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Original Paper

Determination of Radiosensitivity in Established and Primary Squamous Cell Carcinoma Cultures Using the Micronucleus Assay

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In this study, the cytokinesis-block micronucleus assay (CBMN) was used to measure radiosensitivity in three established cell lines (SCC-61, V175 and V134) and 10 primary cell cultures of squamous cell carcinoma (SCC) of the head and neck. Assessment involved optimisation of the assay to determine cytochalasin-B (CB) concentration and sampling time postirradiation. A much closer correlation between dose–response data measured in the clonogenic and micronucleus assays was found when the micronucleus assay was performed under standardised conditions for each cell line (2 μg/ml CB: 48 h postirradiation) instead of predetermined optimised assay conditions. This indicates that, for these SCC cell lines, the CBMN assay may be able to predict *in vitro* radiosensitivity. To be of clinical use in predicting radiosensitivity, the CBMN assay also needs to be evaluated with primary cell cultures. In this study, no relationship between micronucleus frequency at 2 or 6 Gy and patient clinical outcome 12 months following surgery and radiotherapy was seen. Similarly, no association between patient outcome and tumour stage, nodal stage and histology was observed. These CBMN assay data from the primary cell cultures are presently inconclusive as a measure of patient tumour radiosensitivity. © **1997 Published by Elsevier Science Ltd. All rights reserved.**

Key words: micronucleus assay, tumour cell radiosensitivity, predictive assay, squamous cell carcinoma, DNA damage

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INTRODUCTION

ONE ASPECT of recent radiobiological research has been the development of an assay or assays capable of predicting patients response to radiotherapy, with the belief that an individualised approach to radiation therapy could be derived from data obtained from patient tumour characteristics [1, 2].

The measurement of *in vitro* radiosensitivity has been used widely to try and predict tumour radioresponsiveness [3], particularly in carcinoma of the uterine cervix [4] and the head and neck region [5, 6], using surviving fraction at 2 Gy (SF_2) as the prime indicator. West and colleagues have reported a correlation between SF_2 measurements and the outcome of cervical cancer radiotherapy using a soft agar cloning system [3]. In the case of head and neck cancers, using a population growth assay, preliminary correlations between SF_2 and local control in patients treated with surgery and postoperative radiotherapy were disappointing, although a trend was noted of greater radioresistance in tumours that failed [5]. Girinsky and associates [6], also using a population growth assay, showed no significant correlation between SF_2 and local control, although they did conclude that the linear quadratic alpha term (α), derived from the clonogenic survival curve, identified a small group of patients at a significantly higher risk of local failure. Hence, the differences associated with measurements of intrinsic tumour radiosensitivity are potentially a source of variation in local tumour control.

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To date, the assay of choice for predicting radiosensitivity from patient tumour biopsy samples has been the measurement of clonogenic cell survival using a soft agar system. However, the long duration, low plating efficiencies and clumping artefacts are major disadvantages associated with the assay [7, 8]. Speed and simplicity are important requirements of any predictive test for patient radiosensitivity. Since the early 1980s the micronucleus assay has been proposed as a potential alternative to the clonogenic assay as a predictor of radiosensitivity in a number of tumour cell types [9, 10]. The assay itself is technically simple, rapid, and requires only a period sufficient for one mitosis for results to be obtained.

Micronuclei (MN) arise from acentric chromosomal fragments or whole chromosomes which are not incorporated into the daughter nuclei at mitosis, and are observed as minute bodies excluded from the main nucleus in the cytoplasm of interphase cells. The most commonly used experimental approach is the cytokinesis-block micronucleus assay (CBMN), first described by Fenech and Morley in 1985 [11]. This technique allows identification of proliferating cells after the completion of only one mitosis by virtue of their binucleate appearance. The formation of these binucleate cells (BNC) is brought about by the addition of cytochalasin-B (CB), an inhibitor of cytokinesis [12], immediately postirradiation.

In order to establish a test for the prediction of radiosensitivity in primary tumour cultures or cultured tumour cell lines, the assay needs to be thoroughly investigated. The rate of MN induction and their expression as a function of radiation dose, CB dose and postirradiation culture time requires careful analysis [13, 14]. Optimisation of the CBMN assay for maximal MN frequency and BNC expression postirradiation must be explored.

The relationship between MN formation and radiation dose has been examined as a possible indicator of radiosensitivity for tumour cells. Encouraging reports of a correlation between micronucleus formation and cell reproductive death measured clonogenically in established tumour cell lines *in vitro* have been shown [10, 15–17]. However, several authors have reported little or no correlation between data obtained from the CBMN and colony forming (CF) assays when using established tumour cell lines of widely differing origin and radiation sensitivity [14, 18, 19].

The use of the micronucleus assay to determine *in vitro* radiosensitivity of primary biopsy material and thereby to individualise radiotherapy treatment for cancer patients has also been investigated [1, 20]. Subsequently, Wandl and associates [21] showed a linear correlation between clonogenic survival and micronucleus frequency in 8 out of 10 primary renal cell carcinoma cultures. A more generalised study, using a spectrum of human tumours grown in primary culture, showed a close correlation between the micronucleus frequency following 2 Gy (MNF_2) and patient response to radiotherapy [22]. In a recent study, Zolzer and associates, considering both MN frequency and S-phase fraction during a course of radiotherapy, were able to predict reliably patient prognosis in cervical carcinoma patients [23]. In contrast, other workers have found no correlation between clonogenic survival and MNF_2 in human malignant melanoma and ovarian carcinoma primary cell cultures [24].

In addition to an evaluation of the micronucleus assay using established tumour cell lines of widely differing origin and radiation sensitivity [14], we have been investigating established and primary tumour cultures derived from a single tumour type—squamous cell carcinoma (SCC) of the head and neck. Our aim has been to determine whether the variability in terms of clonogenic lethal lesions (LL) associated with a given number of MN/BNC observed in our panel of tumour cell lines [14] was observed in cell lines from a single tumour type. In addition, we wanted to assess the practicality of applying the CBMN assay to primary cultures in a routine clinical setting in order to measure radiation responsiveness and determine whether this data could predict patient outcome after radiotherapy. This paper presents our findings.

