benign bronchial epithelium, the precise character, extent and timing of which are not well defined.

Objectives: In this study we comprehensively defined molecular cytogenetic changes in bronchial cells that may precede lung carcinoma using spectral karyotyping (SKY).

Methods: SKY was applied to cultured benign bronchial cells from 43 high-risk smokers without carcinoma, 14 patients with concurrent lung carcinoma, and 14 never-smoker healthy volunteers.

Measurements and Main Results: The proportion of cells displaying numeric or structural anomalies/total number of metaphase cells was calculated for each case and was referred to as the chromosomal abnormality index. Mean chromosomal abnormality indices were 15.8, 10.1, and 0.7% for patients with cancer, high-risk smokers, and never-smokers, respectively. Clonal abnormalities were found in 17 (40%) of the high-risk smokers without carcinoma and 7 (50%) of the patients with carcinoma, but in none of 14 (0%) never-smokers. Chromosomal gains observed by SKY were confirmed in interphase cultured cells or paraffin sections of biopsy specimens by fluorescence *in situ* hybridization in 11 of 13 cases for which appropriate probes were available. In 6 of 57 high-risk patients or those with carcinoma, identical clonal abnormalities were dispersed at multiple bronchial sites and were admixed with nonclonal cells.

Conclusions: Clonal and single-cell chromosomal abnormalities are frequent in benign bronchial epithelium during lung carcinogenesis, indicating that chromosomal missegregation and other chromosomal rearrangements occur before overt malignancy.

Keywords: FISH; lung cancer; premalignant conditions; spectral karyotype

Lung cancer is one of the few tumors in which a cause—exposure to tobacco carcinogens—has been definitively established, but this information has been difficult to translate into significant clinical advance. Although there has been a reduction in cigarette smoking in recent years and an associated reduction in mortality, lung cancer remains by far the most lethal of all cancers in the United States in both men and women (1), and is a growing worldwide problem (2). Moreover, quitting smoking only gradually reduces the risk of lung cancer, in part because of the irreversibility of the genetic changes that occur in the airways as result of exposure to tobacco smoke. It is therefore crit-

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Chromosomal damage is known to occur during lung carcinogenesis, but the extent, timing, clonality, and relationship to cancer and smoking are not known.

What This Study Adds to the Field

Clonal and single-cell chromosomal abnormalities are frequent in benign bronchial epithelium during lung carcinogenesis, indicating that chromosomal missegregation and other chromosomal rearrangements occur before overt malignancy.

ically important that the nature and timing of the cellular and molecular effects of tobacco smoke on bronchial epithelium (BE) be thoroughly understood to identify biomarkers and devise intervention strategies that might reduce the persisting morbidity and mortality from lung cancer (3).

Lung cancer, like cancers of other organs, is thought to be the result of a multistep process in which a series of genetic and epigenetic alterations result in a malignant phenotype. These genetic alterations include chromosomal rearrangements. The karyotype of most lung cancers is complex, with a variety of abnormalities occurring in non-small cell (4) and small cell carcinomas (5). Compelling evidence demonstrating that these chromosomal changes are associated with chromosomal instability, rather than merely being random, nonpersisting chromosomal missegregations, has recently been assembled (6).

In premalignant epithelial lesions, chromosomal abnormalities have rarely been studied. Using fluorescence *in situ* hybridization (FISH) analysis of interphase cells, chromosome 7 trisomy (7, 8), and gains and losses for two to four centromere or single gene sequences (9) have been reported. These tests, however, measure only changes in copy numbers of specific chromosomes or allelic loci. Karyotypic analyses have been even rarer, probably due to the challenges of low proliferative rate of normal and premalignant epithelium *in vitro*. However, methods for the *in vitro* culturing of bronchial cells have been improving (10, 11), and bronchial epithelial culture is now routine in many research laboratories. Clonal and nonclonal cytogenetic abnormalities have been described by Sozzi and colleagues in bronchial cells using G-banding analysis (12–14). In these studies, nine patients were reported with structural chromosomal abnormalities, including deletions in 3p, 7p, 17p, and dicentric chromosomes.

The development of new multicolor karyotyping technologies has permitted more thorough and accurate chromosome evaluation in tumor and nonneoplastic cells, even in metaphase spreads with poor chromosomal morphology. One of these techniques is spectral karyotyping, which is based on deconvolution

(Received in original form September 15, 2006; accepted in final form June 25, 2007)

Supported in part by National Cancer Institute grants U01-CA85070 (Early Detection Research Network), P30-CA46934 (Cancer Center Support Grant), and P01-CA58187 (Specialized Program of Research Excellence in Lung Cancer).

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Am J Respir Crit Care Med Vol 176, pp 505–512, 2007

Originally Published in Press as DOI: 10.1164/rccm.200609-1329OC on June 28, 2007

Internet address: www.atsjournals.org

of signal from mixtures of fluorescent labels to obtain a pseudo-color image (15). This technology can provide a complete karyotypic profile and potentially could delineate chromosomal rearrangements that are important in lung carcinogenesis. This technology has been successfully applied to lung carcinoma (16), showing a large variety of chromosomal abnormalities in non-small cell lung cancers (NSCLCs). The objectives of this study then were to use spectral karyotyping (SKY) to obtain a comprehensive profile of chromosomal abnormalities that may precede or accompany the occurrence of invasive carcinoma in the lung and to identify targets for a diagnostic screening tool in populations at risk for lung cancer.

