Deficiency of a novel mismatch repair activity in a bladder tumor cell line

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ABSTRACT

We demonstrate here that a cell line derived from a bladder cancer is defective in strand-specific mismatch repair. The mismatch repair deficiency in this cell line is associated with microsatellite instability and blocks an early step in the repair pathway. Since the addition of a known mismatch repair component hMutS α , hMutS β , hMutL α , replication protein A or proliferating cellular nuclear antigen could not restore mismatch repair to the mutant extract, the bladder tumor cell line is likely to be defective in an uncharacterized repair component. However, the repair in the mutant extract could be complemented by a partially purified activity derived from HeLa nuclear extracts. Therefore, in addition to revealing that a loss of mismatch repair function is associated with bladder cancer, this study provides information implicating a new mismatch repair activity.

INTRODUCTION

DNA mismatch repair (MMR) is an important mutational avoidance system. It maintains genomic stability by correcting mismatches generated in normal DNA metabolism (reviewed in 1–5) and by mediating DNA damage-induced apoptosis (reviewed in 6). It has been shown that defects in human MMR cause genome-wide alterations, including instability of simple repeated sequences. Germ-line mutations of several MMR genes, especially *MSH2* and *MLH1*, are the genetic basis for the majority of hereditary nonpolyposis colorectal cancers (HNPCC), also called Lynch syndrome (reviewed in 1,7,8). In addition, a loss of function in these genes, either by mutation or by epigenetic modification, is probably responsible for certain types of sporadic tumors displaying microsatellite instability (MSI), which include colorectal, gastric, ovarian and endometrial tumors (9–18).

Bladder cancer is the fifth most common cancer among Americans. More than 50 000 cases are diagnosed yearly, and >11 000 patients die each year from advanced metastatic cancer (19). Bladder cancer is frequently associated with genomic instability, including changes at the chromosomal level and the nucleotide level. Cytogenetic studies have identified many chromosomal aberrations such as chromosome loss,

translocations and amplifications (reviewed in 20). As in HNPCC patients, MSI has been detected in patients with bladder tumors (21–24). However, the underlying pathogenic mechanism for bladder cancer is still unknown.

Since MSI is considered a hallmark of MMR deficiency, it seemed possible that bladder tumors with MSI may be caused by MMR defects. To explore this possibility, we determined the MMR proficiency of two bladder cancer cell lines using an *in vitro* functional assay, and demonstrate that one of the cell lines is, indeed, deficient in strand-specific MMR. Additionally, complementation experiments indicate that the cause of the MMR deficiency in this cell line is not due to a defective hMutS α , hMutS β , hMutL α , replication protein A (RPA) or proliferating cellular nuclear antigen (PCNA), proteins known to be required or associated with human MMR. Hence, this study suggests that, like HNPCC and other sporadic tumors, bladder tumors are also associated with MMR deficiency; however, unlike the majority of HNPCC cancers, the cause of the MMR deficiency in this bladder cancer cell line is likely to be a defect or defects in uncharacterized repair components.

MATERIALS AND METHODS

Cell lines, nuclear extracts and purified proteins

Two bladder tumor cell lines (J82 and 5637) were purchased from the American Type Culture Collection (Manassas, VA). The J82 cell line was cultured in Eagle's minimum medium (Gibco/Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (HyClone, Logan, UT). The 5637 line was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. Colorectal tumor cell line H6, lymphoblastoid cell line MT1 (obtained from P. Modrich, Duke University) and ovarian tumor cell line 2774 (from K. Orth, University of Michigan) were cultured as described (10,25,26). HeLa S₃ cells were purchased from the Cell Culture Center (Minneapolis, MN). Nuclear extracts of all cell lines were prepared as previously described (25,27). hMutS α , hMutS β and hMutL α were purified from nuclear extracts of HeLa S₃ as described (28–31). Purified RPA and PCNA were obtained from John Turchi (Wright State University, Dayton, OH) and Yue Xiong (University of North Carolina, Chapel Hill, NC), respectively. All purified proteins were of >98% purity as judged by Coomassie-stained SDS–PAGE gels.