MATERIALS AND METHODS

Established cell lines

Culture conditions. The three cell lines used in this study, V134, V175 and SCC-61, are all human squamous cell carcinoma cell lines derived from tumours of the head and neck. The SCC-61 cell line, kindly donated by Dr A.C. Begg (Amsterdam, The Netherlands), has been previously described [25, 26]. The V134 [14] and V175 cell lines were both derived at Velindre Hospital from patient tumour biopsy samples. Tumour cells were maintained as monolayer cultures in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) containing 10% fetal calf serum (Globepharm), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma). The culture medium for the SCC-61 cell line was supplemented with hydrocortisone (0.4 μg/ml, Sigma). The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were routinely subcultured by the addition of 0.02% trypsin/0.05% ethylenediaminetetra-acetic acid (EDTA) (Sigma) in phosphate-buffered saline (PBS, Unipath). Experiments were performed using cells harvested in log phase growth.

Cytochalasin-B. Cytochalasin-B (CB) powder (Sigma), was dissolved in dimethylsulphoxide (Sigma) and stored at −20°C at a concentration of 10 mg/ml. When required, an aliquot was withdrawn, thawed, diluted in culture medium and sterilised by filtration (0.22 μm Acrodisc, Gelman).

Irradiation. All irradiations were performed at room temperature in oxic conditions using a ^{137}Cs gamma source of 0.66 MeV energy. The dose rate was 0.86 Gy/min. Irradiations were usually completed within 20 min. Cells were irradiated either as single-cell suspensions for cloning experiments or as monolayer cultures for CBMN experiments.

Clonogenic cell survival. Exponentially growing cells were irradiated in suspension and plated into 25 cm^2 flasks (Nunclon) in 5 ml of culture medium. Triplicate flasks were set up for non-irradiated controls and for each radiation dose. Surviving cells were allowed to grow for 10–12 days prior to fixing and staining. Resultant colonies were fixed in 4% formaldehyde (BDH) in PBS and were stained with a 1% solution of crystal violet (BDH). Colonies consisting of more than 50 cells were counted.

Cell survival after exposure to cytochalasin-B. Cells were seeded into flasks and incubated for 24 h in culture medium before addition of CB at doses ranging from 0 to 6 μg/ml. Following a further 24 h incubation, the cells were harvested and set up, as described above, for monolayer cloning. Triplicate flasks were set up for control cultures (unexposed to CB) and for each CB dose. Colonies consisting of more than 50 cells were scored.

Table 1. Origin of biopsy samples, classification of the tumours according to their TNM status, histological differentiation and relative staining intensities of cellular outgrowths to the antibody cocktail (AE1/3 and MNF116)

Cell culture number	Stage	Histology	Tumour origin	Staining intensity
124	T_2N_0	Poorly differentiated SCC	Carcinoma anterior floor of mouth	++
134	T_2N_0	Well differentiated SCC	Carcinoma retromolar trigone	+++
161	T_2N_1	Well differentiated SCC	Carcinoma anterior floor of mouth	+
166	T_2N_1	Moderately differentiated SCC	Carcinoma retromolar trigone	+++
168	T_3N_0	Moderately differentiated SCC	Carcinoma oral commissure	+++
170	T_2N_0	Well differentiated SCC	Carcinoma buccal mucosa	++
172	T_2N_0	Moderately differentiated SCC	Carcinoma upper alveolus	+
175	T_2N_0	Moderately differentiated SCC	Carcinoma lateral tongue	+++
176	T_2N_1	Moderately differentiated SCC	Carcinoma floor of mouth	++
187	T_2N_1	Well differentiated SCC	Carcinoma base of tongue	++

+++, majority of cells intensely stained; ++, majority of cells moderately stained; +, majority of cells weakly stained. SCC, squamous cell carcinoma.

Cytokinesis-block micronucleus assay (established cell lines). In the optimised CBMN assay, micronucleus dose–response measurements were set up so that fixation of the cultures coincided with the time of maximum binucleation and micronucleus expression for that cell line. The micronucleus frequency (MN/BNC) was defined as the mean number of micronuclei (MN) per binucleate cell (BNC). Single-cell suspensions were plated onto sterile 15 mm glass cover-slips (Chance Propper) in 3.5 cm petri dishes (Nunclon) at a density between 4×10^4 and 1×10^5 cells per dish. The cultures were incubated for at least 2 h to allow the cells to adhere to the cover slips. The petri dishes were then irradiated in triplicate at room temperature. Once all irradiations were complete, CB diluted in fresh medium was added to the cultures which were then returned to the incubator. At daily intervals postirradiation, triplicate cultures were fixed with cold Carnoys solution (3:1 v/v methanol:glacial acetic acid) (BDH). Non-irradiated control cultures were set up with or without CB. Cells were subsequently stained with 0.01% acridine orange and 0.1 mg/ml mithracin as described previously [14].

In the standardised CBMN assay, culture conditions were fixed so that all cultures were exposed to CB at concentration of 2.0 μg/ml and cells were fixed and stained (as described above) at 48 h postirradiation.

Primary cell cultures

Culture conditions. Tumour biopsy samples were received from patients ($n = 10$) undergoing radical reconstructive surgery for head and neck cancer. Surgical samples were transported by courier in air-tight polythene bags and were received 1–2 h postsurgery. Individual patients details are described in Table 1. All biopsy samples were from squamous cell carcinomas (SCC).

The tissue was washed twice with PBS and any necrotic material removed. The samples were then chopped into small pieces (1–2 mm in diameter) and placed into 25 cm^2 flasks (Nunclon) with 2 ml of a supplemented DMEM medium (Table 2) [27]. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Flasks were viewed daily for any epithelial outgrowth. Any contamination of the culture by fibroblast cells was removed using differential trypsinisation [28]. This was achieved by addition of 0.02% trypsin/0.05% EDTA (1 ml) for 1–2 min to the flasks which resulted in selective detachment of the fibroblasts whilst the epithelial cells remained attached.

