Identification of Small and Non-Small Cell Lung Cancer Markers in Peripheral Blood Using Cytokinesis-Blocked Micronucleus and Spectral Karyotyping Assays

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Keywords

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Abstract

Small cell lung cancer (SCLC) is a highly aggressive form of lung cancer. There is an urgent need to develop tools to identify individuals at high risk of developing SCLC. We have previously reported that the cytokinesis-blocked micronucleus (CBMN) assay is a strong predictor of non-small cell lung cancer (NSCLC). Here, we investigate the sensitivity of the CBMN endpoints as predictors of SCLC risk. We conducted the CBMN assay on SCLC patients ($n = 216$), NSCLC patients ($n = 173$), and healthy controls ($n = 204$). Per sample, 1,000 binucleated cells (BN) were scored, and 3 endpoints, micronuclei (BN-MN), nucleoplasmic bridges (BN-NPB), and nuclear buds (BN-BUD), were recorded. Spectral karyotyping was also conducted on SCLC patients ($n = 116$) and NSCLC patients ($n = 137$) to identify genomic regions unique to each disease. Significantly higher levels of CBMN endpoints were observed in both cancer groups compared to controls. BN-NPBs were significantly higher among SCLC patients

compared to NSCLC patients ($p < 0.001$). Chromosomes 5 and 17 were associated with BN-MN, and chromosomes 5, 18, 20, and 22 were associated with BN-NPBs in SCLC patients. Given the high frequency of chromosome aberrations observed in SCLC, events such as reinsertion of the micronucleus and chromothripsis may be potential mechanisms for the genetic instability in these patients.

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Approximately 85% of all lung cancers are non-small cell lung cancer (NSCLC) while the remaining 15% are of the small cell lung cancer (SCLC) histological subtype. When independently considered from NSCLC, SCLC is the fifth leading cause of cancer mortality in the United States [Argiris and Murren, 2001]. SCLC may be differentiated from NSCLC by its increased doubling time, exponential growth fraction, and the development of metastases at an earlier time. SCLC is one of the most aggressive and lethal human cancers. Most SCLC cases are diagnosed after metastatic spread of the disease, and only 5% of patients survive beyond 5 years after diagnosis [Worden and Kalemkerian, 2000; Cooper and Spiro, 2006] as compared to 18% for NSCLC [Ferlay et al., 2010].

Similar to NSCLC, smoking is a major risk factor for development of SCLC with more than 90% of patients with SCLC being elderly, current or former, heavy smokers, and the risk rises with increasing duration and intensity of smoking [Devesa et al., 2005]. In industrialized countries, the annual incidence of SCLC has decreased over the past 30 years, partly attributed to changes in smoking patterns and diagnostic criteria. Staging of SCLC was originally based on the extent of the disease into “limited disease” or “extensive disease.” Median survival without treatment has been reported as 2–4 months [Greene, 2006; Rami-Porta et al., 2007]. Further, the major reproducible prognostic factor remains disease extent.

An early event in carcinogenesis is the induction of genomic instability that enables an initiated cell to evolve into a cancer cell by achieving a greater proliferative capacity [Fenech, 2002b]. It has been reported that *TP53* mutations are present in approximately 75–90% of SCLC, resulting in a highly complex disease at the molecular level [D’Amico et al., 1992; Miller et al., 1992]. Moreover, studies have found that SCLC is associated with a high incidence of chromosomal changes in the form of deletions in specific chromosomes such as 3p, 4q, 5q, 10q, 13q, and 17p [Petersen et al., 1997], as well as 9p and 3q [Zhao et al., 2005].

Cytogenetic biomarkers have been strongly validated as markers of cancer risk in both cohort and nested case-control studies [Tucker and Preston, 1996; Liou et al., 1999; Bonassi et al., 2000, 2004; Smerhovsky et al., 2001], reflecting the outcome of both the genotoxic effects of carcinogens and individual cancer susceptibility. Assuming that the mechanisms for the induction of chromosomal damage are similar in different tissues, the extent of chromosomal damage evaluated in peripheral blood lymphocytes (PBLs) and other surrogate tissues likely reflects the level of damage in cancer-prone tissues and in turn cancer risk [Norppa et al., 2006]. Studies of paired lung tumors and PBLs from the same patients have demonstrated that genomic instability in the tumors corresponded well to PBL chromosomal changes and suggested that chromosomes commonly altered in both tumors and PBLs represent early events necessary for tumor initiation. Whereas, those chromosomes commonly rearranged in tumors only represent later events in the sequence of cancer development and progression [Pathak et al., 1991; Dave et al., 1995; Gjayee et al., 1997]. Further support for the value of PBL chromosomal changes as markers of early detection is provided through studies of adenomatous polyp patients and their asymptomatic first-degree relatives [Dave et al., 1993].

