DNA double strand break repair in human bladder cancer is error prone and involves microhomology-associated end-joining

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ABSTRACT

In human cells DNA double strand breaks (DSBs) can be repaired by the non-homologous end-joining (NHEJ) pathway. In a background of NHEJ deficiency, DSBs with mismatched ends can be joined by an error-prone mechanism involving joining between regions of nucleotide microhomology. The majority of joins formed from a DSB with partially incompatible 3’ overhangs by cell-free extracts from human glioblastoma (MO59K) and urothelial (NHU) cell lines were accurate and produced by the overlap/fill-in of mismatched termini by NHEJ. However, repair of DSBs by extracts using tissue from four high-grade bladder carcinomas resulted in no accurate join formation. Junctions were formed by the non-random deletion of terminal nucleotides and showed a preference for annealing at a microhomology of 8 nt buried within the DNA substrate; this process was not dependent on functional Ku70, DNA-PK or XRCC4. Junctions were repaired in the same manner in MO59K extracts in which accurate NHEJ was inactivated by inhibition of Ku70 or DNA-PKcs. These data indicate that bladder tumour extracts are unable to perform accurate NHEJ such that error-prone joining predominates. Therefore, in high-grade tumours mismatched DSBs are repaired by a highly mutagenic, microhomology-mediated, alternative end-joining pathway, a process that may contribute to genomic instability observed in bladder cancer.

INTRODUCTION

A range of complex DNA lesions can be produced in response to ionising radiation or radiomimetic chemicals. One of the most toxic lesions is the double strand break (DSB) which, if it remains un repaired, is lethal to the cell (1). Repair of DSBs can be undertaken by two main pathways; homologous recombination (HR) and non-homologous end-joining (NHEJ). These pathways are distinct in that HR copies homologous DNA sequences from sister chromatids resulting in error-free repair whilst NHEJ joins the broken DNA ends in a process that may result in the loss of a small number of terminal nucleotides (2). HR is the prominent pathway for repair of DSBs during late S/G2 phases of the cell cycle when sister chromatids are present, whereas in G0/G1 and early S-phases NHEJ predominates (3).

The joining of DNA termini by NHEJ is initiated by the binding of the Ku heterodimer (Ku70 and Ku86) and subsequent association with the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) (4). This DNA-PK complex may be involved in the protection/alignment of DNA ends and facilitation of ligation by recruitment of the XRCC4/ligase IV complex. Many in vitro assays of NHEJ measure the ability of cell-free extracts or purified recombinant proteins to join compatibly ended DNA substrates generated by restriction nuclease digestion; such complementary DNA ends are joined in an efficient and accurate manner without loss of terminal nucleotides (5,6). However, in vivo, radiation-induced DSBs are often chemically modified, staggered and/or comprise partially or completely incompatible DNA ends that require modification to produce ligatable ends before joining can take place (1). The recruitment of proteins involved in the processing of DNA ends depends on the type of modification required. Removal of chemically modified DNA ends can be undertaken by APE1 (7) or TDP1 (8). The resection of staggered ends requires exonuclease activity for the removal of terminal nucleotides; Mre11, which functions as a complex with Rad50 and Nbs1 (M/R/N) in mammalian cells (9), DNase III (10) and Wrn (11) are 3’–5’ exonucleases whilst Artemis has 5’–3’ exonuclease activity and acquires endonuclease activity when phosphorylated by DNA-PK (12); each of these proteins have been found at the DNA repair synapse. The cleavage of residual DNA overhangs remaining after exonuclease resection can be undertaken by endonucleases including Fen-1 (13). Finally, gap-filling is thought to be performed by DNA polymerases such as μ (14) or λ (15). Such end processing may result in small losses of sequence information at the resultant junctions such that NHEJ is potentially a mutagenic process.

In addition to the classical NHEJ pathway there is evidence that an alternative, Ku-independent mechanism for the end-joining of double stranded DNA molecules with incompatible DNA ends can be utilized in cells (16). Several studies in the Ku86-deficient rodent cell line xrs6 (17–20) revealed that DNA substrates with non-matched ends were joined at a...
much lower frequency than compatible-ended substrates. The repair junctions in these experiments were formed with decreased accuracy, with extensive deletion of nucleotides at DNA ends and base-pairing interactions occurring using small regions of microhomology at internal sites on the DNA substrate. Similar types of deletions and microhomology-driven repair were observed in the joining of incompatible DNA substrates with fractionated Xenopus oocyte (21) and calf thymus (22) cell extracts both lacking Ku and also in a strain of Saccharomyces cerevisiae in which Ku70 had been deleted (23). In addition, end-degradation of the non-complementary ends of double stranded transposon elements was observed in XR-V15B Ku86-deleted hamster cells (24). Microhomology-driven error-prone end-joining also occurs in mammalian cells that are deficient for the other classical NHEJ proteins XRCC4 (19) and ligase IV (25). Therefore, alterations in the function of NHEJ can reduce DSB repair fidelity and indicate a possible role in maintaining chromosome integrity and stability.

