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A Case-Control Study of Nonrandom Distribution of Bleomycin-induced Chromatid Breaks in Lymphocytes of Lung Cancer Cases[1]

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ABSTRACT

We used a case-control study design to determine the association between bleomycin-induced chromatid breaks and the risk of lung cancer in general and by specific histopathological types. Lymphocytes from primary blood cultures of 78 controls and 75 cases with 4 histopathological types of lung cancer were treated with 0.03 unit/ml bleomycin for 5 h, and the frequency of induced chromatid breakage and the locations of the breaks were determined in Q-banded preparations. After adjustment for their length, the larger chromosomes had more breaks than the smaller chromosomes in both cases and controls. The cases had significantly more breaks on chromosomes 4 and 5 than the controls did, with odds ratios (ORs) of 4.9 [95% confidence limits (CL), 2.0, 11.7] and 3.9 (95% CL, 1.6, 9.3), respectively. When the lung cancers were classified by histopathological type, adenocarcinomas had significantly more breaks on chromosomes 4 and 5, with ORs of 3.0 (95% CL, 1.0, 8.7) and 3.5 (95% CL, 1.2, 10.7), respectively. For squamous cell carcinoma, the ORs were significantly elevated for breaks on chromosomes 2, 4, and 5 with ORs of 3.5 (95% CL, 1.0, 11.7), 10.2 (95% CL, 2.5, 41.9), and 7.9 (95% CL, 1.9, 32.8). For small cell carcinoma, breaks on chromosomes 2 and 4 showed significantly increased ORs of 33.2 (95% CL, 2.2, 513.3) and 20.4 (95% CL, 1.7, 250.1), respectively. However, no specific chromatid breaks were detected in cases with large cell carcinoma. When the frequency of chromatid breaks at specific regions was calculated, breaks at 4p14, 4q27, 4q31, 5q21–q22, 5q31, and 5q33 were significantly more common in lung cancer cases than in controls. Lung cancer risk had a dose-response relationship with breaks on chromosomes 4 and 5. Cigarette smoking had a strong interaction with breaks on chromosomes 2, 4, and 5. The findings suggest that the susceptibility of particular chromosome loci to mutagenic damage may be a risk factor for specific types of lung cancer.

INTRODUCTION

Lung cancer is the paradigm of gene-environment interaction. The fact that only 10–15% of cigarette smokers develop lung cancer implies that host genetic factors may modulate susceptibility to tobacco carcinogenesis (1). One measure of this cancer susceptibility may be mutagen sensitivity, which can be measured by an assay developed by Hsu *et al.* (2) that quantifies bleomycin-induced chromatid breakage in cultured lymphocytes. Combining cytogenetic and epidemiological data, Spitz *et al.* (3) showed that mutagen sensitivity was a strong and significant risk factor for malignancies of the upper aerodigestive tract and a predictor of risk for the development of second malignancies of the head and neck (4). Chromosomal breaks, if located in a vital gene locus or loci, could induce expression of a quiescent gene or inactivate a tumor suppressor gene and thus lead to carcinogenesis. In an ongoing molecular epidemiological case-control study of lung cancer, we applied cytogenetic techniques to test whether the frequency and location of these induced breaks were nonrandom and whether the susceptibility of particular chromosome loci to mutagenic damage is a risk factor for lung cancer. The role of exposure to relevant carcinogens such as tobacco smoking was also examined.

MATERIALS AND METHODS

Study Subjects. The cases and controls are a subset derived from an ongoing study of lung cancer in minority populations. The cases were 75 newly diagnosed patients with histologically confirmed lung cancer who were of self-reported African American or Mexican American ancestry and who had not begun radiotherapy or chemotherapy. The patients were identified from The University of Texas M. D. Anderson Cancer Center and from county, community, and Veterans Administration hospitals in the Houston and San Antonio metropolitan areas. Twenty-eight cases had adenocarcinoma, 28 had squamous cell carcinoma, 11 had small cell carcinoma, and 8 had large cell carcinoma. The controls were a convenience sample of 78 healthy subjects recruited from Houston and San Antonio community centers, cancer-screening programs, churches, and employee groups by using a frequency-matching approach, *i.e.*, by age (±5 years), sex, and ethnicity.

