

Comet assay measures of DNA damage are predictive of bladder cancer cell treatment sensitivity *in vitro* and outcome *in vivo*

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Bladder cancer patients suffer significant treatment failure, including high rates of recurrence and poor outcomes for advanced disease. If mechanisms to improve tumour cell treatment sensitivity could be identified and/or if tumour response could be predicted, it should be possible to improve local-control and survival. Previously, we have shown that radiation-induced DNA damage, measured by alkaline Comet assay (ACA), correlates bladder cancer cell radiosensitivity *in vitro*. In this study we first show that modified-ACA measures of cisplatin and mitomycin-C-induced damage also correlate bladder cancer cell chemosensitivity *in vitro*, with essentially the same rank order for chemosensitivity as for radiosensitivity. Furthermore, ACA studies of radiation-induced damage in different cell-DNA substrates (nuclei, nucleoids and intact parent cells) suggest that it is a feature retained in the prepared nucleoids that is responsible for the relative damage sensitivity of bladder cancer cells, suggestive of differences in the organisation of DNA within resistant vs. sensitive cells. Second, we show that ACA analysis of biopsies from bladder tumours reveal that reduced DNA damage sensitivity associates with poorer treatment outcomes, notably that tumours with a reduced damage response show a significant association with local recurrence of non-invasive disease and that reduced damage response was a better predictor of recurrence than the presence of high-risk histology in this cohort. In conclusion, this study demonstrates that mechanisms governing treatment-induced DNA damage are both central to and predictive of bladder cancer cell treatment sensitivity and exemplifies a link between DNA damage resistance and both treatment response and tumour aggression.

Bladder cancer is a common malignancy in both the UK[†] and USA[‡] with 90% of tumours being transitional cell carcinomas (TCC). A total of 75–85% of patients initially present with the disease confined to the bladder mucosa (stage Ta, Tis) or submucosa (stage T1), termed non-muscle-invasive disease (NMID), whilst the remaining 15–25% have muscle-

invasive disease (MID; stages T2–4), often with lymph node involvement or distant metastasis.

NMID is managed primarily by transurethral resection of the bladder tumour (TURBT) plus immediate intravesical chemotherapy, with further adjuvant treatment (*i.e.*, further intravesical chemotherapy or immunotherapy) administered

Key words: bladder cancer, DNA damage, Comet assay, treatment, cell sensitivity, recurrence

Additional Supporting Information may be found in the online version of this article

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[†]UK statistics taken from <http://info.cancerresearchuk.org/cancerstats/types/bladder/incidence/>

[‡]USA statistics taken from <http://www.bcan.org/about/press/facts/>

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What's new?

Treatment failure in bladder cancer is associated with poor survival, so the ability to sensitize tumor cells to treatment or to predict tumor response could have major impacts on patient outcome. Here, alkaline Comet assay (ACA) measures of bladder cancer cell DNA damage caused by chemotherapy were correlated with *in vitro* cancer cell chemosensitivity. Furthermore, reduced ACA measures of *ex vivo* radiation-induced damage was associated with poor treatment outcomes for non-muscle invasive disease following resection, based on ACA analysis of bladder tumor biopsies. The findings suggest that there is a link between resistance to DNA damage and both tumor treatment response and tumor aggression in bladder cancer.

on the basis of assessed clinical and pathological factors.¹ However, even in seemingly adequately treated cases there is a high rate of recurrence, suggesting that current risk estimates are inadequate and that if high-risk cases could be identified in advance, more aggressive treatments could be considered.

Chemotherapy is the principle modality for managing metastatic bladder cancer^{2,3} and has evolved with the application of novel agents and more complex regimens. However, despite more than doubling median survival^{4,5}, more than 80% of these cases result in death; consequently, more effective treatment options and strategies are urgently required.

Patients with non-metastatic MID and eligible for curative treatment have, following any neoadjuvant chemotherapy[§], the choice of radical surgery or organ-preserving treatments. In many institutions, particularly in the US, radical surgery (cystectomy) is considered the gold-standard treatment for non-metastatic MID (T2-4a).⁶ However, this is a major procedure with a significant risk of operative mortality and adverse effects on quality of life. Radical radiotherapy (RT), delivered as a monotherapy or increasingly as concurrent-chemoradiotherapy (CRT),^{7,8} is the mainstay of bladder-sparing regimens, achieving similar cure rates to cystectomy^{8,9}; indeed, in a recent phase three trial reported by James and coworkers, concurrent-CRT (fluorouracil+mitomycin-C+RT) significantly improved locoregional control compared with RT alone.⁸ RT/CRT avoids the trauma of major surgery and is a primary treatment for patients deemed unfit for surgery. However, RT is itself associated with dose-related complications, and whilst ~50% of patients receiving radical RT achieve local-control with acceptable bladder function, the remaining patients suffer local recurrence requiring salvage cystectomy.^{10,11} Radical RT and cystectomy have never been compared in a randomised controlled trial¹² and, as there is currently no accurate means of predicting treatment response, treatment choices are largely governed by patient preferences and physician bias.¹³

Given the above circumstances, if tumour response could be predicted in advance, it might be possible to improve both local-control and overall survival rates by selecting the most appropriate treatment for those patients with correspondingly sensitive/responsive tumours. Furthermore, if key mecha-

nisms governing bladder TCC treatment resistance could be identified, this might reveal new therapeutic targets to enhance tumour cell killing.

Previously, we have shown that induction and repair of radiation-induced DNA damage, as measured by the alkaline Comet assay (ACA), correlates bladder cancer cell radiosensitivity *in vitro* with the extent of comet formation strongly correlating with cell killing ($R^2 > 0.95$).^{14,15} In addition, cells from human bladder tumour biopsies revealed a wide range of predicted radiosensitivities as determined by ACA.¹⁵ Overall, these studies demonstrated ACA to be a good predictive measure of bladder cancer cell radiosensitivity at clinically relevant doses, with potential clinical application.

In the current study, first we further investigate ACA-based measures of DNA-drug damage sensitivity in relation to bladder cancer cell chemosensitivity and highlight cellular/nuclear factors governing bladder cancer cell treatment sensitivity. Second, we report the findings of a study analysing clinical samples to establish the nature of correlations between ACA measures of DNA damage sensitivity and treatment outcome.

Material and Methods**Reagents**

All reagents were purchased from Sigma unless stated otherwise. Liberase blendzyme-3 (28 Units/ml) was purchased from Roche. Cisplatin was provided by the Pharmacy Department, Leicester Royal Infirmary, UK.

Cell lines and cell culture conditions

The six bladder cancer cell lines used were purchased from the American Tissue Culture Collection and the European Collection of Cell Culture. All the cell lines were authenticated using the AmpFISTR[®] Identifier[®] PCR Amplification Kit (Applied Biosystems), in April/May 2011 and Dec 2012 at Paterson Institute for Cancer Research, Manchester UK, and also were determined to be free of mycoplasma contamination ('Mycoplasma Experience'). All cells were cultured as described previously.¹⁵

Preparation of nuclei and nucleoid bodies

Cell nuclei were prepared using the initial cell lysis stage of a Qiagen DNA preparative kit (DNA Midi Kit, Qiagen Ltd) according to the manufacturer's instructions. Nucleoid bodies were prepared *via* the lysis stage of the ACA protocol. This involved an additional wash step (X 3), with phosphate

[§]For the last 5 years, standard treatments for non-metastatic MID have been neoadjuvant chemotherapy followed by radical cystectomy or radical RT.

buffered saline (PBS), immediately after the lysis step in the standard ACA protocol.