The prognosis of patients with muscle invasive transitional cell carcinomas (TCCs) is poor, with a 5 year survival rate of 50% (26). These tumours, which are generally of high grade, carry a large number of genomic deletions and amplifications in addition to inactivating mutations in the p53 and retinoblastoma genes. Ionizing radiation and radiomimetic agents are used in the radical treatment of bladder tumours, as an alternative to the removal of the bladder, which results in the long-term cure of a proportion of patients; however, little is known about the efficiency of DSB repair processes in the bladder and what factors might underlie the high frequency of chromosomal instability in bladder tumours. One factor contributing to this lack of understanding is the difficulty in routinely establishing primary cell lines from bladder tumour epithelial cell material and their resistance to manipulation, e.g. transfection. Furthermore, for the normal bladder epithelium (urothelium) very little tissue can be obtained, however, urethelial cells can be cultured in vitro. Consequently, studies investigating bladder tumour repair biology have to be undertaken on cell-free extracts prepared from fresh tumour and normal primary cells.

To address the role of DNA end-joining in the repair of DSBs in bladder tumours we measured the end-joining characteristics of cell-free extracts prepared from high grade bladder tumours and normal human urothelial (NHU) cells in comparison with those prepared from the NHEJ proficient human glioblastoma cell line MO59K (5). The DSB substrates used contained 3' single stranded overhangs of 4 nt which were generated by BstXI restriction nuclease digestion allowing the creation of incompatible DNA ends. We found that the majority of partially incompatible DSBs were joined in an accurate, NHEJ-dependent manner by MO59K and NHU extracts. In contrast, the repair of partially incompatible DSBs by bladder tumour extracts was carried out through a distinct pathway that was error-prone, resulted from the deletion of terminal nucleotides and joining at regions of microhomology, and was not dependent on Ku70, DNA-PK or XRCC4. In extracts which accurate NHEJ was inhibited by the addition of Ku70 inactivating antibodies or lack of active DNA-PKcs, repair of DSBs resulted in junctions that resembled those observed in bladder tumour extracts. These results suggest that there is a preference for an alternative error-prone end-joining pathway over the classical NHEJ pathway in high grade bladder tumours.

**MATERIALS AND METHODS**

**Urothelial tissue**

Bladder tumours BT1 and BT8 (TCC grade 3, pT2), BT9 (TCC grade 3, pT1), BT7 (squamous cell carcinoma, grade 3, pT2) and NHU cells from pathologically healthy ureter were obtained by surgical resection from patients at St James’s University Hospital, Leeds. All patients gave informed consent.

**Cell culture**

The MO59K and MO59J cell lines were obtained from Professor S. C. West, Cancer Research UK London Research Institute, Clare Hall Laboratories, South Mimms, UK. Cells were maintained at 37°C and 5% CO₂ in Hams F12:DMEM (1:1 v/v) culture medium (GibcoBRL Invitrogen, Paisley, UK), supplemented with 10% fetal calf serum (Harlan SeraLab, Loughborough, UK), 1 mM L-glutamine (GibcoBRL), non-essential amino acids (0.05 mM) and sodium pyruvate (0.1 mM). Primary NHU epithelial cells were isolated and cultured in keratinocyte serum free medium supplemented with cholera toxin, epidermal growth factor (EGF) and bovine pituitary extract (GibcoBRL) as previously described (27) and were only used at passage number of four or below.

**Cell-free extract preparation**

Cell extracts were prepared as previously described (27) using approximately 5 × 10⁷ MO59K or MO59K and 5 × 10⁷–10 × 10⁷ NHU cells. Bladder tumour tissue biopsies were dissected initially to remove obvious blood clots andstromal tissue, then minced in RPMI medium and washed in phosphate-buffered saline (PBS) three times to remove as many erythrocytes as possible. Minced tissue (200 mg to 1 g) was washed and then homogenized in hypotonic buffer (final volume 0.2–1 ml) and mixed with 0.5 vol of high salt buffer. Ultracentrifugation (Beckman Optima TL) was performed for 40 min at 212 000 g at 4°C using thick-walled polycarbonate microfuge tubes and adaptors for a Beckman TLA100.4 rotor. Protein concentration was assessed using the Coomassie assay protein reagent kit according to manufacturer’s instructions (Pierce Biotechnology, Milwaukee, WI).