Immunohistochemistry. All head and neck SCC cell cultures were tested prior to experimentation to establish that cellular outgrowth from the biopsy pieces was of epithelial origin. Two monoclonal antibodies were used in this assessment. One was reactive against both acid and base cytokeratins (AE1 and AE3; ICN) and the other directed at a surface antigen specific to epithelial tumour cells (MNF 116, DAKO). In all cases, the action of the antibody specificity was confirmed by the staining of a known positive (HeLa-S3) and a known negative culture (normal human fibroblasts). The epithelial nature of the cells was scored under fluorescence after application of a FITC conjugate (DAKO). The scoring method was based on the relative staining intensity to the monoclonal antibody cocktail of each early cell outgrowth. A score of +++ represented a strongly stained culture of uniform intensity, ++ represented a moderately well-stained culture of uniform intensity and + a weakly staining culture. No primary cell culture was found to be negative to these epithelial markers. Table 1 details the score for each early cell culture against the monoclonal antibody cocktail. It also summarises the histology and site of origin of each biopsy sample assessed.

Cytokinesis-block micronucleus assay (primary cultures). When a given monolayer of epithelial cells was clearly established, the cells were harvested with 0.02% trypsin/0.05% EDTA in PBS. They were subsequently seeded on to 1 cm^2 cell culture membranes (Sterilin) [29]. The membranes were sterilised by exposure to UV irradiation (254 nm) and placed in 24-well multiwell plates (Nunclon).

Table 2. Constituents of supplemented culture medium used for primary cell culture

DMEM	70%
Ham's nutrient F12	25%
Fetal calf serum	5%
Hydrocortisone	0.4 μg/ml
Transferrin	5 μg/ml
Insulin	5 μg/ml
Tri-iodothyronine	2.0×10^{-11} M
Adenine	1.8×10^{-4} M
Penicillin	100 U/ml
Streptomycin	100 μg/ml
Gentamycin	10 μg/ml
Amphotericin-B	20 μg/ml

DMEM, Dulbecco's Modified Eagle's Medium.

Cells were seeded at a density of 4×10^4–1×10^5 cells/membrane (in 0.5 ml medium) and allowed to attach. The membranes were then irradiated at room temperature in triplicate and immediately refed with fresh medium (0.5 ml) containing CB (2 μg/ml) in all cases. The membranes were incubated for a further 48 h prior to fixation. Unirradiated control cultures were set up with and without CB. Cells were fixed using cold Carnoy's solution and stained with acridine orange (0.0025%, BDH) in M/15 phosphate buffer (pH 6.0) for 1.5 min. The cells were rinsed in buffer (×2) and the membranes mounted in buffer under a glass cover slip (Chance Propper) on a glass slide. It was found that the use of a single stain (acridine orange) was adequate for use on the primary cultures and provided preparations of equal quality when compared to the mithracin/acridine orange stain. No attempt was made to optimise the CB concentration or incubation time postirradiation for each biopsy sample.

Scoring and analysis

Both the single-stain (acridine orange) and double-stain (acridine orange/mithracin) protocols produced BNC with bright, well-defined nuclei and MN within a much paler cytoplasm. A fluorescence microscope (Leitz Dialux) at a magnification of ×40 was used to assess all BNC. At each dose point, 300 BNC were scored for the number of MN randomly by one observer giving the mean number of MN/BNC. The percentage of cells that were binucleate was also determined. In all cases, the mean micronucleus frequency (MN/BNC) in unirradiated controls containing CB was subtracted from that of the irradiated cultures. Each experiment in the study was performed a minimum of three times.

Criteria for scoring micronuclei

Only BNC with well-preserved cytoplasm were considered for analysis. MN were scored only if they conformed to the criteria set out by Bush and McMillan [18]. These were: (i) MN must be clearly separate from and no greater than 1/3 the volume of the main nuclei, and (ii) the morphology and staining properties of the MN were similar to those of the main nuclear material.

RESULTS

Established tumour cell lines

Cell survival measured in the CF assay. The radiation dose–survival curves for the three head and neck cell lines are shown in Figure 1. The data shown are from pooled experiments ($n \geqslant 3$). The data were fitted by a linear quadratic function for each cell line by means of a least-squares Marquardt algorithm [30]. Cell lines characteristics (doubling time, ploidy, cloning efficiency (CE)) and survival

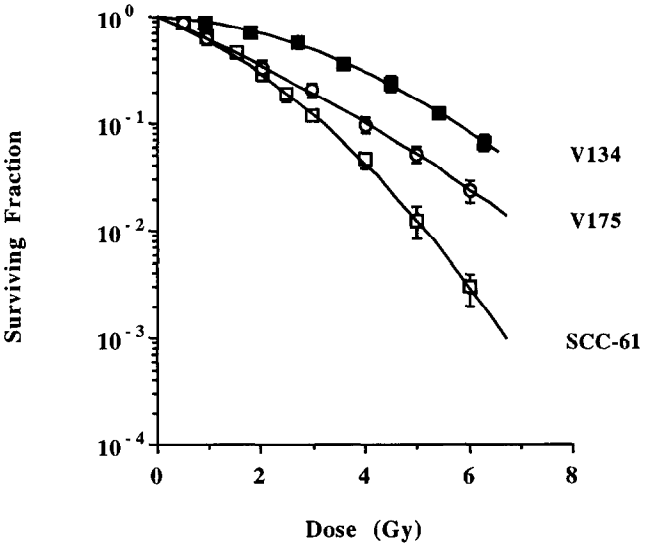

Figure 1. Radiation survival curves for the three head and neck cell lines as determined by the CF assay and fitted by a linear quadratic function. Cell lines are as follows; V134 (■), V175 (○) and SCC-61 (□). In this figure and all subsequent figures, the error bars represent one standard error of the mean and are shown if greater than the symbol.

curve parameters (α, β, SF_2) are shown in Table 3. The SF_2 value was derived by solving the equation $SF_2 = e^{-(\alpha D + \beta D2)}$ for $D = 2$ Gy. A fairly wide variation in radiation sensitivity was observed in these cell lines with SF_2 values of 0.25 (SCC-61), 0.36 (V175) and 0.69 (V134).

