Clone	DNA sequence of TSCE105 mutants around junction site	Deletion Size (bp)
	1st site 2nd site	
perfect	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCTGTTATCCCTA GGTCTGTGCAGACTGC	
2412	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCTGTTATCCCTA GGTCTGTGCAAACTGC	-356 (0)
2429	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCTGTTATCCCTA GGTCTGTGCAAACTGC	-356 (0)
2445	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCTGTTATCCCTA GGTCTGTGCAAACTGC	-356 (0)
2465	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCTGTTATCCCTA GGTCTGTGCAAACTGC	-356 (0)
2703	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCTGTTAT-CCTA GGTCTGTGCAAACTGC	-357 (-1)
2650	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCTGT----CCCTA GGTCTGTGCAAACTGC	-359 (-3)
2393	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCTG-----CCCTA GGTCTGTGCAAACTGC	-360 (-4)
2453	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTA---------TCCCTA GGTCTGTGCAAACTGC	-364 (-8)
2434	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC AT---------ATCCCTA GGTCTGTGCAAACTGC	-365 (-9)
2345	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCT---------A GGTCTCTGCAAACGGC	-365 (-9)
2689	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCT---------A GGTCTCTGCAAACGGC	-365 (-9)
2714	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCT---------A GGTCTCTGCAAACGGC	-365 (-9)
2764	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCT---------A GGTCTCTGCAAACGGC	-365 (-9)
2444	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCT---------A GGTCTCTGCAAACGGC	-365 (-9)
2446	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCT---------A GGTCTGTGCAAACTGC	-365 (-9)
2424	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCT---------A GGTCTGTGCAAACTGC	-365 (-9)
2304	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC AT----------CCCTA GGTCTGTGCAAACTGC	-367 (-11)
2442	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCTGT-------- --------GCAAACTGC	-372 (-16)
2443	TCCGGGCCAAATGTCCGGAGTTGTCAGATCC ----------TA GGTCTGTGCAAACTGC	-372 (-16)
2402	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCTGTTAT------ ----------AACTGC	-372 (-16)
2425	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCTGTTATC----- ----------TGC	-374 (-18)
2435	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ATTACCCTGTT-------- ----------GC	-378 (-22)
2713	TCCGGGCCAAATGGCCGGAGTTGTC-------- ---------- ----TGTGCAAACTGC	-384 (-28)
2735	TCCGGGCCAAATGGCCGGAGTTGTCAGATCC ---------- ----------CTGC	-378 (-22)
2405	TCCGGGCCAAATGGCCG---------- ---------- —TCTGTGCAAACGGC	-392 (-36)
2437	TCCGGGC---------- ---------- ----------AAACTGC	-409 (-53)

Fig. 5 – DNA sequences at the NHEJ repair junction around the I-SceI junction site in TSCE5 cells. "Perfect" is the DNA sequence when two I-SceI sites join perfectly and create a new I-SceI site (highlighted in orange). Sequences in black are upstream of the first I-SceI site and those in blue are downstream of the second I-SceI site. A total of 26 TSCE105 mutants with deletions combining two I-SceI sites are shown. Underlining indicates a new I-SceI recognition sequence produced by error-free NHEJ. Red indicates microhomologous sequences at junctions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

and do not require terminal processing. Perfect joining by NHEJ probably skips the second step. Naturally occurring DSBs produced by oxidative stress, ionizing radiation, and DNA-damaging agents, however, do not have directly ligatable DNA ends and need some form of nucleolytic processing [7,9]. Their repair by NHEJ results in deletions, even if it works properly. In the present study, the size of the deletions caused by NHEJ, however, were relatively small. No recovered TSCE5 or TSCE105 mutants exhibited large deletions or translocations similar to those frequently observed

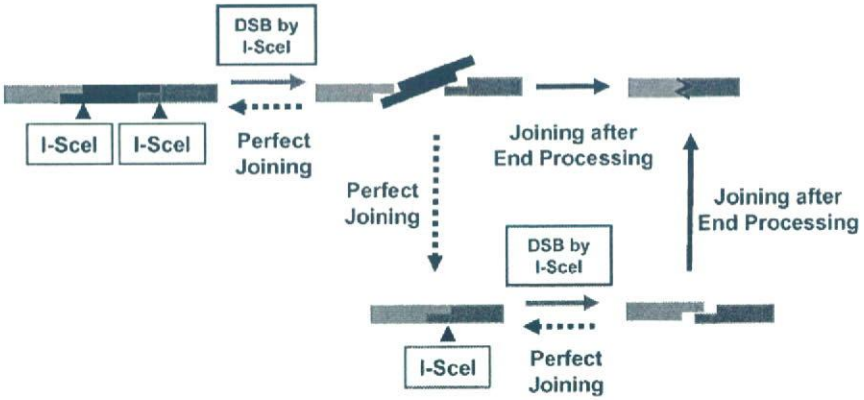

Fig. 6 – A model for NHEJ generating deletions in TSCE105 cells. When a DSB is repaired by perfect joining, an I-SceI site newly generates and is cleaved again. The rare DSB that is joined after exonuclease processing converts to a deletional mutation and accumulates in the cell population.

at the chromosome level in cancer cells [4]. This suggests that NHEJ helps maintain genomic integrity in mammalian cells by repairing DSBs as well as by preventing many deleterious alterations.

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REFERENCES

[1] K.K. Khanna, S.P. Jackson, DNA double-strand breaks: signaling, repair and the cancer connection, Nat. Genet. 27 (2001) 247–254.
[2] K.D. Mills, D.O. Ferguson, F.W. Alt, The role of DNA breaks in genomic instability and tumorigenesis, Immunol. Rev. 194 (2003) 77–95.
[3] D.C. van Gent, J.H. Hoeijmakers, R. Kanaar, Chromosomal stability and the DNA double-stranded break connection, Nat. Rev. Genet. 2 (2001) 196–206.
[4] J.H. Hoeijmakers, Genome maintenance mechanisms for preventing cancer, Nature 411 (2001) 366–374.
[5] E. Van Dyck, A.Z. Stasiak, A. Stasiak, S.C. West, Binding of double-strand breaks in DNA by human Rad52 protein, Nature 398 (1999) 728–731.
[6] S.P. Jackson, Sensing and repairing DNA double-strand breaks, Carcinogenesis 23 (2002) 687–696.
[7] K. Valerie, L.F. Povirk, Regulation and mechanisms of mammalian double-strand break repair, Oncogene 22 (2003) 5792–5812.
[8] P.A. Jeggo, DNA breakage and repair, Adv. Genet. 38 (1998) 185–218.
[9] E. Pastwa, J. Blasiak, Non-homologous DNA end joining, Acta Biochim. Pol. 50 (2003) 891–908.
[10] M. Honma, M. Izumi, M. Sakuraba, S. Tadokoro, H. Sakamoto, W. Wang, F. Yatagai, M. Hayashi, Deletion, rearrangement, and gene conversion; genetic consequences of chromosomal double-strand breaks in human cells, Environ. Mol. Mutagen. 42 (2003) 288–298.
[11] K. Maasho, A. Marusina, N.M. Reynolds, J.E. Coligan, F. Borrego, Efficient gene transfer into the human natural killer cell line, NKL, using the Amaxa nucleofection system, J. Immunol. Methods 284 (2004) 133–140.
[12] M. Honma, L.S. Zhang, M. Hayashi, K. Takeshita, Y. Nakagawa, N. Tanaka, T. Sofuni, Illegitimate recombination leading to allelic loss and unbalanced translocation in p53-mutated human lymphoblastoid cells, Mol. Cell Biol. 17 (1997) 4774–4781.
[13] E.E. Furth, W.G. Thilly, B.W. Penman, H.L. Liber, W.M. Rand, Quantitative assay for mutation in diploid human lymphoblasts using microtiter plates, Anal. Biochem. 110 (1981) 1–8.
[14] O. Gresch, F.B. Engel, D. Nesic, T.T. Tran, H.M. England, E.S. Hickman, I. Korner, L. Gan, S. Chen, S. Castro-Obregon, R. Hammermann, J. Wolf, H. Muller-Hartmann, M. Nix, G. Siebenkotten, G. Kraus, K. Lun, New non-viral method for gene transfer into primary cells, Methods 33 (2004) 151–163.
[15] J. Essers, H. van Steeg, J. de Wit, S.M. Swagemakers, M. Vermeij, J.H. Hoeijmakers, R. Kanaar, Homologous and non-homologous recombination differentially affect DNA damage repair in mice, EMBO J. 19 (2000) 1703–1710.
[16] S.P. Jackson, P.A. Jeggo, DNA double-strand break repair and V(D)J recombination: involvement of DNA-PK, Trends Biochem. Sci. 20 (1995) 412–415.
[17] J.M. Stark, M. Jasin, Extensive loss of heterozygosity is suppressed during homologous repair of chromosomal breaks, Mol. Cell Biol. 23 (2003) 733–743.
[18] P. Bertrand, D. Rouillard, A. Boulet, C. Levalois, T. Soussi, B.S. Lopez, Increase of spontaneous intrachromosomal homologous recombination in mammalian cells expressing a mutant p53 protein, Oncogene 14 (1997) 1117–1122.
[19] G.S. Boehden, N. Akyuz, K. Roemer, L. Wiesmuller, p53 mutated in the transactivation domain retains regulatory functions in homology-directed double-strand break repair, Oncogene 22 (2003) 4111–4117.
[20] C. Richardson, J.M. Stark, M. Ommundsen, M. Jasin, Rad51 overexpression promotes alternative double-strand break repair pathways and genome instability, Oncogene 23 (2004) 546–553.
[21] W.E. Bradley, A. Belouchi, K. Messing, The aprt heterozygote/hemizygote system for screening mutagenic agents allows detection of large deletions, Mutat. Res. 199 (1988) 131–138.
[22] H.L. Liber, D.W. Yandell, J.B. Little, A comparison of mutation induction at the tk and hprt loci in human lymphoblastoid cells; quantitative differences are due to an additional class of mutations at the autosomal tk locus, Mutat. Res. 216 (1989) 9–17.
[23] J. Dahm-Daphi, P. Hubbe, F. Horvath, R.A. El Awady, K.E. Bouffard, S.N. Powell, H. Willers, Nonhomologous end-joining of site-specific but not of radiation-induced DNA double-strand breaks is reduced in the presence of wild-type p53, Oncogene 24 (2005) 1663–1672.
[24] J. Guirouilh-Barbat, S. Huck, P. Bertrand, L. Pirzio, C. Desmaze, L. Sabatier, B.S. Lopez, Impact of the KU80 pathway on NHEJ-induced genome rearrangements in mammalian cells, Mol. Cell 14 (2004) 611–623.
[25] D. van Heemst, L. Brugmans, N.S. Verkaik, D.C. van Gent, End-joining of blunt DNA double-strand breaks in mammalian fibroblasts is precise and requires DNA-PK and XRCC4, DNA Rep. (Amst.) 3 (2004) 43–50.
[26] Z.E. Karanjawala, U. Grawunder, C.L. Hsieh, M.R. Lieber, The nonhomologous DNA end joining pathway is important for chromosome stability in primary fibroblasts, Curr. Biol. 9 (1999) 1501–1504.
[27] P. Ahnesorg, P. Smith, S.P. Jackson, XLF interacts with the XRCC4-DNA ligase IV complex to promote DNA nonhomologous end-joining, Cell 124 (2006) 301–313.
[28] D. Buck, L. Malivert, R. de Chasseval, A. Barraud, M.C. Fondaneche, O. Sanal, A. Plebani, J.L. Stephan, M. Hufnagel, F. le Deist, A. Fischer, A. Durandy, J.P. de Villartay, P. Revy, Cernunnos, a novel nonhomologous end-joining factor, is mutated in human immunodeficiency with microcephaly, Cell 124 (2006) 287–299.
[29] S. Burma, D.J. Chen, Role of DNA-PK in the cellular response to DNA double-strand breaks, DNA Rep. (Amst.) 3 (2004) 909–918.
[30] Z.E. Karanjawala, N. Adachi, R.A. Irvine, E.K. Oh, D. Shibata, K. Schwarz, C.L. Hsieh, M.R. Lieber, The embryonic lethality in DNA ligase IV-deficient mice is rescued by deletion of Ku: implications for unifying the heterogeneous phenotypes of NHEJ mutants, DNA Rep. (Amst.) 1 (2002) 1017–1026.

