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Chromosome-wide aneuploidy study (CWAS) in workers exposed to an established leukemogen, benzene

Luoping Zhang*, **Qing Lan[1]**, **Weihong Guo**, **Alan E.Hubbard**, **Guilan Li[2]**, **Stephen M.Rappaport**, **Cliona M.McHale**, **Min Shen[1]**, **Zhiying Ji**, **Roel Vermeulen[1]**, **Songnian Yin[2]**, **Nathaniel Rothman[1]** and **Martyn T.Smith**

Division of Environmental Health Sciences, School of Public Health, University of California, Berkeley, CA 94720, USA, [1]Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Department of Health and Human Services, Bethesda, MD 20892, USA and [2]Institute of Occupational Health and Poison Control, Chinese Center for Disease Control and Prevention, Beijing, 100050 China

*To whom correspondence should be addressed. Division of Environmental Health Sciences, School of Public Health, University of California, Berkeley, CA 94720-7360, USA. Tel: +1 510 643 5189; Fax: +1 510 642 0427; Email: luoping@berkeley.edu

Evidence suggests that *de novo*, therapy-related and benzene-induced acute myeloid leukemias (AML) occur via similar cytogenetic and genetic pathways, several of which involve aneuploidy, the loss or gain of chromosomes. Aneuploidy of specific chromosomes has been detected in benzene-related leukemia patients as well as in healthy benzene-exposed workers, suggesting that aneuploidy precedes and may be a potential mechanism underlying benzene-induced leukemia. Here, we analyzed the peripheral blood lymphocytes of 47 exposed workers and 27 unexposed controls using a novel OctoChrome fluorescence *in situ* hybridization (FISH) technique that simultaneously detects aneuploidy in all 24 chromosomes. Through this chromosome-wide aneuploidy study (CWAS) approach, we found heterogeneity in the monosomy and trisomy rates of the 22 autosomes when plotted against continuous benzene exposure. In addition, statistically significant, chromosome-specific increases in the rates of monosomy [5, 6, 7, 10, 16 and 19] and trisomy [5, 6, 7, 8, 10, 14, 16, 21 and 22] were found to be dose dependently associated with benzene exposure. Furthermore, significantly higher rates of monosomy and trisomy were observed in a priori defined 'susceptible' chromosome sets compared with all other chromosomes. Together, these findings confirm that benzene exposure is associated with specific chromosomal aneuploidies in hematopoietic cells, which suggests that such aneuploidies may play roles in benzene-induced leukemogenesis.

Introduction

Therapy-related and *de novo* melodysplastic syndromes (MDS) and acute myeloid leukemias (AML) are considered to be similar diseases based on common underlying cytogenetic and genetic pathways (1,2). There is an extensive evidence that the well-known leukemogen, benzene (3,4), induces MDS and AML via similar pathways (4). Several of the pathways-characterizing MDS/AML involve aneuploidy, the loss or gain of chromosomes and a common feature of human cancer (5,6). Around 70% of hematopoietic cancers exhibit loss or gain of one or more chromosomes (6). Loss of whole chromosomes 5 or 7 (monosomy, −5/−7) or partial loss of the long arms of the two chromosomes (5q−/7q−) and gain of whole chromosome 8 (trisomy, +8) are the most common unbalanced aberrations in AML and MDS (7,8). Chromosomal aneuploidy, including that of chromosomes 5 and 7, has been detected not only in benzene-related leukemia and preleukemia patients (9) but also in healthy workers with current exposure to benzene (9–13). This suggests that aneuploidy precedes and may be a potential mechanism underlying benzene-induced leukemia.

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CWAS, chromosome-wide aneuploidy study; FISH, fluorescence in situhybridization; IRR, incidence rate ratio; MDS, melodysplastic syndromes.

Studies of the effects of benzene metabolites *in vitro* and of benzene-exposed workers indicated an apparently selective effect of benzene on certain chromosomes. The benzene metabolites, hydroquinone and 1,2,4-benzenetriol, were shown to induce the loss and long arm deletion of chromosomes 5 and 7 (14,15), as well as trisomy at chromosomes 7, 8 and 21 (15) in human lymphocytes *in vitro*. Aneuploidy of select chromosomes in benzene-exposed workers revealed monosomy of chromosomes 5, 7 and 8 but not chromosomes 1 and 21 in earlier studies (10,13), and monosomy and trisomy of all seven additional chromosomes [2, 4, 6, 11, 12, 14 and 18] examined in a follow-up study, with the loss and gain of chromosome 6 being especially noteworthy (12). These studies, while valuable, analyzed chromosomes hypothesized to play a potential role in benzene-induced AML, based on their involvement in AML and MDS as described in the literature.

To conclusively determine whether benzene induces specific or random aneuploidy in exposed individuals, an agnostic method is necessary to simultaneously assess relative levels of aneuploidy in all human chromosomes. To this end, we developed a novel chromosome-wide aneuploidy study (CWAS) approach, comprising a triple-color painting fluorescence *in situ* hybridization (FISH) method called OctoChrome FISH, which allows for the simultaneous detection of aneuploidy in all 24 chromosomes on a single eight-square slide. We previously applied this CWAS approach to examine aneuploidy in the lymphocytes of six exposed workers (>5 p.p.m. benzene) and five unexposed controls (11). Our pilot results revealed significant increases in the rates of monosomy of chromosomes 5, 6, 7 and 10 and trisomy of chromosomes 8, 9, 17, 21 and 22, compared with all other chromosomes, in the exposed workers.