Clonogenic cell survival following CB exposure. The three head and neck cell lines were all treated with a 24 h exposure to CB. Their subsequent survival was assessed clonogenically (Figure 2) and the data are from pooled experiments ($n \geqslant 3$). Exposure to CB was clearly toxic to the cells with a surviving fraction at 2 μg/ml CB of 0.50 (SCC-61), 0.43 (V175) and 0.27 (V134). The most sensitive cell line to radiation, SCC-61, displayed an exponential decrease in survival as a function of increasing CB dose. The other two lines (V134 and V175), although showing an initial sensitivity to CB (0–2 μg/ml), had an essentially biphasic response.

BNC frequency in non-irradiated cultures

Time course experiments were undertaken to determine the incubation time in CB that produced the maximum expression of BNC for each cell line. Using a cell line-dependent CB concentration added on day 0, unirradiated cells divided to become binucleate over a short period of time (Figure 3) with the maximum frequency occurring after 48 h

Table 3. Summary of SCC cell lines and their clonogenic survival data

Cell line	Doubling time (h)	Clonogenic plating efficiency (% ±1 SE)	Ploidy	SF_2	$\alpha(Gy^{-1})$ (±1 SE)	$\beta(Gy^{-2})$ (±1 SE)
V134*	28.8	5.67 ± 0.25	Polyploid‡	0.69	0.070 ± 0.032	0.058 ± 0.007
V175*	35.7	2.82 ± 0.23	Hyperdiploid	0.36	0.454 ± 0.031	0.027 ± 0.007
SCC-61†	32.75	24.13 ± 1.18	Hyperdiploid	0.25	0.564 ± 0.036	0.066 ± 0.007

*Derived at Velindre Hospital, Cardiff, U.K. †Kindly donated by A.C. Begg, Amsterdam, The Netherlands. ‡E.M. Parry, personal communication.

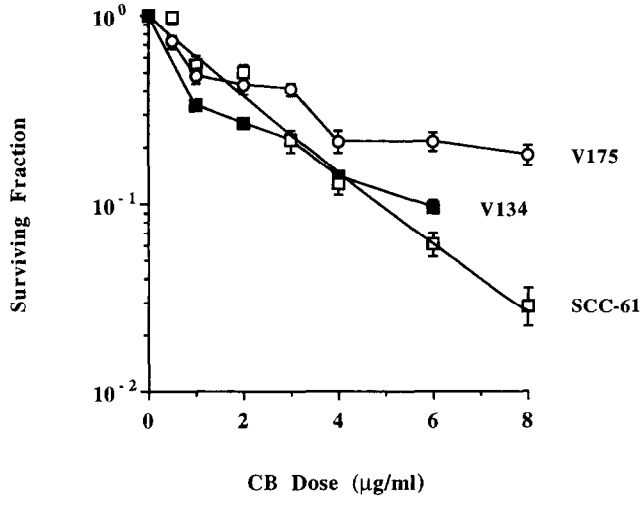

Figure 2. Clonogenic cell survival following 24 h exposure to CB. Dose–response data shown for V134 (■), V175 (○) and SCC-61 (□). The data have been fitted with a single exponential function (SCC-61) or a simple line plot (V134 and V175).

in each case. The SCC-61 (52.8%) and V134 (48.3%) cell lines expressed as higher peak percentage than the V175 cell line (37.1%). In all cases, a rapid decrease in the %BNC followed. This was due principally to the formation of polynucleate cells within the population. In samples not exposed to either radiation or CB, the BNC frequency was never greater than 6% over the time period studied and the number of MN within these BNC was relatively low (<10% contained MN).

BNC and MNF in irradiated cultures

Following irradiation, the pattern of MN and BNC expression was assessed in cultures over time (Figure 4). Both the V134 and SCC-61 cell lines showed maximum binucleation after 72 h in contrast to their unirradiated controls

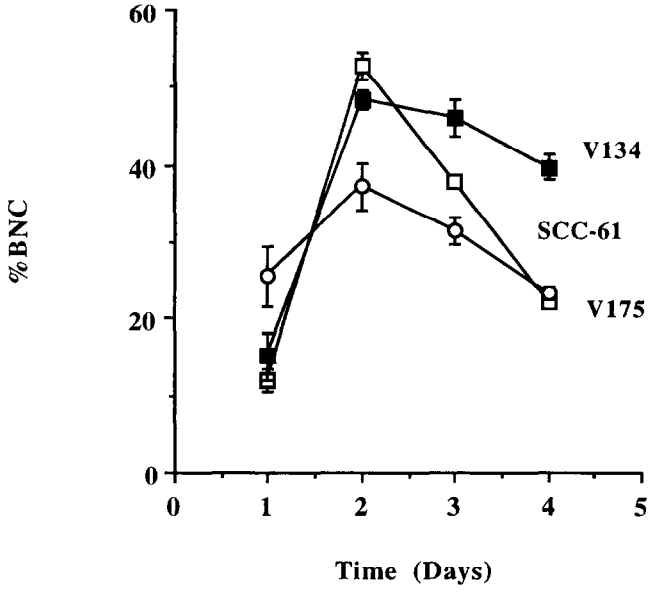

Figure 3. The percentage BNC population in non-irradiated cell cultures as a function of culture time. CB was added on day 0. Data shown are for V134 (■), V175 (○) and SCC-61 (□).

