

Cytokinesis-Blocked Micronucleus Assay as a Novel Biomarker for Lung Cancer Risk

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Abstract

In this case-control study, we modified the cytokinesis-block micronucleus (CBMN) assay, an established biomarker for genomic instability, to evaluate susceptibility to the nicotine-derived nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) by measuring the frequency of NNK-induced chromosomal damage endpoints (micronuclei, nucleoplasmic bridges, and nuclear buds) per 1,000 binucleated lymphocytes. Spontaneous and NNK-induced chromosomal damage were significantly higher in lung cancer patients compared with controls. Forty-seven percent of cases (versus 12% of controls) had ≥ 4 spontaneous micronuclei, 66% of cases (and no controls) had ≥ 4 spontaneous nucleoplasmic bridges, and 25% of cases (versus 5% of controls) had ≥ 1 spontaneous nuclear bud ($P < 0.001$). Similarly, 40% of cases (versus 6% of the controls) had ≥ 5 NNK-induced micronuclei, 89% of cases (and no controls) had ≥ 6 induced nucleoplasmic bridges, and 23% of cases (versus 2% of controls) had ≥ 2 induced nuclear buds ($P < 0.001$). When analyzed on a continuous scale, spontaneous micronuclei, nucleoplasmic bridges, and nuclear buds were associated with 2-, 29-, and 6-fold increases in cancer risk, respectively. Similarly, NNK-induced risks were 2.3-, 45.5-, and 10-fold, respectively. We evaluated the use of CBMN assay to predict cancer risk based on the numbers of micronuclei, nucleoplasmic bridges, and nuclear buds defined by percentile cut points in controls. Probabilities of being a cancer patient were 96%, 98%, and 100% when using the 95th percentiles of spontaneous and NNK-induced micronuclei, nucleoplasmic bridges, and nuclear buds, respectively. Our study indicates that the CBMN assay is extremely sensitive to NNK-induced genetic damage and may serve as a strong predictor of lung cancer risk. (Cancer Res 2006; 66(12): 6449-56)

Introduction

Lung cancer is the leading cause of cancer mortality in the United States, and there is an urgent need to improve outcome by identifying and validating markers to predict risk and allow early diagnosis (1). A crucial early event in carcinogenesis is the induction of the genomic instability phenotype, which enables an initiated cell to evolve into a cancer cell by achieving a greater proliferative capacity (2). It is well known that cancer results from an accumulation of multiple genetic changes that can be

mediated through chromosomal changes and that have the potential to be cytogenetically detectable (3). It has been hypothesized that the level of genetic damage in peripheral blood lymphocytes reflects the amount of damage in the precursor cells that lead to the carcinogenic process in target tissues (4). Evidence that cytogenetic biomarkers are positively correlated with cancer risk has been strongly validated in recent results from both cohort and nested case-control studies showing that chromosome aberrations are a marker of cancer risk (5–9), reflecting both the genotoxic effects of carcinogens and individual cancer susceptibility.

The cytokinesis-block micronucleus (CBMN) assay in human lymphocytes is one of the most commonly used methods for measuring DNA damage because it is relatively easier to score micronuclei than chromosome aberrations (2). Micronuclei originate from chromosome fragments or whole chromosomes that fail to engage with the mitotic spindle and therefore lag behind when the cell divides. Compared with other cytogenetic assays, quantification of micronuclei confers several advantages, including speed and ease of analysis, no requirement for metaphase cells, and reliable identification of cells that have completed only one nuclear division. This prevents confounding effects caused by differences in cell division kinetics because expression of micronuclei, nucleoplasmic bridges, or nuclear buds is dependent on completion of nuclear division (10). Because cells are blocked in the binucleated stage, it is also possible to measure nucleoplasmic bridges originating from asymmetrical chromosome rearrangements and/or telomere end fusions (11, 12). Nucleoplasmic bridges occur when the centromeres of dicentric chromosomes or chromatids are pulled to the opposite poles of the cell at anaphase. In the CBMN assay, binucleated cells with nucleoplasmic bridges are easily observed because cytokinesis is inhibited, preventing breakage of the anaphase bridges from which nucleoplasmic bridges are derived; thus, the nuclear membrane forms around the nucleoplasmic bridge. Both micronuclei and nucleoplasmic bridges occur in cells exposed to DNA-breaking agents (13, 14). In addition to micronuclei and nucleoplasmic bridges, the CBMN assay allows for the detection of nuclear buds, which represent a mechanism by which cells remove amplified DNA and are therefore considered a marker of possible gene amplification [reviewed by Fenech (14)]. The CBMN test is gradually replacing the analysis of chromosome aberrations in lymphocytes because micronuclei, nucleoplasmic bridges, and nuclear buds are easy to recognize and score and the results can be obtained in a shorter time (15).