Epidemiological Data Collection. The epidemiological data were collected by personal interviews (5). After informed consent was obtained, a structured interview of approximately 45 min was conducted by trained interviewers/phlebotomists in English or Spanish. Data were collected on sociodemographic characteristics, recent and past tobacco use, and other life style habits by using standardized questions, when available. Ten ml of blood were drawn into heparinized tubes for cytogenetic analyses.

Chromosome Analysis. Standard lymphocyte cultures were established as described previously (2). On the third day of incubation, the cultures were treated with 0.03 unit/ml bleomycin for 5 h. During the last h, the cells were treated with 0.04 μg/ml Colcemid to arrest them in mitosis before they were harvested for conventional air-dried preparations. All prepared slides were coded and stained with Giemsa. For mutagen sensitivity analyses, breaks were counted in 50 metaphases and expressed as the average number of breaks per cell. Individuals with ≥1 break/cell were recorded as mutagen sensitive. To evaluate the location of the breaks, the first 25 consecutively identified aberrant metaphases were identified and photographed. These slides were then stained with quinacrine dihydrochloride for chromosome banding. The same metaphases were then rephotographed to compare them with the Giemsa-stained cells to identify the locations of the chromatid breaks. The locations and the break frequency for each chromosome in every sample were recorded. Since the slides were coded the case status was not known when the slides were read.

Statistical Analysis. Specific chromosomal breaks were categorized by using the 75th percentile value in the control group, selected *a priori*, as the arbitrary cutoff point. (We repeated the analysis using other cutoff points and found that the results did not change substantially). We also evaluated the specific band locations of the induced breaks. To determine which chromosomes were most likely to break after bleomycin exposure, the observed number of breaks in lung cancer cases and controls was compared with the expected number of breaks calculated according to the chromosome length (6). Partial correlation coefficients were calculated to evaluate the relationship between variables. To test for significant associations among specific chromosomal breaks, mutagen sensitivity, tobacco use, and cancer risk, univariate ORs[4] were calculated as estimates of the relative risk. Ninety-five % CLs were computed by the method of Woolf (7). Simple stratified analysis was used to

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[4] The abbreviations used are: OR, odds ratio; CL, confidence limits.

test for interaction between specific chromosomal breaks and smoking status. Logistic regression was conducted with STATA statistical software (8). A stepwise variable selection procedure was used to establish the best-fitting logistic model.

RESULTS

Distribution of Selected Demographic Variables. Selected demographic characteristics of the cases and controls are summarized in Table 1. Since we are using a frequency-matching approach for control selection in this ongoing study, perfect matching has not yet been achieved, but there were no significant differences in age, sex, and ethnicity between cases and controls. The mean age for the cases was 59.3 years, compared with 60.6 years for the controls. Small cell lung cancer was significantly more frequently diagnosed in the male cases than in the female cases (22% *versus* 0; $P < 0.05$), while women (50%) were more likely than men (30%) to be diagnosed with adenocarcinoma (data not shown). There were no differences in histological distribution by ethnicity or by stage. The cases had significantly fewer years of education than the controls did ($P < 0.05$).

Predictably, there were significant differences in smoking status between cases and controls. Only 9.3% of the cases had never smoked, compared with one-half of the controls. There was a 8.8-fold increased risk associated with ever smoking ($P < 0.05$; data not shown). For men the OR associated with ever smoking was 31.3 (95% CL, 4.0, 245.2) and for women the OR was 4.4 (95% CL, 1.4, 14.0). The cases were also significantly heavier smokers in terms of pack years smoked (44.2 *versus* 12.0) and number of cigarettes smoked per day (24 *versus* 8) ($P < 0.001$). The ORs were significantly elevated for squamous cell carcinoma (OR, 26.2), small cell carcinoma (OR, 11.8), and adenocarcinoma (OR, 3.2). There were no ethnic differences in risk estimates for smoking.