The cytokinesis-blocked micronucleus (CBMN) assay in human lymphocytes is one of the most commonly used cytogenetic methods for measuring DNA damage [Fenech, 2000, 2007]. The CBMN assay is a multi-end-point assay that assesses DNA damage endpoints (in the form of binucleated cells with micronuclei [BN-MN], nucleoplasmic bridges [BN-NPBs], and nuclear buds [BN-BUDs]) as well as other cellular events (such as necrosis, apoptosis, and cell proliferation) simultaneously. BN-MN are identified as chromosome fragments or whole chromosomes that fail to engage with the mitotic spindle and therefore lag behind when the cell divides. BN-NPBs originate from asymmetrical chromosome rearrangements and/or telomere end fusions [Umegaki and Fenech, 2000; Stewenius et al., 2005]. BN-BUDs represent a mechanism by which cells remove amplified DNA and are therefore considered a marker of possible gene amplification through a breakage-fusion-bridge cycle [Fenech, 2002a]. We have previously reported that the CBMN DNA damage endpoints are strong predictors of NSCLC susceptibility [El-Zein et al., 2006, 2008, 2014; McHugh et al., 2013] with a positive predictive value of 91% among smokers.

Although the more recent National Lung Screening Trial signaled the use of low-dose computed tomography imaging for early detection of overall lung cancers, the finding for patients with SCLC was contradictory as 86% of the 125 patients with SCLC identified through early detection programs had advanced disease at diagnosis [Byers and Rudin, 2015]. To address the lack of current methods with proven efficacy for the early detection of SCLC, we investigated the sensitivity of the CBMN endpoints as a predictor of SCLC risk. Furthermore, we broadened our investigation by using molecular cytogenetic techniques to map the regions in the genome associated with SCLC.

Materials and Methods

Study Population

SCLC cases, NSCLC cases, and cancer-free controls were recruited from an on-going NIH-funded lung cancer case-control study at The University of Texas MD Anderson Cancer Center. SCLC cases ($n = 216$) were consecutive patients with newly diagnosed, previously untreated, histologically confirmed SCLC with no age, gender, or ethnicity restrictions. NSCLC cases ($n = 196$) were matched to the SCLC cases on age (± 5 years) and gender. All lung cancer patients were treated in the Thoracic Surgery, Thoracic Oncology, or Pulmonary Units at the University of Texas MD Anderson Cancer Center. Healthy controls ($n = 229$) were recruited from the Kelsey-Seybold Clinic, a multi-specialty practice in

the Houston metropolitan area. Medical history, family history of cancer, smoking habits, and occupational history were obtained through an interviewer-administered risk-factor questionnaire and by the review of an institutional electronic patient history database. For all participants, former smokers were individuals who had smoked at least 100 cigarettes in their lifetime but quit at least 12 months prior to lung cancer diagnosis (for cases) or prior to the interview (for controls). Data on smoking history included smoking duration, number of cigarettes smoked per day and pack years smoked (e.g., computed by multiplying smoking duration [in years] by the number of cigarettes smoked per day and then dividing by 20). Upon informed consent, a 10-mL blood sample was drawn into coded heparinized tubes from all study participants for the assays.

