

Leukaemia-specific chromosome damage detected by comet with fluorescence *in situ* hybridization (comet-FISH)

Patricia A. Escobar, Martyn T. Smith, Ananth Vasishtha,
Alan E. Hubbard and Luoping Zhang*

Division of Environmental Health Sciences, School of Public Health, 140
Warren Hall, University of California, Berkeley, California 94720, USA

Acute myeloid leukaemia (AML) is associated with exposure to benzene and treatment with chemotherapeutic agents. It is thought to arise from damage to specific regions of DNA, resulting in chromosome rearrangements or loss. For instance, a deletion on the long arm of chromosome 5 [e.g. del(5q31)] is common in AML patients previously treated with alkylating agents, such as melphalan, or exposed to benzene. Translocations of the *MLL* gene at 11q23 are frequently observed in AML arising from treatment with topoisomerase II inhibitors, such as etoposide. Our goal was to determine whether or not breakage at 5q31 and 11q23 is selectively induced by these chemical agents. To address this question, the comet assay combined with fluorescence *in situ* hybridization (comet-FISH) was used to detect DNA breakage in the specific chromosomal regions in an *in vitro* model. TK6 lymphoblastoid cells were exposed to melphalan, etoposide or the benzene metabolite, hydroquinone (HQ), at various concentrations. HQ, melphalan and etoposide induced DNA breaks at both 5q31 and 11q23 chromosome regions in a dose-dependant manner. However, HQ produced significantly more DNA damage at 5q31 than at 11q23. Etoposide produce slightly more DNA damage at 11q23 and melphalan had a somewhat greater effect at 5q31, but not significantly so. Thus, HQ and melphalan act similarly, perhaps explaining some similarities between benzene- and alkylating agent-induced AML. Comet-FISH also appears to be a useful approach for detecting and comparing damage to specific chromosome regions of significance in leukaemogenesis.

Introduction

Leukaemogenesis is a complex, multi-step process that involves transformation of a haematopoietic progenitor cell through the induction of several types of chromosomal aberrations and gene mutations followed by clonal expansion (1,2). The cause of most types of leukaemia is largely unknown, but several factors have been directly implicated, including inherited mutations, ionizing radiation and chemical exposure (3,4). Chemotherapeutic treatment with alkylating agents and topoisomerase II inhibitors can cause therapy-related acute myeloid leukaemia (AML) (5–7). However, depending on the agent used, patients display different cytogenetic characteristics (5,6).

AML induced by alkylating agents, such as melphalan, usually develops 7–10 years after exposure and commonly displays a deletion of all or part of chromosomes 5 and 7.

Previous *in vitro* studies have shown that melphalan preferentially induces breaks and structural aberrations in chromosomes 5, 7, 11 and 17 (8–10). Deletion of 5q31 was also detected in multiple myeloma patients treated with melphalan (11). Topoisomerase II inhibitors, such as etoposide, induce AML with a shorter latency period of 1–5 years after exposure and are usually associated with balanced translocations involving chromosome band 11q23, the location of the *MLL* gene (7,12). Topoisomerase II enzymes catalyse breakage of double-stranded DNA during replication, transcription and chromosomal segregation. Topoisomerase II inhibitors stabilize the complex of topoisomerase II, leaving a broken DNA 5' strand end, slowing ligation, triggering double-strand break repair (illegitimate recombination) and, in some instances, apoptosis (12).

Occupational exposure to benzene also induces leukaemia including AML (13,14). Benzene is metabolized in the liver to its primary metabolite phenol, which is hydroxylated to hydroquinone (HQ) (15). HQ can be further oxidized in the bone marrow to 1,4-benzoquinone (16,17). This pathway is currently thought to play a major role in benzene-induced leukaemia. Leukaemias resulting from benzene exposure show many types of chromosomal aberrations including the loss of all or part of chromosomes 5 and 7, such as del(5q31) (18–20). 1,4-Benzoquinone has also been reported to be a topoisomerase II inhibitor and may affect the *MLL* gene at chromosome band 11q23 (21,22). In the present study, we have attempted to determine if leukaemogenic chemicals induce damage specifically at the 5q31 and 11q23 regions involved in leukaemogenesis, and critically to determine if HQ acts more like melphalan or etoposide.

The comet assay (single-cell gel electrophoresis) is a simple method for measuring DNA single- and double-strand breaks in eukaryotic cells. The more DNA breaks produced, the greater the fluorescent intensity of the comet tail relative to the head. These DNA breaks may be induced by oxidative stress (23–25). Recently, the comet assay has been combined with the specificity of fluorescence *in situ* hybridization (FISH) (26,27) to allow simultaneous detection of overall DNA damage and DNA breaks in specific regions of the genome. The use of a fluorescent-labelled DNA probe homologous to the region of interest in conjunction with comet (comet-FISH) permits the detection of DNA breaks within that region. Comet-FISH was introduced in 1997 (26) to study the spatial distribution of specific chromosome sequences and chromatin fibres in comet cells. Subsequently, it was used to examine initial DNA damage and subsequent repair in the *TP53* gene region of bladder carcinoma cells after gamma irradiation exposure (28,29). It has recently been applied to study damage in a number of specific genes (28–32).