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Microsatellite instability analysis

MSI analysis was performed as described (25,32). Single-cell clones of J82 and 5637 cells were made in 96-well plates from a limiting dilution of a clonal population of cells. Genomic DNA was isolated from cells representing ~25 doublings. The DNA samples were amplified by PCR using microsatellite markers BAT25, D5S246, D8S135, CHLC.GGAA4D07 and Mfd257. Standard PCRs (10 μ l) contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 200 μ M each deoxynucleotide triphosphate, 0.2 μ Ci of [α - 32 P]dCTP, 1 μ M each primer, 0.1 μ g of DNA and 1 U of *Taq* DNA polymerase. The $MgCl_2$ concentration (from 0.5 to 2.0 mM) was adjusted for each primer pair for optimal amplification. PCR was performed in a Thermal cycler (Perkin Elmer) for 35 cycles of 1 min each at 94, 55 and 72°C, and completed with a 4-min extension at 72°C. PCR products were denatured and fractionated in a denaturing 6% polyacrylamide gel and detected by exposing to an X-ray film or a Phosphor imager (Storm System).

DNA heteroduplex substrates and MMR assay

Heteroduplex substrates containing a single base-base mismatch or an insertion/deletion mispair were constructed from the 6.4 kb-f1MR bacteriophage series as described (25,33). Each DNA substrate contains a site-specific strand break either 125 bp 5' to the mismatch (the *Sau*96I site in the complementary strand) or 181 bp 3' to the heterology (the *gpII* site in the viral strand) [see (25) for details]. The mismatches lie within overlapping recognition sites of two restriction endonucleases (*Hind*III and *Xho*I) so that the substrates are resistant to cleavage by either enzyme. Thus, strand-specific repair can be scored as conversion of the substrate to the repaired, homoduplex product, which is sensitive to hydrolysis by the restriction enzymes. Unless otherwise indicated, *in vitro* MMR assays were performed as described (25) in 15 μ l reactions containing 50 μ g of nuclear extract and 100 ng of substrate at 37°C for 15 min. Complementation experiments were performed using 50 μ g of bladder cell extract complemented with either 50 μ g of nuclear extract from a characterized, MMR-defective cell line or 50 ng of purified hMutS α , hMutS β , hMutL α , RPA or PCNA.

RESULTS

MSI and MMR deficiency in a bladder tumor cell line

To determine whether bladder tumor cell lines are associated with MMR deficiency, we determined MSI and MMR activity in two bladder tumor cell lines, J82 and 5637. Genomic DNA was purified from single-cell clones of each cell line and used to amplify microsatellite markers BAT25, D5S246, D8S135, CHLC.GGAA4D07 and Mfd257. The results, shown in Figure 1, demonstrate that there was a striking difference between 5637 and J82 subclones in the patterns observed with the CHLC.GGAA4D07 marker (GGAA repeats), although no differences were identified with the other four markers (data not shown). Many different alleles were found in the various 5637 subclones, but all J82 subclones exhibited only the alleles observed in the parent J82 cells (Fig. 2). These results indicate that 5637 cells exhibit MSI, but with a low frequency, i.e. MSI-low (34).

Figure 1. Microsatellite instability in 5637 cells. DNA from single-cell clones of 5637 (A) or J82 (B) was used as template to amplify the CHLC.GGAA4D07 marker (containing GGAA repeats). The PCR products were subjected to electrophoresis and visualized by autoradiography. Each number represents a subclone. (A) Clones 1, 9 and 14 showed only the parental alleles, while the other clones contained new alleles. (B) All clones exhibited the same pattern of alleles that are observed in the parental clone.

Figure 2. MMR deficiency in 5637 cells. Repair assays were performed in reactions containing 100 ng of 6.4 kb circular heteroduplex substrate and 50 μ g of nuclear extract of either 5637, J82 or both, as indicated. Lanes 1–3, repair of 5' G–T heteroduplex substrate containing a strand break 125 bp 5' to the mismatch was evaluated by *Bsp*106 and *Hind*III as described (27). Lanes 4–6, repair of the CA insertion/deletion heteroduplex substrate containing a strand incision 181 bp 3' to the heterology was scored by *Bsp*106 and *Xcm*I as described (25). In both cases, *Bsp*106 cleaves both substrate and product DNA, whereas *Hind*III and *Xcm*I only cleave repaired molecules. Thus, repaired DNA yields the two small fragments as indicated.

Nuclear extracts were prepared from both the J82 and 5637 cell lines and tested for their ability to correct 6.4-kb circular heteroduplex substrates containing a G–T mismatch or a dinucleotide (CA) insertion/deletion mispair using an *in vitro* repair assay (25,27). The G–T substrate contains a strand break 5' to the mismatch while the insertion/deletion heteroduplex possesses a strand break 3' to the heterology. These two substrates allow the determination of MMR proficiency for substrate and orientation specificities. Figure 2 shows the repair of these substrates by the J82 and 5637 nuclear extracts.