Distribution of Chromosomal Breaks. Fig. 1 illustrates the mean observed number of breaks in the lung cancer cases and in controls compared with the number expected based on the chromosome length. In cases and controls the larger chromosomes (1–8 and X) were more susceptible to bleomycin-induced breaks than were the smaller chromosomes (14–22 and Y).

Table 1 *Distribution of selected characteristics of lung cancer cases and controls*

Characteristics	Cases	Controls	*P*
Ethnicity			
Mexican American	14 (18.7)[a]	18 (23.1)	
African American	61 (81.3)	60 (76.9)	0.503
Sex			
Mexican American			
Male	10 (71.4)	12 (66.7)	
Female	4 (28.6)	6 (33.3)	0.773
African American			
Male	40 (65.6)	36 (60.0)	
Female	21 (34.4)	24 (40.0)	0.526
Mean age (yr)			
Total	59.3 ± 11.3[b]	60.6 ± 15.1	0.550
Mexican American	65.5 ± 7.5	64.4 ± 11.5	0.757
African American	57.9 ± 11.6	59.5 ± 15.9	0.533
Education (yr)	9.6 ± 4.0	11.6 ± 4.4	0.007
Smoking			
Status			
Never	7 (9.3)[a]	37 (47.4)	
Former	34 (45.3)	20 (25.6)	<0.001
Current	34 (45.3)	21 (26.9)	<0.001
No. of cigarettes/day	23.5 (14.4)[b]	8.1 ± 11.8	<0.001
Years smoked	34.6 (15.5)	14.7 ± 17.1	<0.001
Pack years	44.2 (29.1)	12.0 ± 20.2	<0.001

[a] Numbers in parentheses, percentage.
[b] Mean ± SD.

The crude and adjusted ORs associated with breaks on particular chromosomes are summarized in Table 2. Cases exhibited significantly more breaks on chromosomes 4 and 5 than controls did (ORs, 4.9 and 3.9, respectively) after adjustment for age, sex, ethnicity, and years smoked. None of the other 22 chromosomes showed significantly more breaks in the cases than in the controls. When the lung cancers were classified by histopathological type, there were significantly more breaks on chromosomes 4 and 5 for adenocarcinomas (adjusted ORs, 3.0 and 3.5, respectively). For squamous cell carcinoma, the adjusted ORs were significantly elevated for chromosomes 2, 4, and 5 (3.5, 10.2, and 7.9, respectively). For small cell carcinoma, the adjusted ORs were markedly elevated for chromosomes 2 and 4 (33.2 and 20.4, respectively). However, no specific chromosomal breaks were noted in patients with large cell carcinoma. When chromosomal breaks were dichotomized by using the median value of the number of breaks for each chromosome in the control group as an arbitrary cutoff point, similar results were obtained (data not shown).

There were significant differences in overall mutagen sensitivity between cases and controls (data not shown). The mean break per cell values were 1.2 for the cases and 0.8 for the controls ($P < 0.001$). No significant differences were found in mean breaks per cell by histology of lung cancer or smoking status. The univariate OR for mutagen sensitivity (dichotomized at 1 break/cell) was 4.4 (95% CL, 2.2, 8.8). The final logistic regression model included the variables age, sex, ethnicity, mutagen sensitivity, breaks on chromosome 4, breaks on chromosome 5, and years smoked. Breaks on chromosomes 4 (OR, 4.7; 95% CL, 1.9, 12.2) and 5 (OR, 2.6; 95% CL, 1.0, 6.9) were significant predictors of lung cancer risk. Mutagen sensitivity (dichotomized at 1 break/cell) and cigarette smoking were also associated with significantly elevated risk (data not shown). After adjustment for age, sex, ethnicity, and smoking, the ORs associated with breaks on chromosomes 2 and 18 were not statistically significant.