Cytokinesis-Blocked Micronucleus Assay

Cytogenetic cultures for the CBMN assay were prepared using peripheral blood from all cases and controls following the method described by Fenech and Morley [1985] and the International Collaborative Project on Micronucleus Frequency in Human Populations [Fenech et al., 2003]. Lymphocyte cultures were prepared in duplicate for each study participant. Each culture contained 2.0×10^6 cells in 5 mL RPMI 1640 medium supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 10% fetal bovine serum, 2 mM L-glutamine (Gibco-Invitrogen, Carlsbad, CA), and 1% phytohemagglutinin (Remel, Lenexa, KS) and was incubated at 37°C. At 44 h after initiation, cytochalasin B (final concentration 4 µg/mL) was applied to block the cells in cytokinesis (Sigma, St. Louis, MO). Following incubation, the cells were fixed in 3:1 methanol:glacial acetic acid, dropped onto clean microscopic slides, air-dried, and stained with Giemsa stain. Using the scoring criteria delineated by the HUMN Project [Fenech et al., 2003], 1,000 binucleated cells per sample were scored blindly to the case/control status using a Nikon E-400 light optical microscope. Binucleated cells were then evaluated for the frequency of micronuclei (BN-MN), nucleoplasmic bridges (BN-NPB), and nuclear buds (BN-BUD).

Spectral Karyotyping Analysis

In order to identify the specific chromosomes involved in BN-MN, BN-NPB, and BN-BUD formation among SCLC and NSCLC, we performed spectral karyotyping (SKY) analysis on study participants for whom sufficient culture material was available for further analyses (SCLC, $n = 116$ and NSCLC, $n = 137$). For purposes of probe conservation, cultured cells from cases and controls were dropped onto a small 2×2 cm localized area on the slides. Human SKY kit (Applied Spectral Imaging [ASI], Israel) was used, following the manufacturer's recommendations. Briefly, slides were pretreated with pepsin, to reduce the background in the fluorescence, followed by 2 washes with solution A ($1 \times$ PBS/MgCl₂) and a second wash with solution B ($1 \times$ PBS/MgCl₂ and 1% formaldehyde) for 10 min each. Slides were then denatured in 70% formamide at 75°C for 2–3 min. In parallel, a human SKY probe was denatured at 80°C for 7 min, and incubated at 37°C for 60 min. Subsequently, 5 µL of the denatured probe was added to a predetermined area on the slides that contained cells with BN-MN and BN-NPBs. The hybridization was carried out for 48 h at 37°C in a humidity chamber, and the excess of the probe was removed. The hybridized slides were stained using anti-digoxin and Cy5-streptavidin and Cy5.5 antibody. The slides were then counterstained with DAPI in

an antifade solution (ASI). Twenty-five images per sample were captured and analyzed using the SKY paint probes multispecies software (ASI). This software allows the identification of the chromosomes predominantly involved in the formation of the chromosome alteration events based on pixel color.

Statistical Analysis

SAS 9.3 (Windows version 6.1.7601) was used to perform descriptive statistics, t tests, and logistic regression analyses. Unpaired t tests were used to compare differences of age, smoking status, pack years smoked, years smoked, number of cigarettes, and the CBMN endpoints (BN-MN, BN-NPB, BN-BUDs) between the 3 study groups (SCLC, NSCLC, and controls) yielding 2 testing p values based on equality of variance. The method pooled t test was used for equal variance comparison and the Satterthwaite t test was used for unequal variance comparison. The method Folded F test was performed to examine the equality of variance. An unequal variance t test was based upon a significant p value of the F test. A Kruskal-Wallis test of difference was also performed.

Exact logistic univariate regression, adjusted by age, sex, smoking status, pack years, year smoked, and/or number of cigarettes was performed in the SCLC cases, NSCLC cases, and controls on CBMN endpoints (BN-MN, BN-NPB, BN-BUDs). Based on these findings, a set of significant covariate(s) and variables were then selected to run the multivariate logistic regression using stepwise, forward, backward, and score methods to confirm the significance of variable effects. To control for multiple testing bias, a false discovery rate estimation of combined 87 p values from all the tests above was performed. The procedure of *MUTTEST* in SAS and the *fdrtool* package in R software (version 3.0.3) were both used for the estimation. Both identified a cutoff p value of 0.0247 for the family-wise significance.