More than 80% of lung cancers are attributed to tobacco exposure. However, because only a fraction of long-term smokers (~15%) will develop lung cancer in their lifetime (16), it is proposed that genetic factors play a role in individual susceptibility. An individual's DNA repair capacity may play a significant role in modifying risk for cancer. The tobacco-specific nitrosamine

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4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a strong pulmonary carcinogen and a potent inducer of lung adenocarcinoma, now the leading lung cancer subtype in the United States (17). Studies on the metabolism of NNK have shown that it induces cross-links in DNA; interacts with DNA, forming different types of adducts; and increases the frequency of chromosome aberrations (18, 19). Hecht (20) reported that DNA adducts derived from NNK are present at a higher level in lung tissues from lung cancer patients than controls, and metabolites of NNK are found in the urine of people who use tobacco products or are exposed to environmental tobacco smoke. The repair kinetics for NNK-induced genetic damage has not been clearly elucidated but may involve several DNA repair pathways, including base excision and nucleotide excision repair pathways (21). In the current study, we used the CBMN assay to measure NNK-induced micronucleus, nucleoplasmic bridge, and nuclear bud frequencies and conducted a pilot proof-of-principle case-control study to evaluate whether NNK-induced damage was associated with lung cancer risk.

Materials and Methods

Study population. Cases and controls for this analysis were accrued from an ongoing molecular epidemiologic study on susceptibility markers for lung cancer. Cases ($n = 139$) were consecutive patients with newly diagnosed, previously untreated, histologically confirmed lung cancer patients. All cases were recruited from The University of Texas M.D. Anderson Cancer Center, with no age, gender, ethnicity, tumor histology, or disease stage restrictions. Healthy controls ($n = 130$) were recruited from the Kelsey-Seybold Clinics, Houston's largest private multispecialty physician group. Controls were matched to the cases on age (± 5 years), gender, ethnicity, and smoking status (current and former). Data related to the subjects' medical history, family history of cancer, smoking habits, and occupational history were obtained through an interviewer-administered risk-factor questionnaire and by review of an institutional electronic patient history database. The institutional review boards at both The University of Texas M. D. Anderson Cancer Center and Kelsey-Seybold Clinics approved this study. After giving informed consent, all study participants donated a 10-mL blood sample, which was drawn into coded heparinized tubes.

Peripheral blood lymphocyte cultures for CBMN test. The CBMN test was done using the cytochalasin B technique described by Fenech and Morley (22) and following recommendations from the International Collaborative Project on Micronucleus Frequency in Human Populations (HUMN Project) to measure micronuclei, nucleoplasmic bridges, and nuclear buds in untreated cells and NNK-treated cells. Duplicate lymphocyte cultures were prepared for each study subject. Each culture contained 2.0×10^6 cells in 5 mL RPMI 1640 supplemented with 100 units/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, 10% fetal bovine serum, and 2 mmol/L L-glutamine (Life Technologies-Invitrogen, Carlsbad, CA) and 1% phytohemagglutinin (Remel, Lenexa, KS). For the cultures treated with NNK, 24 hours after initiation, the peripheral blood lymphocytes were centrifuged and the supernatant growth medium was removed and reserved. The peripheral blood lymphocytes were resuspended in 5 mL serum-free RPMI 1640 supplemented with 0.24 mmol/L NNK (a dose previously used by us and others; refs. 23–26; CAS no. 64091-91-4, National Cancer Institute, Midwest Carcinogen Repository, Kansas City, MO; purity, >98%) and incubated at 37°C in the presence of 5% CO₂ for 2 hours. Next, the peripheral blood lymphocytes were washed twice with serum-free RPMI 1640, transferred to clean tubes, and reincubated for 48 hours in the reserved supernatant. At 44 hours after initiation, cells were blocked in cytokinesis by adding cytochalasin B (Sigma, St. Louis, MO; final concentration, 4 $\mu\text{g}/\text{mL}$). Similarly, cultures for the determination of spontaneous damage (untreated cells) were handled in the same manner, with the exception of treatment with NNK. The total incubation time for all cultures was 72 hours. After incubation, the cells were fixed in 3:1 methanol/

glacial acetic acid, dropped onto clean microscopic slides, air-dried, and stained with Giemsa stain. For each sample, 1,000 binucleated cells were scored blindly using a Nikon (Lewisville, TX) E-400 light optical microscope following the scoring criteria outlined by HUMN Project (2, 22, 27); the numbers of micronuclei, nucleoplasmic bridges, and nuclear buds per 1,000 binucleated cells were recorded. For quality control, 20% of the slides were randomly selected and blindly rescored and the results were compared with the original scoring.

Statistical analysis. All analyses were done using the Intercooled Stata 8.0 statistical software package (Stata Corp., College Station, TX). Pearson's χ^2 test was used to test for differences between cases and controls in terms of gender, alcohol consumption, and family history of cancer. Student's t test was used to test differences in mean age and average number of cigarettes smoked per day. We used the nonparametric Wilcoxon's rank-sum test (continuous) and the Pearson's χ^2 test (categorical) to compare the distribution of spontaneous and NNK-induced micronuclei, nucleoplasmic bridges, and nuclear buds between cases and controls. We also constructed dot charts using S-Plus (version 6.2, Insightful Corp., Seattle, WA, 2003) to compare the distribution of spontaneous and NNK-induced micronuclei, nucleoplasmic bridges, and nuclear buds between cases and controls. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated to provide an estimate of the risk of lung cancer associated with the number of spontaneous and NNK-treated micronuclei, nucleoplasmic bridges, and nuclear buds per 1,000 cells. Unconditional multivariable logistic regression analysis was used to control for confounding by age, gender, alcohol consumption, smoking status, and years smoked.