Drug and radiation treatment

For drug treatment, cells from exponentially growing cultures were prepared in suspension (1×10^5 cells/ml). For ACA-based measures of drug-induced DNA crosslinks and MTT assay assessment of cell viability, the suspended cells were treated with 0, 50, 100 and 200 μM cisplatin or mitomycin-C in 1 ml culture medium at 37°C for 1 hr with shaking. After drug treatment, the cells were pelleted (200g for 5 min), washed three times with 1 ml PBS and processed for modified-ACA or MTT (see below). For clonogenic assay of cell survival, the suspended cells were treated with either 0–40 μM cisplatin or 0–15 μM mitomycin-C in 1 ml of culture medium at 37°C for 1 hr with shaking. The cells were pelleted and washed as described previously and then processed for survival analysis (see below). Cells, nuclei and nucleoid bodies were irradiated embedded in agarose gels on microscope slides (see below).

Human bladder tumour sample acquisition and processing

Samples of human bladder cancer tissue were acquired from patients (following the obtaining of informed consent) undergoing TURBT (mid-2006 to late-2009); acquisition was approved by the Leicestershire, Northampton and Rutland Research Ethics Committee (REC; ref nos. 05/Q3501/96; UHL Nos. 9907). Tumour stage and grade was assessed *via* the appropriate clinical, radiological or histological assessments. Samples were transported to the laboratory in culture medium (RPMI 1640, ca. 10 ml) on ice immediately following resection. Only exophytic areas of the tumour were sampled, avoiding subepithelial layers of the bladder wall. The tumour material was finely chopped, placed in 2 ml Liberase blendzyme-3 (0.028 Units/ml) in RPMI 1640 medium and incubated at 4°C for at least 16 hr. The sample was then filtered through a 100 μm cell sorter sieve and the filtrate washed three times with PBS. Cells expressing human CD326 were selected using MACS CD326 (EpcAM) microbeads according to the supplier's instructions (Miltenyi Biotech Ltd); CD326 is widely expressed on epithelial cells including associated tumour cells. The resulting single cells were counted and their general integrity assessed (trypan blue); samples reporting low values (<70% blue cells) were discarded. Slides were prepared and ACA immediately undertaken to assess the *ex vivo* radiation-induced DNA damage levels (see below). To account for variation between experiments, additional slides were prepared using an established lymphoblastoid cell line (Raji); these were co-irradiated and analysed by ACA in parallel to determine a relative ACA gradient response (RAGR) for the individual sample (see below).

ACA analysis

ACA for the assessment of radiation-induced and crosslink drug-induced DNA damage. The Comet assay (also known as single-cell gel electrophoresis) is a sensitive microscopy-

based method for the detection of DNA damage at the level of individual cells.¹⁶ The assay involves the lysis of agarose-embedded cells to generate nucleoids, which are then subjected to electrophoresis so forming a 'comet'; with the extent of DNA in the comet's tail (reported as mean Olive tail moment (MOTM)) being proportional to the level of damage present.¹⁷

The alkaline version of the assay (ACA) detects immediate strand break damage arising from both single and double strand breaks plus alkali labile sites^{18,19}; drug-induced crosslinks are not detected by standard ACA. However, the ACA has been modified to enable detection of crosslinks, whereby the extent of crosslink formation is assessed as the relative drug-induced reduction in DNA migration of X-ray-induced strand break damage (relative to the irradiated non-drug-treated controls). This modified version of the assay has been shown able to detect DNA crosslinks from a variety of agents both *in vitro* and *in vivo*.^{20,21}

Slide preparation and X-ray irradiations. All of the following steps were conducted under low-light to prevent additional DNA damage. Approximately 15,000 cells (cultured or biopsy-derived), nuclei or nucleoid bodies were irradiated embedded in agarose gels on microscope slides as described previously.¹⁵ Duplicate slides were irradiated on ice, using a Pantak X-ray machine operated at 250 kV constant potential (HVL of 1.2 mm Cu) at a dose-rate of 1 Gy/minute. To generate dose response curves, doses of 2–6 Gy were used. For the assessment of DNA drug-induced crosslink formation 5 Gy X-irradiation was used to introduce a fixed level of DNA breaks (see above).

Single-cell gel electrophoresis. The individual steps of lysis, electrophoresis, comet image capture and data analysis were performed as described elsewhere.^{21,22} The MOTM was calculated for each comet by the Komet® software (Andor Technology). For the *in vitro* data, MOTM was measured from two to four independent experiments and their average is presented as mean \pm S.E.M. Crosslinking was expressed using the formula: %decrease MOTM = $[1 - (\text{MOTM}_{\text{di}} - \text{MOTM}_{\text{cu}}) / (\text{MOTM}_{\text{ci}} - \text{MOTM}_{\text{cu}})] \times 100$; where MOTM_{di} is the MOTM of the drug treated irradiated sample, MOTM_{cu} the MOTM of the untreated unirradiated control and MOTM_{ci} is the MOTM of the untreated irradiated control.²⁰ For the *ex vivo* irradiated human tumour biopsies, MOTM was measured from four replicate gels (50 cells each) and the data (mean \pm S.E.M.) plotted as dose response curves (data not shown). These were used to derive a measure of damage sensitivity for each individual tumour sample; this was defined as the 'relative ACA gradient response' (RAGR), which was calculated by dividing the gradient of the dose response curve for the tumour specimen by that obtained for the co-analysed Raji control cells.

Survival and viability assays

Cell reproductive integrity was assessed by clonogenic assay as described previously.¹⁵ Cell viability was assayed using the Microtetrazolium (MTT) assay²³ with the following modifications. The cells were treated with 12 mM MTT solution, at