Results

The demographic characteristics of the SCLC cases, NSCLC cases, and controls are summarized in Table 1. All the SCLC cases ($n = 216$) were non-Hispanic Whites versus 88 and 89% of the NSCLC cases and the controls, respectively. Therefore, all the analyses were limited to non-Hispanic Whites, resulting in sample sizes of 173 for the NSCLC cases and 204 for controls. The SCLC cases, NSCLC cases, and controls did not differ significantly in terms of gender with 45.8, 47.4, and 51.5% of males, respectively ($p > 0.05$). All 3 groups smoked approximately the same number of cigarettes per day (mean number of cigarettes per day $\pm$ SE for SCLC cases: 25.9 ± 14.2 , for NSCLC: 25.9 ± 12.9 , and for controls: 24.8 ± 13.0 ; $p > 0.05$). The duration of smoking was significantly less among the SCLC cases (mean $\pm$ SE of 35.8 ± 9.8 years) as compared to 39.9 ± 11.5 years among the NSCLC cases ($p < 0.0003$) and 37.5 ± 9.8 years for the controls ($p < 0.08$).

Overall, both SCLC cases and NSCLC cases exhibited significantly higher values of all DNA damage endpoints

Table 1. Demographic characteristics of the SCLC cases, NSCLC cases, and controls

Variables	SCLC	NSCLC	Controls	<i>t</i> test <i>p</i> values		
				SCLC vs. controls	NSCLC vs. controls	SCLC vs. NSCLC
Age, years ^a	63.8±7.7	63.5±8.6	63.8±7.1	0.9857	0.6836 ^b	0.6971
Median age	65	63	64			
Gender				0.2490	0.4321	0.7591
Male, <i>n</i> (%)	99 (45.8)	82 (47.4)	105 (51.5)			
Female, <i>n</i> (%)	117 (54.2)	91 (52.6)	99 (48.5)			
Smoking status ^c				0.9775	0.8297	0.8467
Current, <i>n</i> (%)	140 (65)	114 (66)	133 (66)			
Former, <i>n</i> (%)	75 (35)	58 (34)	69 (34)			
Pack years ^a	46.9±30.0	50.7±27.7	46.8±28.0	0.9780	0.1765	0.1973
Median number	40	45	40			
Smoking duration, years ^a	35.8±9.8	39.9±11.5	37.5±9.8	0.0839	0.0309^b	0.0003^b
Median number	36	40	39			
Cigarettes per day, <i>n</i> ^a	25.9±14.2	25.9±12.9	24.8±13.0	0.3948	0.4314	0.9552
Median number	20	20	20			

^a Values for SCLC, NSCLC, and controls are given as mean ± SE. ^b Unequal variance *t* test. ^c Smoking status was measured at 3 levels, “current,” “former,” and “never.” Due to the extremely small number of “never” (2 in the control group and 1 in both the NSCLC and SCLC groups), this category was dropped off the data set in the analysis using smoking status.

Significant *p* values are in bold.

than the non-cancer controls ($p < 0.0001$). Data on CBMN endpoints, frequencies by age, gender, smoke years, and smoking status are summarized in Table 2 and showed no significant differences among the comparison groups.

The mean number of BN-MN in both the SCLC cases and NSCLC cases was significantly different when compared to controls (mean ± SE = 3.74 ± 0.98 and 3.79 ± 0.96 vs. 1.43 ± 0.68 respectively, $p = 0.0001$). However, there were no significant differences between the SCLC cases and NSCLC cases ($p = 0.57$). Figure 1A shows that approximately 95% of SCLC cases had >2 MN/1,000 binucleated cells (range 1–6 BN-MN/1,000 cells), compared with 98% of NSCLC cases (range 1–6 BN-MN/1,000 cells) and 2.6% of control participants (range 0–4 BN-MN/1,000 cells), and that 97.5% of the control participants had 0 or 1 MN, compared with only 5% of the SCLC cases and 2% of the NSCLC cases, respectively ($p < 0.001$).

The mean number of BN-NPB was significantly different between the 3 comparison groups, where the mean ± SE for SCLC cases was 4.39 ± 1.19 as compared to 4.05 ± 0.95 in NSCLC cases ($p = 0.0014$) and 0.70 ± 0.62 in con-

trols ($p < 0.001$). Figure 1B demonstrates that approximately 94% of SCLC cases had >2 NPBs/1,000 binucleated cells (range 1–7 NPBs/1,000 cells), similar to the NSCLC cases (range 1–6 NPBs/1,000 cells) and 1% of control participants (range 0–3 NPBs/1,000 cells), and that 93% of the control participants had 0 or 1 NPB, compared with only 1.8% of the SCLC cases and 0% of the NSCLC cases, respectively ($p < 0.001$).