Results

Demographics and the study population. The demographic characteristics of the 139 cases and 130 controls are summarized in Table 1. Cases and controls did not differ significantly in terms of gender. However, on average, the cases were 2.5 years younger (mean age \pm SE, 58.4 \pm 0.41) than the controls (mean age \pm SE,

Table 1. Distribution of selected host characteristics by case-control status

Variable	Case patients ($n = 139$)	Control subjects ($n = 130$)	P^*
Age (y), mean \pm SE	58.4 \pm 0.41	60.9 \pm 0.32	<0.001
Gender, n (%)			
Men	97 (69.8)	93 (71.5)	0.752
Women	42 (30.2)	37 (28.5)	
Family history of cancer, n (%)			
No	106 (76.3)	101 (77.7)	0.780
Yes	33 (23.7)	29 (22.3)	
History of alcohol use, n (%) [†]			
Yes	91 (65.5)	65 (50.0)	0.003
No	47 (33.8)	59 (45.4)	
Cigarette smoking, mean \pm SE			
No. years smoked [‡]	42.1 \pm 0.50	37.7 \pm 0.84	<0.001
No. cigarettes smoked per day [§]	30.0 \pm 1.38	29.2 \pm 1.21	0.651

NOTE: All study subjects are self-reported Caucasians and current smokers.

* P s were derived from the χ^2 test for categorical variables and Student's t test for continuous variables. All P s are two sided.

[†]Data were missing for 1 case and 6 controls.

[‡]Data were missing for 1 case.

[§]Data were missing for 6 cases and 2 controls.

Table 2. Overall spontaneous and NNK-induced frequencies of micronuclei, nucleoplasmic bridges, and nuclear buds per 1,000 binucleated cells by age, gender, and years smoked in lung cancer cases and controls

	Case mean \pm SE (median)	Control mean \pm SE (median)
Spontaneous		
Overall		
Micronuclei	3.41 \pm 0.13 (3.0)	1.98 \pm 0.12 (2.0)
Nucleoplasmic bridges	4.14 \pm 0.10 (4.0)	0.57 \pm 0.07 (0.0)
Nuclear buds	0.28 \pm 0.04 (0.0)	0.05 \pm 0.02 (0.0)
Males		
Micronuclei	3.47 \pm 0.17 (3.0)	1.95 \pm 0.13 (2.0)
Nucleoplasmic bridges	4.13 \pm 0.12 (4.0)	0.57 \pm 0.08 (0.0)
Nuclear buds	0.29 \pm 0.05 (0.0)	0.06 \pm 0.03 (0.0)
Females		
Micronuclei	3.26 \pm 0.25 (3.0)	2.05 \pm 0.18 (2.0)
Nucleoplasmic bridges	4.17 \pm 0.16 (4.5)	0.57 \pm 0.13 (0.0)
Nuclear buds	0.26 \pm 0.07 (0.0)	0.0 \pm 0.0 (0.0)
Age \leq 62 y		
Micronuclei	3.44 \pm 0.15 (3.0)	1.97 \pm 0.14 (2.0)
Nucleoplasmic bridges	4.02 \pm 0.12 (4.0)	0.55 \pm 0.09 (0.0)
Nuclear buds	0.25 \pm 0.04 (0.0)	0.05 \pm 0.02 (0.0)
Age >62 y		
Micronuclei	3.34 \pm 0.27 (3.0)	1.98 \pm 0.18 (2.0)
Nucleoplasmic bridges	4.44 \pm 0.16 (5.0)	0.60 \pm 0.11 (0.0)
Nuclear buds	0.39 \pm 0.10 (0.0)	0.05 \pm 0.03 (0.0)
Years smoked \leq 32		
Micronuclei	3.50 \pm 0.42 (3.5)	1.88 \pm 0.21 (2.0)
Nucleoplasmic bridges	3.50 \pm 0.38 (3.0)	0.42 \pm 0.12 (0.0)
Nuclear buds	0.13 \pm 0.13 (0.0)	0.0 \pm 0.0 (0.0)
Years smoked >32		
Micronuclei	3.43 \pm 0.14 (3.0)	2.00 \pm 0.13 (2.0)
Nucleoplasmic bridges	4.13 \pm 0.10 (4.0)	0.61 \pm 0.09 (0.0)
Nuclear buds	0.29 \pm 0.04 (0.0)	0.06 \pm 0.03 (0.0)
History of alcohol use: yes		
Micronuclei	3.47 \pm 0.16 (3.00)	2.00 \pm 0.15 (2.00)
Nucleoplasmic bridges	4.19 \pm 0.13 (4.00)	0.58 \pm 0.11 (0.00)
Nuclear buds	0.29 \pm 0.05 (0.00)	0.07 \pm 0.03 (0.00)
History of alcohol use: no		
Micronuclei	3.32 \pm 0.23 (3.00)	1.88 \pm 0.16 (2.00)
Nucleoplasmic bridges	4.02 \pm 0.16 (4.00)	0.55 \pm 0.10 (0.00)
Nuclear buds	0.27 \pm 0.07 (0.00)	0.03 \pm 0.02 (0.00)
NNK induced		
Overall		
Micronuclei	4.32 \pm 0.10 (4.0)	2.62 \pm 0.10 (3.0)
Nucleoplasmic bridges	8.12 \pm 0.19 (8.0)	2.38 \pm 0.09 (2.0)
Nuclear buds	1.07 \pm 0.09 (1.0)	0.12 \pm 0.03 (0.0)
Males		
Micronuclei	4.37 \pm 0.13 (4.0)	2.63 \pm 0.11 (3.0)
Nucleoplasmic bridges	8.07 \pm 0.23 (8.0)	2.27 \pm 0.10 (2.0)
Nuclear buds	1.07 \pm 0.11 (1.0)	0.12 \pm 0.03 (0.0)
Females		
Micronuclei	4.19 \pm 0.17 (4.0)	2.51 \pm 0.23 (2.0)
Nucleoplasmic bridges	8.24 \pm 0.32 (8.0)	2.68 \pm 0.19 (2.0)
Nuclear buds	1.07 \pm 0.12 (1.0)	0.11 \pm 0.05 (1.0)
Age \leq 62 y		
Micronuclei	4.22 \pm 0.11 (4.0)	2.58 \pm 0.14 (2.0)
Nucleoplasmic bridges	8.11 \pm 0.23 (8.0)	2.41 \pm 0.11 (2.0)
Nuclear buds	1.12 \pm 0.11 (1.0)	0.13 \pm 0.04 (0.0)