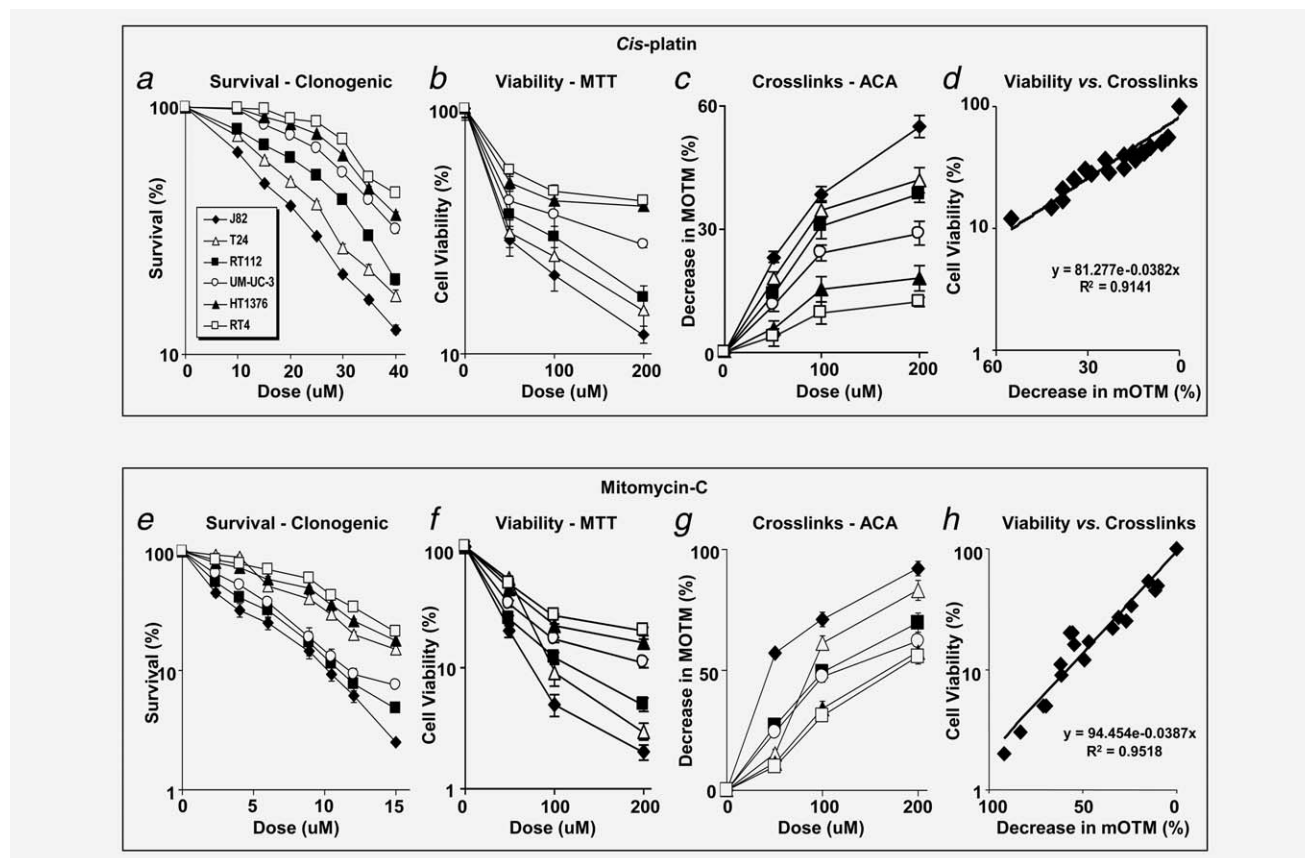


Figure 1. Comparison of clonogenic survival, cell viability and drug-induced crosslink formation in six bladder cancer cell lines exposed to cisplatin or mitomycin-C. The six cell lines investigated (see figure 1a; (J82 ●; T24 △; RT112 ■; UM-UC-3 ○; HT1376 ▲; RT4 □)) were treated for 1 hr with the doses of drug indicated and then assessed for: (a and e) cell survival as determined by clonogenic assay; survival was determined as the number of colonies formed following drug exposure; (b and f) cell viability as determined by MTT assay; (c and g) the extent of drug-induced crosslink formation, as measured by decreases in mean Olive tail moment (MOTM), as determined by modified-ACA. For a and e, b and f and c and g each data point is the mean of three independent experiments \pm S.E; statistical analysis of the data was undertaken (Tukey one-way ANOVA) and significant differences ($p < 0.05$) were observed between the most 'sensitive' (J82) and 'resistant' (RT4/HT1376) cells at all of the doses tested for all the assays undertaken (clonogenic, MTT, and crosslink-ACA). (d and h) Correlation between the decrease in MOTM as a measure of drug-induced crosslink formation and cell viability; the data are fitted with an exponential trend line and the slope and correlation coefficient deduced.

37°C, for 1 hr and the resultant formazan crystals dissolved in 100 μ l DMSO. All experiments were performed on at least three separate occasions.

Statistical analysis

The mean RAGR value for all tumour samples tested was used as the cut-off for Kaplan-Meier analysis; this cut-off was confirmed as the optimum for determination of time to local recurrence by using Cutoff Finder (<http://molpath.charite.de/cutoff/>). Kaplan-Meier curves were compared using both the Log-Rank (Mantel-Cox) test and the more stringent Gehan-Breslow-Wilcoxon test.

Results

Assessment of bladder cancer cell chemosensitivity *in vitro*

Figure 1 shows clonogenic survival, cell viability, the extent of drug-induced crosslink formation and the latter's correla-

tion with cell viability for all six cell lines following either cisplatin (Figs. 1a-1d) or mitomycin-C treatment (Figs. 1e-1h). Figures 1a and 1e show the survival responses, with the cells being more sensitive to mitomycin-C. The cell lines encompass a range of chemosensitivity with J82 being the most sensitive and RT4 and HT1376 the most resistant. Compared with RT112 and UM-UC-3 cells, T24 cells were relatively more sensitive to killing by cisplatin than mitomycin-C and interestingly demonstrated a relatively chemoresistant response towards mitomycin-C at low doses but a more chemosensitive response at higher doses.

Figures 1b and 1f show the cell viability responses (MTT assay) with the cells again being more sensitive to mitomycin-C. Again, the cell lines encompass a range of chemo-responses, with J82 being the most sensitive cell line and HT1376 and RT4 the most resistant. Again, T24 shows a relatively resistant response at the lowest dose of mitomycin-C (50 μ M) but a more sensitive response at higher doses (100–200 μ M).

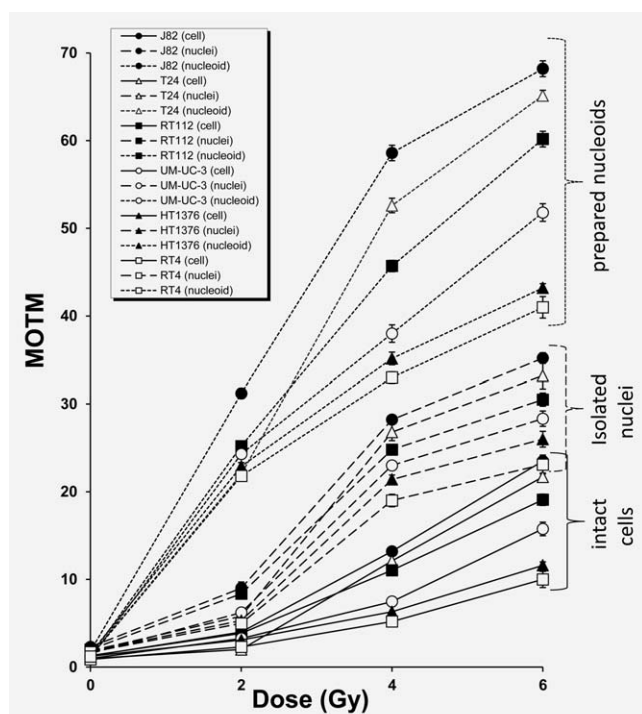


Figure 2. The extent of initial comet formation for prepared nucleoid bodies (lightly dashed lines...) vs. isolated nuclei (heavily dashed lines---) vs. intact cells (solid lines—) of the six cell lines investigated (J82 ●; T24 △ RT112 ■, UM-UC-3 ○; HT1376 ▲; RT4 □) over a dose range of 0–6 Gy, as determined by ACA. Each data point is the mean of three independent experiments \pm S.E.