Here, we have used comet-FISH to compare the DNA damaging effects of melphalan, etoposide and HQ specifically at bands 5q31 and 11q23. Our *a priori* hypothesis was that the alkylating agent melphalan would produce more damage at

*To whom correspondence should be addressed. Tel: +510 643 5189; Fax: +510 642 0427; Email: luoping@berkeley.edu

band 5q31 than at 11q23 and, conversely, that the topoisomerase II inhibitor etoposide would cause more breakage at band 11q23 than at 5q31. For the benzene metabolite HQ, it was unclear which location, if any, would be preferred as it has been described as a topoisomerase II inhibitor (21), but is also known to cause deletion of 5q31 (20,33). In order to test these hypotheses, we exposed a commonly used model, the human lymphoblastoid cell line, TK6, to different concentrations of melphalan, etoposide and HQ and then applied comet-FISH with probes specific for chromosome bands 5q31 and 11q23.

Methods

Human cells and culture conditions

Human lymphoblastoid cell line TK6 was obtained from the American Tissue Culture Collection (Rockville, MD). TK6 was cultured in RPMI-1640 medium with L-glutamine supplemented with 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA) and 10% foetal bovine serum (Omega Scientific Inc., Tarzana, CA). Cells were incubated at 37°C, in a 5% CO₂ atmosphere.

Chemical treatment and cytotoxicity

The TK6 cells were exposed to various concentrations of HQ (Aldrich, Milwaukee, WI), melphalan and etoposide (Sigma, St Louis, MO). Dimethyl sulfoxide was used as the vehicle for melphalan and etoposide and was present in cell cultures at a final concentration of 0.25%. HQ was dissolved in RPMI. When TK6 cells were in exponential growth phase, they were treated with these three chemicals for 1 h and then harvested for cell viability test, cell cycle analysis, comet assay and comet-FISH. All treatments were performed in duplicate for each dose and each experiment was repeated three times. Cytotoxicity was measured utilizing the Trypan Blue exclusion assay. The cells were mixed with Trypan blue 0.04% (Gibco, Carlsbad, CA) in a 1:1 ratio and the percentage of dead cells was determined for each chemical treatment at various concentrations. The percentage of dead cells was <10% at non-cytotoxic doses.

Cell cycle analysis

An aliquot of the cell suspension was mixed with cold Hanks balanced saline solution (HBSS) without Ca⁺⁺ and Mg⁺⁺ (Invitrogen) and washed twice. Then, cold 70% ethanol was added to the cells and left at 4°C overnight. The cells were washed with HBSS, and RNase A (1 mg/ml) and propidium iodide (PI) (34) were added. The solution was stored at 4°C until analysis. The cell cycle analysis was performed on a Beckman-Coulter EPICS XL-MCL. Using the intensity of PI fluorescence as a measure of the total DNA content of individual cells, cell cycle populations were quantified from a standard count of 10 000 cells. Cell cycle analysis was performed at 1 and 24 h after exposure.

Comet assay

The alkaline comet assay was performed according to Singh *et al.* (23) with some modifications. Briefly, super-frosted microscope slides were pre-coated by dipping each slide into 1% normal melting agarose and air-dried overnight. Seventy-five microlitres of low-melting agarose (Fisher Scientific, Pittsburgh, PA) mixed with 1 × 10⁵ cells was placed on the pre-coated slide. Several slides were prepared at each dose to be used for both comet and comet-FISH assays. A glass coverslip was placed on top and slides were kept at 4°C for 10

min to allow the gel to solidify. Then a second layer of low-melting agarose was added and the slides were kept at 4°C for another 10 min to allow the gel to solidify. Slides were placed in cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma base and 1% Triton X-100, pH 10) and kept at 4°C until electrophoresis was performed. The Trizma base was from Sigma and other reagents in the lysing solution were purchased from EM Scientific (Darmstadt, Germany).

Unwinding was performed in an alkaline buffer (300 mM NaOH and 1 mM EDTA, pH 13) for 25 min and electrophoresis was run in the same alkaline buffer at 8°C at 0.73 V/cm, for 35 min. After electrophoresis, the slides were neutralized with 0.4 M Tris, pH 7.5. The gels were dehydrated with 100% ethanol, air-dried overnight and stored in a dry place before being processed for comet or comet-FISH analysis. All slides were blind-coded before they were stained with SYBRTM Green (Molecular Probes, Eugene, OR). The cell scoring was performed under a ×25 objective on a fluorescence microscope using an FITC filter. Twenty-five randomly chosen cells per slide and two slides per dose were analysed with the CometScan (MetaSystems, Germany) imaging software.

Comet-FISH

Comet slides from solvent controls and two concentrations of each of the three chemicals tested were subjected to FISH analysis. The slides were immersed for 20 min in absolute ethanol. All slides were hybridized with two specific chromosome probes, 5q31 and 11q23 (Vysis, Downers Grove, IL), each in separate spots outlined with a diamond pencil. Dual-colour human chromosome band probes 5q31 (200 kb) and 11q23 (350 kb) were used. To each spot, 10 µl of probe mixture was added (2 µl chromosome probe, 1 µl of salmon sperm DNA and 7 µl hybrid solution from Vysis). Coverslips (22 × 22 mm) were placed over each spot and sealed with rubber cement. An automatic denaturation and hybridization procedure was performed using the HyBriteTM denaturation/hybridization system (Vysis). Cells and probes were denatured at 68°C for 10 min and hybridized at 37°C for 72 h. After hybridization, the rubber cement was carefully removed and the slides were placed in water for a few seconds to allow the coverslips to slip off and prevent gel rupture. The slides were washed in 50% formamide/2 × SSC at 45°C for 3 min and 0.1 × SSC at room temperature for 2 min, three times. The cells were counter-stained with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI). The hybridization signals were observed with a fluorescence microscope under triple band-pass filter for DAPI/FITC/Texas Red (excitation at 405, 490 and 570 nm, respectively; emission at 460, 525 and 635 nm, respectively). In each spot, only the spectrum orange signal was taken into account for the comet-FISH analysis because they represented the 5q31 and 11q23 chromosome bands.