**DNA substrates**

Substrates with either compatible or incompatible 3' overhangs of 4 nt were prepared by BstXI (endonuclease cleavage site 5'-CCAN₃TGG) digestion of the following constructs; a 1.2 kb region of ADNA was amplified using PCR primers containing flanking restriction sites for either EcoRI or Xbal (underlined) and different internal BstXI sites (boldface).

(i) 5'-GGAAATCCACTAAGGGTGTTGCAGCTGCTTCACGAAACTC, (ii) 5'-GCTCTAGACCCACCTTATGGAATCTTTCTATACCTAC, (iii) 5'-GCTCTAGACCCACCAAAATGGGATCTCCCTTTTCTACTACCTAC, (iv) 5'-GCTCTAGACCCCCAAATGGAATCTCCCTTTTCTACTACCTAC. The PCR products were digested with EcoRI and Xbal and cloned into pGEM3zf + DNA (Promega, USA) such that digestion of...
each resultant recombinant with BstXI yielded a 3.2 kb plasmid and a 1.2 kb λ fragment with either compatible (primers i + ii) or incompatible (i + iii and i + iv) ends. DNA fragments were gel-purified using spin columns (Qiagen, UK) and resuspended to 5 ng/μl.

DNA end-joining assay
End-joining reactions (20 μl) were carried out with 40 μg protein extract and 20 ng DNA substrate in the presence of 50 mM HEPES pH 8.0; 40 mM KOAc; 0.5 mM Mg(OAc)2; 1 mM ATP; 1 mM DTT and 0.1 mg/ml BSA at 37°C for 2 h unless otherwise stated. Where indicated protein samples were pretreated with wortmannin (10 μM final concentration; Sigma, UK), anti-Ku70 (1:20 to 1:50 dilution; Abcam, Cambridge, UK) or anti-XRCC4 antibody (1:50 dilution; Serotec, Oxford, UK) for 10 min on ice before use in end-joining reactions. Control joining by T4 ligase (New England Biolabs, Beverly, MA) was performed using the manufacturer’s recommended buffer at 37°C for 2 h. Samples were incubated with RNase A (80 μg/ml) for 10 min and then protein was removed by incubation with proteinase K (2 mg/ml) and 0.5% (w/v) SDS for 10 min and extraction with Tris-buffered phenol/chloroform/isoamyl alcohol. Analysis was performed by agarose (0.7%) gel electrophoresis and SYBR Green I staining. Images were collected and quantified using a Molecular Imager FX with SYBR Green I detection setting and Quantity One version 4.1.1 software (Bio-Rad, UK).

PCR and sequencing
For analysis of joined products 2× volume end-joining reactions were ethanol-purified and amplified using AmpliTaq Gold (Applied Biosystems, UK) in the presence of 1.5 mM MgCl2 and internal plasmid primers pFOR (5'-CCGGCG- ACGTGGCCGAGAAG) and pREV (5'-GACTGGAAAGC GGGCAGTGAG) for 35 cycles (1 min 94°C, 30 s 58°C, 1 min 72°C, full length product size 551 bp). PCR products were gel-purified, cloned using TOPO TA cloning kit (Invitrogen) and colonies of resulting recombinants were amplified using PCR with TOPO vector primers T3 (5'-ATTACCCCT- CACTAAAAGGA) and T2 (GGCGATTGAATTTAGCCG- CGCG) for 35 cycles (1 min 94°C, 30 s 55°C, 1 min 72°C, full length product size 625 bp), and sequenced directly using BigDye Terminator version 1.1 (Applied Biosystems) and the pFor or T3 primer. Deletions of >500 bp were not detectable due to the position of the primers used for PCR and those between 450 and 500 bp generally did not yield enough sequence for the junction to be unambiguously determined. PCR amplification of DNA substrates which had not been subject to end-joining reactions yielded no product.

Immunodepletion
Samples of cell-free extract (100 μg) in the presence of 50 mM HEPES pH 8.0; 40 mM KOAc; 0.5 mM Mg(OAc)2; 1 mM ATP; 1 mM DTT and 0.1 mg/ml BSA were precleared by mixing with 20 μl protein G PLUS-Agarose (Insight Biotechnology Ltd, UK) at 4°C for 30 min. After centrifuging at 1000 g the supernatant was mixed with 10 μg (1:50 dilution) of anti-Ku70 antibody (ab87, Abcam, UK) and 20 μl protein G PLUS-Agarose and constantly agitated at 4°C overnight. The supernatant was retained and the agarose was washed five times with PBS and eluted in 50 μl sample buffer containing 20% (v/v) glycerol; 4% (w/v) SDS; 120 mM Tris–HCl, pH 6.8; 0.001% (w/v) bromophenol blue and 100 mM DTT by heating to 95°C for 5 min. Samples were analysed for Ku70 by western blotting as described below.