There was a dose-response relationship between lung cancer risk and the number of induced breaks on chromosome 5, with 0–1 breaks as the referent category (Table 3). The ORs for individuals with 2, 3, and 4 or more breaks on chromosome 5 were 3.3, 15.3, and 26.6, respectively. The trend test by linear regression analysis was significant ($P < 0.0001$). A similar dose-response relationship was also suggested for chromosome 4. None of the other chromosomes exhibited such a dose-response relationship.

To evaluate the possible interaction between the induced breaks and cigarette smoking, we performed stratified analysis (Table 4). The referent category was nonsmokers and subjects with fewer breaks than the 75th percentile of the controls. After adjustment by age, sex, and ethnicity, the OR for smoking in the absence of sensitivity to chromosome 4 breaks was 8.1; for breaks on chromosome 4 in nonsmokers the OR was 3.9. In the presence of both factors, the OR was 128.8. This combined OR suggests that there is a synergistic interaction between cigarette smoking and breaks on chromosome 4 that is greater than multiplicative. A similar effect was suggested for cigarette smoking and breaks on chromosomes 2 and 5. None of the other chromosome breaks showed this effect. However, in the logistic model, the interaction term was not statistically significant (probably due to sample size limitations).

Specific Breaks as Risk Factors. Our next objective was to identify specific chromosome loci involved in the breaks. Of the 339 bands analyzed, only 7 were associated with significantly increased lung cancer risk after adjustment by age, sex, ethnicity, and years smoked (Table 5). These bands were 4p14, 4q27, 4q31, 5q21, 5q22, 5q31, and 5q33.

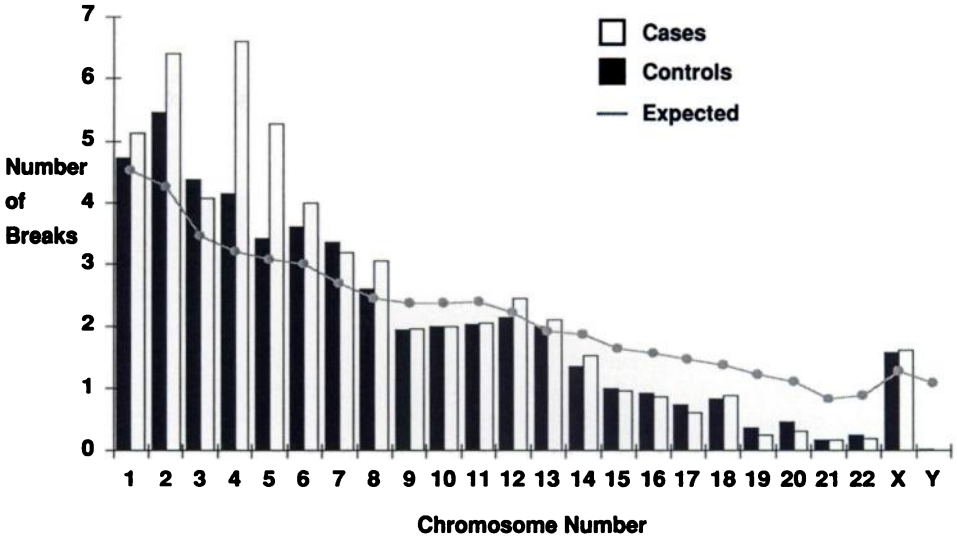

Fig. 1. Average number of observed *versus* expected bleomycin-induced chromatid breaks in lung cancer cases and matched controls. *Expected*, expected number, based on chromosomal length.