METHODS

Patients

A total of 71 patients contributed benign bronchial epithelial samples to this study. Fourteen patients (20%) underwent surgical resection for lung carcinoma at the University of Colorado Health Sciences Center and the Denver Veterans Administration Medical Center. Forty-three subjects (60%) were enrolled in a fluorescence bronchoscopy clinical trial designed to evaluate the detectability of abnormal BE in high-risk smokers and to correlate the histologic features of BE with biomarker expression. Requirements for entry into this trial included a smoking history of more than 30 pack-years, evidence of airway obstruction with FEV₁ less than 70% of the predicted value, and moderate dysplasia or worse on sputum cytology. Subjects meeting these criteria were offered fluorescence bronchoscopy using a Xillix laser-induced fluorescence emission bronchoscope (Xillix, Inc., Richmond, BC, Canada). Finally, 14 (20%) healthy volunteers who had never smoked (<1 pack lifetime exposure) and had no known occupational exposures served as control subjects. All specimens were obtained after informed consent under protocols approved by the Colorado Combined Institutional Review Board.

Table 1 summarizes demographics, smoking status, and clinical data for the study population subsets. Never-smokers were generally younger and more frequently female than the smokers and patients with cancer. The high-risk smokers were on average heavier smokers than were patients with cancer. This was due to the inclusion among the patients with cancer of four never-smokers and one patient with a smoking history of less than 0.3 pack-years. We found no differences in the frequency and character of SKY abnormalities among patients with cancer who were smokers and patients with cancer who were self-reported never-smokers. Patients with lung cancer were therefore analyzed as one group regardless of smoking history. The tumors of the patients with lung cancer included a variety of histologic types, which are listed in Table 2.

BE Collection and Processing

Benign BE was obtained either from endobronchial biopsies or from cross-sections of large bronchi near the surgical margins of resections. Patients undergoing laser-induced fluorescence emission bronchoscopy

were biopsied at multiple suspicious and normal-appearing sites (range, 3–42) at one or more bronchoscopic examinations. When technically possible, two biopsies were taken at the same site, one for histologic examination and one for culture. Biopsies from one or two sites from each subject were used for the primary cultures. The remaining biopsies were fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Histology of each biopsy specimen was graded for dysplasia according to a modification of the World Health Organization classification (17). Dysplasia grade at the bronchial site nearest the site used for culture was compared with the SKY results corresponding to that site. Bronchial cross-sections adjacent to the culture sites from resection specimens were similarly classified according to the World Health Organization classification.

To obtain endobronchial cells, biopsies 1–2 mm × 1 mm in size were placed intact in a T25 culture flask containing BE growth medium (BEGM; Clonetics, Inc., Walkersville, MD). Epithelial monolayers from the explanted biopsies were allowed to grow to a diameter of 1 cm or for approximately 10 days. From the surgical resection specimens, cross-sections of bronchus penultimate to the surgical margin were minced and then digested with Dispase solution (bacillus-derived neutral metalloprotease; Becton Dickinson, Franklin Lakes, NJ) after removal from the resection specimen. Individual cells were allowed to attach to Biocoat T25 flasks in BEGM culture medium (Clonetics, Inc.) and grown up to 90% confluence or cultured for 10 days. All cultured cells processed in this way grew as substrate-adherent monolayers, which are 100% cytokeratin-positive on immunohistochemical staining, as previously described (11).

To obtain metaphase cells for hybridization, cultured epithelial cells were removed from flasks by trypsinization (0.05% trypsin/ethylenediaminetetraacetic acid + 1% polyvinylpyrrolidone; Invitrogen, Carlsbad, CA) and washed three times in Hank's balanced salt solution. A total of 10,000 cells suspended in 500 µl BEGM medium were then pipetted into 4 small Petri dishes, each containing a glass coverslip (22 × 22 mm). Cells were then allowed to reattach and grow for another 4 days. At the end of this period, colcemid (0.02 µg/ml) was added to each dish for 4 hours. Ethidium bromide, to a final concentration of 5 µg/ml, was added during the last hour. The culture medium was removed, and hypotonic salt solution (2 ml of 0.4% sodium citrate: 0.4% KCl, 1:1) was added at 37°C for 20 minutes. Then, 1 ml of Carnoy's fixative (methanol:acetic acid, 3:1) was added. After 2 minutes, the hypotonic/fixative solution was aspirated, and three changes of Carnoy's fixative were added. Coverslips were then removed from the culture dish and air dried. One or two coverslips from each specimen were selected for the SKY study and stored in a desiccator at room temperature for up to 7 days until processed. Remaining coverslips from each specimen were frozen and stored at –70°C.