Comet-FISH scoring

We designed a 4 × 4 table to classify and evaluate the comet-FISH results (Figure 4). The figure describes four categories that quantify site-specific DNA breakage by the number of DNA probe signals (I-IV) and four categories that describe the localization of the probe signal in the comet (head, head and tail or only tail) in relation to the presence of a comet tail which correlates with overall DNA damage (A-D). A total of 150-300 cells were randomly scored for each chemical (50-100 cells per dose). Each cell was categorized according to Figure 4 and the cumulative counts were used for the data analysis. The comet-FISH experiments were repeated three times.

Statistical analysis

The comet parameter used for data analysis was the percentage of DNA in the tail (% tail intensity) and linear regression analysis was performed to determine dose–response relationship shown as a P_{trend} .

The data generated from the comet–FISH assay, using Figure 4, were categorical and were analysed using an order logistic regression, which returns odds ratios (ORs) for an increase in the outcome by one category (35). The ordered logistic regressions analysis assessed the distribution of the chromosome probe signals among the different cell damage categories. Because there were multiple measurements per slide, our inference was based on robust standard errors (36), which adjusts for the residual correlation of measurements made on the same slide. The analyses were performed to determine which chromosomes were more affected by the chemicals of interest, and so we included interaction terms (dose \times chromosome). For all analyses, we treated dose categorically by including the appropriate dummy variables in the model. For instance (using the data in Table III as an example), consider the following ordered logistic regression model [see Models for Ordinal Scales in McCullagh and Nelder (35) for equivalent notation]:

$$\log\left(\frac{\gamma_j(\text{Dose}, \text{Chrom})}{1 - \gamma_j(\text{Dose}, \text{Chrom})}\right) = \theta_j - [\beta_1 I(\text{Dose}=25) + \beta_2 I(\text{Dose}=50) + \beta_3 I(\text{Chrom}=11) + \beta_4 I(\text{Dose}=25)I(\text{Chrom}=11) + \beta_5 I(\text{Dose}=50)I(\text{Chrom}=11)],$$

where $\gamma_j(\text{Dose}, \text{Chrom}) = P(Y \leq j | \text{Dose}, \text{Chrom})$, Y is the ordered outcome (probe signals: $0 \rightarrow 1-2$, $2 \rightarrow 3-4$, $3 \rightarrow 5-7$, $4 \rightarrow \geq 8$) and $I(\text{statement}) = 1$ if ‘statement’ is true, 0 otherwise. In this model, the test of the null $H_0: \beta_4 = 0$ tests whether the effect (OR for moving up one category) of HQ 25 versus 0 is the same for both chromosomes 11 and 5; likewise, the test of the null $H_0: \beta_5 = 0$ tests the equality of the OR’s for 50 versus 0. We used the Wald test of these coefficients (based on the robust standard errors) to derive the P -values reported below in the Results.

Results

Overall DNA damage measured using the comet assay

Exposure of human TK6 cells to three chemical leukaemogens, HQ, melphalan and etoposide, produced a significant increase in overall DNA damage, represented as an increase of the % tail intensity. HQ (up to 75 μM) caused increasing DNA damage ($P_{\text{trend}} = 0.04$) without significant cytotoxicity (Figure 1A). Melphalan ($P_{\text{trend}} = 0.006$) and etoposide ($P_{\text{trend}} = 0.005$) also caused DNA breakage with increasingly more DNA being present in the comet tails, without cytotoxicity at doses up to 25 μM (Figure 1B and C, respectively). Of the three compounds, etoposide was the most effective at producing DNA damage with % tail intensity content approaching 70% at doses above 5 μM .

Cell cycle analysis

Cell cycle analysis by flow cytometry revealed that HQ produced S-phase arrest at concentrations ≥ 25 μM (Figure 2A). Low doses of melphalan produced G₂-phase arrest, whereas higher doses resulted in S-phase arrest (Figure 2B). At