Table 2. Overall spontaneous and NNK-induced frequencies of micronuclei, nucleoplasmic bridges, and nuclear buds per 1,000 binucleated cells by age, gender, and years smoked in lung cancer cases and controls (Cont'd)

	Case mean \pm SE (median)	Control mean \pm SE (median)
Age >62 y		
Micronuclei	4.54 \pm 0.22 (4.0)	2.75 \pm 0.15 (3.0)
Nucleoplasmic bridges	8.15 \pm 0.33 (8.0)	2.35 \pm 0.14 (2.0)
Nuclear buds	0.90 \pm 0.17 (1.0)	0.10 \pm 0.05 (0.0)
Years smoked \leq32		
Micronuclei	3.63 \pm 0.32 (4.0)	2.33 \pm 1.02 (2.0)
Nucleoplasmic bridges	9.25 \pm 0.96 (8.3)	2.36 \pm 0.19 (2.0)
Nuclear buds	1.13 \pm 0.44 (1.0)	0.06 \pm 0.04 (0.0)
Years smoked >32		
Micronuclei	4.33 \pm 0.10 (4.0)	2.69 \pm 0.12 (3.0)
Nucleoplasmic bridges	8.07 \pm 0.19 (8.0)	2.39 \pm 0.09 (2.0)
Nuclear buds	1.08 \pm 0.01 (1.0)	0.13 \pm 0.04 (0.0)
History of alcohol use: yes		
Micronuclei	4.37 \pm 0.14 (4.00)	2.61 \pm 0.15 (2.00)
Nucleoplasmic bridges	8.14 \pm 0.24 (8.00)	2.32 \pm 0.12 (2.00)
Nuclear buds	1.04 \pm 0.11 (1.00)	0.11 \pm 0.04 (0.00)
History of alcohol use: no		
Micronuclei	4.26 \pm 0.14 (4.00)	2.54 \pm 0.15 (3.00)
Nucleoplasmic bridges	8.04 \pm 0.32 (8.00)	2.40 \pm 0.13 (2.00)
Nuclear buds	1.09 \pm 0.18 (1.00)	0.14 \pm 0.04 (0.00)

60.9 \pm 0.32; $P < 0.001$). Twenty-four percent of the patients self-reported a family history of cancer in first-degree relatives compared with 22% of the controls ($P = 0.780$). Cases had, on average, smoked cigarettes for 42.1 years compared with 37.7 years for controls ($P < 0.001$), but both groups smoked about the same number of cigarettes per day (mean number of cigarettes per day \pm SE, 30.2 \pm 1.38 for cases and 29.2 \pm 1.21 for controls; $P = 0.651$).

Frequencies of cytogenetic endpoints by case-control status. Overall, the lung cancer cases exhibited significantly higher values of all cytogenetic end points than the controls (Table 2). The P s for all Wilcoxon's rank-sum tests were < 0.001 .

Frequencies of spontaneous cytogenetic endpoints. Data on spontaneous micronucleus, nucleoplasmic bridge, and nuclear bud frequencies by age, gender, and years of smoking are summarized in Table 2. Table 3 shows that $\sim 47\%$ of the cases had ≥ 4 micronuclei per 1,000 binucleated cells compared with 12% of control subjects and that 40% of the control subjects had 0 or 1 micronucleus compared with only 9% of the cases ($P < 0.001$). The mean number of spontaneous micronuclei \pm SE was significantly higher in the cases (3.41 \pm 0.13) than in the control subjects (1.98 \pm 0.12; $P < 0.001$). Similarly, $\sim 66\%$ of the cases had ≥ 4 nucleoplasmic bridges compared with none of the control subjects, whereas 86% of the controls had 0 or 1 nucleoplasmic bridge compared with none of the cases ($P < 0.001$). Furthermore, the number of spontaneous nucleoplasmic bridges was significantly higher in the cases (mean \pm SE, 4.14 \pm 0.10) than in the controls (mean \pm SE, 0.57 \pm 0.07; $P < 0.001$). With regard to frequency of nuclear bud distribution, $\sim 95\%$ of the controls exhibited no spontaneous buds compared with 73% of cases. Only 5% of the controls had 1 spontaneous bud compared with 25% of the cases ($P < 0.001$). Overall, the average number of spontaneous buds was

Table 3. Distributions and risk estimates of lung cancer for spontaneous and NNK-induced micronuclei, nucleoplasmic bridges, and nuclear buds