SKY

The human SKY probe cocktail and other reagents for hybridization and immunochemical detection were provided by Applied Spectral Imaging (ASI, Migdal HaEmek, Israel). Three fluorophores (fluorescein isothiocyanate [FITC], rhodamine, and Texas red) and two haptens (biotin and digoxigenin) were used to generate the 24-chromosome painting probe. The probe was annealed to blocking sequences by denaturing for 10 minutes at 75°C and incubation at 37°C for 1 hour to reduce nonspecific background labeling. Labeling was performed first by incubation of cells on coverslips in pepsin (0.012 mg/ml in 0.01 M HCl) for 2–3 minutes at 37°C followed by 1% formaldehyde/50 mM MgCl₂ at room temperature for 10 minutes. Chromosomal DNA was

TABLE 1. DEMOGRAPHIC FEATURES OF STUDY POPULATION

Characteristics	Carcinoma	High-Risk Smokers	Normal/Never Smokers
No. of Subjects	14	43	14
Age, yr, median (range)	68 (46–82)	62 (43–83)	51 (37–69)
Sex, % male	57	74	36
Pack-years, median (range)	39 (0–135)	66 (30–358)	0
% With dysplasia	17	28	0

Demographic features of patients with lung cancer; high-risk smokers and never-smokers differed somewhat, because median age and proportion of male subjects were smaller in the never-smoker group. These unavoidable differences were thought to have had minimal effect on frequency of spectral karyotyping abnormalities.

TABLE 2. TUMOR HISTOLOGY OF CANCER PATIENTS

Associated-Tumor type	No.
Squamous Cell	6
Adenocarcinoma	5
Bronchioloalveolar	1
Carcinoid tumor, classical type	2
Total	14

Histologic classification of tumors from patients with cancer reported in this study.

then denatured in 70% formamide/2× saline sodium citrate (SSC) at 70°C for 1–2 minutes, and 10 μ L droplets of probe were applied onto a clean microscope slide. Coverslips were inverted over these areas, which were sealed with rubber cement, and hybridized for 2 d at 37°C in a dry chamber. To wash the unbound reagents, three consecutive 5-min incubations were performed in 50% formamide/2× SSC and 2× SSC at 45°C, followed by a 2-minute wash in 4 × SSC/0.1% Tween 20 at room temperature. The biotin hapten was detected with avidin/Cy5; digoxigenin hapten was detected by indirect immunofluorescence with mouse anti-digoxigenin antibody plus sheep anti-mouse/Cy5.5 (ASI). Coverslips were counterstained with 0.15 μ g/ml 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) in Vectashield mounting medium (Vector, Burlingame, CA). Metaphases were randomly selected all over the coverslip area and imaged using the SD200 SpectraCube system with the SKY-1 optical filter (Chroma Technology, Brattleboro, VT), mounted on an Olympus BX60 microscope (Olympus Corp., Tokyo, Japan). SKYView version 1.6.2 software (Applied Spectral Imaging, Vista, CA) was used for image analysis. Electronically inverted DAPI images (band images) were used to identify deletions and intrachromosomal rearrangements, as well as to assign the chromosome breakpoints.

Karyotype analyses were performed according to International System for Human Cytogenetic Nomenclature criteria (18) blinded to patient identity. Clonal abnormalities were identified by the presence of the same chromosomal lesion in multiple metaphases by SKY alone or by the presence of the same numerical abnormality by both SKY and interphase FISH in tissue sections or cultured cells. The overall extent of chromosomal alteration was estimated by calculating percentage of cells displaying numerical or structural chromosomal anomalies, except for single-cell losses, chromosomal breaks, and polyploidy. The estimate is referred to in this study as chromosomal abnormality index (CAI). Single-cell losses, chromosomal breaks, and polyploidy were excluded from the index because they were dependent on the technical procedures performed, including cell culture, which resulted in an increase in polyploidy, and the chromosomal spread technique, which may have caused chromosomal breakage and loss (*see* NUMERICAL ABNORMALITIES IN PRIMARY CULTURES OF BRONCHIAL CELLS).

Interphase FISH

In 16 high-risk smokers, 4 patients with lung carcinoma, and 1 never-smoker control subject, in whom chromosomal losses or gains were observed by SKY, dual-target, dual-color interphase FISH assays were performed using DNA probes addressing the specific regions or chromosomes with abnormal metaphase results. Control cells in these FISH experiments were obtained from patients without specific abnormalities. For all specimens, interphase FISH assays were performed on coverslips, which were prepared from cell samples grown at the same time but independently from the SKY-tested samples. For seven of these specimens, biopsy sections from the lung site nearest to that analyzed by SKY were tested. Nine probes were used in the dual-target FISH assays, including chromosome enumeration probe (CEP) 3 spectrum orange (SO), CEP 7 spectrum green (SG), CEP 8 SO, CEP 18 SO, locus-specific indicator (LSI) EGR-1 SO/D5S721, D5S23 SG, CEP X SG/CEP Y SO probes (Vysis/Abbott Molecular, Downers Grove, IL), and a cosmid contig mapped at 3p21.31 labeled with FITC (A98 and H59 cosmids [19]).

