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Heterogeneity in human tumour hypoxic fraction using the comet assay

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Summary The alkaline comet assay has previously been used to estimate the fraction of radiobiologically hypoxic cells in tumours from patients undergoing palliative radiotherapy for advanced breast and head and neck cancer. Results obtained from fine needle aspirate biopsies (FNABs) using this method indicate considerable heterogeneity in hypoxic fraction between tumours. Heterogeneity between 3 aspirates taken from the same 10 tumours immediately following single doses of 3.5 to 5 Gy is now examined. Results indicate that a single fine needle aspirate is reasonably representative for DNA damage and DNA content. However, difficulties were encountered in obtaining an adequate sample of tumour cells after the final radiation treatment. The average hypoxic fraction decreased from 14% after the first dose to 9% after the last dose, and in 3 tumours which could be evaluated after both the first and last fraction, the hypoxic fraction decreased in two but increased in the third. Rejoining of DNA strand breaks was observed between sequential aspirates indicating that pooling of samples for analysis may not be advisable using this method.

Keywords: human tumour hypoxia; comet assay; reoxygenation

The alkaline comet assay, a method based on gel electrophoresis of individual cells, has previously been used to provide a measure of hypoxic fraction in advanced breast cancers and head and neck tumours of patients undergoing palliative radiotherapy (Olive *et al.*, 1993*a*). The comet assay detects DNA single-strand breaks with excellent sensitivity (Fairbairn et al., 1995), and it is this property which is used to detect cells which are radiobiologically hypoxic at the time of treatment (Olive and Durand, 1992). Hypoxic cells sustain about three times less DNA single-strand breaks than well-oxygenated cells, and moreover, the relation between oxygen concentration and DNA damage is the same as the relation between oxygen concentration and cell killing (Chapman *et al.*, 1974; Zhang *et al.*, 1995). While microelectrodes are considered the 'gold standard' for measuring human tumour oxygenation, the comet method has several important advantages over this and other methods used to estimate tumour oxygenation: (1) it is the only method which is currently able to estimate the fraction of radiobiologically hypoxic cells present in solid human tumours at the time of irradiation, (2) the response of individual cells (not necrotic material) is measured, (3) hypoxic fraction is measured at the time of irradiation so it will reflect tumour perfusion at that time and (4) hypoxic fraction measured by this method is independent of cell size, energy status, or bioreductive capacity. As with other methods, however, it does not provide an indication of the clonogenic potential of the hypoxic cells which is the true measure of radiobiological hypoxic fraction.

Previous results obtained from fine needle aspiration biopsy (FNAB) indicate considerable heterogeneity in hypoxic fraction between tumours, with values ranging from $<1\%$ to greater than 40% hypoxic cells (Olive *et al.*, 1993*a*). We now address two questions: (1) can FNAB provide a sample representative of the tumour and (2) can the comet assay be applied to detect changes in hypoxic fraction following radiotherapy? In previous studies, several FNABs were pooled in an effort to improve the reliability of the measurement. However, by analysing these aspirates independently, information can be obtained on degree of intratumour heterogeneity in DNA content and hypoxic fraction.

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Methods

FNAB was performed on ten patients undergoing palliative radiotherapy primarily for advanced breast cancer (5), head and neck cancer (2), metastatic renal cell carcinoma (1), metastatic lung cancer (1) and metastatic leiomyosarcoma (1). Immediately following the first and last doses of 3.5-5 Gy fractions delivered over 4-14 days, 3 aspirates were taken from the superior, medial, and inferior regions of the tumour nodule. In all cases, less than 5 min elapsed from the end of radiation to the end of aspiration. Additional results from earlier studies in which tumours received 1-10 Gy were also analysed; the low doses are from samples taken following irradiation of the first field in a 2-3 field treatment plan. The needles were rinsed in phosphate-buffered saline (PBS) on ice, and without further manipulation, approximately 2×10^4 single cells or nuclei were embedded in 0.75% low gelling temperature agarose and pipetted onto a microscope slide. Slides were placed in lysis solution for 1 h (1M NaCl, 0.03M NaOH and 0.2% sarkosyl), then rinsed in 0.03 M NaOH, 2 mM EDTA for 1 h to remove salt and detergent and electrophoresed in 0.03M NaOH, 2 mM EDTA for 25 min at 0.6 volts cm^{-1} (i.e. 20 volts for our gel electrophoresis unit in which the electrodes are 33 cm apart). Comets were stained with 2.5 $\mu g\ ml^{-1}$ propidium iodide for 15 min, and comet images (800-1000 per aspirate) were collected and analysed using a fluorescence image processing system as previously described (Olive and Durand, 1992; Olive *et al.*, 1993*a*). Total image fluorescence was taken as a measure of DNA content, and mean or modal tail moment was used as an indicator of DNA damage. Hypoxic fraction was determined by iterative fitting of histograms of tail moment with two normal distributions representing the aerobic and hypoxic populations (Olive and Durand, 1992; Olive *et al.*, 1993*a*,*b*; Olive, 1994).

