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Exposure to smoky coal combustion emissions and leukocyte Alu retroelement copy number

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Abstract

Household air pollution (HAP) from indoor combustion of solid fuel is a global health burden that has been linked to multiple diseases including lung cancer. In Xuanwei, China, lung cancer rate for non-smoking women is among the highest in the world and largely attributed to high levels of polycyclic aromatic hydrocarbons (PAHs) that are produced from combustion of smoky (bituminous) coal. Alu retroelements, repetitive mobile DNA sequences that can somatically multiply and promote genomic instability have been associated with risk of lung cancer and diesel engine exhaust exposure. We conducted analyses for 160 non-smoking women in an exposure assessment study in Xuanwei, China with a repeat sample from 49 subjects. Quantitative PCR was used to measure Alu repeat copy number relative to albumin gene copy number (Alu/ALB ratio). Associations between clusters derived from predicted levels of 43 HAP constituents, 5-methylchrysene (5-MC), a PAH previously associated with lung cancer in Xuanwei and was selected *a priori* for analysis, and Alu repeats were analyzed using generalized estimating equations. A cluster of 31 PAHs reflecting current exposure was associated with increased Alu copy number (β:0.03 per standard deviation change; 95% confidence interval (CI):0.01,0.04; *P*-value = 2E-04). One compound within this cluster, 5-MC, was also associated with increased Alu copy number (*P*-value = 0.02). Our findings suggest that exposure to PAHs due to indoor smoky coal combustion may contribute to genomic instability. Additionally, our study provides further support for 5-MC as a prominent carcinogenic component of smoky coal emissions. Further studies are needed to replicate our findings.

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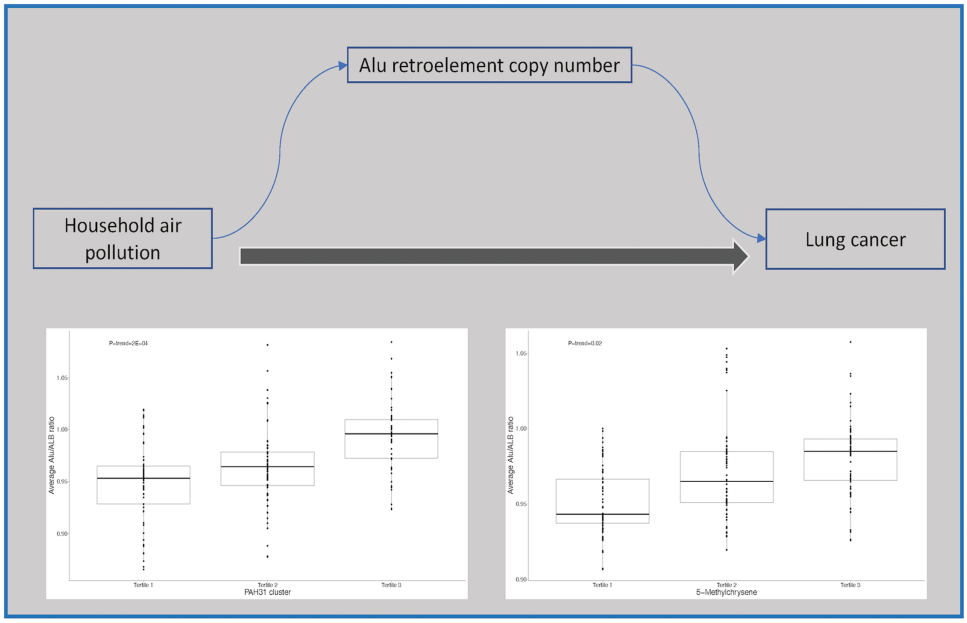
Graphical Abstract



Abbreviations: DEE, diesel engine exhaust; HAP, household air pollution; 5-MC, 5-methylchrysene; PAHs, polycyclic aromatic hydrocarbons.

Introduction

Household air pollution (HAP) is a major global health burden in low- and middle-income countries (1–3). HAP is mainly caused by incomplete combustion of solid fuels (e.g. coal, wood and biomass) used for cooking and/or heating in the home (1,2,4), and affects about half of the world's population (5,6). In Mainland China, it is estimated that over 450 million individuals still use solid fuels (7).