Figures 1c and 1g show the level of drug-induced DNA crosslink formation as determined by the modified-ACA protocol. The extent of crosslink formation (measured as the % decrease in MOTM; see Materials and Methods) reveals that the most chemosensitive cell line J82 has the highest level of cisplatin and mitomycin-C induced crosslink formation, and the most chemoresistant cell lines RT4 and HT1376 the lowest level of induced crosslink formation. For the cisplatin treatments, the rank order for crosslink formation matches the rank order of cell killing (clonogenic assay) and cell viability (MTT assay) for all six lines. For the mitomycin-C treatments the rank order of crosslink formation matches the rank order of cell viability for all six lines and this includes the noted unusual behaviour of T24 being relatively resistant at low doses and relatively more sensitive at high doses. Furthermore, the rank order of clonogenic killing noted at the higher mitomycin-C doses used (10–15 μ M) approximates the rank order of crosslink formation at the lowest dose used (50 μ M) with J82, RT112, and UM-UC-3 being the more sensitive cell lines and HT1376, RT4 and T24 the more resistant.

As both the modified-ACA and MTT assays were performed over the same dose range, the relationship between the extent of crosslink formation and cell viability was determined. Figures 1d and 1h are plots of the relationship between the individual measures of relative crosslink formation (measured as the % decrease in mean Olive tail

moment) and the individual measures of cell viability (MTT) for all six cells for all the exposure doses used of cisplatin (Fig. 1d) and mitomycin-C (Fig. 1h). For both drugs a high degree of correlation exists between the two sets of measures for all six cell lines ($R^2 = 0.9141$ for cisplatin and $R^2 = 0.9518$ for mitomycin-C). There were no associations noted between any of the cells known genotypes (mutation status) and phenotypes (stage and grade original tumour; EMT status) and their chemo/radiosensitivity (data not shown).

Assessment of radiogenic DNA damage sensitivity in different bladder cancer cell-DNA substrates

Figure 2 compares radiation-induced comet formation for nucleoids, isolated nuclei and intact cells of the six cell lines, over a dose range of 0–6 Gy. The plots reveal clear dose response curves exhibiting 1.5–2-fold and 3–4-fold higher measures of comet formation for the nuclei and nucleoids, respectively, compared with the corresponding intact parent cells. In addition, for each individual cell line the relative levels of comet formation noted for the nuclei and nucleoids maintain the same rank order as noted in the intact parent cells. Finally, in all three substrate-systems, the T24 cell line exhibits a relatively low measure of comet formation at low dose (2 Gy) but relatively greater measures at higher doses (4 and 6 Gy) (Fig. 2).

For the intact cells, after 2 Gy there is a significant difference for the measures of MOTM only between the most radiosensitive (J82 and RT112) and radioresistant (T24 and RT4) cell lines ($p < 0.05$, Tukey One-way ANOVA); however, for both the nuclei and nucleoid systems after 2 Gy there is significant difference between the measures of MOTM for a greater number of the nuclei and nucleoid systems (see Supporting Information Table S1).

Analysis of clinical samples

Patient demographics and clinicopathological data. Samples of bladder cancer tissue were acquired from patients undergoing TURBT. Patient demographics and associated clinicopathological data were recorded (Supporting Information Table S2).

ACA assessment of tumour cell DNA damage sensitivity. The 67 bladder tumour samples were collected; 7 tumours were not TCC, 7 tumours did not withstand disaggregation (delivering <70% general integrity (trypan blue)), 3 samples were not analysed due to equipment failure (Pantak X-ray unit broken/malfunction) and 1 sample yielded insufficient material for analysis. Consequently, 49 bladder tumour samples were successfully analysed by ACA and the follow-up data collected and assessed.

Radiation-dose dependent increases in MOTM were assessed for each sample and the RAGR calculated (see methods). The determined RAGRs were ranked; these showed an approximate fivefold response range with a mean response of 1.1 (Fig. 3). Whilst there was a possible trend towards cells

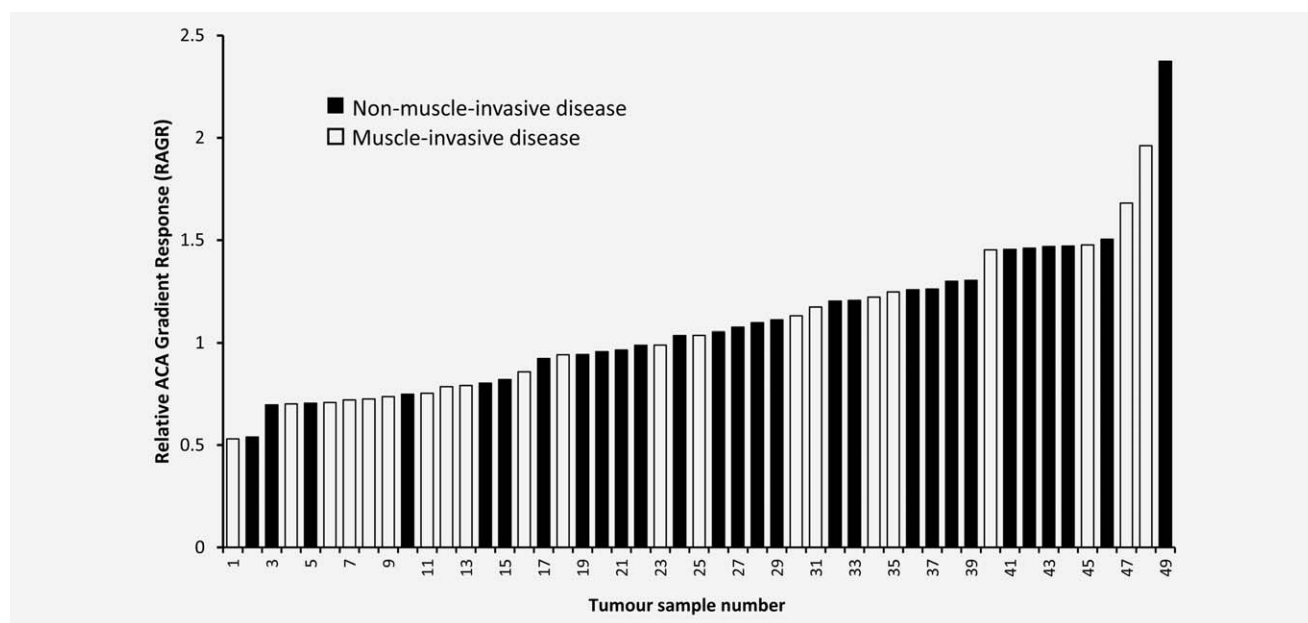


Figure 3. The relative tumour cell DNA damage sensitivity in biopsies from bladder transitional cell carcinomas. Forty nine samples were isolated from muscle-invasive (shaded) and non-muscle-invasive (unshaded) TCC biopsies. The isolated tumour cells were exposed to radiation (0–6 Gy) *ex vivo* and the resulting DNA damage measured using ACA. The gradient of the dose response curve was obtained and standardised against a co-analysed control cell line (Raji); this data was used to identify a relative ACA gradient response (RAGR) for each individual sample, which was used to rank them as to their DNA damage sensitivity.

derived from MID having lower RAGRs this did not reach significance when samples from NMID were compared with MID (Mann–Whitney *U* test, $p > 0.05$).