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Short communication

Sensitivity of the erythrocyte micronucleus assay: Dependence on number of cells scored and inter-animal variability

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Abstract

Until recently, the *in vivo* erythrocyte micronucleus assay has been scored using microscopy. Because the frequency of micronucleated cells is typically low, cell counts are subject to substantial binomial counting error. Counting error, along with inter-animal variability, limit the sensitivity of this assay. Recently, flow cytometric methods have been developed for scoring micronucleated erythrocytes and these methods enable many more cells to be evaluated than is possible with microscopic scoring. Using typical spontaneous micronucleus frequencies reported in mice, rats, and dogs we calculate the counting error associated with the frequency of micronucleated reticulocytes as a function of the number of reticulocytes scored. We compare this counting error with the inter-animal variability determined by flow cytometric scoring of sufficient numbers of cells to assure that the counting error is less than the inter-animal variability, and calculate the minimum increases in micronucleus frequency that can be detected as a function of the number of cells scored. The data show that current regulatory guidelines allow low power of the test when spontaneous frequencies are low (e.g., ≤0.1%). Tables and formulas are presented that provide the necessary numbers of cells that must be scored to meet the recommendation of the International Working Group on Genotoxicity Testing that sufficient cells be scored to reduce counting error to less than the inter-animal variability, thereby maintaining a more uniform power of detection of increased micronucleus frequencies across laboratories and species.

Keywords: Erythrocyte micronucleus assay; Flow cytometry; Binomial counting error; Inter-animal variability; Power calculation

1. Introduction

The ability of the *in vivo* erythrocyte micronucleus assay to detect small increases in the spontaneous background frequency of micronucleated cells in a group of animals (or study subjects) is limited by either the binomial counting error, when the number of cells scored gives small numbers of scored events (micronucleated cells) [1–4], or by inter-animal variation, when that variation is so large that it obscures a small, but real, increase. Furthermore, the sensitivity to detect small increases in the micronucleus frequency in an individual animal is limited at small micronucleus counts by the binomial counting error and at higher counts by the spontaneous variability in that individual animal over the time span in

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which measurements are made. Recognizing these facts, the working group on the *in vivo* micronucleus assay organized by the International Workshops on Genotoxicity Testing (IWGT) has recommended that, whenever possible, sufficient cells should be scored to reduce the counting error to less than the variability in MN frequency between individual animals (for comparison of values in different treated groups) [5].

Prior to the development of flow cytometric scoring methods, the number of cells scored was generally limited by the practical consideration of the number of cells that could be scored in a reasonable period of time by a microscopist, and therefore the minimum number of cells recommended to be scored in current regulatory guidelines is generally less than that required to discern differences between individual animals. Flow cytometric methodologies now make it practical to reduce the counting error to very small values [6–8], allowing, for the first time, reliable determination of the inter- and intra-animal variation in the spontaneous micronucleus frequency.

We summarize below experimentally determined mean and variability among animals in the spontaneous frequency of micronucleated reticulocytes (MN-RETs) in peripheral blood reticulocytes in the Sprague–Dawley rat, CD-1 mouse, and beagle dog, and in bone marrow reticulocytes in the Sprague–Dawley rat, and compare this inter-animal variability with the microscopic counting error associated with the current regulatory recommendations for scoring bone marrow or peripheral blood reticulocytes in these species. From these values, we determine the minimum increase in group mean frequencies of MN-RETs that can be detected in these species, tabulate the minimum increases that can be detected as a function of the number of RETs scored, and identify the numbers of cells that need to be scored to meet the IWGT recommendation that sufficient cells should be scored such that the error in individual animal MN-RET frequencies is less than the inter-animal variability.

2. Inter-animal variability

The inter-animal variability of the percentage of MN-RETs among RETs (no. of MN-RET/no. of RETs scored × 100) in the peripheral blood of Sprague–Dawley rats, CD-1 mice, and purpose-bred beagle dogs, and also in the bone marrow of Sprague–Dawley rats after removal of nucleated cells on a cellulose mini-column as described by Romagna [9] and Weiner et al. [10], was estimated by scoring 20,000 reticulocytes using the flow cytometric method described by Dertinger et al. [11–13]. These data are summarized in Table 1. The data are taken from

Table 1
Mean and inter-animal variation of the micronucleated reticulocyte frequency in the peripheral blood (PB) and bone marrow (BM) of Sprague–Dawley rats, Swiss mice, and Beagle dogs

Species	Strain/breed	Tissue	Mean %MN-RET	S.D. of %MN-RET	Inter-animal %CV[a](%)	No. of animals	No. of experiments.	References
Rat	SD	PB	0.11	0.045	41	15	3	[14]
Rat	SD	BM	0.23[b]	0.059[b]	26	190	38	[21] Fiedler, personal communication
Mouse	CD-1	PB	0.20	0.070	35	79	9	[15]
Dog	Beagle	PB	0.31	0.092	30	22	4	Manuscript in preparation

[a] %CV = S.D./mean × 100%.
[b] Bone marrow %MN-RET values were determined by separation of nucleated cells on a mini-cellulose column [9,22], with subsequent scoring of the MN-RET frequency among 20,000 RETs by the same flow cytometric procedure used for analysis of peripheral blood.

previously reported studies in these species [14,15]. Details of the experimental methodology are reported in the previous publications. As is discussed below, scoring 20,000 RETs results in a sufficient number of events (MN-RETs) that the error associated with individual animals does not exceed approximately 50% of the inter-animal variability of spontaneous MN-RET frequencies in the respective species. The inter-animal % coefficients of variation (%CV = S.D. / mean × 100%) of the MN-RET frequencies were 41% for the rat, 35% for the mouse, and 30% for the dog.

Table 2 presents the binomial error in the count of MN cells in an individual animal obtained by scoring 2000, 4000, 8000, or 20,000 RETs as a function of the spontaneous frequency of MN-RETs. It should be noted that the spontaneous frequency in rodent bone marrow or peripheral blood reported by different experienced laboratories has ranged from 0.05% in rat (see individual laboratory values in Ref. [14]) to a mean value of 0.2% in the mouse [15,16] and 0.31% in the beagle dog. Since the counting error depends on the background rate and the number of cells scored, we have tabulated values over the range of spontaneous frequencies commonly reported in rodents and recently observed in the beagle dog (manuscript in preparation). As can be seen in Table 2, when 2000 cells are scored (the recommended number in the current OECD, FDA, and EPA regulatory guidelines [17–19]) the error in the counts observed in individual animals is substantially greater than the variation between animals (Table 1). When the spontaneous frequency is 0.1%, approximately 6000 cells would need to be scored to reduce the error in the individual animal count to less than the inter-animal variability observed in the rat.

Table 2
Counting error (standard deviation (S.D.) and coefficient of variation) of individual animal values of MN-RET frequency as a function of true spontaneous frequency and number of RETs scored

True %MN-RET	No. of RETs scored per animal	S.D. of count	Counting error %CV
0.05	2,000	0.050	100
	4,000	0.035	71
	8,000	0.025	50
	20,000	0.016	32
0.10	2,000	0.071	71
	4,000	0.050	50
	8,000	0.035	35
	20,000	0.022	22
0.20	2,000	0.100	50
	4,000	0.071	35
	8,000	0.050	25
	20,000	0.032	16
0.30	2,000	0.122	41
	4,000	0.086	29
	8,000	0.061	20
	20,000	0.039	13

3. Sensitivity to increases above the spontaneous frequency

Table 3 summarizes the minimum increases above the spontaneous frequency that can be detected in groups of five animals (the minimum currently recommended in OECD, FDA, and EPA guidelines [17–19]) as a function of the number of target cells scored (in this case RETs) and observed spontaneous frequency (in this case %MN-RETs among RETs). Minimum detectable increases in MN-RET frequencies at $p \leq 0.05$ or ≤ 0.01, with 90% or 95% power were determined using Monte Carlo simulations. Specifically, to reflect inter-animal variability, five binomial probabilities were randomly selected from a normal distribution with the following mean, μ_0, and standard deviation, σ, combinations: (μ_0, σ) = (0.05%, 0.02%), (0.10%, 0.045%), (0.20%, 0.070%), or (0.30%, 0.092%). For a given fold-increase, f, a second set of five binomial probabilities were randomly selected from a normal distribution with mean, $\mu_1 = \mu_0 f$, and the same σ given above. Using the five binomial probabilities from the spontaneous mean group, five MN-RETs frequencies were randomly generated from binomial distributions, with n = number of RETs scored, 2000, 4000, or 20,000. Such selection from a binomial distribution introduces the binomial counting error. Five MN-RET frequencies were similarly generated using the five binomial probabilities from the increased mean group. A one-tailed Mann–Whitney test was then performed on these 10 counts, comparing the spontaneous group to the increased group, and the p-value was noted as to whether it was 0.05 or less and/or 0.01 or less. This was repeated 3000 times and the percentages of the 3000 'samples' for which the p-value was 0.05 or less and 0.01 or less were calculated. The process was repeated over a series of increases, f, at increments of 0.1, to determine the first point at which the power exceeded 90 or 95%. We obtained very similar results (not shown) by generating the five binomial probabilities from beta distributions having the above combinations of μ_0, μ_1, and σ.