Lung cancer rates in never-smoking women of rural Xuanwei and Fuyuan counties in southwestern China are among the highest in the world (1,8,9). Households in Xuanwei and Fuyuan are heavily exposed to hazardous levels of various toxic constitutes of HAP, such as polycyclic aromatic hydrocarbons (PAHs), from combustion of smoky (bituminous) coal used for cooking and heating in the home (2,8,10). PAHs are organic compounds that are emitted from the combustion of fuels and are known for their carcinogenic and genotoxic characteristics (11). In a recent comprehensive epidemiologic study of specific HAP constituents in Xuanwei, we found evidence that lung cancer risk was strongly related to a cluster of PAHs, particularly 5-methylchrysene (5-MC) (12). However, the mechanism of action of HAP and its constituents in the pathogenesis of never-smoking lung cancer is unclear.

Alu retroelements, named after a conserved Alu restrictions site (AGCT) shared by most members of this family of repetitive mobile DNA sequences, are ~300 base pairs in length and compose about 10.7% of the human genome (13). AluY ('Young') is the youngest of the three Alu retroelement major families and exhibits the greatest tendency for active retrotransposition (14), which is the ability for Alu sequences to be transcribed into RNA, reverse-transcribed into DNA, and then inserted ('jump') into different genomic regions and thereby increase in copy number. Increased Alu retrotransposition has been shown to disrupt gene expression, alter protein expression and function (15,16), alter epigenetic regulation (17), as well as disrupt DNA architecture (18). As such, Alu retroelements may be an important form of genetic variation. Further, Alu retroelement copy number (Alu copy number) may be a marker reflecting genomic instability (19). Studies have linked Alu retroelements to chronic diseases, such as breast and prostate cancer (18). Notably, we recently found that higher Alu copy number is associated with increased risk of lung cancer in the prospective Prostate, Lung, Colorectal and Ovarian Cancer (PLCO) Screening Trial (20).

In one of the first human studies to explore the association between environmental genotoxic agents and Alu copy number, we recently found that workers exposed to diesel engine exhaust (DEE), a known lung carcinogen (21,22), had increased Alu copy number (23). To further explore whether Alu retroelements are influenced by environmental pollutants and better understand the carcinogenic effect of HAP and its constituents, we investigated the association between exposure to solid fuel and increase in Alu copy number among never-smoking women in Xuanwei, China.

Material and methods

Study population and design

The study population has been previously described in detail (24,25). Briefly, we conducted our analyses in the cross-sectional Xuanwei Exposure Assessment Study, which collected data on household air pollutants and exposures that may be related to the combustion of solid fuels through cooking or heating in the home. The study included 163 healthy women from 30 villages across Xuanwei and Fuyuan counties in Yunnan province who were enrolled between August 2008 and June 2009. A maximum of five households were selected in each village based on the following criteria: (i) having a stove that utilized solid fuel; (ii) the residence was more than 10 years old; (iii) household used the same equipment for cooking or heating for the last 5 years; and (iv) household included a healthy non-smoking woman aged 20–80 years who was responsible for cooking.

In-person interviews were conducted by two trained personnel and information on demographics, anthropometric and household characteristics was collected. An activity questionnaire was used to record the women's household activity during measurement, and a questionnaire was used to collect information on household stove and ventilation type, cooking activities, heating practices, type of coal mine that supplied household fuel and fuel usage during various points in the woman's life. Two air measurements 24 hours apart were conducted, and blood samples were collected on the second day.

Estimation of individual household air pollutant exposures

Exposure assessment has been previously described in detail (10,12,24,26,27). Briefly, measurements of particulate matter with an aerodynamic diameter ≤ 2.5 μm ($PM_{2.5}$), black carbon (BC, measured by the proxy marker of $PM_{2.5}$ absorbance), PAHs, nitrogen dioxide (NO_2) and sulfur dioxide (SO_2) were collected over two sequential 24-hour periods, with about half of subjects visited in a second season to allow for seasonal adjustments. Supervised stepwise predictive linear mixed-effect models were generated for each pollutant and applied to self-reported histories of stove and fuel use, treating village and individual subject as random effects. A total of 43 individual air pollutants were predicted and are listed in Supplementary Table 1 is available at *Carcinogenesis* Online.