Results

Variability in DNA damage following irradiation

In Figure 1a, DNA damage measured for approximately 25 human tumour aspirates is compared with dose-response curves obtained for 3 cultured cell lines and cells from 5 murine normal tissues irradiated on ice to inhibit strand break rejoining. Modal tail moment is shown rather than mean tail moment to avoid discrepancies resulting from the presence of hypoxic cells or from the impact of heavily damaged cells. The slope of the dose-response curve of the human tumours is reduced about 30% compared with cells

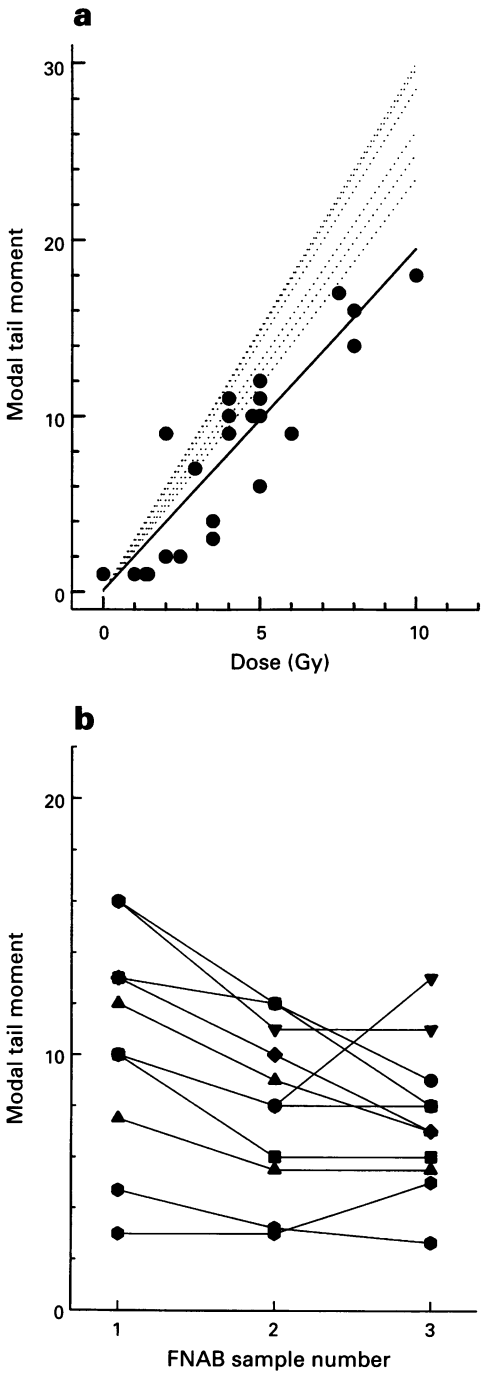

Figure 1 Modal tail moment of comets, a measure of DNA damage, for cells from FNABs taken immediately after irradiation. (**a**) The solid line is the linear best-fit through the filled circles showing the response of 22 separate human tumours. Dotted lines show the response of representative cultured cells and cells from normal tissues of mice irradiated at 4°C to inhibit repair. (**b**): Three FNABs were taken sequentially from each of 11 tumours within 5 min after radiotherapy. A P value of 0.01 was obtained using a paired t-test to compare the modal tail moment for the first and third samples.

irradiated on ice, indicative of the rapid rejoining of strand breaks occurring during and following irradiation; the half-time for rejoining of single-strand breaks for tumour cells cultured under optimum conditions is generally less than 5 min, although this value increases to 6–9 min for murine tumour cells allowed to repair *in vivo* (Olive *et al.*, 1994); a longer rejoining time may also apply to human tumours (Olive *et al.*, 1993*a*).