For the line labeled "∞" in Table 3, there is no counting error; rather, the variability in frequencies is due to inter-animal variation alone. If we assume that inter-animal variation is normally distributed, the minimum difference between μ_1 and μ_0, $\delta = \mu_1 - \mu_0$, detectable using five animals per group with significance level α

Table 3
Minimum detectable increases in MN-RET frequency in groups of five animals as a function of spontaneous frequency and number of RETs scored

Spontaneous frequency (%MN-RET)	No. of RETs scored	Minimum detectable fold-increase in spontaneous frequency			
		With 90% probability		With 95% probability	
		At $p \leq 0.05$	At $p \leq 0.01$	At $p \leq 0.05$	At $p \leq 0.01$
0.05 (S.D. = 0.020)	2,000	4.5	6.8	5.6	9.3
	4,000	3.5	5.5	4.0	6.3
	20,000	2.3	3.1	2.4	3.4
	∞	1.8	2.1	1.9	2.2
0.10 (S.D. = 0.045) (rat PB)	2,000	3.3	4.8	4.1	6.4
	4,000	2.9	4.2	3.2	4.7
	20,000	2.2	3.0	2.4	3.2
	∞	1.8	2.0	2.1	2.4
0.20 (S.D. = 0.070) (mouse PB)	2,000	2.7	3.9	3.0	4.5
	4,000	2.3	3.2	2.4	3.5
	20,000	1.9	2.5	2.2	2.7
	∞	1.7	2.0	1.8	2.1
0.20 (S.D. = 0.059) (rat BM)	2,000	2.7	3.9	2.9	4.4
	4,000	2.2	3.1	2.4	3.3
	20,000	1.8	2.3	1.9	2.5
	∞	1.6	1.8	1.7	1.9
0.30 (S.D. = 0.092) (dog PB)	2,000	2.4	3.4	2.6	3.7
	4,000	2.1	2.8	2.2	3.0
	20,000	1.8	2.3	1.9	2.4
	∞	1.6	1.8	1.7	1.9

Values for the cases of infinite cell counts are calculated based on the observed inter-animal variability (standard deviation from Table 1) for the species stated, assuming no counting error; the inter-animal variability for frequency 0.05% is assumed to be 0.02%. The detectable increase depends on the relative magnitudes of both the counting error and the inter-animal variability. Although counting error can be reduced by scoring more RETs, the minimum detectable increase cannot go below a bound determined by the inter-animal variability (i.e., the value given in the infinite cell count rows). Species entries correspond to the approximate spontaneous frequency and associated inter-animal standard deviation in the species specified in Table 1.

and power $1-\beta$ is [20]:

$$\delta = (t_\alpha + t_\beta)\sigma\sqrt{\frac{2}{5}}.$$

Here, t_α and t_β are the critical values from the $5+5-2=8$ degree of freedom t-distribution having upper tail probabilities α and β, respectively. The minimum detectable fold-increase over the spontaneous group is then

$$f = \frac{\mu_1}{\mu_0} = \frac{\mu_0 + \delta}{\mu_0} = 1 + \frac{\delta}{\mu_0}.$$

While spontaneous MN-RET frequencies determined from counting 2000 RETs from different animals are not often normally distributed, it has been our experience that spontaneous frequencies determined from counting 20,000 RETs from different animals are approximately normally distributed. Therefore, the assumptions of normality that we made above are most likely reasonable.

It should be noted that even if the counting error of the MN-RET frequency in each individual animal could be eliminated, the sensitivity of detection of changes in the observed mean group frequency would still be limited by the inter-animal variability (represented in Table 3 by the line in which an infinite number of cells is scored). It is clear that the regulatory assay as currently conducted is relatively insensitive to changes in the spontaneous frequency, especially when the spontaneous frequency is low. For example, when the spontaneous frequency is 0.05% and only 2000 RETs are scored, even a 6.8-fold increase would fail to be detected at a confidence level of $p \leq 0.01$ in 10% of experiments conducted. Even at the more commonly reported spontaneous frequency of 0.1% a 4.8-fold increase would fail to be detected 10% of the time at this same confidence level. The use of flow cytometric scoring to achieve a sufficient cell count to allow individual animal frequencies with adequate certainty (i.e., certainty of the individual value relative to the inter-animal variation) would increase the sensitivity

Table 4
Number of reticulocytes required to be scored to reduce counting error to less than the observed inter-animal coefficient of variation

Spontaneous frequency (% MN-RET)	Species/tissue with this approximate spontaneous frequency	Inter-animal %CV[a]	No. of RETs to be scored to reduce counting error %CV to		
			Equal the inter-animal %CV	50% of the inter-animal %CV	20% of the inter-animal %CV
0.05	Rat BM & PB(microscopy, some reports)	NA[b]	NA	NA	NA
0.10	Rat PB (data cited above)	41	5943	23,772	148,573
0.20	Mouse BM & PB	35	4074	16,294	101,837
0.30	Dog	30	3693	14,770	92,315

[a] Experimentally determined inter-animal %CV by flow cytometric scoring of 20,000 peripheral blood RETs, at the approximate spontaneous frequency tabulated.
[b] Inter-animal %CV has not been determined at the spontaneous frequency of 0.05%; no reported experiments have scored sufficient cells to determine the inter-animal variability.

such that a doubling of a spontaneous frequency of 0.1% among 20,000 RETs scored would be detected nearly 90% of the time at a confidence level of $p \leq 0.05$. It should also be noted that, regardless of the spontaneous frequency, the sensitivity achieved by scoring 20,000 RETs is close to the optimal sensitivity that could be achieved if no counting error were present.

4. Number of reticulocytes required to be scored to reduce counting error to less than inter-animal variability

Table 4 summarizes the number of cells required to be scored to reduce the counting error of individual animal values (Table 2, %CV) to the observed inter-animal variation or less (Table 1, %CV). These numbers were calculated by setting a multiple ($m = 1.0$, 0.5, or 0.2) of the inter-animal %CV equal to the binomial counting error %CV and solving for the required sample size, n. Mathematically, if p is the percent of MN-RETs among all RETs within an animal, then

$$\%\mathrm{CV}_{\text{binomial error}} = \frac{\sqrt{p(1-p)/n}}{p} = m \times \%\mathrm{CV}_{\text{inter-animal}}.$$

Solving for n, we get

$$n = \frac{1-p}{p(m \times \%\mathrm{CV}_{\text{inter-animal}})^2}.$$

The numbers of RETs required are prohibitively laborious to obtain by conventional microscopic scoring, but are easily achieved by automated procedures such as flow cytometry.

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References

[1] B.H. Margolin, B.J. Collings, J.M. Mason, Statistical analysis and sample-size determinations for mutagenicity experiments with binomial responses, Environ. Mutagen. 5 (1983) 705–716.
[2] B.H. Margolin, K.J. Risko, The statistical analysis of in vivo genotoxicity data: case studies of the rat hepatocyte UDS and mouse bone marrow micronucleus assays, in: J. Ashby, F.J. de Serres, M.D. Shelby, B.H. Margolin, M. Ishidate Jr., G.C. Becking (Eds.), Evaluation of Short-term Tests for Carcinogens: Report of the International Programme on Chemical Safety's Collaborative Study on In Vivo Assays, vol. 1, Cambridge University Press on Behalf of WHO, Cambridge, 1988, pp. 1.29–1.42.
[3] M.A. Kastenbaum, K.O. Bowman, Tables for determining the statistical significance of mutation frequencies, Mutat. Res. 9 (1970) 527–549.
[4] M. Hayashi, I. Yoshimura, T. Sofuni, M. Ishidate Jr., A procedure for data analysis of the rodent micronucleus test involving a historical control, Environ. Mol. Mutagen. 13 (1989) 347–359.
[5] M. Hayashi, J.T. MacGregor, D.G. Gatehouse, D.H. Blakey, S.D. Dertinger, L. Abramsson-Zetterberg, G. Krishna, T. Morita, A. Russo, N. Asano, H. Suzuki, W. Ohyama, D. Gibson, *In vivo* erythrocyte micronucleus assay III. Validation and regulatory acceptance of automated scoring and the use of rat peripheral blood reticulocytes, with discussion of non-hematopoietic target cells and a single dose-level limit test, Mutat. Res. 627 (2007) 10–30.
[6] N. Asano, D. Torous, C. Tometsko, S. Dertinger, T. Morita, M. Hayashi, Practical threshold for micronucleated reticulocyte