Overall, due to the rarity of the events, the average number of BN-BUDs was not different among the 3 comparison groups with a range of 0–1 BN-BUDs in SCLC cases and controls as compared to 0–2 in NSCLC cases. No substantial differences were detected when the frequencies of BN-MN, BN-NPBs, and BN-BUDs were stratified by gender, age, number of years smoked, number of cigarettes per day, or disease stage.

When the BN-MN data were analyzed as a continuous variable, using multivariate logistic regression, there was a 36.11-fold increase in SCLC risk (95% CI, 17.20–75.80) and a 172.95-fold increase in NSCLC risk (95% CI, 55.15–542.33) for each 1-unit increase in micronucleus frequen-

cy. Similarly, when BN-NPBs were analyzed as a continuous variable, there was a 25.26-fold increase in SCLC risk (95% CI, 11.12–57.35) and a 70.5-fold increase in NSCLC risk (95% CI, 17.70–280.79) for each 1-unit increase in BN-NPBs. Comparing the BN-NPBs among SCLC versus NSCLC revealed a 32% increase in risk for each 1-unit increase in BN-NPBs (Table 3).

A total of 2,900 cells were scored using SKY imaging for SCLC and 3,425 cells for NSCLC to identify a chromosome-specific involvement in the formation of BN-MN, BN-NPBs, and BN-BUDs in the 2 lung cancer groups. Fewer chromosomes were involved in BN-MN formation in the SCLC (chromosomes 1, 3, 5, 8, 13, 17, 20–22, and X) as compared to NSCLC (chromosomes 1, 3, 4, 6, 8, 10, 11, 13–16, 18, 21, 22, and X) group. BN-MN involving chromosomes 1, 3, 8, 21, 22, and X were seen in both SCLC and NSCLC cases; however, the frequency of BN-MN formation was significantly higher in the SCLC cases as compared to the NSCLC cases. Figure 2A shows the distribution of each of these chromosomes by disease status. The percentage of observed events was 5.4, 5.7, 3.6, 1.9, and 1.9% for SCLC versus 2.2, 2.6, 1.9, 1.0, and 1.1% for NSCLC for chromosomes 1, 3, 8, 21, and 22, respectively. The percentage of observed events for the X chromosome was slightly higher among the NSCLC cases. BN-MN involving chromosomes 5 and 17 were exclusively seen among the SCLC cases, and BN-MN involving chromosomes 4, 6, 10, 11, 13, 14, 15, 16, and 18 were exclusively seen among the NSCLC cases. We further found that cells from all SCLC cases harbored BM-MN originating from chromosomes 1, 3, 5, and 8.

More chromosomes were involved in BN-NPB formation in the SCLC cases compared to NSCLC cases. Chromosomes 2, 4–6, 9, 13, 14, 17–20, 22, and X were detected in BN-NPB formation among SCLC cases, while chromosomes 1–4, 9, 13, 14, 17, 19, and X were involved in BN-NPB formation among the NSCLC cases. The frequencies of BN-NPB involving chromosomes 2, 4, 9, 13, 14, 17, 19, and X observed in both SCLC and NSCLC cases were not significantly different. Figure 2B shows the distribution frequency of each of these chromosomes by disease status. BN-NPB involving chromosomes 5, 18, 20, and 22 were exclusively seen among the SCLC cases. BN-NPB involving chromosomes 1 and 3 were exclusively seen among the NSCLC cases.

Gene amplification as reflected by BN-BUD formation involved only 2 chromosomes (2 and 8) among the SCLC cases. Chromosome 2 was involved in BN-BUD formation among both groups but with a significantly higher frequency (5.5% of cells) among the SCLC cases

Table 2. Overall frequencies of BN-MN, BN-NPBs, and BN-BUDs per 1,000 binucleated cells in SCLC cases, NSCLC cases, and controls

