Chromosome Translocations in Workers Exposed to Benzene

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As benzene has been linked with elevated risk of both acute myeloid leukemia and lymphoma, we explored the effect of benzene exposure on levels of t(8;21), t(15;17), and t(14;18) translocations. Circulating lymphocytes of normal individuals also often contain t(14;18). Quantitative polymerase chain reaction analysis showed that 37 workers with benzene exposure had a decreased level of t(14;18) in their blood with only 16.2% having 10 or more copies of the t(14;18) BCL-2/IgH fusion gene/µg DNA, as opposed to 55% of 20 controls (P = .0063 by Fisher's exact test). This decline may be related to the immunotoxicity to specific subtypes of circulating B-lymphocytes, but the data do not support the use of t(14;18) as a biomarker of increased lymphoma risk in benzene-exposed populations. None of 88 individuals (31 controls and 57 exposed) exhibited detectable t(8;21) transcripts, and while t(15;17) transcripts were detected in two individuals, the result is inconclusive as one was exposed and the other was unexposed.

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Epidemiological studies indicate that benzene exposure is associated with an increased risk of human leukemia and lymphoma (1,2). Chromosomal translocations are thought to be initiating events in leukemia and lymphoma, and specific translocations are used as markers of diagnosis, disease progression, and relapse. As benzene is thought to act by producing chromosomal aberrations and altered cell differentiation (3), we investigated the possibility of using circulating levels of two acute myeloid leukemia (AML)–associated translocations, t(8;21) (4) and t(15;17) (5), and a follicular lymphoma–associated translocation, t(14;18) (6), as biomarkers of early effect for benzene. Evidence for the occurrence of these translocations in normal individuals is limited, with t(8;21) detected in 1 in 496 cord blood samples (7) and t(15;17) detected in healthy volunteers (8). As recently reviewed (9), the prevalence of t(14;18) in peripheral blood lymphocytes of healthy donors is relatively high at ~50% (range 8%–80% depending on the polymerase chain reaction (PCR) methodology employed and target cell population) (9–13) and rises with smoking (14), but the correlation between frequency and age remains unclear (13,15). The biological consequences of these aberrations in healthy individuals are yet to be understood. One possibility is that the frequency of translocation-positive cells represents a biomarker of early effect for hematopoietic carcinogens which could correlate with the cumulative risk of developing t(14;18)-related follicular lymphoma and t(8;21)- and t(15;17)-related AML, and perhaps other cancers.

The two principal technologies for detection of specific chromosome translocations are fluorescence in situ hybridization (FISH) and real-time PCR. FISH distinguishes individual chromosomes by hybridization with sequence-specific, chromosome-painting probes labeled with spectrally nonoverlapping fluorophores. In real-time quantitative PCR (qPCR), a fluorescently labeled probe is hybridized to the amplification target, and fluorescent signal proportional to the input DNA/cDNA is generated during amplification, thereby allowing quantification. qPCR assays the whole peripheral blood mononuclear cell (PBMC) population in G_0, whereas FISH examines a culture-modified population [consisting of mainly mature T lymphocytes stimulated by the mitogen phytohemagglutinin that have gone through two cell divisions (16)]. The two methods also generate different estimates of t(14;18) translocation levels with FISH detecting all possible t(14;18) breakpoints and the real-time assay described here only detecting breakpoints occurring in the major breakpoint region (MBR) of the BCL-2 gene. Although it was previously thought that the majority of BCL-2 breakpoints occur in the MBR (17), it has been shown more recently that many breakpoints occur outside of the MBR and the minor cluster region (18). The ability of FISH to detect more translocation breakpoints is offset by its lower relative sensitivity (300–500 metaphases examined per individual) compared with real-time PCR (millions of cells examined per individual). The t(14;18) translocation can be detected at the genomic level but the breakpoints in one or both fusion partners involved in the t(8;21) and t(15;17) translocations occur in very long introns, making detection of the resultant fusion transcripts more practical than detection of the genomic breakpoint, particularly at low frequencies. We previously reported increased levels of t(8;21) by FISH, in workers highly exposed to benzene, and confirmed the presence of the translocation in one individual by reverse transcription PCR (19). We also recently reported the detection of t(14;18) by FISH in 4/22 individuals highly exposed to benzene but not in controls (N = 44) or in lower-exposed workers (N = 21) (20). However, lack of DNA of suitable quality precluded confirmation of t(14;18) by qPCR. In this paper, we describe analysis of chromosomal translocations

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t(8;21), t(15;17), and t(14;18) by qPCR in individuals from a more recent molecular epidemiology study of benzene exposure.