Based on these preliminary findings, we hypothesized that benzene would induce chromosome-specific aneuploidy in a dose-dependent manner, a finding that would strengthen the role of such aneuploidy as a potential mechanism underlying benzene-induced leukemia. As benzene-induced AML/MDS may be induced by similar cytogenetic and genetic pathways as *de novo* and therapy-related AML (4), such a finding could also clarify the role of aneuploidy in leukemogenesis generally. Therefore, in the present study, we have applied this CWAS approach to assess dose-dependent effects on chromosome-specific aneuploidy in healthy workers exposed to different levels of benzene.

Materials and methods

Study subjects and demographics

Study subjects (n = 74) for the OctoChrome FISH analysis were selected from a large molecular epidemiology study aimed at evaluating hematologic, cytogenetic and molecular endpoints in workers exposed to benzene in Tianjin, China (16). A total of 47 exposed workers came from two shoe factories studied (22 workers exposed to benzene <10 p.p.m. and 25 exposed to ≥10 p.p.m. benzene) and the 27 unexposed controls were from a clothing manufacturing plant located in the same general geographical area as the shoe factories, which includes all 11 subjects (six exposed workers and five controls) previously analyzed by CWAS in our pilot study (11). The control and exposed groups of workers were frequency matched by age, gender, alcohol consumption and current smoking status. Detailed characteristics of the study subjects are summarized in Table I. Institutional Review Boards at the United States National Cancer Institute and the Chinese Center for Disease Control and Prevention, Beijing, China approved the study. Participation was voluntary and written informed consent was obtained. The peripheral blood samples were collected in June 2000.

Benzene exposure assessment

Prior to phlebotomy, benzene and toluene exposure was monitored in each study participant by organic vapor passive monitor badge as described

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Table I. Demographic and exposure characteristics of study subjects from Tianjin, China

Benzene exposure	Subject (n)	Exposure level[a] (p.p.m.)	Age (year)	Gender		Current smoking		Current drinking alcohol		Recent respiratory infections	
				Male	Female	Yes	No	Yes	No	Yes	No
Controls	27	0.035	31.7	12 (44)[b]	15 (56)	10 (37)	17 (63)	7 (26)	20 (74)	3 (11)	24 (89)
<10 p.p.m.	22	4.95 ± 3.61	35.32	5 (23)	17 (77)	2 (9)	20 (91)	3 (14)	19 (86)	5 (23)	17 (77)
≥10 p.p.m.	25	28.33 ± 20.09	35.24	9 (36)	16 (64)	7 (28)	18 (72)	9 (36)	16 (64)	2 (8)	23 (92)

[a]Mean ± SD values.
[b]No. of subjects (%).

previously (17). Individual full shift air monitoring took place about every month >3 months period, resulting in ~3 to 4 personal air measurements per person. Average individual benzene exposure was calculated for the whole observation period and separately for the last month before biological sample collection. Benzene and toluene were not detected in air samples from the control factories (16). The benzene exposure levels (3 months prior to phlebotomy) in exposed workers were 4.95 ± 3.61 p.p.m. (mean ± standard deviation) in the <10 p.p.m. group and 28.33 ± 20.09 p.p.m. in the ≥10 p.p.m. group and < 0.04 p.p.m. (<0.13 mg/m^3) in the unexposed controls (Table I).

OctoChrome FISH and scoring procedure

The OctoChrome FISH protocol provided by CytoCell (Banbury, UK) was performed as described previously for our pilot study (11), on metaphase spreads prepared from 72 h whole blood culture. OctoChrome FISH, a chromosomic approach, allows the simultaneous analysis of 24 chromosomes on a single eight-square slide, in one hybridization. A Multiprobe device carries whole chromosome painting probes, on each of eight squares, for three different chromosomes in three different colored fluorophores, Texas Red, fluorescein isothiocyanate and Coumarin Spectra (red, green and blue), respectively. The chromosomes are arranged on the Multiprobe device in combinations that facilitate the identification of most specific aneuploidy and chromosomal rearrangements related to human leukemia and lymphoma. The fixed lymphocyte metaphases prepared from the blood of benzene-exposed workers and unexposed controls are dropped onto the eight-square slides matched to the Multiprobe device. The simultaneous denaturation of the probes and target DNA, and the use of rapid formamide-free stringency wash after overnight hybridization, simplifies the FISH procedure. After the hybridization, post-washing and 4′,6-diamidino-2-phenylindole staining steps, metaphase spreads on each square of the eight-square slide are scanned and localized automatically using Metafer software (MetaSystems, Germany) and then evaluated on the computer screen. A total of 237 513 metaphase cells (averaging >3000 per subject and ~400 per chromosome) were selected and scored according to the criteria listed in our previous publications (10,14).

Statistical analysis

Negative binomial regression was applied in this study because (i) it is commonly used when the outcome variable is a count, (ii) it can naturally adjust for differences in the denominator (total number of cells tested), (iii) it allows for overdispersion and (iv) it provides interpretable associations between two measures called incidence rate ratios (IRRs) (18), which describe how the aneuploidy frequency increases as the benzene exposure level increases. Of particular interest is the ranking of chromosomes by their 'sensitivity' to benzene exposure. We ranked the chromosomes based on the estimated strength of associations (using IRR and P value) from the 24 regressions.