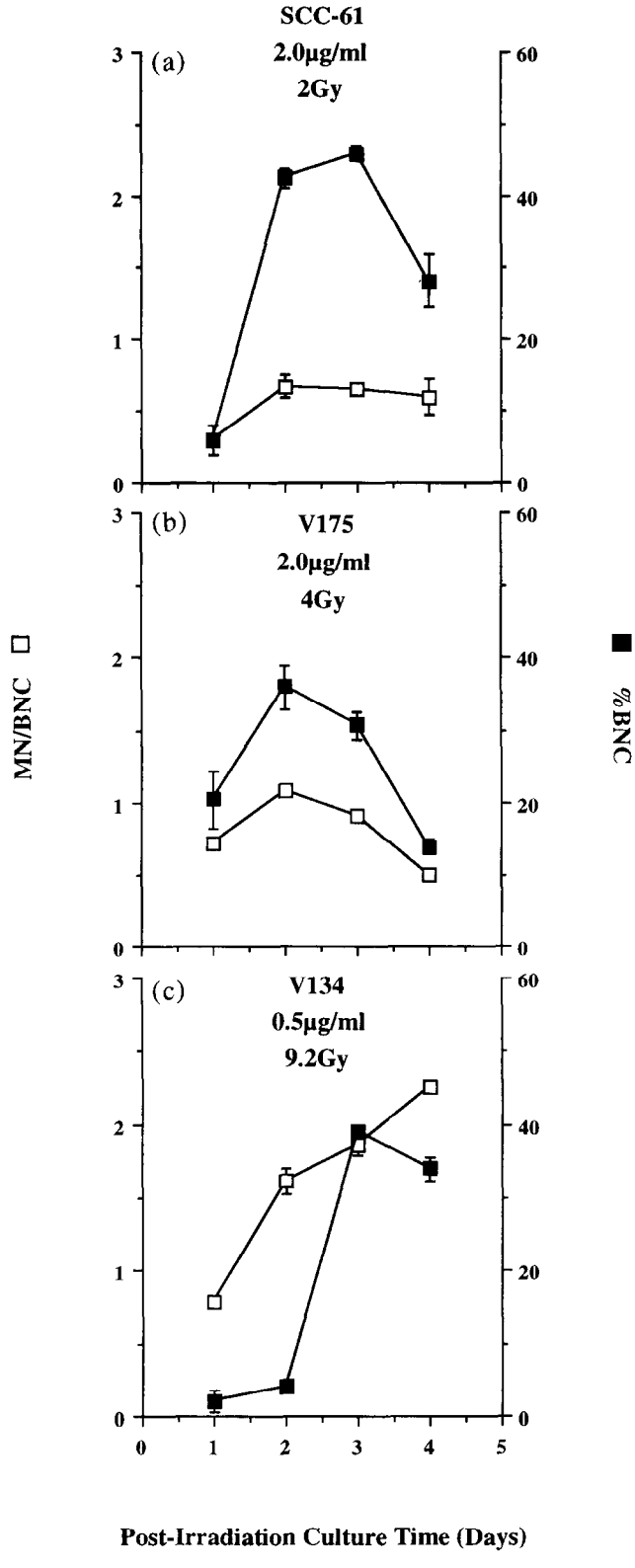

Figure 4. The expression of radiation-induced DNA damage (MN/BNC) (□) and percentage of BNC (■) as a function of postirradiation culture time.

in which maximum binucleation occurred after 48 h (Figure 3). This result indicates a radiation-induced division delay. In contrast, V175 displayed maximal binucleation after 48 h in both unirradiated (Figure 3) and irradiated

cells (Figure 4). As far as MN/BNC was concerned, the peak level in the expression of damage in the V175 and SCC-61 cell lines occurred after 48 h, whereas the V134 failed to reach a maximum level throughout the duration of the experiment (Figure 4).

Effect of CB concentration on MNF

Radiation-induced damage was assessed 48 h (V175 and SCC-61) or 72 h (V134) postirradiation for the three lines studied using two different doses (0.5 and 2.0 μg/ml) of CB (Figure 5). In each case, a significant difference in the frequency of MN/BNC for each CB dose was observed ($P \leqslant 0.001$). For the V175 and SCC-61, the higher CB concentration (2.0 μg/ml) led to increased MN expression as a function of radiation dose, although this was very marginal for the V175. The V134, in contrast, demonstrated a reverse effect with the lower CB concentration (0.5 μg/ml) producing the greater MN/BNC at a given radiation dose.

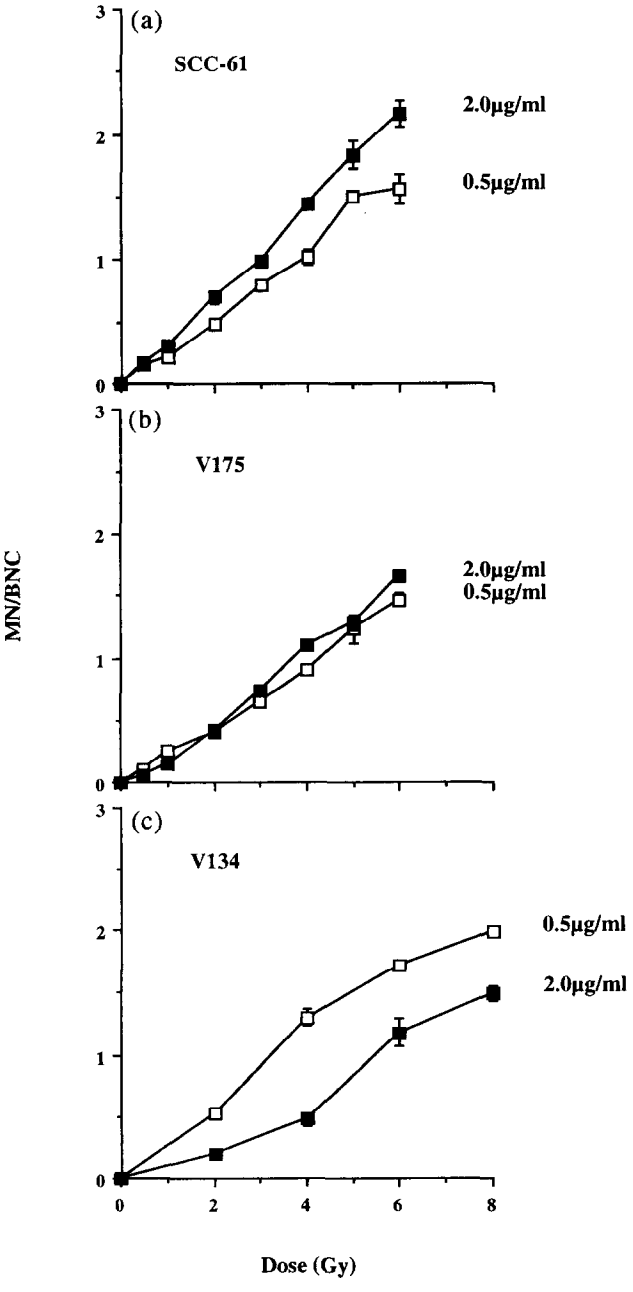

Figure 5. Influence of CB concentration (0.5 μg/ml and 2.0 μg/ml) on the expression of MN/BNC. Radiation dose-response was assessed at the following times: V134—72 h, V175—48 h, and SCC-61—48 h. For all three cell lines a significant difference due to the CB concentration ($P < 0.001$) was observed.