RESULTS
End-joining accuracy depends on DSB compatibility
As the structure of DNA ends may determine the type of DSB repair pathway observed (28) and due to the difficulties arising in comparing results of repair assays undertaken in studies which use cell extracts prepared from distinct species, we measured the end-joining of DNA substrates containing 3’ single stranded overhangs (Figure 1A) which have been published previously (18,21). BstXI sites were engineered into plasmid vector DNA allowing digestion with a single restriction enzyme to yield substrates with either compatible (Co) or incompatible (I2 and I4, containing mismatches of 2 and 4 bases, respectively) ends. Incubation of BstXI digested DNA substrates with T4 ligase indicated that, whilst the compatible DNA substrate could be ligated, both incompatible (I2 and I4) substrates could not be joined without the addition of the appropriate compatible λDNA fragments (Figure 1B). Joining of the compatible-ended substrate using cell-free extract derived from the glioblastoma cell line MO59K, used previously in other NHEJ studies (5), was dependent on magnesium, ATP and the presence of active DNA-PK. Ku70 and XRCC4 proteins, indicative of joining by NHEJ (Figure 1B). DNA substrates with 3’ single stranded overhangs were joined with lower frequency than those with 5’ single stranded overhangs (data not shown) in agreement with other groups (29,30).

In comparison to the Co substrate, I2 and I4 were joined with significantly reduced frequency by the MO59K extract (Figure 2A). Agarose gel analysis of cloned junctions amplified by PCR (Figure 2B) showed that joins formed with Co and I2 were generally without extensive nucleotide loss (full length band size of 625 bp observed) while some PCR products resulting from reactions with I4 were reduced in size. Sequence analysis of the junctions formed with Co and I2 (Figure 2C) revealed that the majority of joins were accurate. The compatible substrate was joined using complementary base-pair interactions on the single strand overhangs as expected. The joins with the I2 incompatible substrate were
Primarily formed using the ‘overlap’ pathway, as previously described (18,21) and involved the sequence of one 4 nt overhang being maintained with the removal of the terminal 3 nt of the other overhang, pairing of the remaining nucleotides and completion of the gap by fill-in synthesis, so the overall sequence of one strand was accurately retained (Figure 2C). In the course of our analysis, occasionally, joins formed with I2 and MO59K extract resulted from the insertion of a single nucleotide (5% of joins formed: 8 out of 146 joins analysed) or deletion of terminal nucleotides (7% of joins formed: 10 out of 146 joins analysed; Table 1). With the I4 incompatible substrate 45% of joins were formed using the overlap pathway. However, 27% of joins were formed using staggered base-pair interactions within the overhangs followed by the fill-in of gaps and the remainder of joins (28%) were formed by large deletion (191–393 bp) of terminal nucleotides with small regions of microhomology (1–5 bp) observed at the resultant junction (Figure 2C). This indicates that a low level of error-prone end-joining occurs in MO59K cells in addition to the dominant, accurate NHEJ pathway.

Compatible DSBs are joined accurately by NHU and the majority of bladder tumours

In order to determine the end-joining characteristics of bladder tumours, DSB repair of compatible ends was compared between cell-free extracts produced from four independent high-grade bladder carcinomas and five NHU primary cell lines. Cultured NHU cells, isolated from ureters of patients without bladder cancer, were chosen as the control tissue as it is impossible to identify and isolate normal bladder epithelium in patients with bladder tumours. One pair of NHU and bladder tumour material was isolated from the same patient (NHU1 and BT1) whilst the other NHU samples were from patients independent from those from whom the bladder tumours were obtained. Most NHU and bladder tumour extracts had slightly reduced joining frequency in comparison with MO59K extract, being able to join DNA substrates to form dimers, and there was some inter-individual variability where NHU3, BT1 and BT7 also produced trimers (Figure 3). As divalent cation concentration is known to be critical for DSB repair processes (31) joining reactions were also performed in the presence of EDTA. Addition of 1 mM EDTA effectively inhibited end-joining observed with the compatible substrate.