DISCUSSION

The results of our current study suggest that bleomycin-induced chromatid breaks in the lymphocytes of lung cancer cases are not randomly distributed but preferentially involve chromosomes 2, 4, and 5. Alterations in chromosomes 4 and 5 have been reported in metastatic lung cancers (9), pleural effusions (10–11), and established lung cancer cell lines (12). Recently, Testa and Graziano (13) reported alterations in chromosome 5 in 16 of 17 lung tumors examined. Losses involving chromosome 5 were noted in 14 cases; 3 involving complete loss of chromosome 5 and 11 involving partial chromosomal deletion. Hosoe *et al.* (14) examined the deletion of chromosome 5q in 59 cases of advanced lung cancer using 12 RFLP markers on 5q. Of 59 lung cancer cases, 48 (81%) had lost some of the 5q loci. The most frequently lost region was 5q21. Wieland and Bohn (15) and D'Amic *et al.* (16) also reported frequent allelic deletions on 5q in lung cancer patients. By introducing a single normal human chromosome 5 or 18 into a human colon carcinoma cell line, Tanaka *et al.* (17) found that the genes on normal chromosomes 5 and 18 functioned as tumor suppressor genes in colon carcinogenesis. Chromosome 5q deletion is a recurring abnormality in malignant myeloid neoplasms (18, 19). Rowley (20) and Strom *et al.* (21) showed that the frequency of partial deletion or loss of 5q is increased in patients with secondary acute myeloid leukemia and acute nonlymphoblastic leukemia. A study by Narod and Dube (22) showed that an occupational history of exposure to chemicals or metals was more common in men with monosomy 5 than in men with a normal karyotype. Therefore, chromosome 5 abnormalities may be related to mutagenic damage such as from cigarette smoking and other carcinogens implicated in lung cancer carcinogenesis.

Chromosome 4 abnormalities are rarely reported in cancer. However, recently Teyssier and Ferre (23) reported that 4q breaks were among the most common karyotypic abnormalities in 203 human primary solid tumors. Using *in situ* hybridization, Donti *et al.* (24) found one or a few copies of c-*myc* translocated onto a novel 4q+ marker chromosome in leukemia cells. Carr and Todd (25) reported that chromosome 4 translocations were present in leukemia. 4q abnormalities have also been reported in squamous cell carcinoma of the skin (26), Hodgkin's disease (27), malignant melanoma (28), head and neck cancer (29), and hepatocellular carcinoma (30).

In this report, we present evidence of a dose-response relationship between chromosome 4 or 5 breaks and risk for lung cancer. These data further support the role of susceptibility to breaks on chromosome 4 and 5 as a risk factor for lung cancer. We also reported a strong interaction between cigarette smoking and susceptibility to

Table 2 *ORs for nonrandom breaks in lung cancer cases*[a]

Chromosome	Carcinoma subtype				
	Adenocarcinoma (N = 28)	Squamous cell (N = 28)	Small cell (N = 11)	Large cell (N = 8)	All (N = 75)
Chromosome 2					
Crude OR (95% CL)	1.5 (0.6, 3.8)	2.3 (0.9, 5.4)	11.3 (2.3, 55.3)[b]	1.7 (0.4, 8.1)	2.3 (1.2, 4.5)[b]
Adjusted OR (95% CL)[c]	1.3 (0.5, 3.7)	3.5 (1.0, 11.7)[b]	33.2 (2.2, 513.3)[b]	1.4 (0.3, 8.2)	1.9 (0.9, 4.4)
Chromosome 4					
Crude OR (95% CL)	2.4 (1.0, 5.8)	4.6 (1.8, 11.5)[b]	12.7 (2.6, 62.8)[b]	1.9 (0.4, 9.2)	3.6 (1.8, 7.1)[b]
Adjusted OR (95% CL)[c]	3.0 (1.0, 8.7)[b]	10.2 (2.5, 41.9)[b]	20.4 (1.7, 250.1)[b]	2.2 (0.4, 13.0)	4.9 (2.0, 11.7)[b]
Chromosome 5					
Crude OR (95% CL)	3.0 (1.1, 7.9)[b]	3.9 (1.4, 10.6)[b]	5.3 (1.1, 25.6)[b]	1.4 (0.3, 6.7)	3.3 (1.7, 6.7)[b]
Adjusted OR (95% CL)[c]	3.5 (1.2, 10.7)[b]	7.9 (1.9, 32.8)[b]	3.1 (0.4, 23.5)	1.3 (0.3, 7.0)	3.9 (1.6, 9.3)[b]
Chromosome 18					
Crude OR (95% CL)	2.7 (1.0, 7.1)[b]	1.0 (0.4, 2.3)	1.3 (0.4, 4.6)	0.4 (0.1, 2.1)	1.3 (0.7, 2.4)
Adjusted OR (95% CL)[c]	2.4 (0.8, 7.1)	1.0 (0.3, 2.9)	1.5 (0.3, 8.7)	0.3 (0.0, 2.0)	1.2 (0.5, 2.7)