CBMN assay under optimised conditions

Optimum assay conditions, i.e. the CB concentration and time point postirradiation where maximal MN/BNC and %BNC was achieved for the three cell lines, were employed to produce radiation dose–response data. The assay was performed using a CB concentration of 2.0 μg/ml (SCC-61, V175) or 0.5 μg/ml (V134) and the cells were fixed at either 48 h postirradiation (V175 and SCC-61) or 72 h (V134). An approximately linear relationship between radiation dose and MN/BNC was observed in all cases (Figure 6a). The more clonogenically radiosensitive cell line (SCC-61, Figure 1) expressed more MN/BNC than the other more resistant lines (V134, V175). The rate of induction of MN/BNC per Gy for each line was 0.366, 0.287 and 0.258 for SCC-61, V175 and V134, respectively.

CBMN assay under standardised conditions

Standard assay conditions (2 μg/ml CB; cultures fixed 48 h postirradiation) were also used to produce radiation dose–response data for the three established SCC cell lines. Again an approximately linear relationship between radiation dose and MN/BNC was observed in all three cell lines (Figure 6b). The more clonogenically radiosensitive cell line (SCC-61, Figure 1) expressed the greater MN/BNC, with the clonogenically radioresistant line (V134, Figure 1) expressing the least MN/BNC. The rate of induction of MN/BNC per Gy for each line was 0.366, 0.287 and 0.162 for SCC-61, V175 and V134, respectively.

Relationship between the CF and CBMN assays

Further analysis of the clonogenic survival data and the CBMN dose–response data enabled us to examine the relationship between the two assays. The number of lethal lesions (LL), determined as $-\ln$ (SF) and derived from the data Figure 1, was plotted against the MN/BNC (under optimal conditions) for each cell line (Figure 6c). From Figure 6c it can be seen that the SCC-61 and V175 plots are significantly different from the V134, such that the first three points of the curve are found to lie outside the 95% confidence band of the combined V175/SCC-61 regression lines. The V175 ($r = 0.996$) and SCC-61 ($r = 0.986$) displayed a linear relationship, whereas the V134 data was fitted with an exponential curve ($r = 0.997$). The number of LLs for each cell line at 1 MN/BNC was 2.23 (SCC-61), 2.17 (V175) and 0.784 (V134).

Figure 6d describes the relationship between the number of lethal lesions and MN/BNC where the CBMN assay was performed under standardised conditions for each cell line (cultures fixed 48 h postirradiation; CB concentration 2.0 μg/ml). In this case, the range in the number of lethal lesions at 1 MN/BNC was much narrower: SCC-61 (LL = 2.23), V175 (LL = 2.17) and V134, fitted with a linear regression ($r = 0.949$; LL = 2.54) (Figure 6d). Nevertheless, the differences between the regression lines remained marginally significant ($P = 0.042$).