Rejoining of DNA strand breaks was sufficiently rapid in these human tumours that an overall decrease of about 30% in DNA damage was observed over the time required to perform FNAB (Figure 1b). Therefore the practice of pooling aspirates to obtain a more representative sample could introduce errors in using this method to detect hypoxic cells. This is illustrated in Figure 2 where 3 FNABs from the same tumour were pooled. All three samples displayed a single population in terms of DNA damage, but when pooled, the histogram was broader and could be fitted to two normal distributions displaced by a factor of 1.8 The calculated hypoxic fraction from this pooled histogram was 0.48.

Adequacy of sample

A single fine needle aspirate provided sufficient cells to perform the comet assay for all tumours following the first fraction. However, cells with either extensive DNA damage (apoptotic or necrotic), or cells with inadequate damage for the dose delivered (circulating leucocytes) were observed in 3/10 tumours following the first fraction and 5/10 tumours after the last fraction.

Heterogeneity between aspirates

Cells from only 3/10 tumours showed obvious heterogeneity in DNA content between the 3 aspirates (e.g. Figure 3, patients 1 and 4). For the remaining tumours, a single aspirate appeared reasonably representative. Five of nine tumours showed an accumulation of cells in the G_2 phase of the cell cycle at the end of treatment relative to the same tumour at the beginning of treatment (e.g. Figure 3, patients 2, 3, and 5).

Hypoxic fraction

After the first dose, 6 tumours evaluated for hypoxic fraction gave a mean and standard deviation of 0.14 ± 0.09. Following the last dose, the hypoxic fraction for 6 tumours was 0.09 ± 13. However, only 3/10 tumours provided adequate samples for measurement of hypoxia both before and after treatment. For 2 tumours containing an average of 12% and 29% hypoxic cells after the first treatment, the hypoxic fraction was negligible following radiotherapy. Figure 4 shows DNA content and hypoxic fraction estimated after the first and last dose for one of these patients. For the third tumour, the percentage of hypoxic cells increased from 11% after the first dose to 29% after the last treatment. The displacement between the peaks for the aerobic and hypoxic distributions averaged 2.5 ± 0.46 ($n = 10$) and included 2 tumours with a displacement of only 1.9–2.0, one of which is shown in Figure 4.

Discussion

The ability of FNAB to provide a representative tumour sample has often been questioned, although it is generally agreed that fine needle aspiration has several advantages over other biopsy methods as it is simple, safe and cost-effective (e.g. Ljung *et al.*, 1994). It is also recognised that experience in performing fine needle aspirates is essential to obtain the best quality biopsy, and multiple biopsies are preferred (e.g. Lee *et al.*, 1993). The process of FNAB can and should involve movement of the needle while aspirating, a practice which will sample a much larger segment of the tumour. In fact, for most of these advanced tumours examined after the first radiation dose, a single fine needle aspirate provided sufficient numbers of tumour cells, and a single aspirate was representative of that tumour. Like many other studies examining tumour heterogeneity in DNA content or oxygenation, it is clear from the results shown in Figure 3 that between-tumour variability is greater than within-tumour variability. However, in 3/9 tumours, some degree

of heterogeneity in DNA content and/or DNA damage between aspirates was observed so that any single aspirate would not be completely representative.

Pooling of aspirates to obtain a more representative sample should be avoided; a histogram prepared from pooled samples could lead to inaccuracies when measuring