Methods

Characteristics of Study Subjects

Peripheral blood was obtained, with written, informed consent, from unexposed controls and individuals occupationally exposed to benzene in factories near Tianjin, China, in 2000 (21,22). The study was approved by Institutional Review Boards at National Cancer Institute and the National Institute of Occupational Health and Poison Control, China, CDC, Beijing. PBMCs were isolated by Ficoll separation within 6 hours of blood collection. RNA was extracted from 94 PBMC samples representing 88 individuals (31 controls, 20 low-exposed [mean 1.8 ppm, median 1.7 ppm benzene], 37 high-exposed [mean 21.9 ppm, median 12.8 ppm], 6 internal quality control samples), using the Qiagen RNeasy kit (Qiagen Inc, Valencia, CA), to screen for t(8;21) and t(15;17). DNA from 20 unexposed controls and 37 individuals exposed to benzene (mean 22.6 ppm, median 13.8 ppm), representing a subset of individuals analyzed for t(15;17) and t(8;21), was extracted using the QIAamp DNA extraction kit (Qiagen Inc) in order to screen for t(14;18).

Detection of Translocations by Real-Time and Nested PCR

Primers, probes, and protocols for t(14;18) (23), t(8;21) (24, 25), and t(15;17) qPCR are detailed in supplemental Tables S1 and S2 available online. DNA quality was confirmed by qPCR of the β-actin gene using 100 ng of DNA (26), and genomic DNA (1 µg) was analyzed for t(14;18). The assay routinely detected three copies of template in 1 µg of background DNA in two out of three replicates and standard curves consistently yielded R^2 values greater than 0.99. For the t(8;21) and t(15;17) assays, cDNA was generated from 1 µg RNA using the Superscript first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA), and integrity of the cDNA was assessed by amplification of β2-microglobulin from 1 µL cDNA (27). For each assay, three replicates for each standard, sample, and control were analyzed by qPCR using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Both assays reproducibly detected 10 copies of t(15;17) or t(8;21) translocation transcripts in 1 µg background RNA (250 000 lymphocyte cells). The standard curve slopes were –3.75 and –3.27 for the t(15;17) and t(8;21) assays, respectively, and the R^2 values were typically >0.99. Nested PCR and DNA sequencing for t(14;18) was carried out as described previously (15). Nested PCR assays for t(8;21) and t(15;17) are detailed in supplementary Tables S3 and S4 available online.

Statistical Methods

Demographic (sex), lifestyle factors (smoking and alcohol drinking), and infection were compared between exposed workers and controls by Pearson chi-square test or Fisher's exact test if the number of subjects in one cell is smaller than five. Age, translocation copies, and categorization of t(14;18) status by copy number were analyzed by Wilcoxon rank-sum test. Spearman correlation between the categorization of t(14;18) status by copy number and benzene exposure status was calculated. Multivariable models (linear regression of the continuous translocation copy variable and logistic regression of translocation number dichotomized into 0 for no events and 1 for any number of events, and 0 vs 1 for 10 or more events) were used to control for age, sex, smoking, alcohol drinking, and other potential confounding factors. All data analysis was carried out using SAS 9.1.3 (SAS Institute Inc, Cary, NC).

Results and Discussion

The t(8;21) and t(15;17) translocations were assayed by real-time and nested PCR assays. Screening 88 individuals by qPCR identified two positives for t(15;17), at a level of three to four transcripts per 250 000 cells. The positive samples, from an exposed individual and an unexposed control, were confirmed by nested PCR (Figure 1). This is only the second report of the presence of t(15;17) in healthy volunteers (8). No individuals were positive for t(8;21). As these translocations are rare in normal individuals (7), screening large populations will be necessary to see the true frequencies.