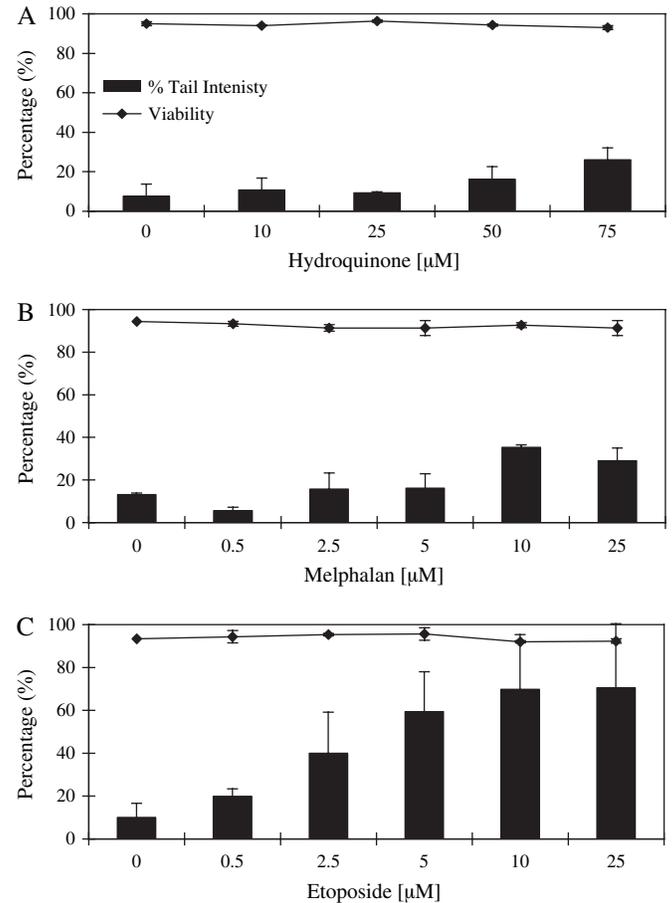


Fig. 1. Cell viability and DNA damage in TK6 cells exposed to (A) HQ, (B) melphalan, and (C) etoposide for 1 h. DNA damage is represented as the percentage of DNA intensity present in the comet tail (% tail intensity). There was a positive dose–response relationship by linear regression analysis for all three chemicals tested: P_{trend} for (A) 0.04, (B) 0.006 and (C) 0.005. Each bar represents the mean % tail intensity of three independent experiments. Error bars indicate standard error of the mean. Cell viability is represented as each diamond point by the percentage of viable cells detected by Trypan blue staining.

the highest (25 μM) dose, however, it produced G₁/S phase arrest increasing the percentage of cells in G₁ phase drastically (Figure 2B). Etoposide caused arrest at G₂/M phase and increasing numbers of cells in G₂ phase with increasing doses (Figure 2C).

Site-specific DNA breaks detected by comet–FISH

FISH probes were targeted to the p and q arms of chromosome 5 at the regions 5p15.5 and 5q31 (Figure 3A). The appearance of probe-specific fluorescent signals in the tail, after HQ exposure at 50 μM (Figure 3B), showed breakage at these locations on the chromosome. Using the scoring criteria described in Figure 4, we quantified the amount of site-specific probe breakage in TK6 cells induced by the three compounds. Figure 4 shows the distribution of the probe fluorescence between the head and tail of the comet, comparing categories A and B with C and D. If all or some of the probe fluorescence is located in the comet tail (categories C and D), this shows significant breakage of the DNA at the probe location. Additional probe signals appearing in the head of the comet could result from aneuploidy, gene amplification and duplication rather than breakage.

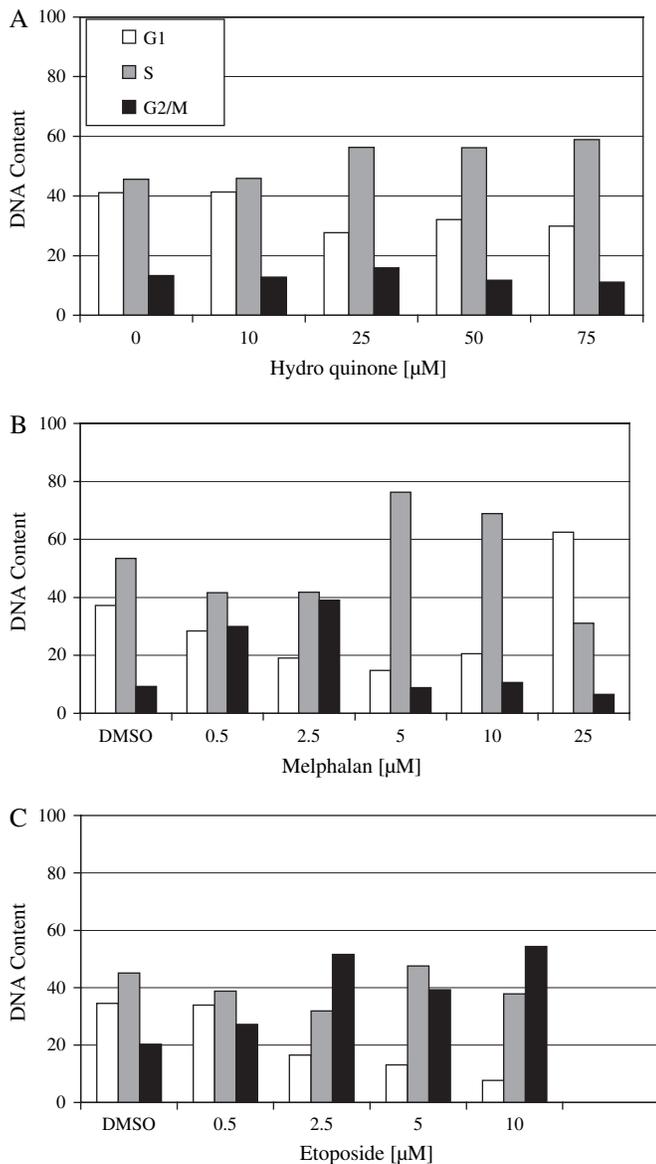


Fig. 2. Cell cycle analysis of TK6 cells exposed to different concentrations of (A) HQ, (B) melphalan and (C) etoposide. Cell cycle profiles were measured by PI staining to determine the percentage of cells in G₁, S and G₂/M phases. Each point represents the mean of at least two independent experiments.