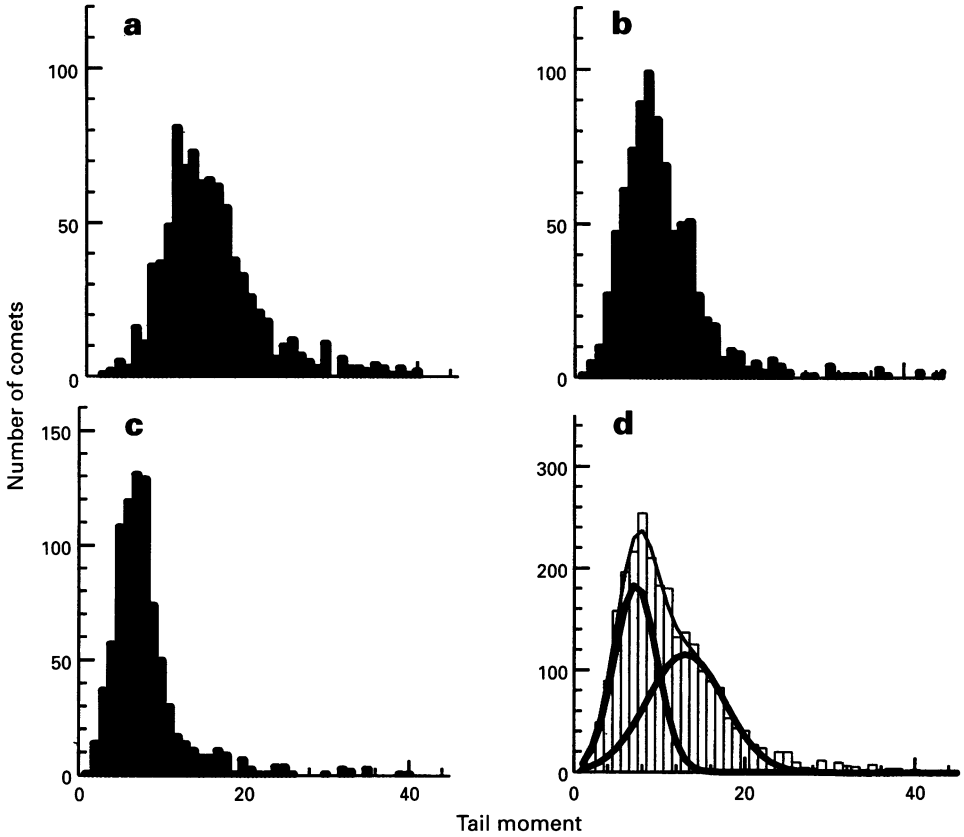

Figure 2 Effect of pooling data from tumour aspirates. (**a**–**c**) The response of 3 FNABs taken sequentially from the same chest wall recurrence of a metastatic breast cancer following irradiation with 4 Gy; none of these samples shows a measurable hypoxic fraction. (**d**) This histogram shows results pooled from panels **a**–**c**, assuming equal numbers of cells per aspirate. Data from this histogram were then fitted to 2 normal distributions as indicated by the lines. The best-fit gave a displacement between the aerobic and hypoxic populations of 1.8 and a hypoxic fraction of 0.47.