The interphase FISH assays were performed as previously described (20, 21). Single band-pass filters for DAPI, Texas red, and FITC, and dual (Texas red/FITC) and triple (Texas red/FITC/DAPI) band-pass filters (Chroma, Brattleboro, VT) were used for examination of hybridized cells. Approximately 400 nuclei were scored in the coverslips, and 200 epithelial nuclei in the tissue sections.

Statistical Analysis

In the interphase FISH studies, the cutoff values for normal frequencies were estimated based on the average + 2 SD of signals per cell found in the specimens used as controls. The cutoff points for the CAI analysis were chosen based on the receiver operating characteristic analysis with fixed 100% specificity and maximized sensitivity. One-way analysis of variance was used to compare CAI across patients with carcinoma, high-risk smokers, and never-smokers. The chi-square test was used to compare differences between frequency distributions.

RESULTS

Cultured bronchial cells grew as a continuous, adherent monolayer without microscopic distinguishing features regardless of histologic appearances of the mucosa from which the cultured cells were biopsied. The monolayers contained occasional mitotic figures. A total of 1,492 metaphase cells were analyzed for this study—an average of 21 metaphases per patient (range, 5–50). Comparable numbers of metaphases were evaluated in carcinoma (mean, 18; range, 7–49), in high-risk smokers (mean, 24; range, 5–50), and in never-smoker control subjects (mean, 15; range, 7–41). Chromosomal abnormalities were observed in 82% of high-risk smokers and in all patients (100%) with carcinoma, regardless of self-reported tobacco exposure. Abnormalities detected included numerical changes (monosomy, trisomy, tetrasomy, and tetraploidy), structural anomalies involving a single chromosome (deletion, inversion, and isochromosome), and structural anomalies involving more than one chromosome (translocation, derivative, and telomeric association). Each specimen showed a small fraction (<10%) of polyploid metaphases. One or two polyploid metaphases were imaged for each specimen, both from test subjects and controls, and were found to be duplications of the normal diploid karyotype.

CAI

A marked difference between CAI of never-smokers and those of patients with cancer or smokers was observed. The mean CAI of normal bronchial cells from never-smokers was 0.7%, whereas it was 10.1% for high-risk smokers and 15.8% for patients with carcinoma (Figure 1). One-way analysis of variance indicated an overall significant difference in the mean CAI among the three groups of subjects ($P = 0.012$). Further comparisons indicated a significant difference in the mean CAI of never-smokers in comparison with high-risk smokers ($P = 0.01$) or patients with carcinoma ($P = 0.003$). No difference in CAI was associated with the presence of dysplasia at the culture site.

Clonal Abnormalities in BE

In cancer and high-risk populations, nearly equal numbers of subjects had clonal abnormalities as had exclusively single-cell

Figure 1. Mean chromosomal abnormality index (CAI) is depicted for each subject group. Significant differences were observed between patients with carcinoma and never-smokers, and between high-risk subjects and never-smokers (analysis of variance), but the difference between high-risk smokers and patients with cancer was not significant. Error bars represent variances of groups.

abnormalities (Figure 2). Half of the patients with cancer had clonal abnormalities in their benign epithelium and half had exclusively single-cell abnormalities. Remarkably, none of the biopsies of benign epithelium from patients with cancer were purely normal diploid cells, whereas 86% of the never-smokers had normal diploid cells only. A total of 40% of high-risk smokers had clonal changes in benign BE, whereas 42% had exclusively single-cell abnormalities. None of the never-smokers harbored clonal abnormalities. The differences in the frequencies of clonal and single-cell abnormalities and diploid cells among the tested groups were highly significant ($P = 2.0 \times 10^{-5}$). Overall, more single-cell abnormalities (190 events) than clonal lesions (52 events) were detected in both carcinoma and high-risk subjects.

Numerical Abnormalities in Primary Cultures of Bronchial Cells

Chromosomal gain was the most common clonal abnormality, with 20 lesions identified in 15 high-risk subjects or patients with cancer (Table 3). It was also the second most common single-cell abnormality, with 26 lesions affecting an additional 15 patients with cancer or high-risk subjects. Clonal gain most frequently affected chromosome 7 (6 cases), followed in order by chromosomes 18 (5 cases), 8 (3 cases), and 5 (2 cases). Single-cell abnormalities were more broadly distributed among the chromosomes. In most subjects, chromosome gain was represented by trisomy. However, tetrasomy for chromosomes 7 and 18 was observed in two patients. Clonal gains of both X and Y chromosomes were also observed.

Whole chromosomal losses (monosomies) were detected as clonal or single-cell abnormalities in 37 of the 57 (65%) patients with carcinoma or high-risk subjects. The most common clonal losses involved chromosomes 21 (3 cases), 10 (2 cases), and 22 (2 cases). Although the number of clonal losses was comparable to the number of clonal gains, the observed number of single-cell losses was almost fourfold greater than the number of gains. There is reason to suspect that this figure may be spuriously high. The methodology used for the creation of the metaphases in this study may have resulted in the displacement of chromosomes from their parental cells, and the cells were thus incorrectly classified as single-cell losses. Confirmatory

FISH testing using available probes failed to confirm loss in interphase cells either in culture or in tissue sections in the case in which this question was addressed (*see below*). Clonal losses, however, were more likely to be a reflection of *in situ* status of the epithelium, as it seems unlikely that the same chromosomes would be randomly displaced in multiple cells. For these reasons, we included clonal losses in the CAI but not single-cell losses (*see METHODS*).