	SCLC cases		NSCLC cases		Controls	
	mean	± SE n	mean	± SE n	mean	± SE n
BN-MN						
Gender						
Male	3.69	±1.02 99	3.76	±1.01 82	1.50	±0.64 105
Female	3.78	±0.96 117	3.82	±0.91 91	1.34	±0.72 99
Age						
≤Median	3.66	±1.05 119	3.97	±1.01 88	1.36	±0.73 107
>Median	3.84	±0.90 97	3.68	±0.90 85	1.51	±0.61 97
Smoking status						
Current	3.75	±0.99 140	3.89	±0.99 114	1.44	±0.69 133
Former	3.71	±0.98 76	3.62	±0.88 58	1.41	±0.67 71
Years smoked						
≤Median	3.73	±0.94 110	3.81	±1.01 104	1.40	±0.71 106
>Median	3.75	±1.03 106	3.77	±0.89 69	1.46	±0.64 98
Overall	3.74	±0.98 216	3.79	±0.96 173	1.43	±0.68 204
BN-NPBs						
Gender						
Male	4.26	±1.22 99	4.01	±0.97 82	0.72	±0.66 105
Female	4.50	±1.16 117	4.08	±0.92 91	0.67	±0.59 99
Age						
≤Median	4.34	±1.14 119	4.01	±0.95 88	0.71	±0.66 107
>Median	4.46	±1.24 97	4.08	±0.94 85	0.68	±0.59 97
Smoking status						
Current	4.41	±1.23 140	3.94	±0.94 114	0.70	±0.59 133
Former	4.37	±1.12 76	4.24	±0.92 58	0.69	±0.69 71
Years smoked						
≤Median	4.55	±1.19 110	4.06	±0.91 104	0.72	±0.60 106
>Median	4.24	±1.17 106	4.03	±1.00 69	0.67	±0.65 98
Overall	4.39	±1.19 216	4.05	±0.95 173	0.70	±0.62 204
BN-BUDs						
Gender						
Male	0.43	±0.50 99	0.45	±0.57 82	0.47	±0.50 105
Female	0.45	±0.50 117	0.41	±0.52 91	0.53	±0.50 99
Age						
≤Median	0.44	±0.50 119	0.45	±0.52 88	0.44	±0.50 107
>Median	0.45	±0.50 97	0.40	±0.56 85	0.56	±0.50 97
Smoking status						
Current	0.42	±0.50 140	0.39	±0.54 114	0.51	±0.50 133
Former	0.49	±0.50 76	0.50	±0.54 58	0.46	±0.50 71
Years smoked						
≤Median	0.48	±0.50 110	0.48	±0.56 104	0.48	±0.50 106
>Median	0.41	±0.49 106	0.35	±0.51 69	0.51	±0.50 98
Overall	0.44	±0.50 216	0.43	±0.54 173	0.50	±0.50 204

versus 0.8% among the NSCLC cases. Chromosome 8 was exclusively involved in SCLC cases, while chromosomes 1, 4, 9–11, 13, 15, 17, 20, and 21 were only involved in the NSCLC cases. Figure 2C shows the distribution frequency of each of these chromosomes by disease status.

Table 3. Risk estimates of SCLC and NSCLC for CBMN endpoints

Variable	SCLC vs. controls		NSCLC vs. controls		SCLC vs. NSCLC	
	OR (95% CI)	<i>p</i> value	OR (95% CI)	<i>p</i> value	OR (95% CI)	<i>p</i> value
BN-MN	36.11 (17.20–75.80)	<0.0001	172.95 (55.15–542.33)	<0.0001	0.94 (0.76–1.16)	0.5554
BN-NPBs	25.26 (11.12–57.35)	<0.0001	70.50 (17.70–280.79)	<0.0001	1.32 (1.09–1.59)	0.0052
BN-BUDs	0.82 (0.56–1.20)	0.3051	0.81 (0.54–1.20)	0.2903	1.00 (0.68–1.49)	0.9938

Bolded *p* values are considered significant according to the cutoff *p* value for family-wise significance from false discovery rate estimation 0.0247.

Fig. 1. Frequency distribution of CBMN endpoints among the study groups. **A** BN-MN distribution among SCLC versus NSCLC and controls. **B** BN-NPB distribution among SCLC versus NSCLC and controls.