induction for low doses of mitomycin C, Ara-C and colchicine, Mutagenesis 21 (2006) 15–20.
[7] J. Grawe, L. Abramsson-Zetterberg, G. Zetterberg, Low dose effects of chemicals as assessed by the flow cytometric in vivo micronucleus assay, Mutat. Res. 405 (1998) 199–208.
[8] D. Torous, N. Asano, C. Tometsko, S. Sugunan, S. Dertinger, T. Morita, Performance of flow cytometric analysis for the micronucleus assay—a reconstruction model using serial dilutions of malaria-infected cells with normal mouse peripheral blood, Mutagenesis 21 (2006) 11–13.
[9] F. Romagna, Current issues in mutagenesis and carcinogenesis; fractionation of a pure PE and NE population from rodent bone marrow, Mutat. Res. 206 (1988) 307–309.
[10] S.K. Weiner, R.D. Fiedler, M.J. Schuler, Development and evaluation of a flow cytometric method for the analysis of micronuclei in rat bone marrow in vivo, Environ. Mol. Mutagen. 43 (2004) 236.
[11] S.D. Dertinger, D.K. Torous, K. Tometsko, Simple and reliable enumeration of micronucleated reticulocytes with a single-laser flow cytometer, Mutat. Res. 371 (1996) 283–292.
[12] S.D. Dertinger, D.K. Torous, N.E. Hall, C.R. Tometsko, T.A. Gasiewicz, Malaria-infected erythrocytes serve as biological standards to ensure reliable and consistent scoring of micronucleated erythrocytes by flow cytometry, Mutat. Res. 464 (2000) 195–200.
[13] S.D. Dertinger, K. Camphausen, J.T. MacGregor, M.E. Bishop, D.K. Torous, S. Avlasevich, S. Cairns, C.R. Tometsko, C. Menard, T. Muanza, Y. Chen, R.K. Miller, K. Cederbrant, K. Sandelin, I. Ponten, G. Bolcsfoldi, Three-color labeling method for flow cytometric measurement of cytogenetic damage in rodent and human blood, Environ. Mol. Mutagen. 44 (2004) 427–435.
[14] J.T. MacGregor, M.E. Bishop, J.P. McNamee, M. Hayashi, N. Asano, A. Wakata, M. Nakajima, A. Aidoo, M.M. Moore, S.D. Dertinger, Flow cytometric analysis of micronuclei in peripheral blood reticulocytes II. An efficient method of monitoring chromosomal damage in the rat, Toxicol. Sci. 94 (2006) 92–107.
[15] D.K. Torous, N.E. Hall, A.H. Illi-Love, M.S. Diehl, K. Cederbrant, K. Sandelin, I. Pontén, G. Bolcsfoldi, L.R. Ferguson, A. Pearson, J.B. Majeska, J.P. Tarca, G.M. Hynes, A.M. Lynch, J.P. McNamee, P.V. Bellier, M. Parenteau, D. Blakey, J. Bayley, B.M. van der Leede, P. Vanparys, P.R. Harbach, S. Zhao, A.L. Filipunas, C.W. Johnson, C.R. Tometsko, S.D. Dertinger, Interlaboratory validation of a CD71-based flow cytometric method (MicroFlow®) for the scoring of micronucleated reticulocytes in mouse peripheral blood, Environ. Mol. Mutagen. 45 (2005) 44–55.
[16] J.A. Heddle, M.F. Salamone, M. Hite, B. Kirkhart, K. Mavournin, J.T. MacGregor, G.W. Newell, The induction of micronuclei as a measure of genotoxicity, Mutat. Res. 123 (1983) 61–118.
[17] OECD, Guideline for the testing of chemicals. Mammalian erythrocyte micronucleus test. Guideline 474, July 1997.
[18] FDA (US Food and Drug Administration), Office of Food Additive Safety, Redbook 2000, Toxicological principles for safety assessment of food ingredients, Updated November 2003, www.cfsan.fda.gov/~redbook/red-toca.html.
[19] EPA (US Environmental Protection Agency), Health Effects Test Guidelines OPPTS 870.5395, Mammalian Erythrocyte Micronucleus Test, Office of Prevention, Pesticides and Toxic Substances (7101) EPA 712-C-98-226, August 1998. www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Series/870-5395.pdf.
[20] G.W. Snedecor, W.G. Cochran, Statistical Methods, 6th ed., The Iowa State University Press, Ames, IA, 1976, pp. 113–116.
[21] R. Fiedler, Pfizer, Inc., Personal communication (2007).
[22] E. Beutler, T. Gelbart, The mechanism of removal of leukocytes by cellulose columns, Blood Cells 12 (1986) 57–64.

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Original Article

Evaluation of statistical tools used in short-term repeated dose administration toxicity studies with rodents

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ABSTRACT — In order to know the different statistical tools used to analyze the data obtained from twenty-eight-day repeated dose oral toxicity studies with rodents and the impact of these statistical tools on interpretation of data obtained from the studies, study reports of 122 numbers of twenty-eight-day repeated dose oral toxicity studies conducted in rats were examined. It was found that both complex and easy routes of decision trees were followed for the analysis of the quantitative data. These tools include Scheffe's test, non-parametric type Dunnett's and Scheffe's tests with very low power. Few studies used the non-parametric Dunnett type test and Mann-Whitney's *U* test. Though Chi-square and Fisher's tests are widely used for analysis of qualitative data, their sensitivity to detect a treatment-related effect is questionable. Mann-Whitney's *U* test has better sensitivity to analyze qualitative data than the chi-square and Fisher's tests. We propose Dunnett's test for analysis of quantitative data obtained from twenty-eight-day repeated dose oral toxicity tests and for qualitative data, Mann-Whitney's *U* test. For both tests, one-sided test with p=0.05 may be applied.

Key words: Statistics; 28-day repeated toxicity study; Rodents; Dunnett's test; Mann-Whitney's *U* test

INTRODUCTION

Short-term repeated oral toxicity study conducted for 14 or 28 days is aimed to (1) predict appropriate doses of test substance for future subchronic or chronic toxicity studies, (2) determine NOELs for some toxicology endpoints and (3) to allow future studies in rodents to be designed with special emphasis on identified target organs (USFDA, 2000). This study also provides information on the possible health hazards likely to arise from repeated exposure over a relatively limited period of time (USEPA, 2000; OECD, 1995). Though these guidelines provide all the information required for the conduct of the study, no information is provided on the appropriate statistical tools to be used to analyze the data obtained from the study. Use of right statistical tool to analyze the data obtained from theses studies is very crucial as the interpretation of the data is mostly based on the results of the statistical analysis.

The statistical tools used to analyze the data obtained from 122 numbers of twenty-eight-day repeated dose oral toxicity tests in rats were examined in the present study. The objective of the study was to know the different statistical tools that are used in these studies and the possible impact of these statistical tools on interpretation of the data. A brief discussion on the use and the property of the different statistical tools used in the studies are also given. The purpose of this article wished for the standardization of statistics and the analysis methods. Finally, the authors made an attempt to suggest statistical techniques that may best suit twenty-eight-day repeated dose oral toxicity studies in rodents.

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MATERIALS AND METHODS

Studies examined

A total number of 122 studies conducted in various test facilities in Japan were examined (MHLW, 2006). The chemical of these examinations was executed with existing chemical substances by the guideline of the Chemical Substance Control Law (1986). The number of studies conducted in each test facility is given in parenthesis: Food and Drug Safety Center, Kanagawa (22), An-Pyo Center, Shizuoka (22), Mitsubishi Chemical Safety Institute Ltd., Ibaraki (18), Safety Research Institute for Chemical Compounds Co., LTD, Hokkaido (15), Bozo Research Center Inc., Shizuoka (12), Research Institute for Animal Science in Biochemistry & Toxicology, Kanagawa (11), Panapharm Laboratories, Kumamoto (10), Nihon Bioresearch Inc., Gifu (9) and National Institutes of Health, Tokyo (3).

Quantitative and qualitative items

Several quantitative and qualitative items are evaluated in twenty-eight-day repeated dose oral toxicity tests in rats, as per the regulatory guidelines. The quantitative items that require statistical analysis are body weight, food consumption, water consumption, leucocytes, erythrocytes, hemoglobin, hematocrit, platelets, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, differential leucocyte counts, prothrombin time, activated partial thromboplastin time, total protein, albumin, albumin/globulin ratio, total bilirubin, alanine aminotransferase, aspartate aminotransferase, γ-glutamic transaminase, alkaline phosphatase, acetylcholinesterase, total cholesterol, tryglycerides, phospholipids, glucose, blood urea nitrogen, creatinine, inorganic phosphorous, calcium, sodium, potassium, chlorides, urine volume, specific gravity of urine, absolute organ weights and relative organ weights. Qualitative items that require statistical analysis are mortality, functional observation battery, clinical signs, urinalysis (color, pH, protein, glucose, ketone bodies, bilirubin, occult blood, urobilinogen, epithelial cells, erythrocytes, leucocytes, casts and crystals) and pathological findings (macroscopic and microscopic). But the regulatory guidelines do not indicate the specific statistical techniques to be used to analyze these data.

Which test to be used - One-sided or two-sided?

When the *t*-test and Dunnett's multiple comparison test (Dunnett's test) are used, the significant difference detection rate of a two-sided test is about 85% as compared with a one-sided test (Kobayashi, 1997a). In toxicological studies, usually a dosed group is compared with the control group. For this comparison, one-sided test is ideal, hence Yoshimura and Ohashi (1992) recommend the one-sided test for comparing a dosed group with the control group.

Is analysis of variance (ANOVA) necessary?

It is a common practice to subject the data, if they are from more than two groups, ANOVA. If ANOVA shows a significant difference among the groups, multiple comparison tests are used to find the significant difference between any two groups. In recent years, several authors suggested that the error of the second kind can be prevented by carrying out direct multiple comparison tests, without subjecting the data to ANOVA (Hamada *et al.*, 1998; Kobayashi *et al.*, 2000a; Sakaki *et al.*, 2000). It may be worth mention in this context that Dunnett (1964) did not recommend ANOVA prior to multiple comparison tests.

Is Bartlett's homogeneity test necessary?

Generally Bartlett's test is used to examine the homogeneity of variance if the number of animals in a group is 10 or more. Therefore, this test is not used in the toxicity studies with dogs, where the number of animals in the group is less. According to Kobayashi *et al.* (1998), Bartlett's test is not required to examine the homogeneity of variance, when the number of animals in a group is less.

Non-parametric type Dunnett's test

The non-parametric Dunnett's multiple comparison test has two techniques - 'joint type' and 'separate type' or Steel's test. When the Steel's test shows the highest dosage correlation, the number of animals required in the dosage groups to detect a significant difference in the low dosage group is four (Inaba, 1994; Kobayashi *et al.*, 1995). On the contrary, 'joint type' needs 15 animals in each group.

Transformation of data

If the data show heterogeneity of variance as per Bartlett's test, sometimes the data are transformed, for example to logarithmic values and then they are subjected to non-parametric tests. According to Finney (1995), "when a scientist measures a quantity such as concentration of a chemical compound in body fluid, his interest usually lies in the scale, perhaps mg/ml, that he has used; he is less likely to be interested in a summary of results relating to a transformed quantity such as the logarithm of blood concentration. If he analyzes in terms of logarithms, encouraged perhaps by an elementary but uncritical statistical textbook or by a convenient software package, he may find significant differences but to express his conclusions in meaningful numbers may be impossible. I do not assert

that a scientist should never transform data before analysis; I urge that data should be transformed only after careful consideration of all consequence". Therefore, transformation should be done cautiously.

Power of Scheffe's test

Use of Scheffé's test is discouraged in recent years because this test may not show a significant difference in the dosage groups even if the dosage groups show a difference of 60-53% compared to control group (Kobayashi *et al.*, 1997b).