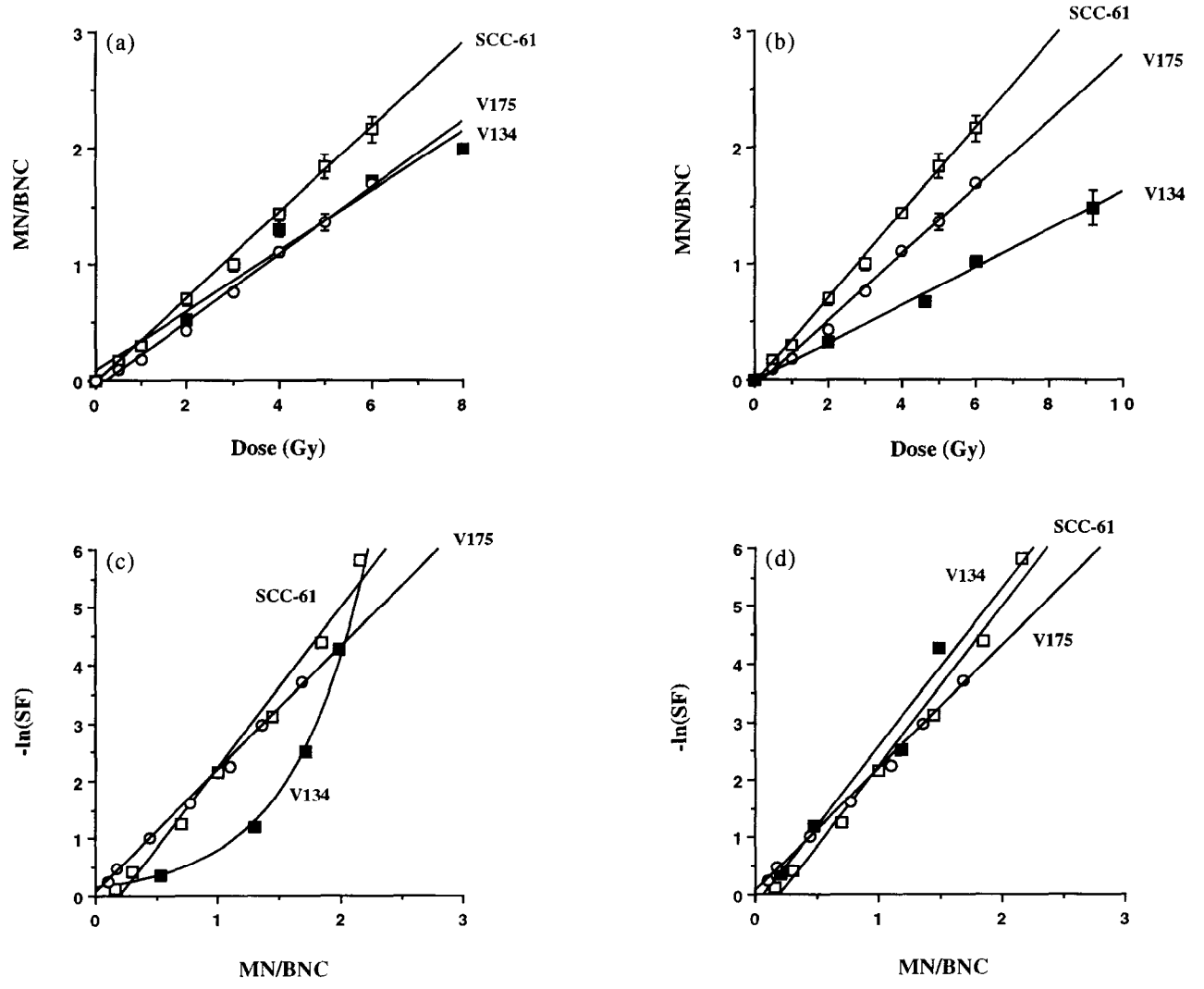

Figure 6. (a) Determination of radiosensitivity using the CBMN assay for the V134 (■), V175 (○) and SCC-61 (□) cell lines under optimised conditions. Linear regression lines have been drawn through all these data. (b) Determination of radiosensitivity using the CBMN assay for the V134 (■), V175 (○) and SCC-61 (□) cell lines under standardised conditions. Again linear regression lines have been drawn through all these data. (c) The relationship between LL and MN/BNC under optimised assay conditions. The data has been fitted using either linear regression (V175 and SCC-61) or exponential lines (V134). (d) The relationship between LL and MN/BNC under standardised assay conditions (2.0 μg/ml CB; cells fixed 48 h postirradiation). Data in all three cases has been fitted with linear regression lines. In (c) and (d) error bars have been omitted for clarity.

Standardised CBMN assay of primary cultures

Radiosensitivity measurements of primary cell cultures were studied using a CB concentration of 2.0 μg/ml, with cultures being fixed and analysed 48 h postirradiation. Data are presented in Figure 7. In all cases, a linear relationship was observed between MN/BNC and radiation dose ($0.997 \geqslant r \geqslant 0.961$). The mean, standard deviation and range of MN/BNC at 2 and 6 Gy for all 10 samples were: 2 Gy (0.316; S.D. = 0.092; 0.190–0.510) and 6 Gy (0.747; S.D. = 0.162; 0.520–1.022). A one-way analysis of variance (ANOVA) shows that there is a significant difference in the MN/BNC measurements between patients at both 2 Gy ($F = 5.694$; $P = 0.0006$) and 6 Gy ($F = 9.741$; $P = 0.0001$). From Figure 7, there is a suggestion that the patients may fall into two groups in terms of their radiation dose response. By combining the data for the upper (134, 166, 170, 172, 187) and lower (124, 161, 168, 175, 176) lines, a significant difference was detected between the mean slopes of the two groups ($F = 48.97$, $P < 0.001$; one-way ANOVA). However, we acknowledge that this finding may possibly be just a totally random distinction between the two data sets.

9 of the 10 patients in the study received no pre-operative radiotherapy. From this group, 7 went on to have conventional postoperative radiotherapy consisting of 55 Gy in 25 fractions over a 5 week period. One patient (172) received a higher dose (60 Gy in 30 fractions over 6 weeks) due to a possible incomplete surgical excision of the tumour. No correlation was observed between the level of radiation-induced DNA damage (MN/BNC) at 2 Gy and 6 Gy and patient clinical outcome (local control or local recurrence) 12 months postsurgery (two-way ANOVA; $F = 0.01$, $P = 0.92$). Further statistical analysis of the data in Table 2 failed to show any association between patient outcome (12 months postsurgery) and tumour stage ($\chi^2 = 3.14$; $P = 0.21$), nodal stage ($\chi^2 = 1.67$; $P = 0.20$) and tumour histology ($\chi^2 = 2.20$; $P = 0.33$).

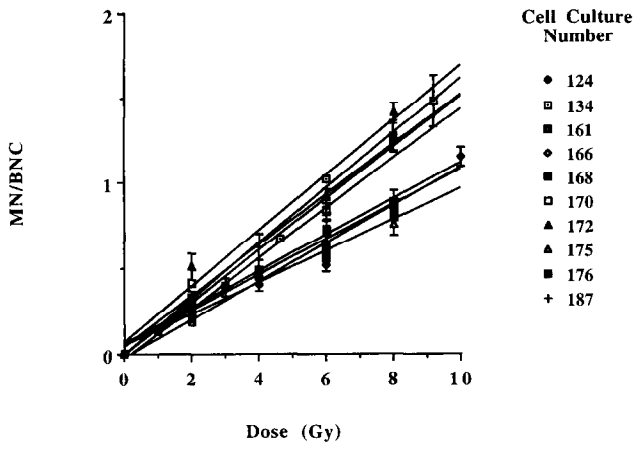

Figure 7. Assessment of primary cell culture radiosensitivity using the CBMN assay under standard conditions (2.0 μg/ml CB; cells fixed 48 h postirradiation). Data for each cell culture has been fitted using linear regression analysis.

DISCUSSION

Assessment of accurate and reliable predictors of tumour and normal tissue response to radiation treatment has become the object of increasing interest. An accurate prediction of response to radiotherapy would allow for an individualised approach to patient therapy. This should then lead to higher locoregional control and higher overall patient survival. This study has investigated the correlation between cell reproductive death and micronucleus formation in three human tumour cell lines of differing radiation sensitivity but of similar origin. Furthermore, the concept of using the CBMN assay as predictor of radiosensitivity has been extended to primary cultures of head and neck tumour biopsy samples. The evaluation of the CBMN assay within one tumour type has been conducted knowing that significant variations in inherent radiosensitivity among SCC cell lines of the head and neck exist [31, 32]. This present study was carried out subsequent to a similar study on tumour cells of widely differing origin and radiosensitivity [14].