Dose response

To test the null hypothesis that all chromosomes have the same association with benzene, we used negative binomial regression and a generalized estimating equation approach (19), which adjusted the inference for potential correlation of monosomy rates made on the same subject but different chromosomes. Specifically, we model, for instance, the rate of monosomy as a log-linear model:

$$\log\big(E(Y|\text{cells, Chrom, Benz})\big) = \log(\text{cells}) + \beta_0 + \beta_1 I(\text{Chrom} = 1) + \cdots + \beta_p I(\text{Chrom} = p) + \alpha\text{Benz} + \cdots + \gamma_p I(\text{Chrom} = p)\text{Benz},$$

where Y is the number of monosomy cells. Note that Benzene is coded 0, 1, and 2 and we are thus estimating a dose response from no to low to high exposure. Then, the test of whether the effect of benzene is the same for all chromosomes is the test of the null:

$$H_0 : \gamma_1 = \gamma_2 = \cdots = \gamma_p = 0,$$

which can be performed using a Wald-type chi-square test.

≥10 p.p.m. versus control and <10 p.p.m. versus control

As for all the global analyses, we perform a Wald test to test the null hypothesis that all chromosomes have the same rate ratio (high over control) comparing high to Control exposure. Specifically, we fit a regression to the monosomy counts, which models the mean (λ) rate of monosomy as a function of chromosome (C) and dose (D).

$$\log(\lambda(C,D)) = \beta_0 + \beta_1 I(C=2) + \beta_2 I(C=3) + \cdots + \beta_{21} I(C=22) + \alpha_0 I(D=\text{high}) + \alpha_1 I(D=\text{high})^* I(C=2) + \cdots + \alpha_{21} I(D=\text{high}) I(C=2).$$

The test of our hypothesis that all chromosomes have the same ratio of rate increase (or decrease) from high to low, we test the null hypothesis that: $H_0 : \alpha_1 = \alpha_2 = \cdots = \alpha_{21} = 0$, which we can do using a Wald test. We account for potential correlation due to repeated measures (each individual contributes monosomy measurements from the 22 chromosomes, excluding the sex chromosomes) by using a generalized estimating equation approach (19) with robust standard errors.

To examine the chromosomes one by one, we perform the following log (linear) regressions on a chromosome by chromosome basis:

$$\log(\lambda(D)) = \beta_0 + \beta_1 D.$$

We also use the Wald statistic to test whether the chromosome is related to dose. ($H_0 : \beta_r \le 0$). We also performed generalized additive model smooths [GAM, loglink; (20)] on a chromosome by chromosome basis, for both monosomy and trisomy versus benzene, to empirically examine the dose response.

Results

In order to determine whether benzene induces chromosome-specific and leukemia-related aneuploidies across a range of exposure levels, we applied a CWAS approach to simultaneously measure the rates of aneuploidy in all 24 chromosomes in the peripheral blood lymphocytes of 74 individuals from an occupational study of benzene exposure. Exposures were categorized as none (n = 27), <10 p.p.m. (n = 22) and ≥10 p.p.m. (n = 25). Detailed characteristics of the study subjects are summarized in Table I.

Heterogeneity in aneuploidy response among all autosomes with increasing benzene exposure level

We determined whether benzene exposure influenced the levels of monosomy and trisomy overall in the 22 autosomes (chromosomes 1–22), at each dose category relative to controls. After adjustment for confounding, a significantly increased rate of trisomy was detected at ≥10 p.p.m. benzene exposure (IRR 1.57, 95% confidence interval 1.05–2.36, P = 0.028). The overall monosomy rate was also increased at ≥10 p.p.m. but was not significant (IRR 1.29, 95% confidence interval 0.89–1.89, P = 0.182).

As the overall effect on aneuploidy could be influenced by differences in the response rates of individual chromosomes, we examined these rates. Heterogeneity in the response of the 22 autosomes, for both monosomy (Figure 1a) and trisomy (Figure 1b) rate, was

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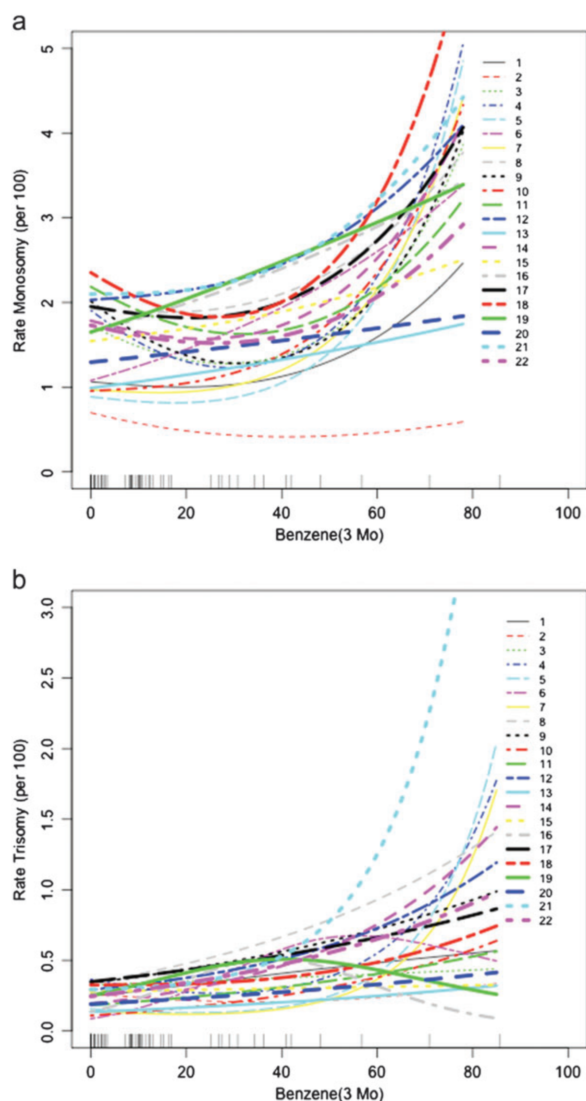