Derivation of cluster prototypes

Given the strong correlation between individual pollutants, we derived exposure prototypes by clustering as previously described (12). Briefly, a hierarchical cluster analysis method was used to identify clusters of the 43 individual air pollutants. Reduction in skewness and improvement in the symmetry of the distribution for individual exposures was achieved through data transformation. The first component score coefficients of a principal component analysis were extracted to derive a cluster prototype for each of the clusters. A larger number of clusters was favored, trading off higher between-cluster prototype correlation to better resemble the individual pollutants belonging to the cluster. A total of 6 cluster prototypes were generated for current pollutant exposure, including a cluster of 31 PAHs (PAH31), a cluster of BC and 6 PAHs (BC and PAH6), a cluster of $PM_{2.5}$ and retene ($PM_{2.5}$ and RET), as well as individual clusters for naphtho(*2,3,k*)fluoranthene (NkF), NO_2 and SO_2. The individual air pollutants included in each exposure cluster are listed in Supplementary Table 1 is available at *Carcinogenesis* Online.

Alu retroelement copy number qPCR assay

A recently developed quantitative polymerase chain reaction (qPCR) assay was used to measure Alu retroelement copy number as previously described (23). Briefly, leukocyte genomic DNA was extracted from a sample of blood collected on the second visit, and monochrome multiplex qPCR was used to measure Alu repeat copy number and single-copy albumin (ALB) gene copy number. The Alu and ALB assays were measured three times for each subject and a unitless ratio between Alu and ALB (Alu/ALB) was calculated. The Alu/ALB ratio represents the average Alu retroelement copy number in the leukocyte genome relative to that of a reference DNA sample. Quality control was conducted on 14 samples from 7 subjects, and the average coefficient of variation was 7.2%.

Statistical analysis

The distribution of continuous variables was assessed using histograms and normality was tested using the Shapiro-Wilks tests. We assessed the association between the 6 clusters and Alu/ALB ratio using generalized estimating equations (GEE) accounting for repeated measurements. We then fit separate GEE models for 5-MC, an individual PAH constituent that has been previously found to have the strongest association with lung cancer risk in previous studies in Xuanwei independent of other PAHs (12), as well as for benzo(*a*)pyrene (BaP), a representative surrogate for levels of other PAHs. We also used GEE to estimate the association between categorical fuel type collected at the time of the home visit and Alu/ALB ratio. All models were adjusted for age, county (Xuanwei or Fuyuan), body mass index (BMI; kg/m^2), education (no education, attended elementary school, graduated elementary school, attended middle school or higher) and socioeconomic status (SES; no luxury items and at least one luxury item, such as a telephone, television, sewing machine, radio, bicycle, watch, motorcycle or tractor). *P*-values < 0.05 were considered statistically significant. All analyses were performed using the R statistical software (version 3.5.1).

All participants provided written informed consent prior to participating in the study. This study was approved by the National Cancer Institute Special Studies Institutional Review Board (#06CN092).

Results

Study population

The characteristics of the study population are shown in Table 1. A total of 160 subjects had available self-reported fuel type and Alu/ALB ratio data, 49 of which had repeated measurements of Alu/ALB ratio. The mean age was 56 [standard deviation (SD) = 14.4] years and the mean BMI was 21.9 (SD = 3.41) kg/m^2. About half of the women reside in Xuanwei county (50.6%), half have at least 1 luxury item (55.0%) and most women did not graduate from elementary school (83.8%). About half of the women used smoky coal at the first visit (50.0%), while the rest used smokeless coal (8.8%), wood or plant (4.4%) and a mix of solid fuels (36.9%).