Confirmation of Numerical Abnormalities by Interphase FISH

The described abnormalities were present in a minority of mitotic cells in each patient (10–30% of cells), except in one case (case 6), for whom 73% of dividing cells were abnormal. To verify whether these changes reflected the presence of a specific subset of abnormal cells or were caused by specimen processing (*in vitro* culture and harvesting), interphase FISH investigations were conducted. A total of 20 high-risk smokers or patients with lung cancer and 2 never-smoker control subjects were selected for these studies, based on the findings of specific losses or gains in the SKY analysis, which were targeted by the centromeric and locus-specific FISH probes in the interphase analysis (Table 4). For these 22 cases, *in situ* cultures matched with the ones used for the SKY karyotyping were tested. In seven cases, sections from formalin-fixed, paraffin-embedded biopsies from tracheobronchial sites close to the cultured site were also tested. Altogether, six probe sets were used in the FISH testing. Specimens from eight subjects were tested with centromeric probe for chromosome 7 (cases 3, 4, 5, 13, 14, 17, 18, and 19); from four subjects with centromere 8 probe (cases 4, 5, 6 and 18); from five subjects (cases 6, 13, 14, 19, and 20) and one control (case 21) with centromere 18 probe; from six subjects (cases 8, 9, 10, 11, 12, and 20) and one control (cases 22) with centromere 3/3p21 probe set; from four patients with 5p15/5q31 probe set (cases 1, 2, 15, and 16), and from one patient with the XY probe set (case 7). All 7 clonal abnormalities and 14/17 single-cell abnormalities for which probes were available were confirmed by FISH, and one example is illustrated in Figure 3. The concordance between SKY and interphase FISH was thus 88% overall, 100% for clonal changes, and 82% for single-cell abnormalities.

Presence of i(5)(q10) was confirmed in cases 2 and 16 by the findings of a significant fraction of interphase nuclei with four copies of the 5q31 signal and two copies of the 5p15 signal. Trisomy 5 was confirmed in case 15. Interphase studies in case 1 detected cells with extra copies of 5p15 signal, compatible with the karyotype findings of extra copies of del(5)(q11.2).

As in the SKY analysis, the frequency of abnormal cells in the interphase studies was generally low (around 10%) for all specimens except for case 6, where abnormal cells exceeded 70% of all other bronchial cells. A higher frequency of gain was detected in interphase than in metaphase cells in cases 5 and 6. Trisomies for chromosomes 7 and 8 were single-cell events in the metaphase analysis of case 5, whereas these abnormalities were present in more than 10% of interphase cells, and tetrasomies 7 and 8 were observed in approximately 5% of these cells. In case 6, trisomy 18 was detected in the majority of metaphase cells by SKY and trisomy 8 was a single-cell event. Trisomy 18 was confirmed in the majority of interphase nuclei. Interestingly, multiple additional copies of chromosome 18, as well as monosomy, trisomy, and tetrasomy for chromosome 8, were also observed in more than 20% of cells. The high frequency detected in interphase nuclei is probably related to the much higher number of cells included in that analysis (~400 nuclei) compared with the metaphase study (average of 17 mitotic spreads).

Figure 2. Graph and table indicate the proportions of subjects whose biopsies contained any clonal or nonclonal spectral karyotyping (SKY) abnormality. Abnormalities were nearly as frequent in high-risk smokers as in patients with lung cancer, but were significantly less frequent in never-smokers (chi-square, $P = 0.00002$).

TABLE 3. CLONAL AND SINGLE-CELL ABNORMALITIES IN BENIGN BRONCHIAL EPITHELIUM BY CLINICAL CONDITION

Abnormality	Carcinoma				High Risk				Totals (Carcinoma + High Risk)			
	Clonal		Single Cell		Clonal		Single Cell		Clonal		Single Cell	
	No. of Abn.	Cases ⁺	No. of Abn.	Cases ⁺	No. of Abn.	Cases ⁺	No. of Abn.	Cases ⁺	No. of Abn.	Cases ⁺	No. of Abn.	Cases ⁺
Gains	7	5	6	5	13	10	20	10	20	15	26	15
Losses	4	3	21	10	11	7	86	25	15	10	107	35
Partial losses	0	0	10	7	10	10	26	16	10	10	36	23
Balanced translocations	0	0	2	2	3	1	4	3	3	1	6	5
Unbalanced translocations	0	0	2	2	3	1	6	3	3	1	8	5
Inversions	0	0	1	1	0	0	4	4	0	0	5	5
Dicentric chromosomes	0	0	1	1	0	0	0	0	0	0	1	1
Telomeric associations	0	0	0	0	1	1	1	1	1	1	1	1
Total Abnormalities	11		43		41		147		52		190	

Definition of abbreviation: Abn. = abnormalities.