Fig. 1. Monosomy and trisomy rates of each of the 22 autosomes (chromosomes 1–22) with continuous benzene exposure. Plot [GAM smoothes (20)] of the (**a**) monosomy and (**b**) trisomy rates of each of the 22 autosomes (chromosomes 1–22) against continuous benzene exposure levels averaged >3 months prior to blood collection. Each chromosome is distinguished by a different color and pattern. Heterogeneity in the response of the 22 autosomes for both monosomy and trisomy rate is apparent from the variation in the slope and shape of the curves. Heterogeneity was confirmed by a likelihood ratio test in which the null hypothesis that all chromosomes responded equally to benzene exposure was rejected with P value < 0.0001.

apparent after plotting smooth aneuploidy for each chromosome against continuous benzene exposure. With regard to monosomy rate, the slope and shape of the curves varied widely among the chromosomes with linear and non-linear (supra, cubic etc.) represented in Figure 1a. Overall, trisomy rates were ∼10-fold lower than monosomy and different curves were exhibited by the different chromosomes. The effect of benzene on trisomy 21 was particularly striking, with a very sharp increase in rate observed at higher benzene concentrations shown in Figure 1b. The null hypothesis that all chromosomes responded to benzene exposure equally was analyzed by likelihood ratio test, and the hypothesis was rejected with P value < 0.0001.

Benzene induces aneuploidy of select chromosomes in a significantly dose-responsive manner

In order to determine, if there was a selective and statistically significant dose-dependent response in the chromosomal damage induced by benzene, the mean monosomy and trisomy frequencies of all 24 chromosomes among the three categories of benzene exposure (≥10 p.p.m., <10 p.p.m. and control) were examined (Figure 2a and b). A statistically significant dose-dependent increase in monosomy rate, across the three exposure categories, of chromosomes 5, 6, 7, 10, 16 and 19 (P < 0.05) was found, as illustrated in Figure 2a. All six chromosomes had IRR values ≥ 1.3 (Table II). Trisomy rates for chromosomes 5, 6, 7, 8, 10, 14, 16, 21 and 22 were significantly increased (P < 0.05), as illustrated in Figure 2b and had IRR values ≥ 1.3 (Table II). Chromosome 21 exhibited the most significant trisomy rate (P = 4.170E-07).

Aneuploidy of specific chromosomes induced by benzene exposure

We considered the aneuploidy IRR of each chromosome in each of the exposed groups (≥10 p.p.m. and <10 p.p.m.), compared with the control group. As shown in Table III, monosomy rates of chromosomes 6, 7, 16 and 19 were significantly increased (P < 0.05) at ≥10 p.p.m. benzene and had IRRs > 1.5. No chromosomes had significantly increased monosomy at <10 p.p.m. benzene (supplementary Table 1 is available at *Carcinogenesis* online). Trisomy rates of chromosomes 6, 10, 14, 16, 19 and 21 were significantly increased (P < 0.05) and had IRRs > 2.0, at ≥10 p.p.m. benzene (Table III). At <10 p.p.m. benzene, the rate of trisomy 10 approached statistical significance (P = 0.06, IRR = 2.35, supplementary Table 1 is available at *Carcinogenesis* online).

Aneuploidy of 'susceptible' chromosome sets induced by benzene

The results described above suggest that specific chromosomal aneuploidies are associated with benzene exposure in a dose-dependent manner, through the analysis of aneuploidy at each chromosome as an independent association with benzene exposure (Table II). We hypothesized that a susceptible chromosome set would show a significant response compared with the set comprising all other chromosomes. Thus, we sought to define a susceptible chromosome set based on association with AML/MDS, benzene-associated leukemia and benzene exposure.

We first selected a candidate set of eight chromosomes [5, 6, 7, 10, 12, 14, 16 and 19] probably to be susceptible to monosomy based on reports in the literature of the association of their loss through monosomy with AML or MDS (7,8,21,22) or with benzene exposure in earlier studies (12,13,15). Several of these chromosomes also exhibited significant induction of monosomy in the dose-response analysis described above in the present study. We called this set of susceptible chromosomes an 'IN'-group. Monosomy induction in the IN-group was compared with that of the group including all remaining chromosomes (OUT-group), by interaction testing, at high and low exposures compared with unexposed controls, Table IV. As hypothesized, the IN-group was significantly more susceptible to monosomy compared with all other chromosomes, at ≥10 p.p.m. (P < 0.001) and <10 p.p.m. (P = 0.04) benzene exposures. Thus, aneuploidy was significantly more probably to be detected in susceptible chromosome sets. As seen in Table IV, analysis with two successively smaller IN-groups (chosen by excluding member chromosomes with less well established relationships to AML and benzene exposure) also showed significantly increased susceptibility to monosomy at ≥10 p.p.m. benzene but no longer showed a significant association at <10 p.p.m.