The DSB repair of the Co substrate was predicted to be accurate as no additional end-processing should be required for ligation to take place. PCR (Figure 4A) and sequence (Figure 4B) analysis of junctions formed with Co and NHU extracts revealed that the majority (between 85 and 100%; Table 1) were formed accurately using the complementary overhangs, with very few deletion-type joins observed. Likewise, the percentage of accurate joins formed with bladder tumour extracts BT7, 8 and 9 was between 60 and 77% of the total, the remainder of joins again resulting from terminal deletions and utilizing regions of microhomology (1–8 bp) (Figure 4B). Unexpectedly, the percentage of accurate joins formed with the BT1 extract was low (26%); junctions were predominantly formed by the deletion of a large number of terminal nucleotides (Table 1) and were associated with larger microhomologies (4–8 bp). For BT1 there was a preference for the deletion-type joins to be formed using an 8 bp microhomology that resulted in the loss of 123 and 4 nt from the 5′ and 3′ ends of the DNA substrate, respectively.

Error-prone joining of incompatible DSBs by bladder tumours

There was a significant difference in the manner in which the I2 incompatible substrate was joined by NHU and bladder tumour extracts. Analysis of joins using I2 (Figure 5; Table 1) showed that similar levels of accurate, overlap joins (63–100%) were observed in the five NHU extracts. In the few junctions arising from deletions, the average size and range were comparable to that observed with NHUs and the compatible substrate, and were not associated with microhomology at the junction, the exception to this was NHU4 where 26% of joins were formed using the 8 bp microhomology observed previously. There was a striking increase in the end-processing of DNA substrate in the bladder tumour extracts. In each bladder tumour extract no accurate joining was observed, with 100% of joins being formed by deletion of terminal nucleotides. The average deletion size was similar.
between tumour extracts (100–125 bp) with a marked preference for the 8 bp microhomology-associated 127 bp deletion. It concerned us that the reduced joining fidelity observed in bladder tumour extracts may arise from the use of solid tissue rather than cultured cells for the preparation of extracts. However, error-prone joining was measured in MO59K extract when fully incompatible substrates were used (Figure 2C). Moreover, accurate joining could be measured in a tumour extract made from a superficial papillary bladder tumour, BT2 (grade 2, pTa), where the majority (72%) of joins formed with I2 were accurate and the remaining joins formed with small deletions (average size 24 bp) that did not utilize Table 1.

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<th>Deletion (bp)</th>
<th>I2 Accurate joins (%)</th>
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<td>15</td>
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*Deletions between 450 and 500 bp did not yield enough sequence for the junction to be unambiguously determined.

Figure 2. Accuracy of end-joining in MO59K extract. (A) End-joining reactions were incubated for 0, 2, 6 or 24 h with the plasmid DNA substrate (1x) indicated. The formation of joined linear dimers, trimers and tetramers are shown (2x, 3x and 4x). Gels shown are representative of two independent experiments. (B) PCR analysis of randomly chosen recombinant clones resulting from the cloning of DNA joins formed in the presence of MO59K extract and Co, I2 or I4 substrates. Joins formed accurately would produce a PCR product (with primers T2 and T3) of 625 bp, junctions formed by the deletion of terminal nucleotides resulted in smaller products. M, molecular mass markers. (C) Sequence analysis of joins formed with DNA substrates Co, I2 and I4, where **** indicates the sequence ATTC, AAAC and TAAG for each substrate, respectively. Top strand of sequence is shown with the join denoted by a vertical bar, inserted nucleotides are underlined and the number of nucleotides deleted from each end of the substrate is indicated. The number of recombinants analysed (clones), the total number of nucleotides deleted or inserted to form the join (del) and microhomology (micro) at junctions are indicated. Joins are termed accurate if sequence information of one strand is retained. Deletions are therefore expressed as the number of nucleotides removed in addition to this processing.
Therefore, as inaccurate repair occurs in cultured cells and accurate joining was apparent in a bladder tumour extract, it is unlikely that the variation in accuracy of joining resulted from fundamental differences between handling cultured cells and tumour tissue. The deletion of terminal nucleotides from DSBs that characterizes repair by bladder tumour extracts indicates the involvement of nuclease activity. The error-prone joining observed in bladder tumour extracts was not caused by high levels of Mg\(^{2+}\) or Mn\(^{2+}\) in the extract as joining was inhibited by 1 mM EDTA (Figure 3) and addition of 5 mM Mg\(^{2+}\), the optimal concentration for the predominant 3' to 5' mammalian exonuclease TREX1 (32,33), to MO59K and NHU end-joining reactions did not result in reduced joining fidelity (data not shown).