The number of specific chromosomal lesions of each type (No. of Abn.) and the cases in which those lesions were observed (Cases⁺) are listed in Table 3. The numbers of abnormalities and positive cases are not identical because there were many instances of multiple chromosomal lesions occurring in the same subject. The most frequent abnormalities were whole chromosomal gains and losses, suggesting that chromosomal missegregation with accompanying changes in gene dosage may be a dominant genetic mechanism in bronchial premalignancy.

Interphase analysis confirmed the presence of a fraction of polyploid cells in all specimens. Polysomy for all tested probes was found in approximately 5% (range, 2–10%) of nuclei in every specimen assayed, including control specimens.

Structural Abnormalities Detected by SKY FISH

Partial losses were clonal events in 10 instances and single-cell events in 36 (Table 2). Repeated deletions were observed at several chromosomal sites, including 1q11–12 (3 cases), 2q21–22

(3 cases), 3p11–21 (5 cases), 5p13–15 (2 cases), 5q11 (3 cases), 6q22 (2 cases), 9q12–21 (4 cases), 11p and q (2 cases), 12q11–13 (2 cases), and 13q21 (2 cases). A clonal deletion with breakpoint in 15q21 associated with a t(15;19)(q21;p13) was found in one patient.

Three clonal reciprocal translocations (Table 5) were identified in a single subject: t(2;4)(q24;q32), t(2;11)(q24;p12), and t(3;6)(p14;q24). A complex three-way translocation, t(1;7;18)(q21;q32;q23), was identified in an additional subject (Figure 4).

TABLE 4. CONFIRMATION OF SPECTRAL KARYOTYPING RESULTS BY INTERPHASE FLUORESCENCE *IN SITU* HYBRIDIZATION

Case No.	Group	SKY Karyotype		Interphase FISH Method		Confirmation	
		Clonal Anomalies	Single-Cell anomalies	Primary Culture	Tissue Section	Clonal	Single Cell
1	Smoker	del(5)(p13), del(5)(q11.2)	del(9)(q12), del(16)(q12)	+5p		Confirmed	
2	Smoker		i(5)(q10), del(9)(p12)	+5q			Confirmed
3	Smoker		+7	+7	+7		Confirmed
4	Smoker	+7	der(2)t(17;20)(q22;p11)	+7, normal 8	+7	Confirmed	
5	Smoker	del(3)(p21.1)	+7, +8, del(1)(q11)	+7, +8			Confirmed (×2)
6	Smoker	+18	+8	+8, +18		Confirmed	Confirmed
7	Smoker	+X		+X		Confirmed	
8	Smoker		+2, inv(9)(p12q13)	del(3)(p21)		New finding	
	Smoker	t(2;4)(q24;q32), t(2;11)(q24;p12), t(3;6)(p14;q24)	del(1)(q11), del(11)(pq)	del(3)(p21)		New finding	
9							
10	Smoker	del(3)(p14), +18	der(9)t(9; 18)(q12;q11)	del(3)(p21)		Confirmed	
11	Smoker		del(3)(p11), del(5)(q13)	del(3)(p21)			Confirmed
	Smoker	del(2)(q21), der(2)t(2;3)(q22;q11)	t(2;3)(q11;q21.3), der(3)t(3; 11)(q11;q11), der(3)t(3;12)(q21 0.3;q13), del(7)(q11), del(12)(q13)	no del(3)(p21)			Unconfirmed
12							
13	Smoker	del(15)(q21) t(15;19)(q21;p13)	+7 +18	Normal 7 +18	Normal 7 +18		Unconfirmed Confirmed
14	Smoker		–7	Normal 7, normal 18			Unconfirmed
15	Smoker	del(2)(q12)	+5, del(5)(q12), del(9)(q12)	+5	+5		Confirmed
16	Smoker		i(5)(q10), del(13)(q21)	+5q			Confirmed
17	Carcinoma	+7		+7	+7	Confirmed	
18	Carcinoma		+7, +8, t(1;4)(q23;q22)	+7, +8	+7, +8		Confirmed (×2)
	Carcinoma		+18, del(12)(q12), del(13)(q14)	Normal 7, +18			Confirmed
19			+18, dic(1;5)(p36;q11), del(3)(p12)	+18, del(3)(p21)	+18		Confirmed
20							
21	Normal		+18	Normal 18			Unconfirmed

Definition of abbreviations: FISH = fluorescence *in situ* hybridization; SKY = spectral karyotyping.

Correlation between SKY and interphase FISH on cultured cells and tissue sections confirmed clonality in seven of seven cases and demonstrated that many (14 of 17) single-cell abnormalities were also clonal. This indicates that SKY, performed by itself on the small number of metaphases usually available in biopsies, underestimates the frequency of clonal lesions.

Figure 3. Composite figure showing spectral image (A), inverted 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) band image (B), fluorescence *in situ* hybridization (FISH) image of interphase cells cultured from the same biopsy on which SKY was performed (C), and FISH image of paraffin section of bronchial biopsy adjacent to the biopsy used for spectral imaging (D). Spectral and inverted DAPI images indicate trisomy 18 (arrows), which is confirmed in FISH images of cells probed for CEP18 (three red signals) and CEP 7 (two green signals) per cell.