Associations between HAP and Alu/ALB ratio

We observed a monotonic increasing relationship between the PAH31 cluster and Alu/ALB ratio among all participants (β = 0.03, 95% CI: 0.01,0.04, *P*-value = 2E-04, per 1 SD change), adjusting for all other exposure clusters and potential confounders (Table 2). We further observed an exposure-response relationship between tertiles of PAH31 and Alu/ALB ratio (P_{trend} = 2E-04) (Figure 1). The observed relationships between the PAH31 cluster and Alu/ALB ratio strengthened when we restricted to individuals who used smoky coal at blood draw (β = 0.07, 95% CI: 0.03,0.11, *P*-value = 0.001, per 1 SD change). We also found an association between NO_2 and Alu/ALB ratio; however, similar to acting as a proxy for traffic-sourced air pollution (28), the compound may act as a surrogate for a HAP component that is not reflected through other measured constituents.

We further observed a monotonic increasing relationship between 5-MC and Alu/ALB ratio among all participants (β = 0.004, 95% CI: 0.001,0.01, *P*-value = 0.02, per ng/m^3), as well as an exposure-response relationship between tertiles of 5-MC and Alu/ALB ratio (P_{trend} = 0.02), adjusting for potential confounders (Table 2/Figure 2). The observed relationships between 5-MC and Alu/ALB ratio strengthened when we restricted to individuals who used smoky coal at blood draw (β = 0.01, 95% CI: 0.003,0.01, *P*-value = 0.001, per ng/m^3). Alu/ALB ratios were similar between smoky coal and smokeless coal users among all participants, for which we did not find significant differences. However, given that the most toxic smoky coal is located in Xuanwei county, we compared Xuanwei smoky coal users with all smokeless coal users and observed a significant increase in Alu/ALB ratio (*P*-value = 0.01) (Figure 3). We further found a weaker association between BaP and Alu/ALB ratio overall (β = 0.001, 95% CI: 0.00001,0.001, *P*-value = 0.05, per ng/m^3), as well as in analyses restricting to smoky coal users (β = 0.002, 95% CI: 0.001,0.002, *P*-value = 2.7E-05, per ng/m^3).

Table 1. Characteristics of participants in the Xuanwei Exposure Assessment Study (*N* = 160)

	N[a] (%)
Age, years, Mean (SD)	56.0 (14.4)
BMI (kg/m^2), Mean (SD)	21.9 (3.41)
SES	
No luxury items	72 (45.0%)
At least 1 luxury item	88 (55.0%)
Education	
No school	104 (65.0%)
Attended elementary school	30 (18.8%)
Graduated elementary school	18 (11.3%)
Attended middle school or higher	8 (5.0%)
County of residence	
Xuanwei	81 (50.6%)
Fuyuan	79 (49.4%)
Fuel type at measurement[b]	
Smoky coal	80 (50.0%)
Smokeless coal	14 (8.8%)
Wood/plant	11 (6.9%)
Mixed fuel[c]	55 (34.4%)
Exposure to 5-MC[d] per ng/m^3-years, Mean (SD)	7.80 (4.2)

[a]A total of 160 subjects with available data on Alu/ALB ratio, out of which 49 have repeated measurements.
[b]Values represent fuel type at first visit.
[c]Mixed fuel type includes a mix of various solid fuels (e.g. wood and smokeless coal).
[d]158 participants had estimates for 5-MC, out of which 48 had repeated measurements. Units are per ng/m^3-year.

Discussion

To investigate the relationship between coal combustion emissions, their constituents, and Alu retroelement copy number, we conducted a molecular epidemiology study with detailed exposure assessment among never-smoking women in rural China. We observed an association between a cluster of 31 PAHs and increased Alu retroelement copy number. We also found an association between current exposure to 5-MC, an individual PAH constituent selected *a priori*, and increased Alu retroelement copy number, whereas a weaker association was observed between BaP, a constituent used as a surrogate for levels of other PAHs and Alu retroelement copy number. Further, we observed an increase in Alu copy number comparing smoky coal users in Xuanwei to all smokeless coal users. To our knowledge, this is the first study to find a link between coal combustion emissions constituents and Alu retrotransposition in humans, which suggests that these constituents may contribute to increased genomic instability.