HQ at 25 and 50 μM caused a highly significant shift in location of the probe fluorescence from the head to the tail for FISH probes specific to both bands 5q31 and 11q23 (Table I). To test our *a priori* hypothesis, we directly compared the HQ-induced specific loci breakage on both bands and found that HQ treatment caused significantly more breakage at 5q31 (OR: 5.34) in the tail than at 11q23 (OR: 2.82) ($P = 0.02$, Table II). Melphalan, at doses that did not produce cytotoxicity or cell cycle changes, induced only modest breakage of probes to bands 5q31 and 11q23 and movement of the probes into the comet tail (Table I). This effect on bands, 5q31 (OR: 1.88) and 11q23 (OR: 1.32), was almost the same, but the OR at 5q31 was slightly more pronounced (Table II). Etoposide produced a large shift of probe fluorescence from the comet head to the tail especially at the higher dose of 2.5 μM . The effect of etoposide was slightly greater on band 11q23 (OR: 9.09) than

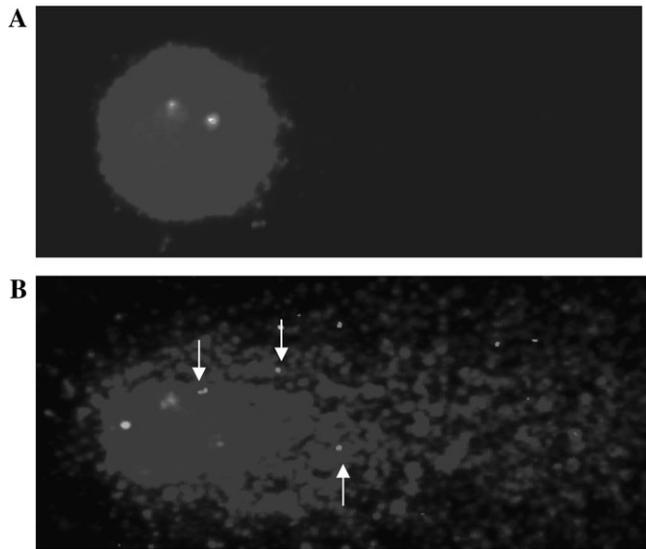


Fig. 3. Comet-FISH staining in human TK6 cells. Human TK6 cells were hybridized with a dual colour probe for chromosome bands 5q31 (42) and 5p15.5 (green). (A) Non-treated control cells. (B) Cells exposed to 50 μM HQ. The arrows show the probe signals located in the comet head and tail.

on 5q31 (OR: 6.79), but this difference was not statistically significant (Table II).

Another method of scoring the amount of site-specific damage in each band is to count the number of probe signals detected by the fluorescent probes in each cell. We therefore counted fluorescent probe spots and categorized them into four categories, I-IV: I (1–2 spots), II (3–4 spots), III (5–7 spots) and IV (>8 spots) (Figure 4). Categorization in this fashion showed that HQ and melphalan caused site-specific damage and a shift from categories I and II to categories III and IV at both 25 and 50 μM (Table III). These two compounds induced a slightly higher shift at 5q31 than at 11q23. However, etoposide tended to induce more damage at 11q23 than 5q31, but these differential effects were not statistically significant (Table IV). Thus, in contrast to spot counting, the categorization of probe fluorescence into head and tail displayed a better assessment of the effects of the three leukaemogens to specific chromosomes.

The data were further classified into two more categories (Figure 4); number of probe signals in comets with no tail (A) and comets with tails (B, C and D), and into what we considered low (AI, AII and BI), medium (AIII, BII, BIII and CII) and high (CIII, CIV, DIII and DIV) effects. The results from these classifications did not provide any additional information over that presented in Tables I and III.

Discussion

Measuring classical chromosomal aberrations in metaphase cells, either by Giemsa staining or FISH, is the most frequently used method to detect chromosomal damage. This method is time consuming and requires skilled personnel. We have explored the potential of comet-FISH as a simpler alternative method to assess damage to specific chromosomal regions.

Comet-FISH was introduced in 1997 and has been recently applied to study damage to the telomeres (37,38) and to specific genes such as *p53*, dihydrofolate reductase (*DHFR*) and the O⁶-methylguanine DNA methyltransferase (*MGMT*)

Table I. Distribution of 5q31 and 11q23 probe signals in the comet head, head and tail or tail, in relation to chemical dose

Treatment	Dose (μ M)	Localization (%) ^a					
		5q31			11q23		
		Head	Head/tail	Tail	Head	Head/tail	Tail
HQ	0	96.67 ^b	3.33	0	86.09	13.25	0.66
	25	30.13	55.13	14.74	35.71	53.90	10.39
	50	20.39	64.47	15.14	39.08	48.34	12.58
Melphalan	0	73	27	0	77	22	1
	0.5	46.67	36	17.33	47.41	47.4	5.19
	2.5	45.75	33.99	20.26	66	34	0
Etoposide	0	80.92	16.45	2.63	78.15	21.19	0.66
	0.5	43.48	51.55	4.97	42.11	53.95	3.94
	2.5	6	69.33	24.67	1.97	58.55	39.48

^aLocalizations are classified from Figure 4 as head = A + B, head/tail = C and tail = D.

^bThe data show the cumulative counts of three independent experiments; in each experiment, approximately 50–100 cells were analysed. The percentage is calculated from 150 to 300 cells representing 100% total.

Table II. ORs and statistical significance after ordered logistic regression analysis comparing the distributions of 5q31 and 11q23 probe signals in the comet head, head and tail or tail

Chemical treatment	OR ^a		P-value ^b
	5q31	11q23	
HQ	5.34	2.82	0.02
Melphalan	1.88	1.32	0.19
Etoposide	6.79	9.09	0.41

^aOR for trends, i.e. the OR for increasing one category of damage from jumping one dose category of the chemical. For example, the OR of 5.34 represents the ratio of odds of comparing one category of damage to the next one higher for changing dose of HQ from 0 to 25 (or 25 to 50) among chromosome 5.

^bComparison between chromosome bands 5q31 and 11q23.

(28–32,34,39). To our knowledge, there are only two studies that used chromosome probes to study specific chromosomal damage (27,40) and these studies used whole-chromosome painting. In the present study, we quantified the amount of DNA damage induced by HQ, melphalan and etoposide in chromosome regions 5q31 and 11q23 using the comet–FISH technology. These chemicals and chromosome locations were chosen because of their relationship to chemically induced leukaemia (5,7).