The frequency of the t(14;18) translocation among 20 healthy nonexposed individuals and 37 workers exposed to benzene, assayed here by a genomic-based qPCR approach, is shown in Table 1. Surprisingly, the mean number of copies of the t(14;18) BCL-2/IgH fusion gene was significantly lower in the exposed workers (22.3 copies/µg DNA) than in the controls (147.6 copies/µg DNA) (P = .0004). Categorization of the data by copy number confirmed that the translocation frequency was statistically significantly greater among the positive individuals from the control group (Table 1). qPCR analysis showed that 37 workers with benzene exposure had a decreased level of t(14;18) in their blood with only 16.2% having 10 or more copies of the t(14;18) BCL-2/IgH fusion gene/µg DNA, as opposed to 55% of 20 controls (P = .0063 by Fisher's exact test).

The finding of a lower level of t(14;18) translocations in benzene-exposed workers is surprising, but we hypothesize that it may be explained by benzene depressing a subtype of circulating B-cells (22,28). In the current study, B-lymphocyte levels were depressed 36% in exposed (>10 ppm) individuals compared with controls.

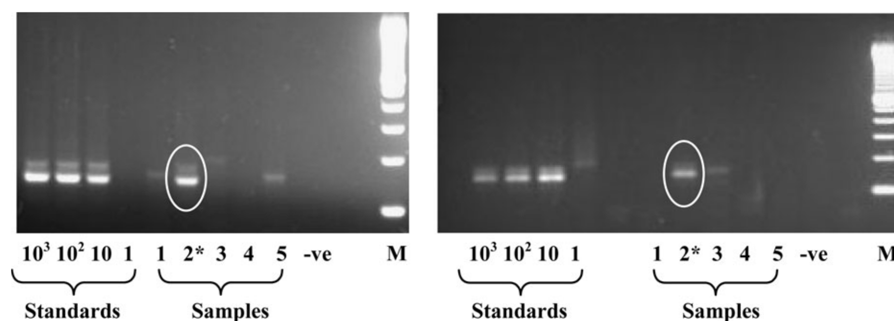

Figure 1. Nested polymerase chain reaction result for t(15;17). Second-round amplification products are shown. Standards are in vitro transcripts generated from cloned breakpoints, −ve = Negative control (human colon RNA), positive samples are circled and denoted by "*."

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Table 1. Translocation t(14;18) detected by real-time PCR in benzene-exposed workers and controls

Characteristics	Control (n = 20)	Exposed (n = 37)	*P* value
Benzene exposure (ppm)	0	22.6 ± 21.44* (13.78)†	
Age			.52‡
Mean ± SD	35.2 ± 7.6	36.7 ± 7.7	
Median	36	37	
Sex			.05§
Male	10 (50%)	9 (25%)	
Female	10 (50%)	28 (76%)	
Currently smoking			.06§
Yes	9 (45%)	8 (22%)	
No	11 (55%)	29 (78%)	
Currently drinking			0.39§
Yes	7 (35%)	9 (24%)	
No	13 (65%)	28 (76%)	
Recent infection			1‖
Yes	1 (5%)	2 (5%)	
No	19 (95%)	35 (95%)	
Translocation copies	147.6 ± 365.2* (17.65)†	22.3 ± 106.1 (0)	.0004‡
t(14;18)¶			0.0002‖
0	4 (20%)	25 (68%)	
1	5 (25%)	6 (16%)	
2	6 (30%)	5 (14%)	
3	4 (20%)	1 (3%)	
4	1 (5%)	0	
t(14;18)¶			0.0063‖
No (0–1)	9 (45%)	31 (84%)	
Yes (2–4)	11 (55%)	6 (16%)	

* Mean ± SD.

† Median, 0 = negative.

‡ Wilcoxon test, 1 = ambiguous.

§ Chi-square test, 2 = positive (10–100 copies).

‖ Fisher's exact test, 3 = positive (100–999 copies).

¶ Categorization of t(14;18) status by copy number, 4 = positive (≥1000 copies).