**Error-prone end-joining is not dependent on functional Ku70, DNA-PK or XRCC4**

To determine the dependence of end-joining in bladder tumours on classical NHEJ factors, reactions were performed in the presence of wortmannin (a DNA-PK inhibitor) or inactivating Ku70 or XRCC4 antibodies. Concentrations of wortmannin (10 \(\mu\)M) or Ku70 (1:50 dilution) and XRCC4 (1:50 dilution) antibodies that inhibited compatible end-joining in MO59K and NHU1 extracts did not inhibit joining in the bladder extract BT1 (Figure 6A). Similarly, joining by extracts BT7, BT8 and BT9 was not inhibited by the addition of Ku70 or XRCC4 antibodies (data not shown). To determine that Ku70 could be completely inhibited by the addition of anti-Ku70 antibodies, MO59K extract was fully depleted of Ku70 by immunoprecipitation (Figure 6B). Likewise, Ku70 could be immunoprecipitated from bladder tumour extracts using the same conditions (data not shown). Analysis of the Ku-dependency of incompatible I2 end-joining by bladder tumour extracts was performed (Figure 6C). In order to accurately detect levels of end-joining, including that which may occur below the sensitivity of SYBR Green I detection, the junctions formed were amplified using PCR and then levels of product formation were compared. Addition of Ku70 antibody, at a concentration in excess of that known to completely immunodeplete Ku70 from cell extracts, significantly reduced joining of I2 by the MO59K and NHU extracts. However Ku70 antibody did not reduce the level of joins formed by the bladder tumour extracts, indicating that formation of joins in bladder tumour extracts did not involve active Ku. As all end-joining reactions were performed at fixed concentrations of protein, Mg\(^{2+}\) and ATP, it was unlikely that differences in the Ku dependency of end-joining arose from experimental conditions affecting Ku–DNA binding interactions (34).
Incubation of MO59K extract with Ku70 antibodies dramatically reduced, but did not completely abolish, joining of the I2 incompatible substrate. Analysis of cloned junctions amplified by PCR (Figure 6D) showed that whilst joins formed with MO59K extract were predominantly accurate, the joins formed in the presence of the Ku70 inactivating antibody resulted from extensive nucleotide loss. Sequence analysis of the junctions formed under these conditions showed that when Ku was inhibited all but one join was formed in an error-prone manner (average deletion size 212 bp), resulting from deletion of terminal nucleotides and with a preference for the 8 bp region of microhomology resulting in the deletion of 127 nt observed previously in bladder tumour extracts (Figure 6E). This type of deletion was not observed when joining was performed in the presence of the same concentration of a mouse monoclonal β-actin antibody (data not shown).

In a further experiment, addition of 10 μM wortmannin along with Ku70 antibody did not result in any further reduction in joining efficiency or accuracy (average deletion size 241 and 235 bp; joining accuracy 0 and 4% for Ku70 antibody alone and Ku70 antibody + wortmannin, respectively) indicating that the residual activity observed in Ku70-inhibited MO59K extract was indeed NHEJ-independent. Joins formed by the 127 bp deletion were also apparent when inactivating Ku70 antibodies were incubated with NHU1 extract and I2 substrate (data not shown).

The type of end-joining occurring in the presence of Ku but the absence of active DNA-PK was measured. Treatment with wortmannin inhibits DNA-PK autophosphorylation and disassociation from DNA ends and so could prevent access of other DNA repair proteins (35) therefore extract from MO59J, a cell line which has a PRKDC gene mutation so rendering DNA-PKcs mRNA unstable, was used. Error-prone end-joining was observed in 82% of joins (average deletion size 62 bp, range 2–311 bp) formed by MO59J with I2 (Figure 6E), however, deletions were generally small, did not use microhomologies and the remaining 18% of junctions were formed accurately. Addition of 10 μM wortmannin to MO59J extract increased error-prone joining of I2 to 94% (average deletion size 124 bp, range 10–415 bp) and complete inhibition of DNA-PKcs in this manner resulted in a striking increase in the use of microhomology at the junctions (Figure 6E); this suggests that there may be residual DNA-PKcs activity in MO59J which is sufficient to promote some accurate end-joining. Thus the absence of functional Ku70 or DNA-PKcs promotes an inefficient error-prone microhomology-driven end-joining pathway, similar to that observed in bladder tumour extracts.