The J82 extract efficiently corrected both the G–T (Fig. 2, lane 1) and the insertion/deletion (Fig. 2, lane 4) substrates, as judged by its ability to convert the circular heteroduplexes into two smaller fragments. However, the conversion of either substrate into the smaller fragments was not observed in nuclear extracts derived from 5637 cells (Fig. 2, lanes 2 and 5). To determine whether a diffusible inhibitor is responsible for the negative result in the 5637 nuclear extract, repair assays were performed by mixing extracts of 5637 and J82 equally. Repair activity of the J82 nuclear extract was not inhibited by the addition of the 5637 extract (Fig. 2, lanes 3 and 6). These results suggest that cell line 5637 is defective in MMR.

5637 cells may be defective in an uncharacterized MMR component

To determine the defective component(s) in 5637 cells, complementation experiments were performed by mixing nuclear extracts of 5637 with those of the known MMR mutant cell lines (such as *MLH1*-defective H6, *MSH2*-defective 2774 and *MSH6*-defective MT1) and measuring MMR proficiency of the G–T heteroduplex substrate. As shown in Figure 3, the 5637 extract is capable of complementing each individual mutant extract in repair of a G–T heteroduplex (Fig. 3, lanes 3–5), indicating that this bladder tumor cell line possesses functional *MSH2*, *MSH6* and *MLH1*. These results were further confirmed by complementation experiments using purified proteins hMutS α (*MSH2*–*MSH6*), hMutS β (*MSH2*–*MSH3*) and hMutL α (*MLH1*–*PMS2*). None of these heterodimers could restore MMR to the 5637 nuclear extract (Fig. 3, lanes 6–8). Since RPA and PCNA have recently been shown to be required for MMR (35–38), purified recombinant RPA or PCNA was used to attempt to reconstitute MMR in the 5637 extract. Again, neither protein could restore MMR to the extract (data not shown). Thus, 5637 cells appear to have no defects in known MMR components hMutS α , hMutS β , hMutL α , RPA and PCNA. Therefore, this mutant cell line is likely to be defective in an uncharacterized MMR component.

The defect in 5637 cells blocks MMR at the step of excision

The MMR reaction involves repair initiation, excision and DNA resynthesis. Previous studies using HeLa nuclear extracts have demonstrated that MMR intermediates can be visualized by mapping excision points that are found on the substrate after the MMR functional assay is carried out in the presence of a specific repair inhibitor (39). Additionally, excision points may also be formed on the substrate by extracts that are defective in MMR (25,26). For example, if an extract is defective in an MMR initiation factor, repair of the substrate will be blocked prior to excision. Thus, the linearized products in this case will have lengths that are equal to that of the linearized (nicked) substrate (3.2 kb for the G–T substrate, which is the distance from the *Bsp*106 site to the nick). However, if repair is inhibited at the step of DNA resynthesis, excision points can be inferred from the sizes of the linearized products: they will have lengths slightly less (<3.2 kb) than the original substrate. In this case, the products have single-strand gaps that span from the strand break up to and beyond the site of the mismatch, to the excision point (39).

Using the repair in HeLa nuclear extracts as a control, Figure 4 demonstrates an analysis of repair intermediates produced in 5637 nuclear extracts from the G–T mismatch substrate in the

Figure 3. 5637 cells are defective in an uncharacterized MMR activity. Complementation repair assays (lanes 2–8) were performed using 100 ng of 5′ G–T substrate, 50 μ g of 5637 nuclear extracts and 50 μ g of nuclear extract from a complementing cell line (lanes 3–5) or 50 ng of purified proteins (lanes 6–8). Lane 1, positive repair in the J82 extract; lanes 9 and 10, ability of hMutL α or hMutS α to restore MMR to the hMutL α -deficient H6 extract or the hMutS α -deficient MT1 extract, respectively. L α , hMutL α ; S α , hMutS α ; S β , hMutS β .