Emerging evidence has shown that Alu retroelements may influence gene expression, genomic architecture, biological function and diseases such as cancer. Prospective nested case-control studies have previously found associations between DNA methylation of Alu sequences and risk of cancer

Table 2. Associations between exposure clusters derived from imputed individual air pollutants and Alu retroelement copy number

	All participants[a] *N* = 158		**Smoky coal users[b] *n* = 80**	
	Estimate (95% CI)[c]	***P*-value**	**Estimate (95% CI) [c]**	***P*-value**
Current exposure cluster				
PAH31[d]	0.03 (0.01,0.04)	**2E-04**	0.07 (0.03,0.11)	**0.001**
SO_2	0.02 (0.00,0.03)	0.05	0.001 (−0.03,0.03)	0.93
NO_2	0.02 (0.00,0.03)	**0.01**	0.02 (0.00,0.04)	0.05
NkF	0.0001 (−0.02,0.02)	0.99	−0.03 (−0.06,0.00)	0.09
$PM_{2.5}$ and RET	−0.01 (−0.02,0.01)	0.54	−0.02 (−0.07,0.03)	0.39
BC and PAH6[d]	−0.01 (−0.03,0.01)	0.26	−0.03 (−0.07,0.01)	0.12
5-MC[e]	0.004 (0.001,0.01)	**0.02**	0.01 (0.003,0.01)	**0.001**
BaP[e]	0.001 (0.00001,0.001)	0.05	0.002 (0.001,0.002)	**2.7E-05**

[a]Analyses were conducted in 158 individuals with non-missing Alu/ALB and cluster data, out of which 48 subjects had a repeated measurement.
[b]Analyses conducted in 80 individuals who exclusively used smoky coal during one of the measurement visits, out of which 18 subjects had a repeated measurement.
[c]GEE were used to estimate the change in Alu retroelement copy number (as reflected by Alu/ALB ratio) per 1 standard deviation increase in each cluster, mutually adjusting for all other clusters, as well as for age, county, body mass index, education and SES.
[d]Clusters containing multiple air pollutants, most of which are PAHs and are listed in Supplementary Table 1 is available at *Carcinogenesis* Online.
[e]Air pollutants identified *a priori* and included in the PAH31 cluster. Estimates are provided per ng/m^3-year. (A) All participants. (B) Smoky coal users.

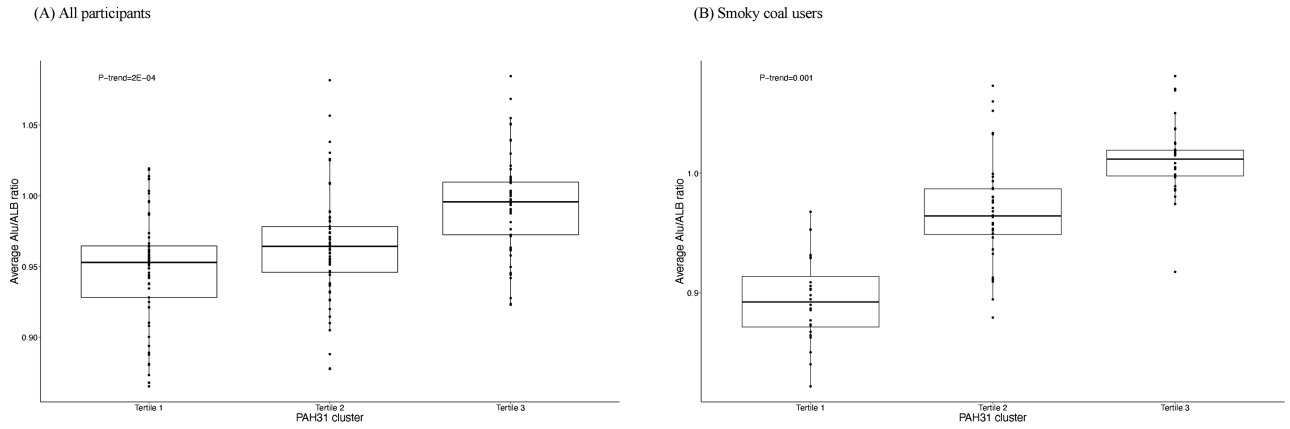


Figure 1. Distribution of Alu/ALB ratio by tertiles of PAH31 cluster derived from modeled ambient pollutants of current exposure in (**A**) all participants (N = 158 with 49 subjects having repeated measurements) and (**B**) participants using smoky coal (n = 80 with 17 subjects having repeated measurement). GEE were used to obtain the P-value estimates, treating PAH31 as a continuous variable, mutually adjusting for other clusters, as well as for age, county, education, body mass index and SES. Individual PAHs included in the PAH31 cluster include 5-MC, BaP among others listed in Supplementary Table 1 is available at *Carcinogenesis* Online. (A) All participants. (B) Smoky coal users.