Discussion

Chromosomal aberrations in the form of aneuploidy, numerical alterations as well as structural alterations are hallmarks of cancer [Masuda and Takahashi, 2002; Bonassi et al., 2011]. It has been shown that cancer cells accumulate a series of genetic changes, some of which can be identified by chromosomal analysis of PBLs [Dave et al., 1993]. In our study, we demonstrate that although both SCLC and NSCLC cases had significantly higher levels of genetic instability (BN-MN, BN-NPB, and BN-BUDs) as compared to healthy cancer-free controls, only the levels of the BN-NPBs were significantly higher among the SCLC cases as compared to the NSCLC cases ($p < 0.001$). While we have previously reported that the multi-endpoint CBMN assay is a sensitive predictor of lung cancer risk [McHugh et al., 2013], to our knowledge, this is the first study to compare the level of genetic insta-

bility between SCLC and NSCLC cases using this multi-endpoint approach.

Comparison of specific chromosome damage within the 2 lung cancer groups revealed repeated involvement of several chromosomes in the pathogenesis of both SCLC and NSCLC. It has been shown that the regions of recurrent genomic alteration frequently harbor genes crucial to the carcinogenic process [Zhao et al., 2005; Ding et al., 2008]. In BN-MN formation, which is reflective of chromosome loss or aneuploidy, chromosomes 1, 3, 8, 21, 22, and X were involved in both SCLC and NSCLC, however, a significantly higher frequency of involvement was observed among the SCLC for chromosomes 1, 3, and 8. Chromosomes 5 and 17 involvement in BN-MN formation was exclusively associated with SCLC, while several chromosomes were exclusively associated with NSCLC. In BN-NPB formation, which is reflective of structural rearrangements, several chromo-

Fig. 2. Frequency of specific chromosome involvement by endpoint and disease status as detected by SKY analysis. **A** Chromosome involvement in BN-MN. **B** Chromosome involvement in BN-NPB. **C** Chromosome involvement in BN-BUD.

somes were associated with both SCLC and NSCLC with similar frequency. BN-NPB formation involving chromosomes 5, 18, 20, and 22 was exclusively associated with SCLC, and chromosomes 1 and 3 were associated with NSCLC. In terms of BN-BUD formation, which is reflective of gene amplifications, only chromosome 8 was exclusive to SCLC.

Several genetic and molecular alterations have been consistently reported in both NSCLC and SCLC, some of which were also noted in our study [Pei et al., 2001; Bal-

sara and Testa, 2002; Masuda and Takahashi, 2002; Haga et al., 2005; Ma et al., 2006; van Meerbeeck et al., 2011]. Chromosome 1 involvement is a common event in lung cancer [Garnis et al., 2005], where it has been shown to harbor members of the WNT and NOTCH families that play a role in various cancers including NSCLC and SCLC [Hassan et al., 2014]. Chromosome 1 also carries a member of the MYC family of oncogenes, MYC lung carcinoma-derived homolog 1 (*MYCL1*), shown to be associated with SCLC [Wistuba et al., 2001]. In chromosome 2, an-

other member of the MYC family, MYC neuroblastoma-derived homolog (*MYCN*), is located. We observed amplifications involving chromosome 2 in both SCLC and NSCLC, however, an approximately 7-fold higher frequency of BN-BUDs was observed among the SCLC cases. In addition, amplification of chromosome 8, which harbors the third member of the MYC family, was observed and was exclusive to the SCLC group. Our findings support previous studies showing the 3 MYC family genes being frequently amplified in SCLCs [Wistuba et al., 2001; Kim et al., 2006; Voortman et al., 2010; Larsen and Minna, 2011; Sos et al., 2012].

Chromosome 3 has historically been characterized by chromosomal breakage near the centromere and other rearrangements in NSCLC [Pei et al., 2001; Balsara and Testa, 2002], while the deletion of the short arm of chromosome 3 has been seen in virtually all SCLC tumors [Franklin et al., 2010]. A number of candidate genes for lung cancer are located on chromosome 3, including the oncogene *PIK3CA* [Qian and Massion, 2008; Schwartz et al., 2011] and the *FHIT* gene [van Meerbeeck et al., 2011] which is reportedly one of the most altered genes in cancer. The *FHIT* gene functions in DNA repair to help maintain genomic stability [Saldivar et al., 2010; Wali, 2010], thereby supporting our finding of a higher frequency of damage on chromosome 3 among SCLC and its involvement in virtually 100% of SCLC patients as compared to 47% of NSCLC patients.