We selected a candidate set of eight chromosomes (5, 6, 7, 8, 10, 14, 16 and 21) probably to be susceptible to trisomy based on reports in the literature of the association of their loss through trisomy with AML or MDS (7,8,23–31) or with benzene exposure in earlier studies (9–13,32). This IN-group was significantly more susceptible to trisomy compared with all other chromosomes at ≥10 p.p.m. benzene (P = 0.005), but not at <10 p.p.m., as shown in Table IV. After exclusion of chromosomes 10 and 16, the IN-group remained significantly more susceptible (P = 0.035) at ≥10 p.p.m. benzene compared with all other chromosomes.

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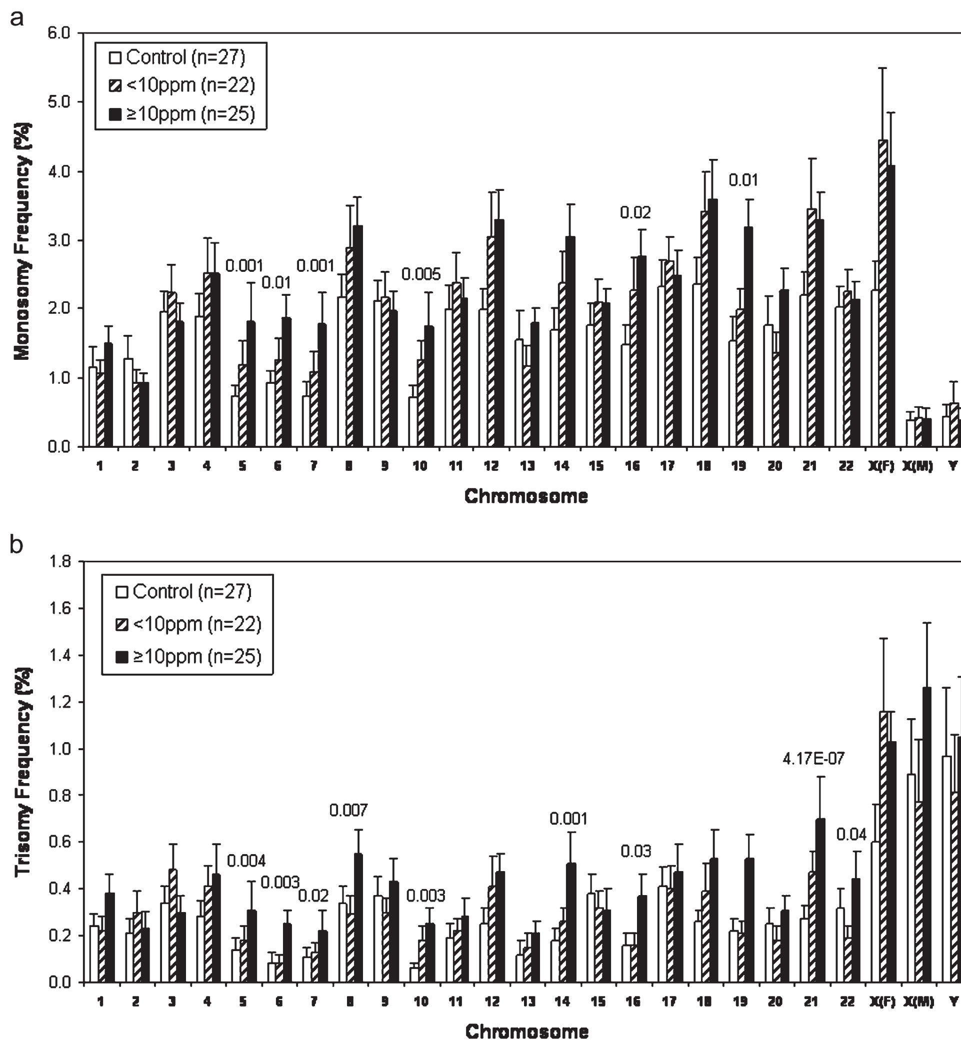

Fig. 2. Mean monosomy and trisomy frequencies of all 24 chromosomes in workers exposed to no, <10 p.p.m. and ≥10 p.p.m. benzene. Mean frequency and standard errors of (**a**) monosomy and (**b**) trisomy for all 24 chromosomes in unexposed controls ($n = 27$) and workers exposed to <10 p.p.m. benzene ($n = 22$) and ≥10 p.p.m. benzene ($n = 25$) are shown. The P_{trend} for chromosomes with statistically significant ($P < 0.05$) dose-dependent increases (detailed in Table II) are shown.

Loss and gain of chromosome X detected in exposed females but not males

We examined aneuploidy effects on the sex chromosomes in females and males separately. As illustrated in Figure 2a, an increase in the mean frequency of loss of chromosome X was apparent in females, but not males, at ≥10 p.p.m. and <10 p.p.m. benzene relative to controls. Less apparent were effects on gain of chromosome X in either gender (Figure 2b) and of loss and gain of chromosome Y in males. Therefore, we assessed the rates of loss and gain of chromosomes X and Y for each gender (supplementary Table 2 is available at *Carcinogenesis* online). In females, after adjusting for confounders, the IRR of loss of chromosome X was 1.55 (95% confidence interval 1.01–2.37) and approached significance ($P = 0.045$) at ≥10 p.p.m. but not at <10 p.p.m. benzene. Gain of chromosome X was not significant at either exposure level in females, after adjustment (supplementary Table 2 is available at *Carcinogenesis* online). In males, rates of loss of chromosomes X and Y were not significantly altered at either dose. Rates of gain of chromosomes X and Y were inconclusive in males, probably due to the smaller number of men studied and the less frequent occurrence of trisomy events.