As DSB repair utilising microhomologies at the resultant junctions was observed in NHEJ-inactivated MO59K cells it was possible that the error-prone repair in bladder tumours arose from a deficiency in a NHEJ component. The levels of NHEJ proteins in bladder tumour extracts were determined by western blotting (Figure 7A). Overall, reduced levels of NHEJ proteins were found in NHU and bladder tumour extracts in comparison to MO59K. The levels of Ku70 and Ku86 were reduced, on average to 55%, in NHU and bladder tumour extracts. DNA-PKcs and ligase IV levels were also lower in both NHU and bladder extracts when compared with MO59K, with ligase IV levels being particularly low in BT7 and BT9 (reduced to 17 ± 8% and 11 ± 5% of MO59K levels, respectively). XRCC4 levels were significantly reduced only in NHU1 (to 51 ± 9% of MO59K levels). These differences could not be correlated with the differences in repair accuracy observed in MO59K, NHU and bladder extracts. Analysis of the M/R/N complex in bladder tumour extracts by western blotting showed that in the BT1 extract Mre11 had a reduced apparent molecular mass (~64 kDa) with no full

**Figure 5.** Error-prone end-joining in bladder tumours with incompatible DNA substrate I2. (A) PCR analysis of randomly chosen recombinant clones resulting from the cloning of DNA joins formed in the presence of incompatible substrate with NHU1 and BT9 extracts. Joins formed accurately produced a full length PCR product of 625 bp with primers T2 and T3 (as indicated by F); junctions formed by the deletion of terminal nucleotides resulted in smaller products. M, molecular mass markers. (B) Sequence analysis of joins formed in NHU1 and bladder tumour (BT1, 7, 8, 9, 2) extracts. Junctions formed using an 8 bp microhomology are shown in bold. "As in Figure 2."
Figure 6. NHEJ-independent end-joining in bladder tumours and Ku70-depleted MO59K. (A) End-joining with the Co substrate in the presence of 10 μM wortmannin (+W), 1:50 anti-Ku70 antibody (– Ku) and 1:50 anti-XRCC4 antibody (– X) as indicated. Plasmid DNA substrate (1×) and joined dimers and trimers (2× and 3×) are shown. Gels shown are representative of two independent experiments. (B) Ku70 levels in 20 μg untreated MO59K sample (extract) and, following immunodepletion of Ku70 using 1:50 anti-Ku70 antibody, protein eluted from sepharose-G beads (IP) and remaining in extract (depleted). (C) PCR analysis of products joined by MO59K, NHU1 and bladder tumour (BT1, 7, 8, 9) extracts and I2 substrate in the presence of 1:20 anti-Ku70 antibody (– Ku). The level of end-joining is expressed as PCR band density in the presence of anti-Ku70 relative to that without antibody (three independent experiments, SD). (D) PCR analysis of randomly chosen recombinant clones containing DNA joins formed with incompatible I2 substrate and MO59K extract pretreated with 1:20 dilution of anti-Ku70 monoclonal antibody (– Ku). Joins formed accurately produced a full length PCR product of 625 bp with primers T2 and T3 (as indicated by F); junctions formed by the deletion of terminal nucleotides resulted in smaller products. M, molecular mass markers. (E) Sequence analysis of joins formed by MO59K and MO59J extract pretreated with 1:20 dilution of anti-Ku70 monoclonal antibody (– Ku) or 10 μM wortmannin (+W). Junctions formed using an 8 bp microhomology are shown in bold, % accurate indicates the proportion of accurate joins formed. *As in Figure 2.
extract from the MO59K human glioblastoma cell line to define the type of DSB end-joining expected in an NHEJ proficient cell and then compared this with the end-joining in extracts prepared from four high grade bladder tumours and five normal urothelial samples. Repair of compatible and partially incompatible DSBs by MO59K was accurate however, DSBs with fully mismatched overhangs could be joined by the non-random deletion of terminal nucleotides at regions of microhomology indicating the presence of an error-prone end-joining mechanism constitutively present at a low level in normal cells. Use of the partially incompatible DSB substrate I2 revealed that while the NHU extracts produced mostly accurate joins, bladder tumour extracts joined with lower fidelity and utilized regions of microhomology within the DNA substrate in a manner that was not dependent on Ku, DNA-PK or XRCC4 and strikingly similar to joins formed in extracts in which Ku70 or DNA-PKcs were not active.