In our present study, chromosome 8 was involved in BN-MN formation among both SCLC and NSCLC cases. A recent study identifying molecular markers that predict clinical outcomes among NSCLC patients reported that chromosome 8 harbored several significant alterations [Baykara et al., 2015]. The most frequently amplified genes on chromosome 8 were *ZNF703* and *PRDM14*. *ZNF703* is a zinc finger protein that when overexpressed increases genetic instability, contributes to tumor aggressiveness [Reynisdottir et al., 2013], and increases lung metastasis rates in breast cancer [Slorach et al., 2011]. Thus, these findings indicate that *ZNF703* may function as an oncogene in different cancers and support our observation of higher genetic instability and BN-BUD formation (reflecting chromosome 8 amplifications) among SCLC cases. Similarly, *PRDM14* has been identified as a susceptibility locus for cancer [Ruark et al., 2013], and its amplification and overexpression was shown to be associated with a more aggressive phenotype and reduced sensitivity to chemotherapy in breast cancer [Nishikawa et al., 2007]. To date, there are no reports addressing the association of *PRDM14* with SCLC, however, given our ob-

servation of BN-BUD formation involving chromosome 8 exclusively among SCLC patients, it is plausible that this gene plays a role in the aggressive phenotype and resistance to chemotherapy in SCLC patients.

Chromosome 17 harbors the tumor suppressor *TP53*, whose inactivation is implicated in various cancers. It has been reported that patients with SCLC have more frequently mutations in *TP53* than do patients with NSCLC [Franklin et al., 2010] which supports our findings of higher frequency of chromosome 17 loss and rearrangements among SCLC cases than in NSCLC cases. Involvement of chromosome 22 is another interesting finding, as a recent study identified a potential oncogene, *CRKL* on 22q11, which may promote tumor cell proliferation, survival, and motility [Kim et al., 2010]. 22q12.2 has been recently identified as a new lung cancer susceptibility locus in a genome-wide association study of Han Chinese [Hu et al., 2011].

A recent study reviewed the fate and consequences of micronuclei and micronucleated cells and summarized the different potential fates as degradation of the micronucleus, elimination of the micronucleus from the cell, persistence in the cytoplasm, reinsertion into the main nucleus, elimination by apoptosis, and chromothripsis (chromosome shattering, within the confined space of the micronucleus, of one or a few chromosomes resulting in a large number of rearrangements) [Hintzsche et al., 2017]. The authors concluded that although the consequences of micronuclei remain unclear, the reinsertion and the persistence of the micronuclei lead to genetic imbalances in the daughter cells that contribute to both initiation and progression of the carcinogenic process. In addition, micronuclei formation is now recognized as a mechanism by which both chromothripsis and chromoanagenesis (complex genomic rearrangements occurring as a single catastrophic event) occur, and are major contributors to cancer initiation and development [Stephens et al., 2011; Crasta et al., 2012].

A recent study on genome-wide identification of genes with amplification and/or fusion in SCLC reported that several genes co-amplified with *MYCL1* and *MYC*, and that these genes were likely to be rearranged and amplified as a result of an immense genomic rearrangement acquired in a single catastrophic event such as chromothripsis [Iwakawa et al., 2013]. The higher frequency of genetic alterations observed in our study among SCLC as compared to NSCLC may therefore be a consequence of any of the different micronuclei fates listed above. Detection of such genetic alterations in the blood of the patients is indicative of early carcinogenic events, and hence

they have the potential of being used as markers for detection of the early stage disease.

To date, SCLC continues to significantly lag behind NSCLC and other cancers in terms of molecular profiling and targeted therapies [Byers and Rudin, 2015]. Therefore, new methods for early detection of SCLC are necessary in order to address these shortcomings. Because our present study substantiates our previous findings that the robust CBMN biomarker assay is a sensitive tool for the identification of individuals at high risk of developing lung cancer, in general, we believe it may be further used to identify lung cancer subtypes such as SCLC through the addition of molecular cytogenetic techniques. Using such an approach may further elucidate the underlying molecular mechanisms associated with SCLC and foster the development of strategies for earlier SCLC detection.

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Statement of Ethics

The study was approved by the Institutional Review Boards of the University of Texas MD Anderson Cancer Center and Kelsey-Seybold Clinic. All study participants signed an informed consent prior to enrollment and were coded using a unique study ID throughout the study.

Disclosure Statement

The authors have no conflicts of interest to declare.

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