[a] Compared to the 75th percentile in controls (N = 78).
[b] $P < 0.05$.
[c] Adjusted by age (as a continuous variable), sex, ethnicity, and years smoked.

breaks on chromosome 4 or 5. In a previous paper (3), we suggested that there was an interaction between cigarette smoking and mutagen sensitivity. Our results also suggest that the larger chromosomes are more susceptible to bleomycin-induced breaks, although the reason for this phenomenon remains unknown.

It is interesting to note that the nonrandom breaks associated with lung cancer risk were located at chromosomal regions that harbor clusters of putative tumor suppressor genes (*MCC* and *APC* at 5q21 and *IRF1* at 5q31), oncogenes (*LCA* at 2q14), immunofactor genes (*IL1A*, *IL1B*, and *IL1RN* at 2q14; *IL2* at 4q25; and *IL3–5*, *IL9*, and *IL13* at 5q31) and growth factor genes (*EGF* at 4q25 and *FGF2* at 5q31). Three of these nine bands harbor the loci of the *IL1A*, *IL1B*, *IL1RN*, *IL2*, *IL3*, *IL4*, *IL5*, *IL9*, and *IL13* genes. It is noteworthy that loss of heterozygosity at the *MCC* and *APC* loci is very frequent in human small cell lung cancer (16) and that *IL2* has unique anticancer properties (31). However, demonstration of breaks in specific regions of critical genes at the cytogenetic level is admittedly rather a crude measurement of the involvement of these genes. Furthermore, we used normal cells (cultured lymphocytes) from lung cancer cases and controls, not target tissue. Nevertheless, identification of such preferential chromosomal breakage may serve as an initial stepping stone for designing more definitive experiments at the molecular level. We are in the process of extending this research to primary cultures of lung cancer tissues by using fluorescence *in situ* hybridization techniques with specific chromosome probes and cosmid clones to confirm these preliminary findings.

There are other limitations in this study. The cases and controls were not population based. However, only newly diagnosed cases from a variety of metropolitan hospitals in Houston and San Antonio were included to minimize selection bias and any misclassification resulting from therapeutic interventions or the disease process. In our analyses, adjusted ORs instead of crude ORs were presented to control for potential confounding from age, sex, ethnicity, and smoking. The reproducibility of the bleomycin assay has been tested in two independent laboratories (2, 32). Both groups have shown that the patterns appear stable over time. Cloos *et al.* (32) demonstrated low mean intraindividual variation (0.08) compared with a mean interindividual variation of 0.35, confirming the earlier work of Hsu (2). All slides were coded before scoring to minimize misclassification. Trizna *et al.*[5] have shown the inter-reader agreement rate was above 86% in 400 samples studied.