Variable	Case patients, n (%)	Control subjects, n (%)	OR (95% CI)* or P [†]
Micronuclei			
Pearson's χ^2 test			
Spontaneous			
0 or 1	13 (9.4)	52 (40.0)	
2 or 3	61 (43.9)	63 (48.5)	
≥4	65 (46.8)	15 (11.5)	<0.001
NNK induced			
1 or 2	5 (3.6)	64 (49.2)	
3 or 4	78 (56.1)	58 (44.6)	
≥5	56 (40.3)	8 (6.2)	<0.001
Multivariate logistic			
Regression analysis			
Spontaneous	139	130	2.06 (1.60-2.65)
NNK induced	139	130	2.32 (2.32-4.80)
Nucleoplasmic bridges			
Pearson's χ^2 test			
Spontaneous			
0 or 1	0 (0.0)	112 (86.2)	
2 or 3	48 (34.5)	18 (13.9)	
≥4	91 (65.5)	0 (0.0)	<0.001
NNK induced			
0 or 2	0 (0.0)	84 (64.6)	
3 or 5	15 (10.8)	46 (35.4)	
≥6	124 (89.2)	0 (0.0)	<0.001
Multivariate logistic			
Regression analysis			
Spontaneous	139	130	29.05 (7.48-112.80)
NNK induced	139	130	45.52 (4.48-422.17)
Nuclear buds			
Pearson's χ^2 test			
Spontaneous			
0	102 (73.4)	122 (95.3)	
1	35 (25.2)	6 (4.7)	
2	2 (1.4)	0 (0.0)	<0.001
NNK induced			
0	48 (34.5)	113 (86.9)	
1	59 (42.5)	15 (11.5)	
≥2	32 (23.0)	2 (1.5)	0.001
Multivariate logistic			
Regression analysis			
Spontaneous	139	130	6.53 (2.37-18.01)
NNK induced	139	130	10.10 (4.67-21.87)

*Adjusted by age, gender, history of alcohol use, number of years smoked, and number of cigarettes smoked per day.

†Ps are derived from the Pearson's χ^2 test and are two sided.

significantly higher in the cases than in the controls (mean \pm SE, 0.28 \pm 0.04 and 0.05 \pm 0.02, respectively; $P < 0.001$). No substantial differences were detected when the frequencies of micronuclei, nucleoplasmic bridges, and nuclear buds were stratified by gender, age, family history of cancer, number of years smoked, number of cigarettes per day, tumor histology, or disease stage. When the spontaneous micronuclei data were analyzed as a continuous variable, using multivariate logistic regression, there was a 2.06-fold

increase in lung cancer risk (95% CI, 1.60-2.65) for each 1-unit increase in micronucleus frequency. Similarly, when spontaneous nucleoplasmic bridges were analyzed as a continuous variable, there was a 29.05-fold increase in lung cancer risk (95% CI, 7.48-112.80) for each 1-unit increase in nucleoplasmic bridges. For the nuclear buds, there was a 6.5-fold increase in lung cancer risk (95% CI, 2.37-18.01) for each 1-unit increase in spontaneous bud frequency (Table 3).

Frequencies of NNK-induced cytogenetic endpoints. Data on NNK-induced micronucleus, nucleoplasmic bridge, and nuclear bud frequencies by age, gender, and smoking status are also summarized in Table 2. Table 3 shows that substantially more cases had ≥ 5 NNK-induced micronuclei per 1,000 binucleated cells than controls (40% versus 6%); conversely, far fewer cases than controls had only 1 or 2 NNK-induced micronuclei (4% versus 49%; $P < 0.001$; Fig. 1A). The differences were even more pronounced for spontaneous and NNK-induced nucleoplasmic bridges. The mean number of NNK-induced micronuclei \pm SE was significantly higher in cases (4.32 \pm 0.10) than in controls (2.62 \pm 0.10; $P < 0.001$). As reported in Table 3, ~89% of the case patients had ≥ 6 NNK-induced nucleoplasmic bridges compared with none of the control subjects. Conversely, 65% of the control subjects had 0 to 2 NNK-induced nucleoplasmic bridges compared with none of the case patients ($P < 0.001$; Fig. 1B). The number of NNK-induced nucleoplasmic bridges was also significantly higher in cases (mean \pm SE, 8.12 \pm 0.19) than in controls (mean \pm SE, 2.38 \pm 0.09; $P < 0.001$).

There were no substantial differences when the frequencies of NNK-induced micronuclei and nucleoplasmic bridges were stratified by gender, age, family history of cancer, number of years smoked, number of cigarettes per day, tumor histology, or disease stage. When the NNK-induced micronuclei data were analyzed as a continuous variable, there was a 2.32-fold increase in lung cancer risk (95% CI, 2.32-4.80) for each 1-unit increase in NNK-induced micronuclei. Similarly, NNK-induced nucleoplasmic bridges showed a 45.52-fold increase in lung cancer risk (95% CI, 4.48-422.17) for each 1-unit increase in frequency. With regard to bud distribution, 23% of the cases had ≥ 2 NNK-induced buds compared with ~2% of controls; 87% of the controls had no NNK-induced buds compared with 35% of the case patients ($P < 0.001$; Fig. 1C). The number of NNK-induced buds was significantly higher in cases than in controls (mean \pm SE, 1.07 \pm 0.09 and 0.12 \pm 0.03, respectively; $P < 0.001$). When the nuclear bud frequency was analyzed as a continuous variable, there was a 10.1-fold increase in lung cancer risk (95% CI, 4.67-21.87) for each 1-unit increase in NNK-induced buds. Similar patterns of difference between cases and controls were observed for micronuclei, nucleoplasmic bridges, and nuclear buds within subgroups of subjects stratified by gender, age, family history of cancer, number of years smoked, number of cigarettes per day, tumor histology, and disease stage (Table 3).