How could DSBs be joined in the absence of classical NHEJ? The joining of DSBs in NHEJ proficient cells has been shown to result in the formation of predominantly accurate joins, where DNA-PK holoenzyme is predicted to stabilize the association of short mismatched overhangs by single nucleotide interactions so allowing fill-in synthesis and ligation to occur (18,29,38). In addition the ligase IV/XRCC4 complex also plays a role in protecting DNA from end-degradation so promoting accurate joining (25). In the absence of classical NHEJ the association of incompatible DNA ends would have to involve the base-pairing of DNA sequences via regions of microhomology within the DNA molecule such that the deletion of non-base-pairing terminal nucleotides would be necessary prior to ligation. The joining of mismatched ends in this way would require the search and recognition of regions of microhomology and resection of DNA substrate by exonucleolytic activity to form ends which would be ligatable. It is possible that bladder tumour extracts could contain increased levels of non-specific nucleases which would result in the random deletion of terminal nucleotides from the DSB; however, the non-random, asymmetric end-processing and use of microhomology by these extracts, and also in extracts in which ether Ku or DNA-PKcs are inactive, points to the involvement of a regulated exonuclease activity. The M/R/N complex contains independent alignment and nuclease functions ideal for DSB processing (39) and, as junctions in this study are formed between sequences found near or at one end of the DSB and those buried some distance from the other end, M/R/N could be involved in the detection and alignment DNA microhomologies prior to resection of 3' overhangs by an as yet unidentified nuclease (40,41).

The reduced joining fidelity observed in bladder tumours could be due to a deficiency for a critical NHEJ factor or increased expression of proteins involved in the error-prone pathway. Western blot analysis of bladder tumour extracts did not identify a common deficiency in NHEJ components or an increase in M/R/N levels that would account for the predominance of error-prone joining, although there was a generalized reduction in the levels of NHEJ components in both NHUs and bladder tumours. The full length Nbs1 protein observed in bladder tumour extracts was accompanied by a smaller immunoreactive protein not apparent in MO59K or NHU. Truncated Nbs-1 has been observed in patients with Nijmegen breakage syndrome and results from translation from an internal
initiation site such that the N-terminal portion of the protein is not expressed (42). However, as N-terminal truncated Nbs1 retains Mre11 and Rad50 binding capacity and this region of the protein is dispensable for DSB repair and survival it seems unlikely that this will impact on the joining fidelity observed in this study (43). Truncation of Mre11 was observed in BT1 the extract that was associated with a particularly high level of error-prone joining of complementary DSBs. A similarly sized truncated Mre11 was observed in the chromosomal instability syndrome AT-like disorder, which predisposes to cancer, and results from a point mutation causing premature termination of Mre11 at the C-terminal (44) and loss of critical C-terminal DNA binding domains (43).

It is possible that whilst the levels of NHEJ components are not altered in bladder tumours compared with NHU the activity of these proteins could be altered. Reduced DNA binding by Ku has been observed in aggressive basal cell carcinoma biopsies compared with normal controls (45). In a separate study the Ku-binding characteristics of seven bladder tumours were measured by gel retardation; five low grade tumours (grade 1–2, pTa–T1) displayed increased DNA binding by Ku whilst two high grade invasive tumours (grade 3, pT3) had a 1.5- to 3-fold decrease (46). The ability of Ku to interact with DNA ends could be altered in the high grade bladder tumours in this study. Similarly, alterations in the activity of DNA-PKcs, the DNA-PK holoenzyme or ligase IV/XRCC4 had a 1.5- to 3-fold decrease (46). The ability of Ku to interact with DNA ends could be altered in the high grade bladder tumours in this study. Similarly, alterations in the activity of DNA-PKcs, the DNA-PK holoenzyme or ligase IV/XRCC4 complex could also affect DNA end-protection and end-joining fidelity. Interestingly, in V(DJ) recombination the RAG-associated post-cleavage complex is thought to guide the bond DNA ends towards classical NHEJ so avoiding aberrant joining by alternative end-joining processes (47). Similar ‘shepherding’ roles for proteins may exist in a non-V(DJ) context to ensure that repair of DSBs is undertaken by accurate NHEJ; alterations in proteins involved in this role could leave broken DNA ends open to repair by a usually minor, less faithful pathway (47). Therefore, in bladder tumour extracts alterations in the function of proteins involved in classical NHEJ could shift the balance away from NHEJ and towards error-prone microhomology-mediated end-joining.

Genomic instability is a hallmark of cancer and the accurate repair of DSBs is essential for maintenance of chromosome integrity. Compromised NHEJ can be compensated for, to a certain extent, by HR (48,49) and remaining cells containing unrepaired breaks are most likely eliminated by apoptosis (50). Loss of p53 function in a Ku null background enhances chromosomal instability, increases joining of translocation breakpoints at regions of microhomology and promotes tumour formation in mice (37,51). Bladder tumours have been found to carry inactivating mutations in p53 or Rb and a large number of genomic deletions/amplifications (26). In this study we show that, bladder tumours utilize error-prone end-joining in preference to accurate NHEJ indicating a deficiency in the classical NHEJ pathway. This highly mutagenic joining of DSBs via sequence microhomologies may contribute to the increased genomic instability observed in high grade bladder cancer.

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