In summary, sensitivity to bleomycin-induced chromatid breaks is a risk factor for lung cancer, and these breaks appear to be nonrandom. The susceptibility of specific chromosome loci to mutagenic damage may predispose individuals to lung cancer. Confirming these findings at the molecular level is the next step.

Table 3 *Dose-response relationship of induced breaks on chromosomes 4 and 5 and lung cancer risk*

No. of breaks	No. Cases	No. Controls	OR (95% CL) Crude	OR (95% CL) Adjusted[a]
Chromosome 4[b]				
0–2	4	18	1.0	1.0
3	5	16	1.4 (0.3, 6.2)	0.9 (0.1, 5.4)
4–5	22	22	4.5 (1.3, 15.5)	7.1 (1.4, 35.7)
6+	44	22	9.0 (2.7, 29.8)	13.9 (3.0, 64.8)
Chromosome 5[c]				
0–1	1	9	1.0	1.0
2	4	15	2.4 (0.2, 25.0)	3.3 (0.2, 46.7)
3	13	16	7.3 (0.8, 65.5)	15.3 (1.2, 192.5)
4+	57	38	13.5 (1.6, 110.9)	26.6 (2.3, 307.0)

[a] Adjusted by age (as a continuous variable), sex, ethnicity, and years smoked.
[b] Values corresponding to the quartiles of breaks on chromosome 4 in controls are 0–2, 3, 4–5, and 6+ breaks.
[c] Values corresponding to the quartiles of breaks on chromosome 5 in controls are 0–1, 2, 3, and 4+ breaks.

Table 4 *ORs for specific chromosomal breaks and smoking status*

Chromosome breaks	Smoking status	No. Cases	No. Controls	OR (95% CL) Crude	OR (95% CL) Adjusted[a]
Chromosome 2					
No	No	6	25	1.0	1.0
No	Yes	31	29	4.5 (1.6, 12.4)	6.0 (1.9, 19.2)
Yes	No	1	12	0.3 (0.0, 3.2)	0.3 (0.0, 4.5)
Yes	Yes	37	12	12.8 (4.3, 38.7)	15.8 (4.4, 56.1)
Chromosome 4					
No	No	3	24	1.0	1.0
No	Yes	28	32	7.0 (1.9, 25.8)	8.1 (2.0, 32.7)
Yes	No	4	13	2.5 (0.5, 12.7)	3.9 (0.5, 33.7)
Yes	Yes	40	9	35.6 (8.8, 144.4)	128.8 (11.9, 1391)
Chromosome 5					
No	No	2	19	1.0	1.0
No	Yes	16	21	7.2 (1.5, 35.7)	8.4 (1.5, 45.5)
Yes	No	5	18	2.6 (0.5, 15.4)	2.6 (0.3, 23.2)
Yes	Yes	52	20	24.7 (5.3, 115.9)	55.5 (8.1, 380.1)

[a] Adjusted by age (as a continuous variable), sex, and ethnicity.

Table 5 *ORs for breaks in specific chromosome bands*

Chromosome band	Univariate OR (95% CL)	Adjusted[a] OR (95% CL)
2q14	3.4 (1.1, 9.8)	2.8 (0.6, 12.5)
4p14	3.3 (1.6, 7.1)	2.9 (1.2, 7.1)
4q25	1.8 (1.0, 3.5)	1.6 (0.7, 3.6)
4q27	3.0 (1.6, 5.8)	3.8 (1.6, 9.0)
4q31	2.2 (1.1, 4.2)	2.8 (1.2, 6.6)
5q21	8.7 (1.9, 39.9)	5.1 (1.0, 25.8)
5q22	3.3 (1.7, 6.4)	4.9 (2.0, 12.0)
5q31	2.8 (1.4, 5.4)	3.2 (1.4, 7.5)
5q33	1.8 (0.9, 3.8)	3.6 (1.3, 10.2)

[a] Adjusted by age (as a continuous variable), sex, ethnicity, and years smoked.

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