Discussion

Aneuploidy is a characteristic of both solid and blood cancers and can put significant stress on the cell (33). It has long been known that benzene (or its metabolites) induces chromosomal aneuploidy *in vitro* and in the peripheral blood lymphocytes of individuals occupationally exposed to benzene (9,10,12–15), but methods employed in earlier studies did not allow systematic chromosome-wide and chromosome-specific analysis. Therefore, we developed a novel CWAS approach and demonstrated in a pilot study that several specific aneuploidies were detected more frequently in the lymphocytes of six benzene-exposed workers than in five unexposed controls (11). Here, for the first time, we have applied this CWAS approach to examine chromosome-specific and dose-dependent aneuploidy in the peripheral blood lymphocytes of a benzene-exposed population ($n = 74$). Although chromosomal studies are typically performed on bone marrow cells in leukemia patients, chromosomal aberrations in cohorts exposed to occupational and/or environmental leukemogens are usually measured in peripheral blood lymphocytes (15). As well as being readily accessible in such studies, peripheral blood lymphocytes are considered a good surrogate for bone marrow effects, as shown by the high concordance between chromosomal abnormalities of chromosomes 5, 7, 8, and 11 detected in bone marrow and peripheral blood lymphocytes of myeloid leukemia patients (34).

In the present study, we found heterogeneity in aneuploidy induction with increasing benzene exposure. Furthermore, statistically significant, chromosome-specific increases in the rates of monosomy [5, 6, 7, 10, 16 and 19] and trisomy [5, 6, 7, 8, 10, 14, 16, 21 and 22] were found to be dose dependently associated with benzene exposure. The

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Table II. Dose-dependent monosomy and trisomy rates of the 22 autosomes among groups exposed to no, <10 p.p.m. and ≥10 p.p.m. benzene[a]

Chromosome	Monosomy			Chromosome	Trisomy		
	IRR[b]	95% CI[c]	*P*-value		IRR[b]	95% CI[c]	*P*-value
7	1.51	1.18–1.92	0.001	6	1.79	1.21–2.65	0.003
5	1.50	1.18–1.91	0.001	21	1.68	1.37–2.05	4.170E-07
10	1.43	1.12–1.83	0.005	14	1.57	1.20–2.06	0.001
19	1.34	1.07–1.67	0.01	5	1.57	1.15–2.13	0.004
6	1.32	1.07–1.64	0.01	10	1.51	1.16–1.98	0.003
16	1.29	1.05–1.58	0.02	16	1.49	1.05–2.12	0.03
1	1.23	0.98–1.55	0.07	7	1.48	1.05–2.08	0.02
12	1.19	0.96–1.47	0.11	8	1.37	1.09–1.72	0.007
13	1.18	0.92–1.52	0.20	22	1.35	1.01–1.80	0.04
14	1.15	0.93–1.43	0.20	12	1.34	1.00–1.79	0.05
20	1.15	0.91–1.44	0.24	11	1.28	0.97–1.70	0.08
17	1.13	0.95–1.35	0.16	9	1.28	1.00–1.62	0.05
18	1.13	0.93–1.37	0.21	4	1.27	0.98–1.64	0.08
21	1.13	0.91–1.40	0.26	1	1.26	0.99–1.60	0.06
8	1.13	0.90–1.41	0.31	13	1.24	0.86–1.79	0.25
15	1.12	0.94–1.34	0.20	19	1.23	0.96–1.56	0.10
3	1.10	0.93–1.32	0.27	17	1.22	0.93–1.60	0.16
4	1.10	0.90–1.35	0.35	18	1.18	0.89–1.58	0.25
9	1.07	0.90–1.27	0.46	20	1.14	0.86–1.52	0.36
22	1.06	0.88–1.26	0.55	3	1.05	0.81–1.37	0.71
11	1.04	0.85–1.26	0.71	2	1.03	0.71–1.48	0.89
2	0.85	0.65–1.10	0.21	15	1.02	0.77–1.35	0.90

[a]Data listed according to IRR values.
[b]IRR, adjusted for age, sex, alcohol, infection status and smoking and for a 20 U increase in exposure.
[c]95% confidence interval.