Power of non-parametric tests using ranked data

In four groups setting with the highest dosage correlation, the minimum numbers of animals required in the low-dose group to detect a significant difference, compared to control, using the statistical tools of Scheffé's type, Dunn's test, Tukey type, Dunnett type, Williams-Wilcoxon test, Steel test and Mann-Whitney's *U* test are 22, 19, 18, 15, 8, 4 and 3, respectively. Therefore, in the twenty-eight-day repeated dose oral toxicity tests in rats, where the number of animals is 5/sex/group, except Steel and Mann-Whitney's *U* tests, other tests are not used. Inaba (1994) made a similar observation on the power of the above tests.

Power of Chi-square and Fisher's tests

When a finding in the animals of a control group is 0, in order to find a significant difference of the finding between the control group (n=5) and dosage group (n=5) by chi-square test, all the 5 animals in the dosage group (n=5) should show the finding, whereas by Fisher's test 4 animals should show the finding. When 1 animal in the control group shows a finding, even if the finding is seen in all the animals in the dosage group, a significant difference is not detected by chi-square test, but it is detected by Fisher's test. In the light of the above it may be stated that power of one-sided Fisher's test is better than the Chi-square test.

Dunnett's test is the expanded version of *t*-tests

Dunnett's test becomes *t*-test when two groups are analyzed (Kobayashi *et al.*, 1997c). Therefore, when comparing the recovery groups in the twenty-eight-day repeated dose oral toxicity tests in rats, where number of the groups is 2, it does not make any difference, whether the analysis is carried out by Dunnett test or *t*-test.

Power of Mann-Whitney's *U* test

This test is generally used for the analysis of pathology data (Kobayashi *et al.*, 1997d). A significant difference by a one-sided test is detected if the calculated *U* value is four or less. Since one-side is expected in studies like twenty-eight-day repeated dose oral toxicity tests in rats, a one-sided Mann-Whitney's *U* test is used to analyze pathology data obtained from these studies.

RESULTS

Quantitative data

Out of 122 studies examined, 79 studies used statistical tools that follow a complicated course (tool numbers; 2, 3, 4, 5, 8, 9, 10, 12, 15, 16 and17) and 43 studies used statistical tools that follow simple course (tool numbers; 1, 6, 7, 11, 13 and14) (Table 1; Fig. 1). The statistical tools describing the method of analyzes, in the case of three or more groups and two groups were mentioned in 6 studies, whereas this description was not found in 11 studies. Only eight studies used trend test (Jonckheere, 1954). In the tool number 10, the significance level of ANOVA and Kruskal-Walis's *H* test were set at p=0.10. For comparing with the control, this tool set the significance level of p=0.05. Tool numbers 13 and 14 did not perform Bartlett's test for testing the homogeneity of variance. Use of one-sided or two-sided test is not indicated in 87 studies. Only one study indicated use of non-parametric test.

Qualitative data

Since urinalysis data were classified into many grades, chi-square test was used to analyze these data in most of the studies. For macro- and microscopic pathological findings, Mann-Whitney's *U* test, Fisher's test and Chi-square test were used. Most of the studies did not indicate the alpha. Only the pathological findings of 3 studies were examined for dose-relationship (Table 2).

Use of a one-sided test was more common than a two-sided test in the case of analysis of both quantitative and qualitative data (Table 3).

DISCUSSION

National Toxicology Program, USA published technical reports of long-term carcinogenicity studies and short-term toxicity tests carried out with more than 500 substances in rat and mouse (NIH, 2006). Most of these studies used the statistical tools almost similar to the ones currently used to analyze the data obtained from the toxicity tests of agricultural chemicals and medical drugs (Kobayashi *et al.*, 2000b).

On examination of 122 studies, it was found that complex and easy courses of analytical techniques were used for the analysis of the quantitative data. These tools may be classified into 4 different categories. Five tools (tool

numbers; 4, 5, 8, 16 and17) are the advanced type of the algorithm, similar to the one developed by Yamazaki *et al.* (1981). These tools include Scheffé's test, non-parametric type Dunnett's and Scheffé's tests with very low power. Six tools (tool numbers; 3, 7, 9, 10, 12 and 15) are again advanced type of algorithm developed by Sano and Okayama (1990), which can be used even if the number of animals in the groups are different. Use of the non-parametric Dunnett type test with low power is also seen in few studies. Mann-Whitney's *U* test was also used (tool number; 9) in 14 studies in order to retain the power. Three tools (tool numbers; 2, 6 and 11) are an improved version of non-parametric type Dunnett's test ('joint type') and Steel's test ('separate type'). Dunnett's or Scheffé's tests is independently used for 3 tools (tool numbers; 1, 13 and 14). Though use of Scheffé's test has the advantage of comparison of groups in various combinations, for example, control+mid dose *vs*.high dose, low dose+mid dose *vs.* high dose, etc., it has extremely low detection power. Hence, this test is not widely used in recent years.

Yoshimura (1987) used Bartlett's test to analyze the difference in distribution of variance among the groups, where number of animals in the group is more than 10. The power of Bartlett's test decreases when the number of animals in the group is less.

Dunnett's test is the expanded version of *t*-tests, hence, it becomes *t*-test when two groups are analyzed by Dunnett's test. Therefore, for the comparison of two groups either Dunnett test or *t*-test can be used.

The most important purpose of applying statistical analysis in toxicity studies is to know whether the items estimated in the experimental group has increased or decreased compared to the control. Therefore, a one-sided test is used. Detection rate of two-sided test is half of the one-sided test, hence it is important to mention in the study report whether a one-sided or two-sided test is used. It may

Table 1. Classification of number of studies based on the statistical tools used for the analysis of quantitative data.

Tool. No.	Description of statistical tools	Number of studies
1	Dunnett's test: Three groups or more; Student or Aspin-Welch's *t*-test: Two groups	5
2	Bartlett's test, ANOVA, Dunnett's test, Kruskal-Walis's *H* test, Steel's test	7
3	Bartlett's test, ANOVA, Dunnett's test, Kruskal-Walis's *H* test, non-parametric type Dunnett's test: Three groups or more; Student or Aspin-Welch's *t*-test: Two groups	9
4	Bartlett's test, ANOVA, Dunnett's test, Scheffé's test, Kruskal-Walis's *H* test, Non-para type Dunnett's test, non-parametric type Scheffe's test: Three groups or more; Student or Aspin-Welch's *t*-test: Two groups	10
5	Bartlett's test, NOVA, Dunnett's test, Duncan's test, Kruskal-Walis's *H* test, non-parametric type Dunnett's test	9
6	Bartlett's test, Dunnett's test, Steel's test	20
7	Bartlett's test, Dunnett's test, non-parametric type Dunnett's test	10
8	Bartlett's test, ANOVA, Dunnett's test, Scheffé's test, Kruskal-Walis's *H* test, non-parametric type Dunnett's test, non-parametric type Scheffé's test	23
9	Bartlett's test, ANOVA, Dunnett's test, Kruskal-Walis's *H* test, Mann-Whitney's *U* test	14
10	Bartlett's test, ANOVA (p=0.10), Dunnett's test, Kruskal-Walis's *H* test (p=0.10), Mann-Whitney's *U* test, When compared with control setting (p=0.05)	1
11	Bartlett's test, Dunnett's test, Steel's test	3
12	Bartlett's test, ANOVA, Dunnett's test, Kruskal-Walis's *H* test, non-parametric type Dunnett's test: Three groups or more; Student's *t*-test or Mann-Whitney's *U* test: Two groups	1
13	Dunnett's test: Three groups or more; *t*-test or Mann-Whitney's *U* test: Two groups	4
14	Dunnett's or Scheffé's tests: Three groups or more; *t*-test or Mann-Whitney's *U* test: Two groups	1
15	Bartlett's test, ANOVA, Dunnett's test, Kruskal-Walis's *H* test, non-parametric type Dunnett's test	3
16	Bartlett's test, ANOVA, Dunnett's test, Jaffé's test, Kruskal-Walis's *H* test, non-parametric type Dunnett's test, non-parametric type Jaffé's test	1
17	Bartlett's test, ANOVA, Dunnett's test, Scheffé's test, Kruskal-Walis's *H* test, non-parametric type Dunnett's test, non-parametric type Scheffé's test: Three groups or more; Student's *t*-test: Two groups	1
	Jonckheere's trend test (Not included in the number of tools)	8
	Total	**122**

Statistical tools used in short-term toxicity studies.