Correlation of bladder tumour cell DNA damage sensitivity with recorded clinical end-points. Kaplan–Meier analysis was used to identify correlations between ACA measures of DNA damage sensitivity and key clinical end-points recorded over 24 months follow-up; this included: overall survival, bladder cancer-specific death, local recurrence and metastasis. The 2-year overall survival for the entire patient cohort was 75.5% and the 2-year bladder cancer-specific survival was 81.6%.

The patients were stratified according to the tumour cell's damage sensitivity, using the RAGR calculated from the radiation response of their excised biopsies, as either being above or below the mean response of 1.1. There was no significant association noted between the extent of DNA damage sensitivity and overall survival or bladder cancer-specific survival for either NMID and MID; indeed, for MID there was no significant association noted between the extent of damage sensitivity and any of the recorded clinical end-points. However, there was a significant association between low tumour cells' damage sensitivity and fewer days to local recurrence (Log-Rank (Mantel-Cox) test $p = 0.0278$; Gehan-Berslow-Wilcoxon test $p = 0.0308$) for NMID (Fig. 4a). Figure 4b shows the rate of local recurrence for patients with NMID for five sub-divisions of RAGR. The proportion of patients not-progressing at 720 days parallels RAGR over the five ranges; no patients with NMID scoring RAGR > 1.5 showed progression two years after TURBT whilst those in the range

0.5–0.74 experienced the earliest time to local recurrence and showed the highest proportion of patients progressing. Finally, no significant correlation was noted between the presence of high risk histology (G3 pT1) and the development of local recurrence for NMID (Fig. 4c).

In addition to the significant association noted between DNA damage sensitivity and time to local recurrence for NMID, for MID there was an apparent association between the development of metastasis and low tumour cell DNA damage sensitivity, though this was not significant (Log-Rank (Mantel-Cox) test $p = 0.0789$; Gehan-Berslow-Wilcoxon test $p = 0.0861$). Furthermore, for all of the assessed correlations there was, collectively, a significant trend for patients demonstrating low measures of damage sensitivity (with RAGR of < 1.1) to suffer a proportionally higher number of adverse events (AE) per number of individuals (*n*) in the analysis group; for RAGR < 1.1 , AE/*n* = 0.414 (mean) \pm 0.139 (SD) whilst for RAGR > 1.1 , AE/*n* = 0.122 \pm 0.108 ($p < 0.001$, paired-*T*-test (SPSS)).

Discussion

Bladder cancer patients suffer significant rates of treatment failure; notably high rates of post-resection recurrence for NMID, significant rates of RT-treatment failure for MID and poor outcomes with chemotherapy for metastatic disease. Consequently, the delineation of key molecular/cellular features responsible for treatment resistance and/or the development of predictive tests to target effective treatments are both highly desirable; the enormous human, emotional and fiscal

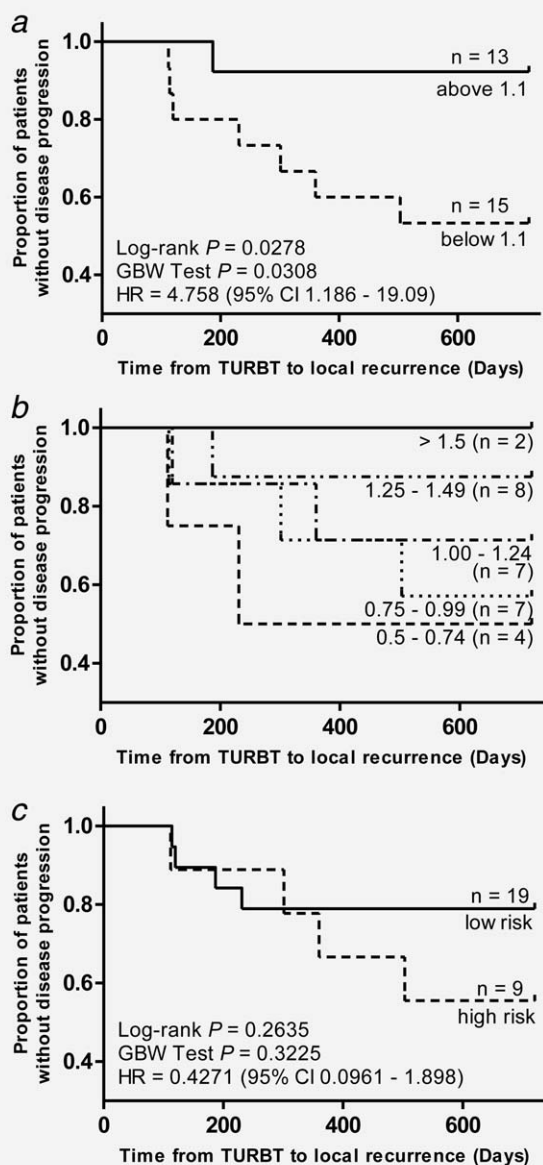


Figure 4. Assessed correlations, as determined by Kaplan-Meier analysis, between the ACA measures of bladder tumour cell DNA damage sensitivity and (a) days to local recurrence for NMID; (b) the rate of local recurrence for patients with NMID for five subdivisions of RAGR and (c) between the presence of high risk histology (G3 pT1) and the development of local recurrence for NMID. Kaplan-Meier curves were compared using both the Log-Rank (Mantel-Cox) test (LgR) and the more stringent Gehan-Berslow-Wilcoxon test (GBW).

burden of bladder cancer further highlights the importance of optimising prognosis and treatment protocols.²⁴

For radiation treatment and many chemotherapeutic drugs, genomic DNA is the primary cellular target for their biological effects. Previously, two independent studies, conducted in our respective laboratories (in Leicester and Ulster), demonstrated ACA measures of DNA damage to be capable of predicting radiation bladder cancer cell survival *in vitro*, with both studies showing a strong inverse correlation

between initial measures of comet formation and clonogenic survival.^{14,15} Intriguingly, for one cell line (T24) there was a notable change in survival response, from being relatively radiation resistant to radiation sensitive with increasing dose; this was mirrored in the ACA responses.

Cisplatin and mitomycin-C-induced damage correlates with bladder cancer cell chemosensitivity *in vitro*

In our current study of the same bladder cancer cells, we first show that modified-ACA measures of cisplatin and mitomycin-C-induced damage correlate cell chemosensitivity *in vitro*. There is approximately the same rank order for chemosensitivity (this study) as noted previously for radiosensitivity¹⁵ and, intriguingly, the differential survival/viability response of the T24 cell line at low and high doses of mitomycin-C (Figs. 1e and 1f) is also reflected in the relative extent of crosslink formation as determined by modified-ACA (Figs. 1g and 1h).