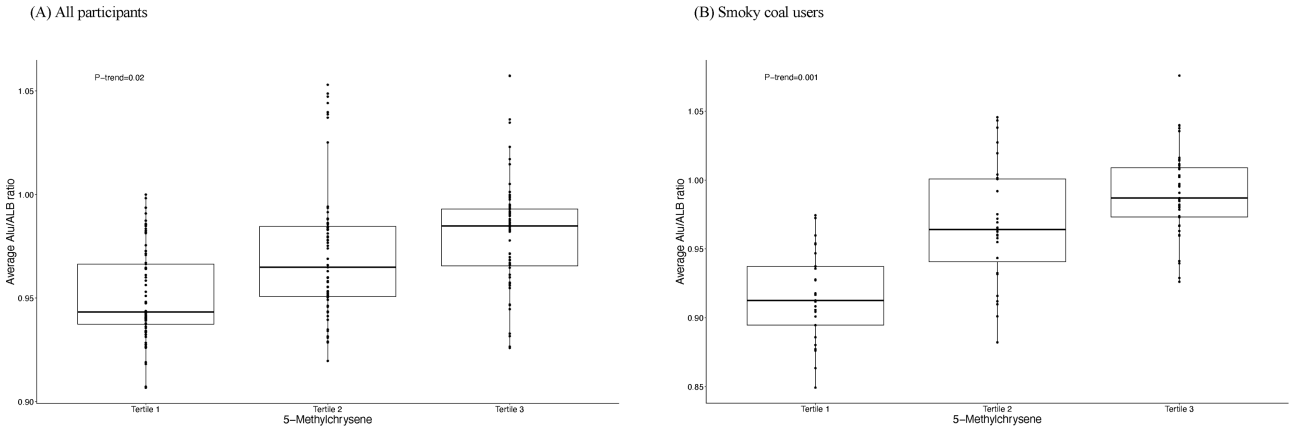


Figure 2. Distribution of Alu/ALB ratio by tertiles of 5-MC exposure in (**A**) all participants (N = 158 with 49 subjects having repeated measurements) and (**B**) participants using smoky coal for current fuel (n = 80 with 17 subjects having repeated measurement). GEE were used to obtain the P-value estimates, treating 5-MC as a continuous variable, adjusting for age, county, education, body mass index and socioeconomic position.