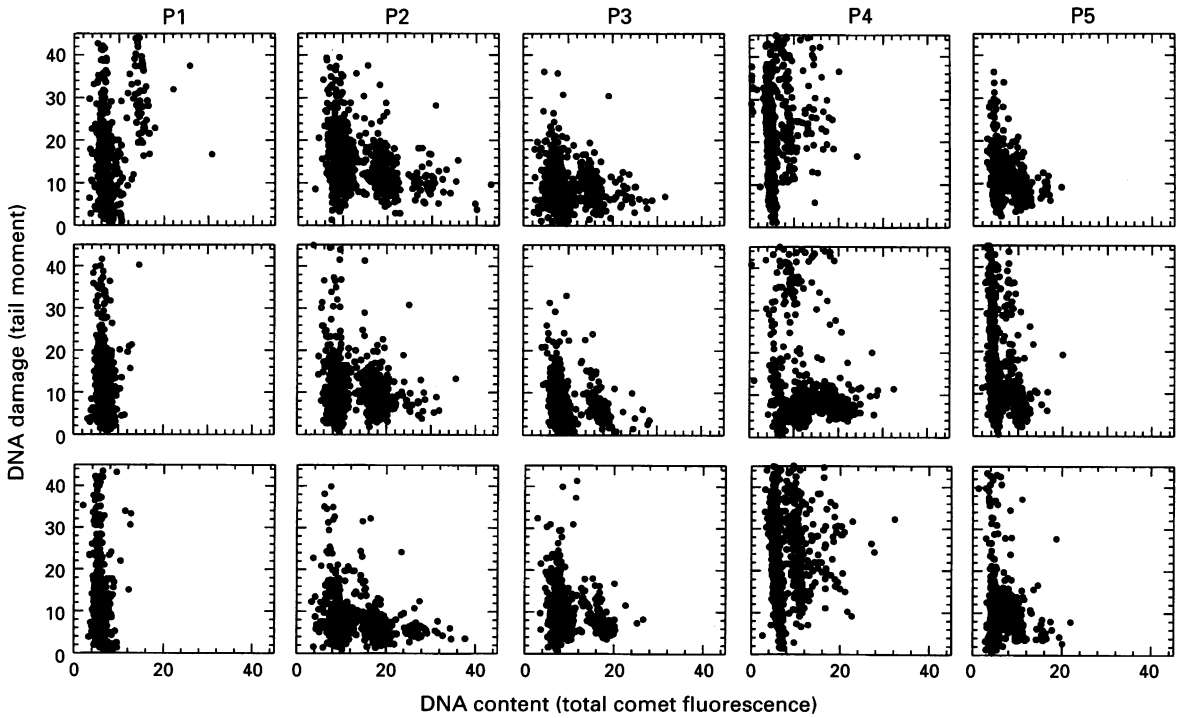

Figure 3 Heterogeneity in response of cells from 3 sequential fine needle aspirates from 5 representative patients. Tumours from patients 1 and 4 were sampled immediately after the first dose. Tumours from patients 2, 3 and 5 were sampled after the last fraction. Each symbol shows the response of an individual cell in the alkaline comet assay.

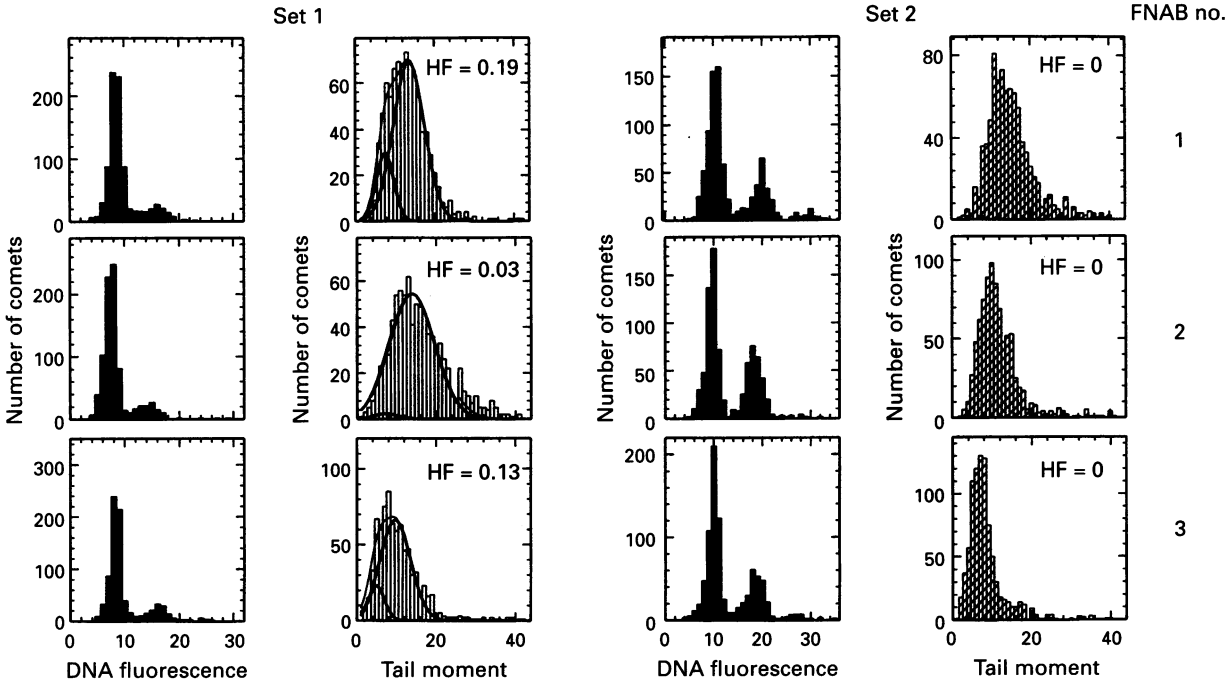

Figure 4 Change in hypoxic fraction after radiotherapy. FNAB was performed on 3 different areas of a breast cancer chest wall recurrence after the first and last treatments (5 × 4 Gy given over 7 days). Note the increase in the proportion of cells in G_2 phase at the end of treatment in response to damage by radiation, and the decrease in the widths of the tail moment histograms after the last treatment.