To ensure quality control, 20% of the slides were randomly selected for blind rescoring. Agreement between the original data and the rescored data was measured using the Cohen's κ statistical test. A statistically significant value of $P < 0.001$ was obtained for both spontaneous and NNK-induced variables, indicating that the agreement between the original and the rescored data was not attributable to random chance.

CBMN assay results as a predictor of case-control status. Using the 75 percentile of the controls as a cutoff, the sensitivity of the CMBN assay was 96.4%, 100%, and 100% for the micronuclei,

nucleoplasmic bridges, and nuclear buds, respectively. The specificity of the assay was 93.0%, 100%, and 100% for the micronuclei, nucleoplasmic bridges, and nuclear buds, respectively. The probabilities that a study subject was a case based on various cut points for the numbers of micronuclei, nucleoplasmic bridges, and nuclear buds were calculated (Table 4). The numbers of micronuclei, nucleoplasmic bridges, and nuclear buds were defined by percentile cut points in the control data. The probability of being a case increased as the percentile cut points increased for high numbers of micronuclei, nucleoplasmic bridges, or nuclear buds. The numbers of spontaneous and NNK-induced micronuclei showed no difference in terms of predictive capacity at the 90th percentile. The numbers of spontaneous and NNK-induced nucleoplasmic bridges showed a slight difference in terms of predictive capacity at the 90th percentile, with the number of induced nucleoplasmic bridges having a better predictive capacity than that of spontaneous nucleoplasmic bridges. The number of NNK-induced nuclear buds showed the highest predictive capacity of the three end points at and above the 85th percentile. On combining all three end points and by using the 75 percentile of the controls as a cutoff, the CBMN assay sensitivity for detecting NNK-induced damage was 96.4%, with 80.8% specificity and 84.3% positive predictive value.

Discussion

In the current study, we tested the sensitivity of the study subjects' lymphocytes to the tobacco-specific nitrosamine NNK, because it represents an important class of carcinogens known to be associated with the development of lung cancer, particularly adenocarcinoma, which is now the leading lung cancer histologic subtype in the United States, having surpassed squamous cell carcinoma (17). Our results show that cases and controls had differential sensitivity to the genotoxic effects of NNK. The lymphocytes from patients with lung cancer were significantly more sensitive to NNK, with 1.6-, 3.4-, and 8.9-fold increases in micronucleus, nucleoplasmic bridge, and nuclear bud frequencies, respectively, over controls. The results of this analysis also show that NNK-induced cytogenetic damage (expressed in terms of micronucleus, nucleoplasmic bridge, and nuclear bud frequencies) seems to be a highly sensitive predictor of lung cancer status.

Tobacco smoke contains an array of potent carcinogens, including polycyclic aromatic hydrocarbons, aromatic amines, and *N*-nitrosamines. Among the polycyclic aromatic hydrocarbons, benzo[*a*]pyrene has been the most extensively studied, and our research to date has focused largely on this carcinogen. Benzo[*a*]pyrene is an effective pulmonary carcinogen, inducing