Table III. Monosomy and trisomy rates of the 22 autosomes in benzene-exposed (≥10 p.p.m.) versus controls[a]

Chromosome	Monosomy			Chromosome	Trisomy		
	IRR[b]	95% CI[c]	*P*-value		IRR[b]	95% CI[c]	*P*-value
6	1.94	1.17–3.20	0.01	6	3.33	1.16–9.53	0.02
19	1.93	1.23–3.03	0.004	16	2.73	1.31–5.69	0.01
7	1.89	1.02–3.48	0.04	14	2.62	1.23–5.59	0.01
5	1.73	0.92–3.25	0.09	10	2.48	1.06–5.77	0.04
10	1.70	0.93–3.13	0.09	21	2.23	1.24–3.99	0.01
14	1.64	1.01–2.65	0.05	19	2.09	1.24–3.53	0.01
16	1.62	1.04–2.53	0.03	13	1.99	0.82–4.80	0.13
12	1.41	0.90–2.21	0.13	18	1.66	0.85–3.25	0.14
18	1.35	0.86–2.11	0.19	11	1.56	0.76–3.19	0.22
21	1.27	0.81–1.99	0.30	12	1.56	0.80–3.01	0.19
1	1.25	0.75–2.06	0.39	4	1.52	0.78–2.96	0.21
8	1.23	0.75–2.01	0.41	8	1.52	0.89–2.59	0.13
20	1.16	0.72–1.87	0.54	1	1.52	0.86–2.67	0.15
4	1.15	0.71–1.88	0.57	22	1.47	0.72–2.98	0.29
15	1.13	0.77–1.67	0.53	5	1.45	0.63–3.35	0.39
13	1.12	0.65–1.94	0.69	7	1.22	0.50–2.99	0.66
17	0.98	0.65–1.48	0.92	20	1.17	0.62–2.21	0.62
22	0.97	0.65–1.45	0.88	9	1.13	0.62–2.06	0.69
11	0.95	0.60–1.50	0.83	2	1.13	0.50–2.56	0.78
3	0.91	0.59–1.39	0.65	17	1.10	0.57–2.10	0.78
9	0.89	0.59–1.35	0.58	3	0.91	0.50–1.64	0.74
2	0.60	0.36–1.00	0.05	15	0.76	0.42–1.38	0.37

[a]Data listed according to IRR values.
[b]IRR, adjusted for age, sex, alcohol, infection status and smoking and for a 20 U increase in exposure.
[c]95% confidence interval.

chromosomes shown to be targeted by benzene in the present study validate earlier studies analyzing the effects of benzene on specific chromosomes. The C group of chromosomes (chromosomes 6–12 and X) was previously reported to be preferentially associated with benzene exposure in earlier studies using traditional cytogenetic methods, as summarized (9). Increased monosomy of chromosomes 5, 6, 7, 10, 16 and 19, was reportedly associated with benzene exposure in exposed workers (11–13) and a dose-dependent effect was found for monosomy 5, 6 and 7 (12,13), across > 31 p.p.m. and ≤ 31 p.p.m. exposure groups. Therefore, the current CWAS approach has refined previously observed dose-dependent effects and revealed additional ones. Regarding trisomy, most of the findings have been previously reported in workers exposed to very high levels of benzene. For example, increased rates of trisomy 5, 6, 7, 8, and 21, with

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Table IV. Aneuploidy rates of the IN-group (susceptible chromosome set) versus the OUT-group (remaining chromosomes) chromosomes

Chromosome group	≥10 p.p.m. benzene versus control					<10 p.p.m. benzene versus control				
	OUT[c]-group		IN[c]-group		*P*-value of interaction	OUT-group		IN-group		*P*-Value of Interaction
	IRR[a]	95% CI[b]	IRR	95% CI		IRR	95% CI	IRR	95% CI	
Monosomy										
5, 6, 7, 10, 12, 14, 16 and 19	1.09	0.75, 1.57	1.83	1.20, 2.82	<0.001	1.02	0.68, 1.52	1.33	0.85, 2.09	0.04
5, 6, 7, 10, 16 and 19	1.15	0.80, 1.65	2.00	1.22, 3.27	<0.001	1.06	0.71, 1.58	1.34	0.81, 2.22	0.18
5, 6, 7 and 10	1.21	0.84, 1.74	2.16	1.18 3.94	0.007	1.07	0.72, 1.60	1.48	0.83, 2.63	0.17
Trisomy										
5, 6, 7, 8, 10, 14, 16 and 21	1.37	0.91, 2.05	2.10	1.32, 3.35	0.005	1.08	0.76, 1.53	1.21	0.81, 1.81	0.46
5, 6, 7, 8, 14 and 21	1.44	0.97, 2.15	2.01	1.23, 3.30	0.035	1.10	0.78, 1.55	1.15	0.76, 1.75	0.75
6, 7, 8 and 21	1.51	1.01, 2.27	1.88	1.14, 3.09	0.20	1.13	0.79, 1.59	1.07	0.69, 1.65	0.76

[a]IRR, adjusted for age, sex, alcohol, infection status and smoking and for a 20 U increase in exposure.
[b]95% confidence interval.
[c]OUT and IN chromosome groups defined a priori based on their association with AML or MDS and benzene exposure in the literature, as described in the text.

dose-response effects for trisomy of chromosomes 6, 7, 8 and 21, were found (9,10,12,13,32). Global analysis of aneuploidy in a pilot study by OctoChrome FISH revealed a significant induction of trisomy at chromosomes 6, 8, 9, 12, 14, 16, 19, 21 and 22 (11). Additionally, trisomy 10 was previously detected at an increased rate in exposed workers though IRR and *P*-value were not available due to the zero count found in the controls (11).