Our *a priori* hypothesis was that the alkylating agent melphalan would produce more damage at band 5q31 than at 11q23 and, conversely, that the topoisomerase II inhibitor etoposide would cause more breakage at band 11q23 than at 5q31. For the benzene metabolite HQ, it was unclear which location, if any, would be preferred as it has been described as a topoisomerase II inhibitor (21), but is also known to cause deletion of 5q31 (20,33). Surprisingly, HQ exposure produced significantly more DNA breakage at 5q31 than at 11q23. Melphalan induced a modest overall effect, but tended to produce more breakage at 5q31 than 11q23 (Table I). Melphalan is a bifunctional alkylating agent that has the capability to induce DNA cross-linking. Cross-linking causes a retardation in DNA migration during the electrophoresis, which could account for the apparently low overall DNA breakage we observed with melphalan in the comet assay (Figure 1). Etoposide induced considerable DNA damage in a dose-dependant fashion, showing more damaging effects on chromosome 11q23 than at 5q31 at the higher dose. Etoposide is a topoisomerase II inhibitor that induces a wide variety of DNA damage, but our findings are in agreement with those of Ng *et al.* (41), which showed an apparent preference for the *MLL* gene at chromosome 11q23. In the studies presented here, etoposide induced more DNA breaks than HQ, and HQ induced more than melphalan (Table III). Melphalan and etoposide showed preferential effects that were in line with our *a priori* hypotheses, but to our surprise there was evidence of

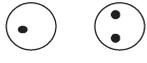
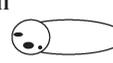
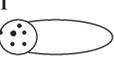
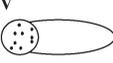
PROBE LOCATION & TAIL PRESENCE	NUMBER OF PROBE SIGNALS			
	1 – 2 Spots [I]	3 – 4 Spots [II]	5 - 7 Spots [III]	\geq 8 Spots [IV]
Probe in Head No Tail [A]	A-I 	A-II 	A-III 	A-IV 
Probe in Head Tail Produced [B]	B-I 	B-II 	B-III 	B-IV 
Probe in Head/Tail Tail Produced [C]	C-I 	C-II 	C-III 	C-IV 
Probe in Tail Tail Produced [D]	D-I 	D-II 	D-III 	D-IV 

Fig. 4. Comet–FISH scoring criteria.

Table III. Distribution of 5q31 and 11q23 probe signals in comets grouped by number of probe signals¹

Chemical treatment	Dose (μM)	Percentage of comets with given number of probe signals (%) ^a							
		5q31				11q23			
		1–2	3–4	5–7	≥ 8	1–2	3–4	5–7	≥ 8
HQ	0	35.33 ^b	54	9.33	1.34	44.37	45.03	9.97	0.66
	25	5.77	39.74	41.03	13.46	12.99	33.12	31.82	22.08
	50	12.50	30.92	42.11	14.47	17.88	38.41	34.44	9.27
Melphalan	0	37	43	19	1	27	46	23	4
	0.5	18	30	36.67	15.33	20.78	38.31	35.06	5.85
	2.5	29.41	30.07	17.65	22.87	32.67	40	22	5.33
Etoposide	0	32.89	51.97	13.16	1.98	45.7	35.1	14.57	4.63
	0.5	22.98	37.27	31.68	8.07	17.76	31.58	42.77	7.89
	2.5	6.67	20	38.67	34.66	2.63	23.68	41.45	32.24

^aDNA probe signals are classified from Figure 4 as I = 1–2 spots, II = 3–4 spots, III = 5–7 spots and IV ≥ 8 spots.

^bThe data show the cumulative counts of three independent experiments; in each experiment, approximately 50–100 cells were analysed. The percentage is calculated from 150 to 300 cells representing 100% total.

Table IV. ORs and statistical significance after ordered logistic regression analysis comparing the distributions of 5q31 and 11q23 probe signals in comets grouped by number of probe signals

Chemical treatment	OR ^a		<i>P</i> -value ^b
	5q31	11q23	
HQ	2.40	2.11	0.71
Melphalan	1.48	0.91	0.39
Etoposide	3.38	3.84	0.52

^aOR for trends, i.e. the OR for increasing one category of damage from jumping one dose category of the chemical.

^bComparison between chromosome bands 5q31 and 11q23.

a greater effect of HQ on 5q31 than at 11q23. This tends to contradict the hypothesis that HQ acts as an etoposide-like topo II inhibitor in one proposed mechanism for benzene-induced leukaemia (21,42). It does, however, agree with the notion that benzene-induced leukaemia mediated through the quinone pathway has similar characteristics to therapy-induced leukaemias produced by alkylating agents in which loss of band 5q31 is a common clonal event (43).

Each of the chemicals tested produced a different pattern of DNA damage at bands 5q31 and 11q23. Typically, comet-FISH data are classified as either head or tail effects. We devised a system to classify the damage using a 4 \times 4 table (Figure 4). This helped us determine the presence of the chromosome probe in the head and/or tail of the comet and to quantify the level of site-specific breakage by measuring the number of specific chromosome signals present. As described in Methods, DNA damage was classified into four categories A, B, C and D that described the localization of probe signal in the comet (head, head and tail or only tail). Categorizing in this manner revealed differences between the chemicals that may be functionally important. We also tried scoring the amount of breakage in each chromosome band by counting the number of signals detected by the fluorescent probes in each cell, dividing them into four categories, I–IV: I (1–2 spots), II (3–4 spots), III (5–7 spots) and IV (>8 spots) (Figure 4). Categorizing by this second procedure revealed fewer differences in the comparison between the three chemicals presented here, but may be useful in future studies.