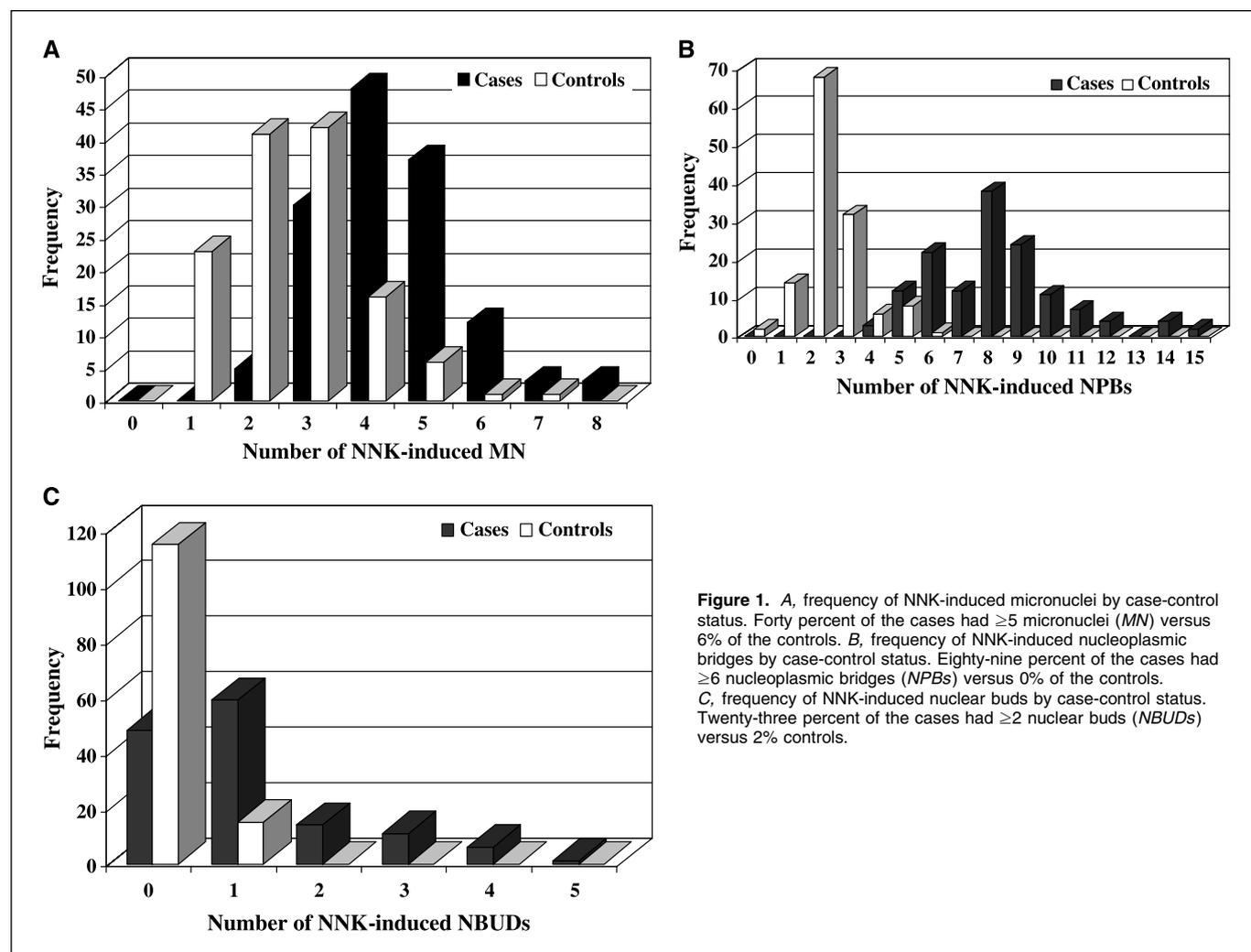


Figure 1. A, frequency of NNK-induced micronuclei by case-control status. Forty percent of the cases had ≥ 5 micronuclei (MN) versus 6% of the controls. B, frequency of NNK-induced nucleoplasmic bridges by case-control status. Eighty-nine percent of the cases had ≥ 6 nucleoplasmic bridges (NPBs) versus 0% of the controls. C, frequency of NNK-induced nuclear buds by case-control status. Twenty-three percent of the cases had ≥ 2 nuclear buds (NBUDs) versus 2% controls.

Table 4. Probability of being a case based on various percentile cut points for the numbers of micronuclei, nucleoplasmic bridges, and nuclear buds in the controls

Percentile of controls	Spontaneous			NNK induced			Spontaneous and NNK induced		
	Micronuclei	Nucleoplasmic bridges	Nuclear buds	Micronuclei	Nucleoplasmic bridges	Nuclear buds	Micronuclei	Nucleoplasmic bridges	Nuclear buds
75	70.1	72.8	86.0	67.0	75.1	85.8	85.6	84.3	82.4
80	70.1	72.8	86.0	67.0	75.1	85.8	85.6	84.3	82.4
85	70.1	72.8	86.0	81.3	75.1	100.0	85.6	89.7	100.0
90	81.3	88.5	86.0	81.3	90.8	100.0	95.6	96.3	100.0
95	81.3	88.5	86.0	87.5	94.4	100.0	95.6	98.2	100.0

predominantly squamous cell carcinoma on intratracheal instillation into rats and hamsters. Tobacco-specific nitrosamines are found in high concentrations in mainstream smoke (28). The most potent carcinogenic member of this group, as shown in experimental animals, is NNK (29). NNK induces lung adenocarcinoma independent of route of administration and in both susceptible and resistant strains of mice (30). The estimated NNK dose of lifetime smokers (2 packs daily for 40 years) is 1.6 mg NNK/kg body weight (31), close to the lowest dose shown to induce lung tumors in rats (1.8 mg; ref. 32). The total level of NNK in mainstream smoke is 3 to 15 times that of benzo[*a*]pyrene (33). Gender differences and DNA repair allelic variants have been reported to modulate the effect of NNK-induced genotoxic damage using the conventional chromosome aberration assay (24, 25) or fluorescence *in situ* hybridization assay using chromosome 1-specific probes in healthy smokers and nonsmoker controls (26). In addition, NNK was shown to be a potent mutagen using both the Ames *Salmonella* assay and the micronucleus test in Swiss mice (34).

The CBMN assay is a genotoxicity assay that provides simultaneous information on a variety of chromosomal damage endpoints that reflect chromosomal breakage, chromosome rearrangements, and gene amplification. In the current study, the frequencies of micronuclei, nucleoplasmic bridges, and nuclear buds were significantly higher in the lung cancer patients than in controls. Cheng et al. reported similar results after evaluating the micronucleus frequency in 42 patients with lung cancer and 55 controls (35). The significantly higher spontaneous micronuclei levels observed in the cases suggest a higher background level of genetic instability in the cancer patients. The effect of smoking on micronucleus frequency in peripheral blood lymphocytes has not been consistent across studies, which generally have been small and not properly designed to detect the effect of smoking as the main outcome measure (36–38). However, a pooled reanalysis of 24 databases (5,710 subjects, of which 1,409 were current smokers) from the HUMN Project revealed that micronucleus frequency was not influenced by the number of cigarettes smoked per day among subjects occupationally exposed to genotoxic agents, whereas a U-shaped curve was observed for nonexposed smokers, with a significant increase of micronucleus frequency in individuals smoking ≥ 30 cigarettes per day (frequency ratio, 1.59; 95% CI, 1.35–1.88; ref. 39). Our results showed no significant association between micronucleus frequency and age, gender, or smoking status, which is in agreement with the results reported by Cheng et al. (35). The current study is, to our knowledge, the first to report a significantly higher frequency of spontaneous nucleoplasmic