The t(14;18) translocation is enriched in or restricted to a subtype of B-cells (11, 12, 29), and this subtype may be more susceptible to the toxic effects of benzene. At least some of the t(14;18)-bearing clones are likely to be memory cells rather than naive B-cells, because most BCL-2/IgH clones are long-lived and persist over several years in the periphery (30). Chronic hepatitis C virus infection can promote either the occurrence or maintenance of t(14;18)$^+$ clones (31). The frequency of t(14;18) translocation-positive cells, but not the prevalence, was increased with increasing plasma tetrachlorodibenzo-*p*-dioxin (TCDD), possibly through alteration of immune parameters and immune response by TCDD (32). As well as depression of the immune system through reduction of B and T lymphocyte numbers, benzene exposure also perturbs their mitogenic responses (33).

Table 1 shows the gender and age characteristics of the population as well as smoking and drinking status. Differences in age, drinking, and recent infection were not significant between the two groups, but there were slightly more smokers among the controls (45%) than the exposed workers (22%), $P = .06$, and smoking has been associated with elevated t(14;18). However, adjusting for age, smoking, alcohol, and other confounding factors by linear and logistic regression resulted in the same negative association of t(14;18) with benzene exposure. Further, results were similar when we excluded subjects with an ambiguous t(14;18) copy number value (Table 1). Overall, our data do not support the utility of circulating lymphocyte t(14;18) levels as a biomarker of early effect for benzene.

Nested PCR was used to confirm the presence of t(14;18) in 10 samples positive by real-time PCR (>10 copies) and to obtain DNA fragments for subsequent sequencing (15). Sequence analysis was performed on five PCR products (with fragment length >300 bp) and each showed a different overall sequence (Table 2), similar to that in follicular lymphoma. In three individuals (4, 5, and 7), BCL-2 was fused to J_H, whereas in the other two (39 and 40) BCL-2 was fused to D_HJ_H. In the translocation from individual 39, N-regions were observed between the BCL-2 breakpoint and the D_H region of the IgH gene, as well as between the D_H and J_H regions of the IgH gene.

The frequency of the t(14;18)–positive lymphocytes with more than 10 copies detected in healthy Chinese individuals (55%) was similar to previous frequencies reported for Caucasian populations (15,34) and higher than that reported for Japanese individuals (16%) (34). The Chinese have a similarly lower level of lymphoma as the Japanese in comparison to Western Caucasian populations. Thus, the apparent correlation between lower t(14;18) levels in Japanese subjects and lower lymphoma rates in that country reported previously requires further investigation in larger sample sizes from Asian populations.

Table 2. BCL-2/IgH breakpoint sequences from five individuals

Sample	Position in Accession AY220759	BCL-MBR breakpoint	N-region	Position in Accession X97051	J_H Breakpoint
4	193535	CCCTCCTGCCC	CCTCTCCGGCTCAAGGCCNGAGA	89762	TACTACTAC
5	193598	AGTGGTGCTTA	ACTGGTTCGACCC	89788	CTGGGGCCAAGGG
7	193599	AGTGGTGCTTAC	TCCCACCGTGT	89768	TACTACTAC
39	193598	AGTGGTGCTTA	TCCCTTCAGCCGGACAAGAACCCCT CTTGACCAA <u>GTATTACTATGATAGTAGTGGTTAT</u> CGTGGAAGTGTGG	89788	CTGGGGCCAAGG
40	193544	CCCTCCTTCCGC	TCCCTACTNGGA <u>ATATTGTAGTAGTACCAGCTGCTAT ACC</u>	89764	CTACTACTACTAC

Diversity (D_H) regions are underlined. MBR = major breakpoint region.

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We recently reported increased t(14;18) translocation in highly exposed workers as measured by FISH (20), which is contradictory to our current findings by real-time PCR. The discrepancy may be explained by the different target cell populations and differential sensitivities of the two assays.

In conclusion, there are limitations to the use of translocations as biomarkers of early effect for hematologic malignancies. Current detection methodologies may lack the necessary sensitivity for their detection at biologically meaningful levels. Also, although translocations are thought to be initiating events in leukemia, their presence alone does not cause leukemia (35). Additional cooperating mutations are required for disease. As illustrated by the t(14;18) findings presented here, the presence of the translocation in unselected cell populations may not be informative due to other effects of long-term exposure on the immune system.

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