Six single-cell reciprocal translocations were found in five subjects and eight unbalanced translocations were found in an additional five subjects. Most translocations and derivatives involved material originating from two chromosomes. Recurrent translocations and derivatives were not detected, either among the patients or between distinct sites in a patient.

TABLE 5. LIST OF TRANSLOCATIONS IDENTIFIED IN SAMPLES FROM 12 INDIVIDUALS

Clonality/Type	Translocation	Subjects		
		Normal	High Risk	Carcinoma
Clonal	Balanced			
	t(2;4)(q24;q32)	0	1	0
	t(2;11)(q24;p12)	0	1	0
	t(3;6)(p14;q24)	0	1	0
Single Cell	Balanced			
	t(1;7;18)(q21;q32;q23)	0	1	0
	t(1;4)(q21;q23,q22)	0	1	0
	t(1;9)(p21;p13)	0	1	0
	t(4;10)(p15.3;q11.2)	0	1	0
	t(9;10)(p23;q21)	0	1	0
	t(15;20)(q;q)	0	0	1
	t(15;19)(q21;p13)	0	1	0
	Unbalanced			
	der(2)t(2;3)(q22;q11)	0	1	0
	der(4)t(3;4)(p7;p15)	0	0	1
	der(3)t(3;11)(q11;q11)	0	1	0
	der(3)t(3;12)(p21.3;q13)	0	1	0
	der(9)t(8;9)(q21;q13)	0	0	1
	der(9)t(18;9)	0	1	0
	der(14)t(12;14)	0	1	0
	der(20)t(17;20)(q22;p11)	0	1	0

Both clonal and single-cell abnormalities were detected. No recurring translocations were found in this small group of subjects.

Figure 4. Composite metaphase figure illustrating inverted DAPI band image (A), spectral image (B) and computer-generated karyotype table (C). A three-way translocation (t 1;7;18) is evident (arrows) in which a portion of the long arm of chromosome 1 is translocated to the long arm of chromosome 7, which, in turn, translocates to chromosome 18. Finally, a short segment of chromosome 18 is translocated to chromosome 1.

Other observed abnormalities included pericentric inversions involving chromosomes 8, 9, and 14 in three high-risk subjects and isochromosome of 5q in one high-risk subject and one patient with carcinoma, and, in all cases, were single-cell events. Dicentric chromosome (single-cell event) and telomeric associations (clonal involving 17q22) were observed in a patient with cancer and a high-risk subject, respectively. Recurrent breaks were detected at 1q12 (six patients), 2q11–12 (five patients), 3p14–21, 5q12, 9q12, and 11q12 (four patients each). Homogeneously staining regions were not observed, although small elements resembling double minutes originated from chromosome 3 were observed in a high-risk subject.

DISCUSSION

This study takes advantage of a technical advance, SKY (15, 22), to more completely characterize chromosomal damage in the benign BE of smokers and patients with carcinoma than has previously been possible. To date, SKY has been used to confirm the high frequency of chromosomal rearrangements that are present in lung cancer cell lines (16, 23) and lung carcinomas (24, 25). The National Cancer Institute has established a website on which lung cancer “SKYGRAMS” derived from SKY data are posted (26). The chromosomal rearrangements documented by SKY in lung carcinoma have been complex and generally nonrecurring among different specimens. These abnormalities have included complete or partial losses, gains, and reciprocal and nonreciprocal translocations (16, 23–25).

Precisely when such abnormalities first occur in the BE during lung carcinogenesis is unknown. Although SKY is a recently introduced technology, chromosomal imbalance was observed in carcinoma and premalignancy many years ago. Boveri is cited as the originator of the concept that abnormal separation of chromosomes during mitosis results in an unbalanced distribution of chromosomes to daughter cells, a condition referred to as aneuploidy (27). Over the past 2 decades, aneuploidy has been well documented in lung carcinomas by flow cytometry and static tissue morphometry, with most studies indicating 50–75% frequency of aneuploidy in NSCLC (reviewed in Reference

28). Aneuploidy also has been documented in the benign airway epithelium of smokers (7, 8, 29–31) and in benign head and neck mucosa (32), where it has been shown to predict the development of squamous carcinoma. The predictive value of aneuploidy in sputum has also recently been reported (33).

The cohort assembled for this study provided a unique opportunity to assess the nature and full extent of tobacco-induced chromosomal damage in benign BE of individuals with and without lung cancer. As observed in invasive tumors themselves, chromosomal damage in the airways of smokers without carcinoma was frequent and heterogeneous. Similar high levels of chromosomal damage were detected in benign airways of patients with cancer and high-risk smokers. Chromosomal abnormalities were observed in 82% of high-risk smokers and in all patients (100%) with carcinoma, regardless of self-reported tobacco exposure. A total of 12 of 14 volunteers with normal bronchial histology and no history of tobacco exposure had purely diploid biopsies. Quantification of chromosomal damage by CAI indicated significantly more damage in the epithelium of both patients with lung cancer ($P = 0.003$) and high-risk smokers ($P = 0.01$) than in never-smokers. CAI was higher in patients with cancer than in high-risk smokers (15.8 vs. 10.1%), but the difference was not significant ($P = 0.21$). The overall findings indicate a high level of chromosome damage in bronchial tissue and suggest that SKY may serve as an appropriate tissue endpoint in assessing levels of smoke-induced genomic damage and the *in vivo* effect of DNA repair mechanisms.