Together, these data demonstrate that benzene induces certain chromosomal aneuploidies in a dose-dependent manner. Many of the significant aneuploidies have been demonstrated in leukemia patients. Loss of whole chromosomes 5 or 7 (−5/−7) or of the long arms of the two chromosomes (5q−/7q−) is the most common unbalanced aberrations in *de novo* and therapy-related MDS and AML (7,8). Trisomy 8 is the most frequent numerical aberration in AML and MDS, occurring at a frequency of 10–15% (23). Trisomy 21 (+21) is the second most common trisomy in AML and MDS, occurring at a frequency of ∼3% (24). Trisomy 6 (25,26), trisomy 10 (27,28), trisomy 14 (29,30) and trisomy 16 (31) have all been reported to be nonrandom numerical anomalies of myeloid disorders. That these benzene-induced and leukemia-relevant aneuploidies occur in healthy workers with current exposure to benzene as demonstrated in the present study, as well as in benzene-related leukemia and preleukemia patients (9), suggests that aneuploidy precedes and may be a potential mechanism underlying benzene-induced leukemia. Aneuploidy of chromosomes 5 and 7 may also be a mechanism underlying therapy-related AML cases, which arise after treatment with the alkylating drugs such as melphalan (35,36).

Benzene is a common environmental pollutant to which almost everyone is exposed through automobile exhaust, gasoline use and smoking (37,38). Benzene is thought to affect the blood-forming system at levels <1 p.p.m., the current United States occupational standard (39), with no evidence of a threshold (40–42). Benzene induces hematotoxicity (16), manifest as decreases in almost all blood cells, even at levels <1 p.p.m. (43). Given the likely lack of a threshold and the multimodal mechanism of action exerted by benzene through its metabolites (4), cumulative effects, such as aneuploidy and other chromosome aberrations acquired through environmental exposure to benzene, may contribute to *de novo* leukemia.

Although our study demonstrated a dose-dependent induction of chromosomal aneuploidy by benzene, effects on individual chromosomes at the lower exposure category (<10 p.p.m.) were statistically insignificant. This is probably due to a lack of power as this exposure category comprised only 22 individuals. Indeed, we recently reported significantly increased chromosome loss in a larger number of workers exposed to <1 p.p.m. (n = 109) compared with controls (n = 139) using a traditional non-banding assay (44). In the present study, we found increased monosomy in a susceptible chromosome set, compared with all other chromosomes, at both ≥10 p.p.m. and <10 p.p.m. benzene. Analysis of aneuploidy in this susceptible chromosome set in an expanded population exposed to low levels of benzene could clarify such effects at low doses. Such a study would also assess the potential application of an aneuploidy signature as a biomarker of benzene exposure and early effect. A susceptible chromosome set with increased trisomy rates relative to all remaining chromosomes at ≥10 p.p.m. but not <10 p.p.m. benzene exposure was determined. As trisomy is a less frequent event than monosomy, a large sample size may increase the power to detect trisomy effects at low levels of exposure. Further clarification of aneuploidy induction at low levels of benzene exposure will help to elucidate its role in benzene-induced leukemogenesis.

It remains to be determined how benzene causes aneuploidy. Aneuploid cells may arise directly from diploid cells through errors in chromosome segregation, as a consequence of incorrect microtubule–kinetochore attachments, or through failure of the spindle checkpoint (45). Dysfunctional telomeres may also play a role (46). As altered expression of multiple genes may play a role in aneuploidy (47), we are analyzing global gene expression datasets that we previously generated from the same pool of benzene exposed and control subjects (48) as in the current study, in conjunction with the aneuploidy data described here, to identify aneuploidy-related genes induced by benzene.

Future studies could explore whether the apparent selectivity of aneuploidy induction in certain chromosomes is a function of benzene-targeting-specific chromosomes or of certain aneuploidies providing a survival or growth advantage to the cell. Only non-lethal aneuploidies are detected by the methodologies employed here and might not represent the full spectrum of targeted chromosomes. Thus, cells with lethal aneuploidies may die off and aneuploidies, which not only permit survival but that also give a growth advantage to the cell, may be preferentially found in individuals exposed to leukemogens. Such aneuploidies subvert normal hematopoietic processes when they occur in a hematopoietic cell and thus cause leukemia. One example is constitutive trisomy 21, which characterizes Down's syndrome and predisposes affected individuals to risk of leukemia. A recent study showed that trisomic doses of several chromosome 21 genes, one of which is *RUNX1*, perturb hematopoietic stem and progenitor cell differentiation leading to the hyperproduction of immature progenitor cells, in Down's syndrome (49). Microarray analyses in AML with trisomies 8 (n = 12), 11 (n = 7), 13 (n = 7) and monosomy 7 (n = 9), as sole changes, revealed altered expression levels of genes located in the affected chromosomal regions (50).

A second possibility is that the three-dimensional organization of the chromosomes in the nucleus, which is dynamic with respect to cell type and differentiation stage, may render some chromosomes more sensitive to loss or gain. This has been shown to influence the development of translocations found in lymphoma and leukemia, as reviewed (51). Although we measured aneuploidies in mature lymphocytes, they could have arisen in hematopoietic stem/progenitor cells; indeed, we have detected aneuploidies induced by another

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candidate leukemogen, formaldehyde, in progenitor cell colonies (52). Future studies are necessary to determine which cells are more sensitive to benzene-induced aneuploidy and the role of chromosome architecture.

Conclusion

We have shown that benzene significantly induces aneuploidy in a chromosome-specific and dose-dependent manner. Investigation of the role of aneuploidy at these chromosomes in benzene-induced leukemogenesis, particularly at low levels of benzene exposure, is warranted.

Supplementary material

Supplementary Tables 1 and 2 can be found at http://carcin.oxfordjournals.org/

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