Cisplatin is widely used for treating a variety of malignant tumours (inc. bladder cancer) and its cytotoxic effects are believed to be due to the DNA damage (mostly in the form of intrastrand and interstrand crosslinks) it induces.²⁵ Mitomycin-C is a bioreductive DNA crosslinking agent used to treat a variety of malignancies including NMID and MID.^{8,26} It would therefore be expected that for both of these agents the greater the level of crosslinks induced, the more likely the cells will die. However, tumour response is heterogeneous; for example, for NMID, a broad spectrum of clinical outcome is observed, even in patients with histologically identical tumours.^{27,28} There is, therefore, a need to develop predictive assays that can accurately forecast tumour chemosensitivity and tailor chemotherapy towards individual patients who are most likely to benefit. Presently we demonstrate that modified-ACA predicts bladder cancer cell chemosensitivity *in vitro*; the appropriateness of the Comet assay as a predictive test (notably of mitomycin-C chemosensitivity) is discussed further below.

Our observations of measures of cisplatin and mitomycin-C-induced damage correlating bladder cancer cell chemosensitivity *in vitro* support a hypothesis that levels of immediate DNA damage are important determinants of cell sensitivity towards these agents. This is supported by several studies. For example, positive correlations have been reported between levels of cisplatin-induced DNA crosslinks (in peripheral blood lymphocytes, used as a surrogate tissue) and good clinical outcome for solid tumours.^{29,30} In addition, Volpato and co-workers have reported good correlation between the induction of mitomycin-C induced DNA interstrand crosslinks (as determined by a modified-ACA protocol, similar to the protocol described here) and cellular response to mitomycin-C for a range of cancer cell lines, including bladder³¹; indeed, in the latter study, the lack of evidence supporting a strong role for repair or apoptosis in mitomycin-C sensitivity³¹ suggests that biochemical events leading to DNA damage formation are key factors in determining cell response.

Analogous radiogenic DNA damage sensitivities in different bladder cancer cell-DNA substrates

As mentioned earlier, for the six cell lines studied, there was approximately the same rank order for chemosensitivity (this study) as for radiosensitivity.¹⁵ In an attempt to delineate factors potentially causative of this noted ranking, the relative radiation DNA damage sensitivities of three distinct bladder cancer cell-DNA substrates (intact cells, prepared nuclei and prepared nucleoid bodies) were investigated using ACA. The observation of higher measures of immediate comet formation for the nuclei and nucleoids (Fig. 2) is in line with expectation, as the preparation of nuclei results in loss of low molecular weight scavengers from the nuclear compartment, whilst the preparation of nucleoids results in the loss of nuclear histone proteins; the loss of either scavenger or histones will result in increased DNA damage sensitivity.^{32,33}

The observation of irradiated nucleoids and nuclei showing the same rank order for comet formation as observed in the intact parent cells suggests that it is a feature present/retained in the nucleoids that is responsible for the relative extent of comet formation of the two other systems. This may reflect differences in the organisation/stability of the DNA in radiation sensitive versus resistant cells, either rendering the DNA intrinsically more damage sensitive in radio-sensitive cells or there being weaker associations between DNA/chromatin and the underlying nuclear matrix through nucleoprotein interactions called matrix associated regions (MARs). MARs allow the maintenance of contiguous looped regions of nuclear DNA of varying superhelical densities.^{34,35} However, altered/deficient MARs may result in weaker attachment, so allowing for the additional release of adjacent/contiguous loops of DNA because of the presence of radiation-induced DNA damage, and this could result in greater measures of comet formation. A consequence of weaker DNA-matrix associations is a lower rewinding ability for DNA supercoils in the presence of DNA damage. Studies of radiation sensitive and resistant cells reveal that heightened radiosensitivity correlates with reduced DNA supercoil rewinding ability, plus the absence of various nuclear matrix proteins.^{36,37} Indeed, similar measures of reduced DNA supercoiling ability in cells derived from human invasive TCC biopsies have been shown to correlate with improved local tumour control after RT.^{38,39} Consequently, it is highly likely that TCC cells of differing radiosensitivity will have altered MARs and/or nuclear matrix protein composition.

ACA assessment of tumour cell DNA damage sensitivity and correlation with clinical end-points

In the second part of the current study, to determine if there is an association between intrinsic damage sensitivity and tumour response to treatment, ACA analyses of *ex vivo* radiation-induced DNA damage in bladder cancer cells derived from biopsied bladder tumours were undertaken and possible correlations sought with patient outcome. A wide range of ACA damage

responses was obtained (Fig. 3). This in itself is important since these differences may reflect actual differences in tumour cell radiosensitivity; with the observation of high degrees of apparent radioresistance (Fig. 3) being likely to contribute to the current high level of RT treatment failure for MID.^{10,40,41}

Whilst there was no significant association noted between the extent of DNA damage sensitivity and either overall survival or bladder cancer-specific survival, there was a significant association between the measures of low bladder tumour cell DNA damage sensitivity and the development of local recurrence for NMID. In addition, there was a significant trend for patients demonstrating low measures of damage sensitivity to generally suffer a proportionally higher number of adverse events. It was also noted that the observation of lower measures of induced damage was also a better indicator of local recurrence than the presence of high-risk histology (G3 pT1) for NMID in this cohort.

DNA damage and bladder cancer cell treatment sensitivity *in vitro* and outcome *in vivo*

The data presented in this study (i) supports a hypothesis that mechanisms which govern treatment-induced DNA damage formation are central to bladder cancer cell treatment sensitivity and (ii) support an association between damage resistance and aggressive tumour phenotype in this cancer model.

In support of (i) we and others have shown that *in vitro* measures of immediate DNA damage correlate both bladder cancer cell radiosensitivity^{14,15} and chemosensitivity³¹ (plus this study). Furthermore, *in vivo*, expression levels of three key DNA damage repair proteins (APE1, XRCC1 and MRE11) predict cancer-specific survival following radical RT in bladder cancer^{42,43} and single nucleotide polymorphisms in DNA repair genes might be prognostic factors in bladder cancer patients treated with CRT⁴⁴ or RT.⁴⁵

In support of (ii), for many cancers, associations have frequently been noted between tumour aggression, damage resistance and resistance to apoptosis, whereby, the more aggressive the cancer, the more resistant the tumour's constituent cells. Intuitively, a low DNA damage sensitive phenotype will render cells resistant to DNA damage-dependent apoptosis; in turn, apoptosis resistance strongly contributes to the clonal expansion of cancer cells and is also important for malignant cells to disseminate and form metastasis.⁴⁶ Intriguingly, SIP1 protein, a regulator of epithelial mesenchymal transition and a proposed marker of tumour cell aggressiveness, has been shown to protect bladder cells from DNA damage induced apoptosis and to be of independent prognostic value in bladder cancer.⁴⁷ A further possible molecular pathway is the interferon/Stat1 pathway recently proposed to have a role in tumour resistance to genotoxic stress and aggressive growth.⁴⁸ Uncovering the nature of an aggressive/treatment-resistant phenotype in bladder cancer may lead to the discovery of new therapeutic targets and prognostic tools.