The comet-FISH approach described here can measure whole-genome DNA damage plus specific chromosomal region damage in a manner that is faster and more streamlined than the classical chromosomal aberration assay. It may also be a useful method for biomarker research in human populations where one wishes to study effects on specific chromosome regions because it does not require metaphase cells for analysis.

Acknowledgements

This study was supported by grants from P42 ES04705 and P30 ES01896 from the National Institute of Environmental Health Sciences (to M.T.S.). We are grateful to Weihong Guo for her laboratory assistance and generous help with the manuscript.

Conflict of interest statement

M.T.S. has received consulting and expert testimony fees from law firms representing both plaintiffs and defendants in cases involving exposure to benzene.

References

- Krivtsov, A. V., Twomey, D., Feng, Z. *et al.* (2006) Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. *Nature*, **442**, 818–822.
- Taki, T. and Taniwaki, M. (2006) Chromosomal translocations in cancer and their relevance for therapy. *Curr. Opin. Oncol.*, **18**, 62–68.
- Smith, M. T., Skibola, C. F., Allan, J. M. and Morgan, G. J. (2004) Causal models of leukaemia and lymphoma. *IARC Sci. Publ.*, **157**, 373–392.
- Deschler, B. and Lubbert, M. (2006) Acute myeloid leukemia: epidemiology and etiology. *Cancer*, **107** (9), 2099–107.
- Larson, R. A. and Le Beau, M. M. (2005) Therapy-related myeloid leukaemia: a model for leukemogenesis in humans. *Chem. Biol. Interact.*, **153–154**, 187–195.
- Pedersen-Bjergaard, J. (2005) Insights into leukemogenesis from therapy-related leukemia. *N. Engl. J. Med.*, **352**, 1591–1594.
- Felix, C. A. (2001) Leukemias related to treatment with DNA topoisomerase II inhibitors. *Med. Pediatr. Oncol.*, **36**, 525–535.
- Mamuris, Z., Dumont, J., Dutrillaux, B. and Aurias, A. (1990) Specific chromosomal mutagenesis observed in stimulated lymphocytes from patients with S-ANLL. *Int. J. Cancer*, **46**, 563–568.
- Mamuris, Z., Gerbault-Seureau, M., Prieur, M., Pouillart, P., Dutrillaux, B. and Aurias, A. (1989) Chromosomal aberrations in lymphocytes of patients treated with melphalan. *Int. J. Cancer*, **43**, 80–86.
- Mamuris, Z., Prieur, M., Dutrillaux, B. and Aurias, A. (1989) The chemotherapeutic drug melphalan induces breakage of chromosomes regions rearranged in secondary leukemia. *Cancer Genet. Cytogenet.*, **37**, 65–77.
- Amiel, A., Yukla, M., Yogeve, S., Manor, Y., Fejgin, M. D. and Lishner, M. (2004) Deletion of 5q31 and 7q31 in patients with stable melphalan treated multiple myeloma. *Cancer Genet. Cytogenet.*, **152**, 84–87.