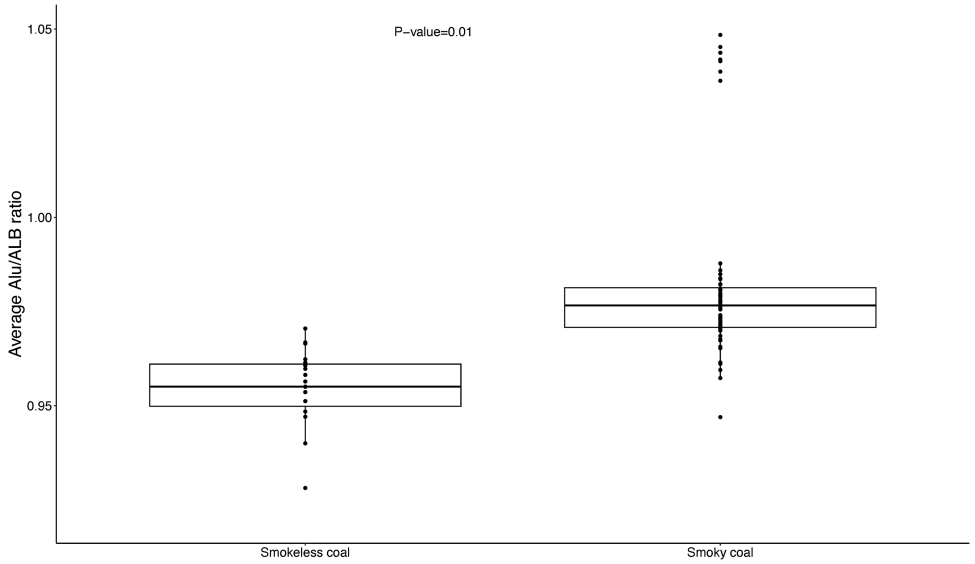


Figure 3. Distribution of Alu/ALB ratio comparing smoky coal users in Xuanwei (most toxic coal) (n = 47 with 13 subjects having repeated measurements) to all smokeless coal users (n = 14 with 5 participants having repeated measurements). GEE were used to obtain the P-value estimate, adjusting for age, county, education, body mass index and SES.

development (29,30). We recently found that higher Alu retroelement copy number was associated with an elevated risk of overall lung cancer in the prospective PLCO Screening Trial (20). These findings were especially notable in lung adenocarcinoma, a histological subtype that is more prevalent among never-smoking women and related to risk factors such as air pollution (31). Furthermore, a recent genome-wide association study found an enrichment of polymorphic Alu insertions in regions of the genome known to be associated with risk of human disease, including some cancers (18).

We previously found that lifetime smoky coal users had a nearly 100-fold increased risk of lung cancer mortality compared with lifetime smokeless coal users (1), and that the increased risk was especially pronounced in early life exposure (32). To identify the specific components of smoky coal that potentially drive this excess risk, we previously identified a cluster of PAHs related to lung cancer risk, from which 5-MC had the strongest association (12). Subsequently, we found that coal combustion emissions, and particularly 5-MC, are associated with urinary mutagenicity (33) and that air pollutants have been shown to induce DNA damage in experimental studies (8,34). Similarly, in our current study we observed that women with higher exposure to a PAH cluster and 5-MC have an increased Alu copy number. Taken together with our previous study linking DEE to increased Alu copy number (23), our findings suggest that the genotoxic constituents in air pollution could potentially contribute to lung carcinogenesis through promotion of genomic instability.

Our study had a number of strengths. First, the study population was composed of exclusively non-smoking Chinese women, which removes the potential confounding that may be caused by sex, race/ethnicity and cigarette smoking. Additionally, our subjects had comprehensive personal air monitoring and we used established statistical methods for exposure prediction, which limits the potential for exposure misclassification. However, this study had some limitations. The sample size was limited, which may have reduced our statistical power to detect modest associations. Furthermore, due to the very high correlation between HAP components, we were not able to further isolate signals of individual HAP components. Second, the subjects in this study were selected to represent the general population of Xuanwei using various types of fuel, reducing the contrast in exposure and therefore further limiting the power to detect associations. However, the wide range of exposure to HAP increased the chances of detecting noteworthy associations.

In summary, this is among the first epidemiologic studies to suggest that exposure to indoor air pollutants may be associated with increase Alu retroelement copy number. Our findings are consistent with those in prior epidemiological studies examining environmental carcinogens, such as occupational diesel exposure, as well as animal studies where exposure to genotoxic compounds resulted in an increase in Alu copy number. However, caution is recommended when interpreting the findings, and results from our study require replication. Further studies on subjects exposed to various fuel types, such as coal, wood and biomass are warranted to better understand the impact of environmental exposures on markers of genomic instability.

Supplementary material

Supplementary data are available at *Carcinogenesis* online.

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Conflict of interest statement: None declared.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Author contributions

B.B., R.V., N.R. and Q.L. developed the study concept, analysis plan and provided general oversight of the project. B.B. conducted statistical analyses and led the preparation of the manuscript. J.W., W.H., R.C., G.D., L.P., Y.Z., B.N., M.R., B.J., J.L., K.Y., D.H., D.S. and Y.H. provided input on the study design, statistical analysis and presentation of the data. B.N., J.L. and K.Y. conducted fieldwork and collected data from participants in the study. All authors reviewed the final version of this manuscript.

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