It was considered necessary to determine the optimal assay conditions prior to dose–response measurements. The cell line data show the effects that varying the parameters within the CBMN assay can have upon the measurement of radiosensitivity *in vitro* (Figures 3–5). All three lines showed diminished clonogenic survival with increasing CB dose, with the most radiosensitive line (SCC-61) being the most CB sensitive. It was clear that the proportion of BNC and the emergence of MN was dependent upon the exposure time to CB, as has been previously shown [10, 14, 24]. Optimal assay conditions were considered to be the time of maximum binucleation and MN/BNC. We assume that at the point of maximum binucleation the measured radiosensitivity is representative of the whole cell population. MN data (Figure 6a and Figure 7) confirm the capability of the CBMN assay to measure DNA damage directly following radiation in both established tumour cell lines and primary tumour cultures.

Our data suggest that with optimised conditions (Figure 6c), the correlation between LL and MN/BNC was close only in two out of the three cell lines (V175 and SCC-61). By simply reverting to standard assay conditions (2.0 μg/ml CB; 48 h), a far closer relationship between LL and MN/BNC was observed (Figure 6d). This was because under optimised conditions (0.5 μg/ml CB; 72 h) the V134 line expressed significantly more MN/BNC than under standard assay conditions. Standard assay conditions required a CB concentration of 2 μg/ml which resulted (for the V134 cell line) in increased cellular toxicity (Figure 2) potentially removing BNC with MN from the analysis as indicated in Figure 5, although the reverse is seen for the SCC-61 and V175 cell lines. The time of performing the assay may well be the crucial factor. For V134, the MN frequency and %BNC showed a sharp decrease between 72 and 48 h (Figure 4). Therefore, radiation-induced damage (MN) may be unable to be fully expressed at 48 h due to the very low level of BNC. This results in the contrasting radiation dose–response plots for the V134 cell line using the CBMN assay under the two sets of conditions (Figure 6a, b). Under the standard assay conditions, the difference between the MN/BNC and LL plots for each cell line remains marginally significant ($P = 0.042$) (Figure 6d). It would appear unlikely that these differences are biologically meaningful. This close correlation, however, between LL and MN/BNC is in conflict with a similar study that used only lymphoblastoid cell lines [19] and also with our previous study using a panel of seven tumour cell lines of widely differing origin and radiosensitivity [14]. However, these data are in agreement with other tumour cell line studies [10, 16, 17] in which radiosensitivity was adequately predicted. These results suggest that the CBMN assay is able to predict adequately *in vitro* radiosensitivity of these three head and neck SCC cell lines as long as assay conditions are standardised. However, as only three cell lines have been analysed so far, this conclusion may be a little tentative but is certainly encouraging.

Our study was extended to primary cell cultures derived from head and neck biopsy material using the standard assay conditions. The CBMN assay using primary biopsy material requires sufficient tissue to provide adequate cellular outgrowth. The majority of samples received after surgery were relatively small (mean weight 0.267 g, S.D. = 0.193). Due to the small size of biopsy tissue, culturing becomes very difficult and therefore impractical to attempt optimisation of the assay. For all 10 patients assessed, a good linear relationship between MN/BNC and radiation dose was observed and there was a significantly wide range of interpatient variability in the slopes (Figure 7). The primary cell data (Figure 7) shows significant differences between expression of radiation-induced DNA damage (MN/BNC) at 2 and 6 Gy, indicating that differences in inherent cellular radiosensitivity exist between patients. Several other *in vitro* studies have also shown these differences in inherent cellular radiosensitivity [32, 33]. However, there was no correlation observed between MN/BNC and whether the patients tumour recurred or was locally controlled.

The CBMN assay results for the primary head and neck cell cultures shows the mean and range in MN/BNC values at 2 and 6 Gy to be: 2 Gy (0.316; S.D. = 0.092; 0.190–0.510) and 6 Gy (0.747; S.D. = 0.162; 0.520–1.022). These mean values of MN/BNC and the spread of data at 2 and 6 Gy are considerably lower than the corresponding range for the three established head and neck cell lines: 2 Gy (mean = 0.445; range 0.196–0.700) and 6 Gy (mean = 1.672; range 1.179–2.16) (Figure 6b), using stan-

dard conditions (2 μg/ml CB; 48 h). This is also true when the data are compared to a panel of tumour cell lines we have previously studied [14]. We also observed a dose-dependent reduction in the fraction of binucleate cells as a function of radiation dose which was especially severe at doses >6 Gy in the primary cultures (data not shown). This large reduction in the %BNC at relatively high radiation doses may be due to the inability of the majority of the cell population to complete karyokinesis and become binucleate. Hence only the most radioresistant subpopulation would be capable of forming BNC. This may, therefore, explain the relatively low MN expression at 2 and 6 Gy and the apparent radioresistance of the ten patient samples as a whole.

This is a preliminary study and so far only a very small number of patients have been assessed. Further patient data are required to provide a more balanced assessment of the CBMN assay as a predictor of radiation response in primary cultures of SCC of the head and neck. At present, there are insufficient patient data to determine the exact relationship between MN/BNC and patient outcome.

In summary, we have found the CBMN assay to be a useful indicator of radiation-induced DNA damage in SCC tumour cells. Within this one tumour type, it appears capable of predicting clonogenic radiosensitivity. It was unable to help predict patient outcome following surgery and radiotherapy, albeit in a very small number of patients. We have only assessed patients having surgery and adjuvant radiotherapy. It is possible that there could be a correlation in patients only having radiotherapy when tumour radiosensitivity could potentially be of greater influence in determining outcome. From the CBMN data, it appears that the CBMN assay alone may not be useful in predicting locoregional control in head and neck cancer patients. It appears likely that a panel of tests is required to predict radiocurability adequately. These would include correlating patient outcome to tumour radiosensitivity [5, 6, 33, 34], consideration of tumour proliferation kinetics [35, 36] and DNA content [37, 38].

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