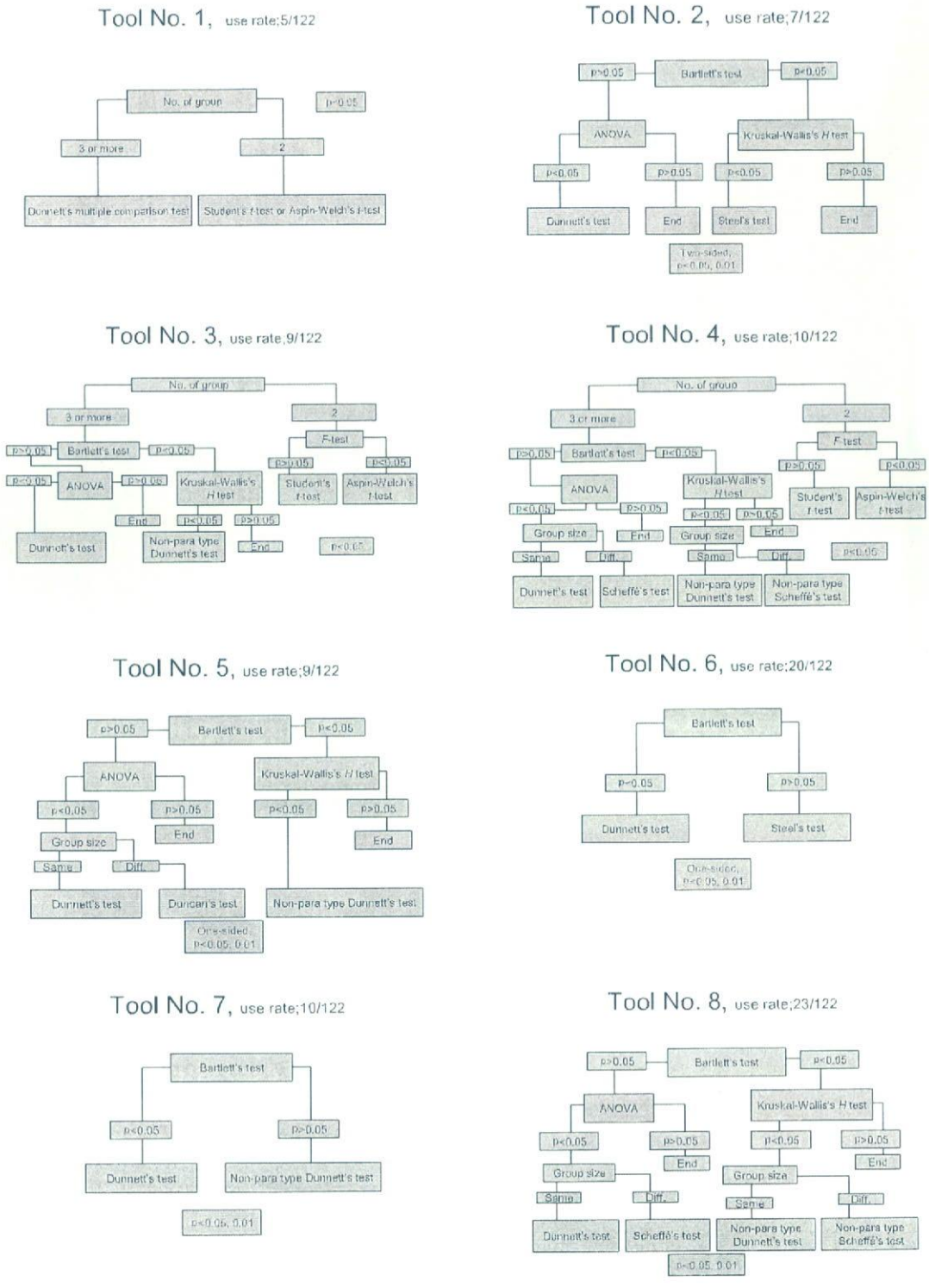

Fig. 1. Classification of number of studies based on the statistical tools used for the analysis of quantitative data.

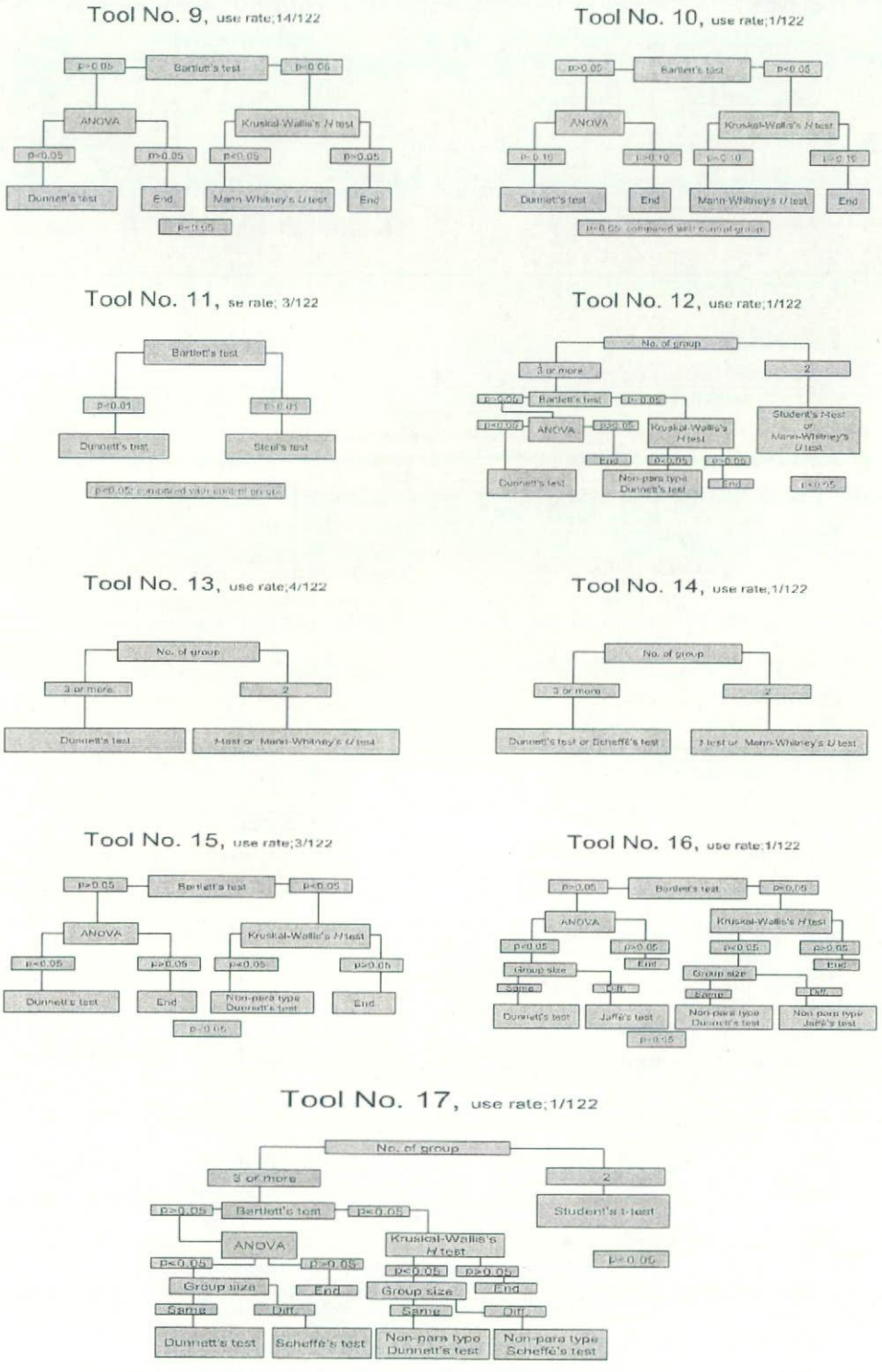

Fig. 1. Continued.

be noted that use of ANOVA causes the error of the second kind. Because of this, some of the recent studies skipped ANOVA in the decision tree and straight away used the statistical tools for *post hoc* comparison (Sumida *et al.*, 2006; Nagano *et al.*, 2006).

For the analysis of qualitative data, chi-square and Fisher's tests do not seem to be appropriate, though Fisher's test is slightly more sensitive than the chi-square test. These two tests do not detect a significant difference between a finding in the dosage group and control group, when all the animals (5/5) show the finding in the dosage group and 2 animals in the control group (2/5). On the other hand, Mann-Whitney's *U* test, which converts the scores into numerical values, detects a significant difference, when the finding in the dosage group is 5/5 and control group is 2/5. Therefore, Mann-Whitney's *U* test has better sensitivity to analyze qualitative data than the chi-square and Fisher's tests. Trend test like Jonckheere test can be used to determine no observed adverse effect level/ no observed effect level (NOAEL/NOAL) in the twenty-eight-day repeated dose oral toxicity tests. The statistical tools used, especially in the case of non-parametric tests, to determine the NOAEL/NOAL may be clearly elaborated in the study report.

We propose Dunnett's test for the analysis of quantitative data obtained from twenty-eight-day repeated dose oral toxicity tests in rodents and for qualitative data, Mann-Whitney's *U* test. For both tests, one-sided test with p=0.05 may be applied.

Table 2. A classification of number of studies based on the statistical tools used for the analysis of qualitative data.

Tool. No.	Description of statistical tools		Number of studies
1	Scored data	Frequency data	6
	Mann-Whitney's *U* test (two-sided, $p<0.05$)	Fisher's test (one-sided, $p<0.05$)	
2	Urinalysis	Pathological findings	7
	Cumulated Chi-square test (two-sided, $p<0.05$, $p<0.01$)	Mann-Whitney's *U* test (two-sided, $p<0.05$, $p<0.01$)	
3	Urinalysis	Pathological findings	13
	Cumulated Chi-square test ($p<0.05$)	Mann-Whitney's *U* test (two-sided, $p<0.05$)Fisher's test (one-sided test, $p<0.05$)	
4	Pathological findings		26
	Fisher's test (one-sided test, $p<0.05$)		
5	Pathological findings		19
	Chi-square test ($p<0.05$)		
6	FOB, urinalysis and differential leucocytes		15
	Kruskal-Wallis's *H* test, Mann-Whitney's *U* test ($p<0.05$)		
7	Urinalysis and pathological findings		9
	Mann-Whitney's *U* test (two-sided, $p<0.05$, $p<0.01$)		
8	Pathological findings		1
	Fisher's test		
9	FOB, sense function test and macroscopic and microscopic findings of pathology		1
	Wilcoxon rank-sum test, Fisher's test and Mann-Whitney's *U* test ($p<0.05$, $p<0.01$)		
10	Pathological findings		4
	Nonparametric type Dunnett's test or non-parametric type Scheffe's test, and Cochran-Armitage's trend test		
11	FOB, sense function test and macroscopic and microscopic findings of pathology		21
	No statistical tool mentioned		
Total			122

Table 3. Use of one-sided or two-sided test for short-term repeated dose administration toxicity studies with rats.

Data	One-sided	Two-sided	No mentioned	Total
Quantitative	22	13	87	122
Qualitative	34	22	70	126

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REFERENCES

Dunnett, C.W. (1964): New tables for multiple comparisons with a control. Biometrics, September, 482-491.

Finney, D.J. (1995): Thoughts suggested by a recent paper: Questions on non-parametric analysis of quantitative data (Letter to editor). J. Toxicol. Sci., **20**, 165-170.

Hamada, C., Yoshino, K., Matsumoto, K., Nomura, M. and Yoshimura, I. (1998): Three-type algorithm for statistical analysis in chronic toxicity studies. J. Toxicol. Sci., **23**, 173-181.

Inaba, T. (1994): Problem of multiple comparison tests for evaluation of enzyme inhibitor X1. Bull. Jap. Soc. Biopharm. Stat., **40**, 33-36.

Jonckheere, A. R. (1954): A distribution-tree k-sample test against ordered alternatives. Biometrika, **41**, 133-145.

Kobayashi, K., Watanabe, K. and Inoue, H. (1995): Questioning the usefulness of the nonparametric analysis of quantitative data by transformation into ranked data in toxicity studies. J.Toxicol. Sci., **20**, 47-53.

Kobayashi, K. (1997a): A comparison of one- and two-sided tests for judging significant differences in quantitative data obtained in toxicological bioassay of laboratory animals. J. Occup. Health, **39**, 29-35.