bridges and nuclear buds in a lung cancer case-control study, thus supporting the hypothesis of breakage-fusion-bridge cycle mechanism of hypermutation during carcinogenesis (reviewed in refs. 2, 13). Gisselsson et al. (40) reported that abnormal nuclear morphology (associated with nucleoplasmic bridges, micronuclei, and nuclear buds) is indicative of significant genomic instability within cells and is a common feature of a wide variety of cancers. An increase in the frequency of these chromosomal damage end points in a surrogate tissue, such as peripheral blood lymphocytes, would imply constitutional sensitivity to genetic damage.

Several epidemiologic studies employing a variety of measures of DNA repair capacity have been done to compare cancer patients and healthy control subjects and thereby assess the role of repair capacity in cancer risk (19). Using a variety of *in vitro* assays, we have shown previously (41–45) that sensitivity to mutagens varies widely between subjects with and without cancer and that this variation translates into interindividual variability in susceptibility to *in vitro* carcinogenic challenge. The mutagen sensitivity assay measures indirectly an individual's DNA repair capacity from cellular damage remaining after an *in vitro* mutagenic exposure and subsequent recovery. This assay, developed by Hsu et al. (46), reflects general and nonspecific impairment of the DNA repair machinery and host genomic stability.

Our current study is, to our knowledge, the first to validate the use of the CBMN assay, with NNK as the challenge mutagen, in a case-control study by testing the sensitivity of this genomic instability biomarker as a predictor of lung cancer risk. Table 4 shows the predictive probabilities for being a case based on various cutpoints for the numbers of spontaneous and induced micronuclei, nucleoplasmic bridges, and nuclear buds, alone and in combination, as defined by the frequency distribution in control subjects. The probability of being a case increased as the percentile cutpoints increased for all cytogenetic end points and the highest probabilities were observed when spontaneous and NNK-induced cytogenetic events were combined. The case-control differences were so striking that we considered, but rejected, alternative explanations. All the cases were enrolled at diagnosis and before initiating treatment; the assays were done blindly and in batches. We rescored randomly selected samples and obtained high levels of reliability. Further, the values did not differ according to smoking duration or intensity and especially not disease stage (lessening the likelihood that this is a tumor marker rather than a marker of risk).

This assay has been used to study susceptibility to other mutagenic agents. Scott et al. (47) showed that individuals who developed breast cancer and their relatives were more sensitive than controls to the DNA-damaging effect of ionizing radiation as

shown by micronucleus frequency. This sensitivity was observed in 10 of 11 cases of BRCA1 mutation carriers and was indicative of a defect in double-strand break repair (48), thus suggesting that this assay is useful not only as a marker of DNA damage but also as a means of measuring the DNA repair phenotype. Umegaki and Fenech (11) recently validated the use of nucleoplasmic bridges as a biomarker of DNA damage in a human B lymphoblastoid cell line (WIL2-NS). Nucleoplasmic bridge frequency in binucleated cells increased up to 20-fold in the WIL2-NS cells relative to control cells in response to agents known to induce DNA strand breaks; the effects were found to be dose dependent. Crott et al. (49) recently reported that the frequency of nucleoplasmic bridges and nuclear buds correlated significantly and negatively with folic acid concentrations, suggesting that these chromosomal end points may be induced by folic acid deficiency. In addition, Kimura et al. (50) showed a significant effect of methylenetetrahydrofolate reductase C677T polymorphism and folic acid concentration on micronuclei, nucleoplasmic bridges, and nuclear buds in human lymphocytes. Recently, our group reported that polymorphisms in genes involved in folate metabolism were associated with lung cancer risk, an effect that may be modulated by dietary nutrient intake (51). We plan on genotyping all of the study subjects in our current study to determine the effect of genetic polymorphisms in the folate pathway on modulating the frequency of the measured

endpoints. In addition, one of the advantages of the CBMN assay is the capability of comprehensively assessing DNA damage through measuring the frequency of apoptosis, necrosis, and number of micronuclei in mononucleated cells. Such variables are available for all the study subjects included in our study and we plan to report our findings as soon as the data analysis is complete.

In summary, our study shows differential sensitivity of peripheral blood lymphocytes from lung cancer patients and healthy controls to NNK-induced genetic damage. The data provide convincing evidence that the CBMN assay is a robust test for detection of this sensitivity and yields results that are a good predictor of lung cancer risk. The simplicity, rapidity, and sensitivity of the CBMN test make it a valuable tool for screening and possibly for prioritizing potential cases for early detection of the disease. This assay seems to give results that yield more accurate predictions than other phenotypic assays also undergoing assessment in this population of lung cancer cases and controls.

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Cytokinesis-Blocked Micronucleus Assay as a Novel Biomarker for Lung Cancer Risk

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