Data presented in this study are also consistent with clinical experience, in that, patients who respond well to neoadjuvant

chemotherapy often respond well to radiotherapy to the bladder. This empirical observation is supported by there being the same rank order for chemosensitivity as for radiosensitivity in the reported cell culture studies (this study and ¹⁵).

Comet assay as a predictor of bladder cancer cell treatment outcome

In a review of biomarkers in bladder cancer,⁴⁹ Bryan and coworkers concluded that, despite significant advances in the molecular understanding of bladder cancer biology, it is unlikely that any single marker will be able to improve prognosis for patients (above and beyond grade and stage), but that a combination of multiple independent markers might more precisely predict the outcome. In this study we measure a single entity (DNA damage). However, this measure encompasses many important molecular, genetic and epigenetic events that dictate/influence a cell's response to treatment. We therefore propose that analysing whole cells (allowing for assessments of both damage formation and repair) from an individual tumour offers a legitimate chance of predicting a tumour's response to treatment. This argument also supports the proposition that the Comet assay can also be used to predict mitomycin-C chemosensitivity. The advantage of this approach is that intact cells express both a variety of proteins/reductases involved in the bioreductive drug activation process and also detoxification pathways and other cellular defense mechanisms. The extent of crosslink formation is likely to represent the balance between these two processes and may more accurately reflect treatment sensitivity in patients. Whilst predicting response to therapy based on analysis of single molecular markers remains an attractive proposition, it is probably too simplistic and 'all-inclusive' cell-based procedures such as the Comet assay represent a realistic way forward.

Forty nine bladder tumour biopsies were analysed, representing an overall success rate of 73% from tumour collection to assay. With the Comet assay being considered a possible tool in the clinical management of cancer,⁵⁰ this success rate compares well with best-case scenarios for the accepted gold-standard lab-assay for determining tumour cell sensitivity, clonogenic survival. However, the main disadvantages of the

clonogenic assay are that a number of weeks are required to obtain a result, and many excised tumours fail to grow/clone.¹⁴

The Comet assay as a potential predictive test has distinct advantages. It is straightforward, rapid, relatively cheap, does not require cell growth and it can be automated; all features that make it suitable for routine testing in a clinical context. The assay has been successfully used to demonstrate that treatment sensitivity can be measured in a range of tumour cell lines including cervical, colon, bladder, prostate, renal, breast, lung and ovarian (reviewed by McKenna *et al.*⁵⁰). Since each cell line in effect represents a different individual, these studies collectively demonstrate that the Comet assay is capable of detecting intrinsic differences in sensitivity between different tumour cells.

Conclusions

We have shown that Comet assay measures of induced damage correlate bladder cancer cell sensitivity *in vitro*, with there being principally the same rank order for chemosensitivity as for radiosensitivity. Furthermore, studies of radiation-induced damage in different bladder cancer cell-DNA substrates suggest that it is a feature of the nucleoid bodies that is responsible for the relative damage sensitivity of bladder cancer cells, suggestive of causative differences in the organisation/stability of the nuclear DNA within resistant/sensitive cell lines. Finally, lower ACA measures of DNA damage sensitivity have been shown to be significantly associated with local recurrence for non-muscle-invasive disease and to broadly correlate with poorer outcomes following bladder tumour treatment. Altogether, the current study demonstrates that mechanisms which govern treatment-induced DNA damage are both central to and predictive of bladder cancer cell treatment sensitivity and identifies/substantiates an association between damage resistance and aggressive tumour phenotype for this cancer type.

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REFERENCES

1. Sylvester RJ, van der Meijden APM, Oosterlinck W, et al. Predicting recurrence and progression in individual patients with stage Ta T1 bladder cancer using EORTC risk tables: a combined analysis of 2596 patients from seven EORTC trials. *Eur Urol* 2006;49: 466–77.
2. Aparicio AM, Elkhouri AB, Quinn DI. The current and future application of adjuvant systemic chemotherapy in patients with bladder cancer following cystectomy. *Urol Clin N Am* 2005; 32:217–30.
3. Raghavan D. Progress in the chemotherapy of metastatic cancer of the urinary tract. *Cancer* 2003;97:2050–5.
4. Raghavan D, Shipley WU, Russell PJ, et al. Biology and management of bladder-cancer—reply. *N Engl J Med* 1991;324:125–6.
5. Loehrer PJ, Einhorn LH, Elson PJ, et al. A randomized comparison of cisplatin alone or in combination with methotrexate, vinblastine, and doxorubicin in patients with metastatic urothelial carcinoma—a cooperative group-study. *J Clin Oncol* 1992;10:1066–73.
6. Parekh DJ, Bochner BH, Dalbagni G. Superficial and muscle-invasive bladder cancer: principles of management for outcomes assessments. *J Clin Oncol* 2006;24:5519–27.
7. Choudhury A, Swindell R, Logue JP, et al. Phase II study of conformal hypofractionated radiotherapy with concurrent gemcitabine in muscle-invasive bladder cancer. *J Clin Oncol* 2011;29: 733–8.
8. James ND, Hussain SA, Hall E, et al. Radiotherapy with or without Chemotherapy in Muscle-Invasive Bladder Cancer. *N Engl J Med* 2012;366: 1477–88.
9. Kotwal S, Choudhury A, Johnston C, et al. Similar treatment outcomes for radical cystectomy and radical radiotherapy in invasive bladder cancer treated at a United Kingdom specialist treatment center. *Int J Radiat Oncol Biol Phys* 2008; 70:456–63.
10. Cooke PW, Dunn JA, Latief T, et al. Long-term risk of salvage cystectomy after radiotherapy for