Kobayashi, K., Ohori, K., Kobayashi, M. and Takeuchi, H. (1997b): Choice of method for statistical analysis of quantitative data obtained from toxicological studies. San Ei Shi, **39**, 86-92 (in Japanese).

Kobayashi, K., Ohori, K., Kanamori, M. and Takeuchi, H. (1997c): Comparison to Dunnett and *t*-test for two groups. Jap. Soc. Occup. Health, Tokai Branch, pp.56-57 (No. 203).

Kobayashi, K., Miura, D., Ohori, K., Kobayashi, M., Kanamori, M. and Takeuchi, H. (1997d): Statistics for grade data of pathological findings. 43th. Tokai-koshueisei-gagai, (No. 3-03).

Kobayashi, K., Miura, D., Watari, N., Yamamoto, T., Shoji, A., Kitajima, S., Shiya, M., An, R., Sho, H. and Takasaki, K. (1998): A view of Bartlett test for small sized animal setting. Shizuoka Experimental Animals Research Association, **25**, 8-13 (in Japanese).

Kobayashi, K., Kanamori, M., Ohori, K. and Takeuchi, H. (2000a): A new decision tree method for statistical analysis of quantitative data obtained in toxicity studies on rodent. San Ei Shi, **42**, 125-129 (in Japanese).

Kobayashi, K. (2000b): Trends of the decision tree for selecting hypothesis-testing procedures for the quantitative data obtained in the toxicological bioassay of the rodents in Japan. J. Environ. Biol., **21**, 1-9.

NIH(2006): http://ntp.niehs.nih.gov/index.cfm?objectid=084801F0-F43F-7B74-0BE549908B5E5C1C

MHLW (2006): http://wwwdb.mhlw.go.jp/ginc/html/db1-j.html

Nagano, K., Umeda, Y., Senoh, H., Gotoh, K., Arito, H., Yamamoto, S. and Matsushima, T. (2006): Carcinogenicity and chronic toxicity in rats and mice exposed by inhalation to 1, 2-dichloroethane for two years. J. Occup. Health, **48**, 424-436.

OECD (1995): Organization for Economic Cooperation and Development. OECD Guideline for the Testing of Chemicals. 407 Adopted 27.07.95. Repeated Dose 28-day Oral Toxicity Study in Rodents. Geneva.

Sakaki, H., Igarashi, S., Ikeda, T., Imamizo, K., Omichi, T., Kadota, M., Kawaguchi, T., Takizawa, T., Tsukamoto, O., Terai, K., Tozuka, K., Hirata, J., Handa, J., Mizuma, H., Murakami, M., Yamada, M. and Yokouchi, H. (2000): Statistical method appropriate for general toxicological studies in rats. J. Toxicol. Sci., **25**, 71-98 (in Japanese).

Sano, M. and Okayama, Y. (1990): Programs of Dunnett's multiple comparison test by one and two sided with high accuracy table. Bull. Jap. Soc. Biopharm. Stat., **32**, 21-44 (in Japanese).

Sumida, K., Saito, K., Oeda, K., Otsuka, M., Tsujimura, K., Miyaura, H., Sekijima, M., Nakayama, K., Kawano, Y., Kawakami, Y., Asamoto, M. and Shirai, T. (2006): Optimization of an animal test protocol for toxicogenomics studies (II). J.Toxicol. Sci., **32**, 47-56.

USEPA (2000): United States Environmental Protection Agency. Health Effects Test Guidelines. OPPTS 870. 3050. Repeated Dose 28-Day Oral Toxicity Study in Rodents. Washington, D.C., USA.

USFDA (2000): United States Food and Drug Administration. Short-Term Toxicity Studies with Rodents. Toxicological Principle for the Safety Assessment of Food Ingredients. Redbook 2000. Rockville, USA.

Yamazaki, M., Noguchi, Y., Tanda, M. and Shintani, S. (1981): Statistical method appropriates for general toxicological studies in rats. J. Takeda Res. Lab., **40** (3/4), 163-187 (in Japanese).

Yoshimura, I. and Ohashi, S. (1992): Statistical Analysis for Toxicology Data. pp23, Chijin-shokan, Tokyo (in Japanese).

Yoshimura, I. (1987): In "Statistical analysis of toxicological data", pp45, Scientist, Tokyo (in Japanese).

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Potassium bromate treatment predominantly causes large deletions, but not GC > TA transversion in human cells

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Abstract

Potassium bromate ($KBrO_3$) is strongly carcinogenic in rodents and mutagenic in bacteria and mammalian cells in vitro. The proposed genotoxic mechanism for $KBrO_3$ is oxidative DNA damage. $KBrO_3$ can generate high yields of 8-hydroxydeoxyguanosine (8OHdG) DNA adducts, which cause GC > TA transversions in cell-free systems. In this study, we investigated the in vitro genotoxicity of $KBrO_3$ in human lymphoblastoid TK6 cells using the comet (COM) assay, the micronucleus (MN) test, and the thymidine kinase (*TK*) gene mutation assay. After a 4 h treatment, the alkaline and neutral COM assay demonstrated that $KBrO_3$ directly yielded DNA damages including DNA double strand breaks (DSBs). $KBrO_3$ also induced MN and *TK* mutations concentration-dependently. At the highest concentration (5 mM), $KBrO_3$ induced MN and *TK* mutation frequencies that were over 30 times the background level. Molecular analysis revealed that 90% of the induced mutations were large deletions that involved loss of heterozygosity (LOH) at the *TK* locus. Ionizing-irradiation exhibited similar mutational spectrum in our system. These results indicate that the major genotoxicity of $KBrO_3$ may be due to DSBs that lead to large deletions rather than to 8OHdG adducts that lead to GC > TA transversions, as is commonly believed. To better understand the genotoxic mechanism of $KBrO_3$, we analyzed gene expression profiles of TK6 cells using Affymetrix Genechip. Some genes involved in stress, apoptosis, and DNA repair were up-regulated by the treatment of $KBrO_3$. However, we could not observe the similarity of gene expression profile in the treatment of $KBrO_3$ to ionizing-irradiation as well as oxidative damage inducers.

Keywords: Potassium bromate ($KBrO_3$); *TK*-mutation; Loss of heterozygosity (LOH); 8-Hydroxydeoxyguanosine (8OHdG); Gene expression profile

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1. Introduction

Potassium bromate ($KBrO_3$) is used as in bread making a flour improver and in the production of fish-pastes. The EU countries now prohibit its use as a food additive because of its carcinogenicity. Japan and the USA, however, permit its use in bread making on the condition that it never remains in the final product. $KBrO_3$ causes tumors, especially in kidney, in rats, and mice after long-term oral administration in drinking water [1–3]. $KBrO_3$ is also genotoxic. It is positive in in vitro genotoxicity tests – including the bacterial reverse mutation assay [1], the chromosomal aberration test conducted in Chinese hamster cells [4], and the mouse lymphoma assay [5] – and in vivo in the micronucleus test (MN) [6,7].

It has been proposed that $KBrO_3$ induces tumors through the production of oxidative damage to DNA. Oxidative DNA damage can cause mutations that contribute to the activation of oncogenes and/or the inactivation of tumor suppressor genes, thereby leading to tumorigenesis [8,9]. 8-Hydroxydeoxyguanosine (8OHdG) is the main form of oxidative DNA damage induced by $KBrO_3$ [10]. It primarily causes GC>TA transversions (as a result of the pairing of 8OHdG with A) and is believed to be responsible for mutagenesis, carcinogenesis, and aging [11,12]. $KBrO_3$ increases 8OHdG DNA adducts in vivo and in vitro [13–15]. However, $KBrO_3$ induces mutations weakly in microbial mutation assays and the *Hprt* mutation assay in mammalian cells, while it induces chromosome aberrations strongly both in vivo and in vitro [1,16,17]. These findings raise the question of whether 8OHdG is required for the mutagenic process involved in $KBrO_3$-induced carcinogenesis.

In the present study, we examined the genotoxic properties of $KBrO_3$ using the comet assay (COM), the MN test, and thymidine kinase (*TK*) gene mutation assays in human lymphoblastoid TK6 cells [18]. Unlike the X-linked hemizygous *HPRT* gene mutation assay, the *TK* mutation assay can detect not only point mutations, but also large scale chromosomal deletions, recombinations, and aneuploidy [19–21]. Most of the genetic changes observed in *TK* mutants occur in human tumors and are presumed relevant to carcinogenesis. We analyzed the *TK* mutants induced by $KBrO_3$ at the molecular level and investigated what kind of mutation predominated. We also profiled global gene expression in TK6 cell exposed to $KBrO_3$ using Affymetrix GeneChip® Expression analysis to understand the genotoxic mechanism of $KBrO_3$.

2. Materials and methods

2.1. Cell culture, chemicals, and treatment

The TK6 human lymphoblastoid cell line has been described previously [22]. Cells were maintained in RPMI 1640 medium (Gibco-BRL, Life Technology Inc., Grand Island, NY) supplemented with 10% heat-inactivated horse serum (JR Biosciences, Lenexa, KS), 200 μg/ml sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cultures were incubated at 37 °C in a 5% CO_2 atmosphere with 100% humidity. $KBrO_3$ (CAS No.7758-01-2) was purchased from Wako Pure Chemical Co. (Tokyo) and dissolved in RPMI medium just before use.

We prepared 20 ml aliquots of cell suspension at a concentration of 5.0×10^5 cells/ml in 50 ml polystyrene tubes. Different concentrations of $KBrO_3$ were added to the tubes, which were then placed on a platform shaker and incubated at 37 °C for 4 h with gentle shaking. At the end of the treatment period, the cell cultures were centrifuged, washed once, and re-suspended in fresh medium. We cultured them in new flasks for the MN assay and *TK* gene mutation assay, or diluted them for plating for survival estimates.

2.2. Genotoxicity assays

After treating cells with $KBrO_3$, we prepared slides for conducting the alkaline and neutral COM assay. The alkaline COM assay was performed as previously reported [23]. For the neutral COM assay, the slide was electrophoresed with chilled neutral solution (pH 8) containing of 90 mM Tris, 2 mM Na_2EDTA, and 90 mM boric acid according to the method by Wada et al. [24]. The COM slides were stained with SYBER green (Molecular Probes, Eugene, OR) and observed by an Olympus model BX50 fluorescence microscope. At least 50 cells were captured by CCD camera, and tail length of the comet was measured. The relationship between $KBrO_3$ treatment and migration was statistically analyzed by the Dunnett test [25].