12. Felix, C. A., Kolaris, C. P. and Osheroff, N. (2006) Topoisomerase II and the etiology of chromosomal translocations. *DNA Repair (Amst.)*, **5**, 1093–1108.
13. Infante, P. F., Rinsky, R. A., Wagoner, J. K. and Young, R. J. (1977) Leukaemia in benzene workers. *Lancet*, **2**, 76–78.
14. Rinsky, R. A., Hornung, R. W., Silver, S. R. and Tseng, C. Y. (2002) Benzene exposure and hematopoietic mortality: a long-term epidemiologic risk assessment. *Am. J. Ind. Med.*, **42**, 474–480.
15. Snyder, R. (2002) Benzene and leukemia. *Crit. Rev. Toxicol.*, **32**, 155–210.
16. Smith, M. T., Yager, J. W., Steinmetz, K. L. and Eastmond, D. A. (1989) Peroxidase-dependent metabolism of benzene's phenolic metabolites and its potential role in benzene toxicity and carcinogenicity. *Environ. Health Perspect.*, **82**, 23–29.
17. Smith, M. T. (1996) The mechanism of benzene-induced leukemia: a hypothesis and speculations on the causes of leukemia. *Environ. Health Perspect.*, **104** (Suppl. 6), 1219–1225.
18. Zhang, L., Eastmond, D. A. and Smith, M. T. (2002) The nature of chromosomal aberrations detected in humans exposed to benzene. *Crit. Rev. Toxicol.*, **32**, 1–42.
19. Zhang, L., Rothman, N., Wang, Y. *et al.* (1998) Increased aneusomy and long arm deletion of chromosomes 5 and 7 in the lymphocytes of Chinese workers exposed to benzene. *Carcinogenesis*, **19**, 1955–1961.
20. Zhang, L., Wang, Y., Shang, N. and Smith, M. T. (1998) Benzene metabolites induce the loss and long arm deletion of chromosomes 5 and 7 in human lymphocytes. *Leuk. Res.*, **22**, 105–113.
21. Eastmond, D. A., Mondrala, S. T. and Hasegawa, L. (2005) Topoisomerase II inhibition by myeloperoxidase-activated hydroquinone: a potential mechanism underlying the genotoxic and carcinogenic effects of benzene. *Chem. Biol. Interact.*, **153–154**, 207–216.
22. Frantz, C. E., Chen, H. and Eastmond, D. A. (1996) Inhibition of human topoisomerase II in vitro by bioactive benzene metabolites. *Environ. Health Perspect.*, **104** (Suppl. 6), 1319–1323.
23. Singh, N. P., McCoy, M. T., Tice, R. R. and Schneider, E. L. (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.*, **175**, 184–191.
24. Tice, R. R., Agurell, E., Anderson, D. *et al.* (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ. Mol. Mutagen.*, **35**, 206–221.
25. Collins, A. R. (2004) The comet assay for DNA damage and repair: principles, applications, and limitations. *Mol. Biotechnol.*, **26**, 249–261.
26. Santos, S. J., Singh, N. P. and Natarajan, A. T. (1997) Fluorescence in situ hybridization with comets. *Exp. Cell Res.*, **232**, 407–411.
27. Rapp, A., Bock, C., Dittmar, H. and Greulich, K. O. (2000) UV-A breakage sensitivity of human chromosomes as measured by COMET-FISH depends on gene density and not on the chromosome size. *J. Photochem. Photobiol. B*, **56**, 109–117.
28. McKelvey-Martin, V. J., Ho, E. T., McKeown, S. R., Johnston, S. R., McCarthy, P. J., Rajab, N. F. and Downes, C. S. (1998) Emerging applications of the single cell gel electrophoresis (Comet) assay. I. Management of invasive transitional cell human bladder carcinoma. II. Fluorescent in situ hybridization comets for the identification of damaged and repaired DNA sequences in individual cells. *Mutagenesis*, **13**, 1–8.
29. McKenna, D. J., Rajab, N. F., McKeown, S. R., McKerr, G. and McKelvey-Martin, V. J. (2003) Use of the comet-FISH assay to demonstrate repair of the TP53 gene region in two human bladder carcinoma cell lines. *Radiat. Res.*, **159**, 49–56.
30. Schaeferhenrich, A., Sendt, W., Scheele, J., Kuechler, A., Liehr, T., Claussen, U., Rapp, A., Greulich, K. O. and Pool-Zobel, B. L. (2003) Putative colon cancer risk factors damage global DNA and TP53 in primary human colon cells isolated from surgical samples. *Food Chem. Toxicol.*, **41**, 655–664.
31. Horvathova, E., Dusinska, M., Shaposhnikov, S. and Collins, A. R. (2004) DNA damage and repair measured in different genomic regions using the comet assay with fluorescent in situ hybridization. *Mutagenesis*, **19**, 269–276.
32. Kumaravel, T. S. and Bristow, R. G. (2005) Detection of genetic instability at HER-2/neu and p53 loci in breast cancer cells using Comet-FISH. *Breast Cancer Res. Treat.*, **91**, 89–93.
33. Stillman, W. S., Varella-Garcia, M. and Irons, R. D. (2000) The benzene metabolite, hydroquinone, selectively induces 5q31- and -7 in human CD34+CD19- bone marrow cells. *Exp. Hematol.*, **28**, 169–176.
34. Amendola, R., Basso, E., Pacifici, P. G., Piras, E., Giovanetti, A., Volpato, C. and Romeo, G. (2006) Ret, Abl1 (cAbl) and Trp53 gene fragmentations in comet-FISH assay act as in vivo biomarkers of radiation exposure in C57BL/6 and CBA/J mice. *Radiat. Res.*, **165**, 553–561.
35. McCullagh, P. and Nelder, J. (1989) *Generalized Linear Models*. CRC Press, London.
36. Zeger, S. L. and Liang, K. Y. (1986) Longitudinal data analysis for discrete and continuous outcomes. *Biometrics*, **42**, 121–130.
37. Hovhannisyann, G., Rapp, A., Arutyunyan, R., Greulich, K. O. and Gebhart, E. (2005) Comet-assay in combination with PNA-FISH detects mutagen-induced DNA damage and specific repeat sequences in the damaged DNA of transformed cells. *Int. J. Mol. Med.*, **15**, 437–442.
38. Arutyunyan, R., Rapp, A., Greulich, K. O., Hovhannisyann, G., Haroutiunian, S. and Gebhart, E. (2005) Fragility of telomeres after bleomycin and cisplatin combined treatment measured in human leukocytes with the Comet-FISH technique. *Exp. Oncol.*, **27**, 38–42.
39. Knobel, Y., Gleib, M., Weise, A., Osswald, K., Schaeferhenrich, A., Richter, K. K., Claussen, U. and Pool-Zobel, B. L. (2006) Uranyl nitratolactate, a stabilized salt of uranium, is genotoxic in nontransformed human colon cells and in the human colon adenoma cell line LT97. *Toxicol. Sci.*, **93**, 286–297.
40. Harreus, U. A., Kleinsasser, N. H., Zieger, S., Wallner, B., Reiter, M., Schuller, P. and Berghaus, A. (2004) Sensitivity to DNA-damage induction and chromosomal alterations in mucosa cells from patients with and without cancer of the oropharynx detected by a combination of comet assay and fluorescence in situ hybridization. *Mutat. Res.*, **563**, 131–138.
41. Ng, A., Taylor, G. M. and Eden, O. B. (2006) Genotoxicity of etoposide: greater susceptibility of MLL than other target genes. *Cancer Genet. Cytogenet.*, **164**, 164–167.
42. Whysner, J., Reddy, M. V., Ross, P. M., Mohan, M. and Lax, E. A. (2004) Genotoxicity of benzene and its metabolites. *Mutat. Res.*, **566**, 99–130.
43. Irons, R. D. and Stillman, W. S. (1996) The process of leukemogenesis. *Environ. Health Perspect.*, **104** (Suppl. 6), 1239–1246.

Received on November 3, 2006; revised on April 10, 2007;
accepted on April 13, 2007