hypoxic fraction because cells determined to be hypoxic based on low numbers of strand breaks may simply be those cells sampled last and thus able to repair more strand breaks before biopsy (Figure 2). Little rejoining of strand breaks occurs after FNAB, even for cells/nuclei returned to complete medium at 37°C (Olive *et al.*, 1993*b*), however the half-time of rejoining of strand breaks *in vivo* is apparently rapid and appears consistent with rates measured in the SCCVII murine tumour (Olive *et al.*, 1994). To estimate the effect of pooling samples, results from 3 sequential FNABs were combined (Figure 2). However, even in this 'worst case scenario' a histogram with a typical displacement of 2.5 between the peaks of the aerobic and hypoxic distributions was not obtained. Instead the displacement was only 1.8, and the fact that the modal tail moment was the same as the position of the hypoxic peak would be suspect. In two tumours, however, one of which is shown in Figure 4, the best fit for the displacement between the aerobic and hypoxic peaks was only 1.9–2.0. This smaller displacement could be indicative of the presence of cells intermediate in hypoxia such as we see in the SCCVII murine tumour (Olive, 1994). Although the smaller displacement makes it much more difficult to obtain an accurate estimate of hypoxic fraction, the greater width of the tail moment histogram is also indicative of the presence of hypoxic cells in the first set of samples from this tumour (Figure 4).

Analysing tumour response after the final dose for some tumours was questionable for 3 reasons: lack of sufficient cells, presence of heavily damaged cells, and lack of cells blocked in G_2 (possibly indicative of the absence of cycling tumour cells in the sample). For other tumours, however, it was possible to obtain information on tumour hypoxia and DNA content from even a single fine needle aspirate biopsy. A main limitation in obtaining a measure of reoxygenation, aside from not knowing the clonogenic capacity of the hypoxic cells, was obtaining a sufficient number of tumour cells from tumours that had received 20 Gy. While this has not been a problem in measuring reoxygenation in the SCCVII murine tumours 24 h after 10 Gy (Olive 1994), or in preliminary experiments in mice using 5 × 4 Gy, examination of the potential of a human tumour for reoxygenation may have to be determined early during the course of therapy.

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References

CHAPMAN JD, DUGLE DL, REUVERS AP, MEEKER BE AND BORSA J. (1974). Studies on the radiosensitizing effect of oxygen in Chinese hamster cells. *Inter. J. Radiat. Biol.*, **26**, 383–389.

FAIRBAIRN DW, OLIVE PL AND O'NEILL KL. (1995). The comet assay: A comprehensive review. *Mutat. Res.*, **339**, 37–59.

LEE TK, WILEY AL, ESINHART JD, RILEY RS, AND BLACKBURN LD. (1993). Variations associated with disaggregations methods in DNA flow cytometry. *Anal. Quan. Cytol. Histol.*, **15**, 195–200.

LJUNG BM, CHEW K, DENG G, MATSUMURA K, WALDMAN F AND SMITH H. (1994). Fine needle aspiration techniques for the characterization of breast cancers. *Cancer*, **74**, 1000–1005.

OLIVE PL. (1994) Radiation-induced reoxygenation in the SCCVII murine tumour: evidence for a decrease in oxygen consumption and an increase in tumour perfusion. *Radiother. Oncol.*, **32**, 37—46.

OLIVE PL AND DURAND RE. (1992). Detecting hypoxic cells in a murine tumour using the comet assay. *J. Natl Cancer Inst.*, **85**, 707–711.

OLIVE PL, DURAND RE, LE RICHE J AND JACKSON SM. (1993*a*). Gel electrophoresis of individual cells to quantify hypoxic fraction in human breast cancers. *Cancer Res.*, **53**, 733–736.

OLIVE PL, JACKSON SM AND DURAND RE. (1993*b*). Predicting tumour response to radiotherapy using the comet assay. In *Prediction of Response in Radiation Therapy: Radiosensitivity and Repopulation*. Paliwal BR, Kinsella TJ, Herbert D and Fowler J. (eds) pp. 65–76. The American Institute of Physics: Woodbury.

OLIVE PL, VIKSE CM AND DURAND RE (1994). Hypoxic fractions measured in murine tumours and normal tissues using the comet assay. *Inter. J. Radiat. Oncol. Biol. Phys.*, **29,** 487–491.

ZHANG H, KOCH CJ, WALLEN CA AND WHEELER KT. (1995). Radiation-induced DNA damage in tumours and normal tissues. III. Oxygen dependence of the formation of strand breaks and DNA-protein crosslinks. *Radiat. Res.*, **142,** 163–168.