We prepared the MN test samples 48 h after treatment, as previously reported [23]. Briefly, approximately 10^6 cells suspended in hypotonic KCl solution were incubated for 10 min at room temperature, fixed twice with ice-cold methanol containing 25% acetic acid, then re-suspended in methanol containing 1% acetic acid. A drop of the suspension was placed on a clean glass slide and air-dried. The cells were stained with 40 μg/ml acridine orange solution and immediately observed with the aid of an Olympus model BX50 fluorescence microscope equipped with a U-MWBV band pass filter. At least 1000 intact interphase cells for each treatment were examined, and the cells containing MN were scored. The MN frequencies between non-treated and treated cells were statistically analyzed by Fisher's exact test [26].

We prepared the *TK* gene mutation assay samples 3 days after treatment. We seeded cells from each culture into 96-well plates at 40,000 cells/well in the presence of 3.0 μg/ml trifluo-

rothymidine (TFT). We also plated 1.6 cells/well without TFT to determine plating efficiency. All plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. After 14 days, we scored colonies on the PE plates and the normal-growing (NG) *TK* mutants on the TFT plates, then re-fed the plates containing TFT with fresh TFT, incubated them for an additional 14 days, and scored them for slow-growing (SG) *TK* mutants. Mutation frequencies, relative survival (RS), and relative suspension growth (RSG) were calculated as previously described [23]. The data of mutant frequencies were statistically analyzed by Omori's method, which consists of a modified Dunnett's procedure for identifying clear negative, a Simpson–Margolin procedure for detecting downturn data, and a trend test to evaluate the dose-dependency [27].

2.3. LOH analysis of TK mutations by polymerase chain reaction (PCR)

To avoid analyzing identical mutants, we performed an additional *TK* mutation assay and isolated *TK* mutants from independent culture after a 4 h treatment with 2.5 mM $KBrO_3$. We confirmed the phenotype of the *TK* mutant clones by re-challenging them with TFT medium. We also determined the growth rate of the clones and confirmed whether they were NG or SG mutants.

Genomic DNA was extracted from the *TK* mutant cells and used as a template for PCR. We conducted the PCR-based LOH analysis of the human *TK* gene as described previously [28]. A set of primers was used to each amplify the parts of exons 4 and 7 of the *TK* gene that is heterozygous for frame shift mutations. A third primer set for amplifying parts of the β-globin was also used as the internal control. We applied quantitative-multiple PCR for co-amplification of the three regions. The PCR products were analyzed with an ABI310 genetic analyzer (PE Biosystems, Chiba, Japan), and were classified into "no LOH", "hemizygous (hemi-) LOH", or "homozygous (homo-) LOH". To determine the extent of the LOH, we analyzed 10 microsatellite loci on chromosome 17q by PCR-based LOH analysis [28]. The results were processed by GenoTyper™ software (PE Biosystems, Chiba, Japan) according to the manufacturer's guidelines.

2.4. Gene expression analysis

Total RNA was isolated from the TK6 cells after 4 h treatment with 2.5 mM $KBrO_3$ and was purified by RNeasy columns (Qiagen, Valencia, CA). We conducted a single cDNA synthesis, cRNA labeling, and cRNA fragmentation according to the manufacturer's recommendations (Affymetrix Inc., Santa Clara, CA) and employed Affymetrix GeneChip Expression analysis. The hybridization mixture for each sample was hybridized to an Affymetrix U133A human genome array. We processed the scanned data using Microarray Suite Software Version 5.0 (Affymetrix Inc., Santa Clara, CA) and imported the data into GeneSpring software (Silicon Genetics, Redwood City, CA). Signal intensity was normalized by per-gene and per-chip, and the ratios were calculated by normalizing $KBrO_3$ sample to the corresponding control sample. We used intensity-dependent (step-wise) selection of significant changes with higher cut-off value for lower signal intensity (1.75-, 2.0-, 2.25-, 2.5-, and 3.5-fold for genes intensity range of >1000, 500–1000, 100–500, 50–100, and 10–50, respectively), and up-regulated genes with a presence call in $KBrO_3$ sample, whereas down-regulated genes with a presence call in the control sample.

3. Results

3.1. Cytotoxicity and genotoxicity of $KBrO_3$

$KBrO_3$ exerted strong and concentration-dependent cytotoxicity in TK6 cells (Fig. 1). It induced approximately 50% cytotoxicity (51% RSG and 44% RS) at 2.5 mM. To investigate whether $KBrO_3$ directly causes DNA damage, we conducted the COM assay. Induction of COM tail after the treatment of in alkaline version was statistically significant 2.5 and 5 mM. In the neutral COM assay, the induction was observed form the lower concentration (Fig. 1). Because the neutral COM is thought to be associated with DNA double strand breaks (DSBs) [29], this result indicates that $KBrO_3$ directly causes DNA damage including DSBs. $KBrO_3$ also induced MN and *TK* mutation in a concentration-dependent manner and their inductions were statistically significant (Fig. 1). At the maximum concentration, it induced both MN and *TK* mutation frequencies about 30 times the control values. Two distinct phenotypic classes of *TK* mutants were generated: NG mutants grew at the same rate as the wild type (doubling time 13–17 h), and SG mutants grew at a slower rate (doubling time > 21 h). NG mutants result from intragenic mutations, while SG mutants result from gross changes (extending beyond the *TK* gene) [20]. $KBrO_3$ predominantly induced SG mutants (Fig. 1), implying that $KBrO_3$ treatment predominantly causes gross structural changes, but not small genetic alterations such as point mutations.

3.2. Molecular analysis of TK mutants

The *TK* mutants were randomly isolated from independent cultures treated with 2.5 mM $KBrO_3$ for 4 h. Table 1 shows the cytotoxicity (RSG), mutation frequency, and proportion of SG mutants induced by $KBrO_3$. We subjected 40 induced mutants to LOH analysis. Of those, 32 (80%) were SG mutants, which corresponded closely to the percentage of SG mutants induced in the assay (74.1%), indicating that the result of LOH analysis reflected the character of the induced

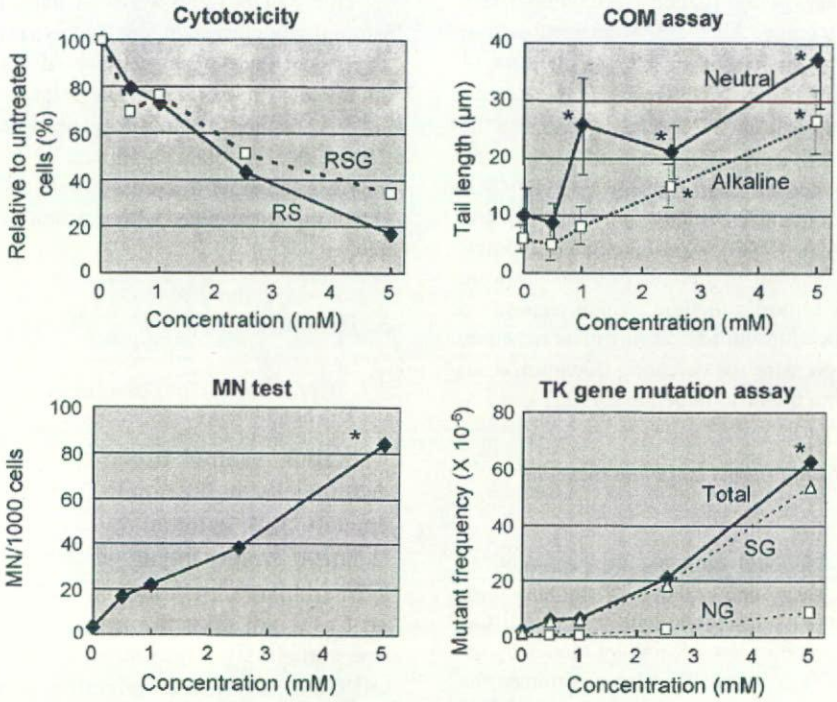

Fig. 1. Cytotoxic (relative survival, RS; relative suspension growth, RSG) and genotoxic responses (COM assay, MN test, and *TK* gene mutation assay) of TK6 cells treated with $KBrO_3$ for 4 h. Asterisk (*) statistically significant in Dunnett's test ($P < 0.05$) in COM assay, and in both pair-wise comparison and trend test ($P < 0.05$) in MN test and *TK* gene mutation assay.

mutations. Table 1 also shows the results of LOH analysis of the induced and spontaneously occurring mutants. The result of molecular analysis of spontaneous *TK* mutants was reported previously [21]. We classified the mutants into three types: non-LOH, hemizygous LOH (hemi-LOH), and homozygous LOH (homo-LOH). In general, hemi-LOH is resulted by deletion and homo-LOH is by inter-allelic homologous recombination [20]. Among the $KBrO_3$-induced mutants, 63% of NG mutants and 84% of SG mutants were hemi-LOH. In spontaneous mutants, on the other hand, majority of NG and SG mutants were non-LOH and homo-LOH, respectively. These results indicated that $KBrO_3$ predominantly induced large deletions. We previously reported the mutational spectra of *TK* mutants in TK6 cells that treated with the alkylating agent ethylmethane sulfonate (EMS), or X-irradiated [20,21]. Fig. 2 shows the comparison of the mutational spectra of spontaneous and induced *TK* mutants by EMS, X-irradiation, and $KBrO_3$. The mutation spectrum induced by $KBrO_3$ was similar to that induced by X-radiation (which also induces LOH, predominantly via deletion [21]) but not by EMS. The majority of the mutations induced by $KBrO_3$ were large deletions, but not point mutations.

Fig. 3 shows the regions of LOH and the distribution of spontaneous, X-ray-induced, and $KBrO_3$-induced

Table 1
Cytotoxic and mutational responses to $KBrO_3$, and the results of LOH analysis of normally growing (NG) and slowly growing (SG) *TK* mutants

Treatment	Cytotoxic and mutational response			LOH analysis at *TK* gene (%)			
	RSG (%)	MF ($\times 10^{-6}$)	% SG	Number	Non-LOH	Hemi-LOH	Homo-LOH
Spontaneous[a]	100	2.19	56	56			
NG mutants				19	14 (74)	3 (16)	2 (11)
SG mutants				37	0 (0)	9 (24)	28 (76)
$KBrO_3$ (2.5 mM)	51	29.4	74	39			
NG mutants				8	3 (37)	5 (63)	0 (0)
SG mutants				31	1 (3)	27 (84)	4 (13)

[a] Data from Zhan et al. [22].