Abnormalities present in more than a single cell were defined as clonal. The presence of clonal abnormalities indicates that chromosomal damage in benign bronchial cells is replicated in daughter cell clones. The most frequently observed clonal abnormality was chromosomal gain. Overall clonal gains were found in 15 of the 57 (26%) patients with carcinoma or those at high risk (see Table 2). This probably is an underestimate of the true frequency of clonal gain, because only a limited number of metaphase cells could be examined and, in 11 cases, gain was confirmed as clonal by interphase FISH performed on tissue sections and coverslips of cultured cells where it had previously been found by SKY to be a single-cell abnormality. The same gain for specific chromosomes was found, not only in cultures established from the same biopsy used for SKY, but also in six cases in cultures prepared from dispersed bronchial sites. Clonal expansion and migration of mutant cells in benign mucosa has previously been reported in a single patient (34). The findings of the present study suggest that the phenomenon is frequent, occurring in at least 10% of high-risk individuals.

Clonal gains were detected in eight different chromosomes, and did not appear to be entirely random, as clonal gains of chromosomes 5, 7, 8, and 18 were detected in multiple cases, whereas gains of other chromosomes were not. The only one of these chromosomes for which prior data is available is chromosome 7. In a FISH analysis, Crowell and colleagues (7) found trisomy 7 in benign bronchial cells from 33% of patients with lung cancer, 12% of smokers, and 47% of uranium miners without cancer. Using probes for chromosome 7, Hittelman has also reported frequent “polysomy” (gain) in the airways of smokers that increases in degree with tobacco exposure (35). Recently, multicolor FISH has been used by Romeo and colleagues (9) to evaluate cultured bronchial cells from high-risk smokers. Increased copy number for one or more of chromosomes 5p15.2, 6, 7p12, and 8q24 was found in 26% of these individuals. It is of interest that the multicolor probe used in the study (LAVysion; Vysis/Abbott Molecular) contains markers for three of the four chromosomes found increased in the present study. This same probe set has been reported to be predictive of incident car-

cinoma in a nested case-control study of high-risk smokers (33). The study indicated an abnormality rate of 83% in subjects with subsequent carcinoma, but only 20% in matched controls.

Thus, whereas SKY is not a practical tool to directly apply to sputum, it does identify candidate chromosomal sequences that could improve the sensitivity of a FISH probe set for sputum screening and risk assessment. The most obvious candidate is a probe for chromosome 18, which proved to be one of the most commonly gained chromosomes. Improvement in sensitivity and perhaps automated processing and analysis could move a FISH-based assay forward to clinical application.

In addition to numerical change, SKY can detect chromosomal structural abnormalities. Several translocations were identified in this study, including 4 clonal translocations in 2 different high-risk smokers and 13 single-cell events in 10 additional subjects. None of the translocations were recurrent. This large number of translocations is indicative of the high level of chromosomal damage in bronchial cells even in the absence of invasive carcinoma. The prognostic significance of the translocations could not be assessed in this small pilot study, nor could it be determined whether the translocations reflected small subsets of premalignant lesions rather than sporadic, random, nonrecurring events. To answer this important question, it will be necessary to study larger cohorts for a longer interval. This question has taken on added urgency with the recent description of the first recurring translocation in a solid tumor, prostate carcinoma (36). None of the translocations identified in this study correspond to the reported unbalanced rearrangement that occurs in prostatic carcinoma (TMPRSS2:ERG) (36), but the likelihood of specific fusion proteins affecting significant numbers of NSCLCs remains an intriguing possibility.

Conclusions

Our intent in this study was to use the SKY technique to comprehensively map the extent of chromosomal damage that occurs in the benign BE of high-risk smokers and patients with lung cancer. We found that (1) chromosomal abnormalities are frequent in benign epithelium of high-risk smokers before carcinoma; (2) SKY abnormalities in benign epithelium may be clonal or nonclonal; (3) chromosomal gain and partial loss are the most frequent abnormalities overall, suggesting that chromosomal missegregation and double-stranded chromosomal breaks are frequent during lung carcinogenesis; (4) clonal changes may occur at more than one site; (5) individual abnormalities may occur alone or in association with different abnormalities, suggesting accumulation of abnormalities after the occurrence of an initial, possibly destabilizing genetic hit; and (6) SKY FISH is a feasible method for comprehensive evaluation of the chromosomal changes in nonmalignant bronchial epithelial cells of high-risk individuals.

Conflict of Interest Statement: M.V.-G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. L.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.L.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. F.R.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.C.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.K. is a collaborator with Y.E.M. on a patent application for the use of prostacyclin analogs for the chemoprevention of cancer. Y.E.M. received \$39,000 for being the site principal investigator for multicenter trials sponsored by Xillix, Inc., in 2003, received \$87,000 from Perceptrix, Inc., in 2004, and received \$60,000 for a single-site trial sponsored by SomaLogic in 2004. J.D.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. W.A.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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