- muscle-invasive bladder cancer. *Eur Urol* 2000;38: 279–85.
11. Quilty PM, Duncan W, Chisholm GD, et al. Results of surgery following radical radiotherapy for invasive bladder-cancer. *Br J Urol* 1986;58: 396–405.
 12. Huddart RA, Hall E, Lewis R, et al. Life and death of spare (selective bladder preservation against radical excision): reflections on why the spare trial closed. *BJU Int* 2010;106:753–5.
 13. Colquhoun AJ, Jones GDD, Al Moneef M, et al. Improving and predicting radiosensitivity in muscle invasive bladder cancer. *J Urol* 2003;169: 1983–92.
 14. McKeown SR, Robson T, Price ME, et al. Potential use of the alkaline comet assay as a predictor of bladder tumour response to radiation. *Br J Cancer* 2003;89:2264–70.
 15. Moneef MAL, Sherwood BT, Bowman KJ, et al. Measurements using the alkaline comet assay predict bladder cancer cell radiosensitivity. *Br J Cancer* 2003;89:2271–6.
 16. Collins AR. The comet assay for DNA damage and repair—principles, applications, and limitations. *Mol Biotechnol* 2004;26:249–61.
 17. Olive PL. DNA damage and repair in individual cells: applications of the comet assay in radiobiology. *Int J Radiat Biol* 1999;75:395–405.
 18. Singh NP, McCoy MT, Tice RR, et al. A simple technique for quantitation of low-levels of DNA damage in individual cells. *Exp Cell Res* 1988;175: 184–91.
 19. Tice RR, Agurell E, Anderson D, et al. Single cell gel/comet assay: Guidelines for in vitro and in vivo genetic toxicology testing. *Environ Mol Mutagen* 2000;35:206–21.
 20. Hartley JM, Spanswick VJ, Gander M, et al. Measurement of DNA cross-linking in patients on ifosfamide therapy using the single cell gel electrophoresis (comet) assay. *Clin Cancer Res* 1999; 5:507–12.
 21. Almeida GM, Duarte TL, Farmer PB, et al. Multiple end-point analysis reveals cisplatin damage tolerance to be a chemoresistance mechanism in a NSCLC model: implications for predictive testing. *Int J Cancer* 2008;122:1810–9.
 22. Duarte TL, Almeida GM, Jones GDD. Investigation of the role of extracellular H₂O₂ and transition metal ions in the genotoxic action of ascorbic acid in cell culture models. *Toxicol Lett* 2007;170:57–65.
 23. Price P, McMillan TJ. The use of non-clonogenic assays in measuring the response of cells in-vitro to ionizing-radiation. *Eur J Cancer* 1994;30A:838–41.
 24. Sievert KD, Amend B, Nagele U, et al. Economic aspects of bladder cancer: what are the benefits and costs? *World J Urol* 2009;27:295–300.
 25. Saris CP, vandeVaart PJM, Rietbroek RC. In vitro formation of DNA adducts by cisplatin, lobaplatin and oxaliplatin in calf thymus DNA in solution and in cultured human cells. *Carcinogenesis* 1996;17:2763–9.
 26. Carther SK CS. Mitomycin C: current status and new developments. New York: Academic Press, 1979.
 27. Tolley DA, Parmar MKB, Grigor KM, et al. The effect of intravesical mitomycin C on recurrence of newly diagnosed superficial bladder cancer: a further report with 7 years of followup. *J Urol* 1996;155:1233–7.
 28. Basu S, Brown JE, Flannigan GM, et al. Immunohistochemical analysis of NAD(P)H: quinone oxidoreductase and NADPH cytochrome P450 reductase in human superficial bladder tumours: relationship between tumour enzymology and clinical outcome following intravesical mitomycin C therapy. *Int J Cancer* 2004;109:703–9.
 29. van de Vaart PJM, Belderbos J, de Jong D, et al. DNA-adduct levels as a predictor of outcome for NSCLC patients receiving daily cisplatin and radiotherapy. *Int J Cancer* 2000;89:160–6.
 30. Schellens JHM, Ma J, Planting AST, et al. Relationship between the exposure to cisplatin, DNA-adduct formation in leucocytes and tumour response in patients with solid tumours. *Br J Cancer* 1996;73:1569–75.
 31. Volpato M, Seargent J, Loadman PM, Phillips RM. Formation of DNA interstrand cross-links as a marker of Mitomycin C bioreductive activation and chemo sensitivity. *Eur J Cancer* 2005;41: 1331–8.
 32. Elia MC, Bradley MO. Influence of chromatin structure on the induction of dna double strand breaks by ionizing-radiation. *Cancer Res* 1992;52: 1580–6.
 33. Milligan JR, Aguilera JA, Ward JF. Variation of single-strand break yield with scavenger concentration for plasmid DNA irradiated in aqueous-solution. *Radiat Res* 1993;133:151–7.
 34. Vogelstein B, Pardoll DM, Coffey DS. Supercoiled loops and eukaryotic DNA replication. *Cell* 1980; 22:79–85.
 35. Pienta KJ, Coffey DS. A structural analysis of the role of the nuclear matrix and DNA loops in the organisation of the nucleus and chromosome. *J Cell Sci* 1984;Suppl 1:123–35.
 36. Malyapa RS, Wright WD, Roti JLR. Radiation sensitivity correlates with changes in DNA supercoiling and nucleoid protein-content in cells of 3 chinese-hamster cell-lines. *Radiat Res* 1994;140: 312–20.
 37. Malyapa RS, Wright WD, Roti JLR. DNA supercoiling changes and nucleoid protein composition in a group of L5178Y cells of varying radiosensitivity. *Radiat Res* 1996;145:239–42.
 38. Vaughan ATM, Anderson P, Wallace DMA, et al. Local-control of T2/3 transitional cell-carcinoma of bladder is correlated to differences in DNA supercoiling—evidence for 2 discrete tumor populations. *Cancer Res* 1993;53: 2300–3.
 39. Lynch TH, Anderson P, Wallace DMA, et al. A correlation between nuclear supercoiling and the response of patients with bladder-cancer to radiotherapy. *Br J Cancer* 1991;64:867–71.
 40. Duncan W, Quilty PM. The results of a series of 963 patients with transitional cell-carcinoma of the urinary-bladder primarily treated by radical megavoltage X-ray therapy. *Radiother Oncol* 1986;7:299–310.
 41. Shipley WU, Rose MA, Perrone TL, et al. Full-dose irradiation for patients with invasive bladder-carcinoma—clinical and histological factors prognostic of improved survival. *J Urol* 1985;134: 679–83.
 42. Choudhury A, Nelson LD, Teo MTW, et al. MRE11 expression is predictive of cause-specific survival following radical radiotherapy for muscle-invasive bladder cancer. *Cancer Res* 2010; 70:7017–26.
 43. Sak SC, Harnden P, Johnston CF, et al. APE1 and XRCC1 protein expression levels predict cancer-specific survival following radical radiotherapy in bladder cancer. *Clin Cancer Res* 2005; 11:6205–11.
 44. Sakano S, Wada T, Matsumoto H, et al. Single nucleotide polymorphisms in DNA repair genes might be prognostic factors in muscle-invasive bladder cancer patients treated with chemoradiotherapy. *Br J Cancer* 2006;95:561–70.
 45. Teo MTW, Landi D, Taylor CF, et al. The role of microRNA-binding site polymorphisms in DNA repair genes as risk factors for bladder cancer and breast cancer and their impact on radiotherapy outcomes. *Carcinogenesis* 2012;33: 581–6.
 46. Glinsky GV, Glinsky VV. Apoptosis and metastasis: a superior resistance of metastatic cancer cells to programmed cell death. *Cancer Lett* 1996;101: 43–51.
 47. Sayan AE, Griffiths TR, Pal R, et al. SIP1 protein protects cells from DNA damage-induced apoptosis and has independent prognostic value in bladder cancer. *Proc Natl Acad Sci USA* 2009;106: 14884–9.
 48. Khodarev NN, Roizman B, Weichselbaum RR. Molecular pathways: interferon/Stat1 pathway: role in the tumor resistance to genotoxic stress and aggressive growth. *Clin Cancer Res* 2012;18: 3015–21.
 49. Bryan RT, Zeegers MP, James ND, et al. Biomarkers in bladder cancer. *BJU Int* 2010;105:608–13.
 50. McKenna DJ, McKeown SR, McKelvey-Martin VJ. Potential use of the comet assay in the clinical management of cancer. *Mutagenesis* 2008;23: 183–90.