presence or absence of aphidicolin, a DNA polymerase inhibitor that blocks MMR resynthesis (27,40). Analysis of products recovered from reaction without aphidicolin (lanes 2 and 3) shows conversion of the 3.2-kb species to a 6.4-kb full-length strand. This indicates that the nicked strand of the substrate had been largely converted to a covalently closed form in both extracts; this is likely to be due to the sealing of the nick on the substrate by a ligase before repair occurs (in the 5637 reaction) or normal repair and ligation (in the HeLa reaction). As expected, the presence of aphidicolin in the reaction containing HeLa extracts led to the accumulation of excision intermediates <3.2 kb, with the major species mapping ~3 kb from the site of *Bsp*106 cleavage (Fig. 4, lane 1). These results confirm previous observations in MMR-proficient nuclear extracts (26,38,39). In 5637 extracts, aphidicolin did not induce the magnitude of excision intermediates observed in HeLa extracts (compare lanes 1 and 4), but a very small population of molecules with lengths slightly less than 3.2 kb (indicated by an arrow) in length are evident. These observations suggest that MMR in 5637 cells is almost completely blocked either before or at the step of excision.

A partially purified activity from HeLa nuclear extracts restores MMR to 5637 extracts

To explore further the possibility that the 5637 repair defect might be due to a simple deficiency of a required MMR component, we tested the ability of a partially purified HeLa nuclear extract to restore MMR to the 5637 nuclear extract. The partially purified fraction was prepared by loading HeLa nuclear extract onto a phosphocellulose column and fractionating the extract via KCl gradient elution, as previously described (25,26). As shown in Figure 5, repair of a G–T heteroduplex substrate was restored to the 5637 nuclear extracts by a high-salt fraction from this column. The fraction that contained this complementing activity also complemented the 5637 extract in repair of the CA insertion/deletion substrate (data not shown). These results suggest that the 5637 bladder tumor cell line is defective in an activity that is required for the bidirectional repair in the human reaction.

Figure 4. Repair in 5637 cells is blocked at the step of excision. Circular G-T heteroduplex (200 ng) containing a complementary strand incision 125 bp 5' to the mismatch was incubated with 100 μ g of HeLa or 5637 nuclear extracts in the presence (A) or absence (N) of 90 μ M aphidicolin. DNA samples were recovered and digested with *Bsp*106, followed by phenol extraction and ethanol precipitation prior to suspension in alkaline loading buffer (0.2 N NaOH, 40 mM EDTA, 30% glycerol, 0.1% bromocresol green). DNA was fractionated at 1.5–2.5 V/cm through 1.5% agarose gel in 30 mM NaOH, 2 mM EDTA at 4°C, and then electrophoretically transferred onto nylon membranes (ICN Biotrans). The membrane was hybridized with [5'-³²P]d(ATGGTTTCATTGGTGACGTT) to visualize the product lengths. After hybridization, the membrane was washed twice (5 min each) with 1 $\times$ SSC (0.15 M NaCl, 15 mM sodium citrate, pH 7.0) containing 0.1% SDS, and then twice with 0.1 $\times$ SSC containing 0.1% SDS. Radioactivity was detected by exposing membranes to an X-ray film. Corresponding indirectly end-labeled intermediates and electrophoretic species are shown to the left of the gel. The 3.2 kb marker indicates the original location of the site-specific strand break and the solid bar represents the oligonucleotide probe. Normal ligated repair products and the ligated substrates map to 6.4 kb, whereas the mismatch and the strand break in the heteroduplex map 3.1 kb (right at the mismatch site) and 3.2 kb 5' to the *Bsp*106 site, respectively. Products <3.2 kb are the MMR-associated excision intermediates.

DISCUSSION

Bladder cancer is one of the most common malignancies among Americans. Like most tumors, bladder tumors are associated with genomic instability, including both structural and numerical chromosomal changes; however, the underlying pathogenic mechanism for this disease is unknown. Recently, a number of studies have shown that a substantial fraction of patients with bladder cancer display frequent alterations in simple repeated sequences (21–24,41–43). Given the correlation between MSI and MMR deficiency in HNPCC syndrome, these observations suggest a possible loss of MMR function in these bladder cancers. Using a functional MMR assay, we have demonstrated that a bladder tumor cell line exhibiting MSI is defective in strand-specific MMR. In fact, Thykjaer *et al.* (44) have recently shown a partial MMR defect in another bladder tumor cell line. These observations, although based on a small sample size, suggest that loss of MMR function may be responsible for development of some bladder cancers.

MMR deficiency has previously been identified in HNPCC and certain types of sporadic cancers, including colorectal, gastric, ovarian and endometrial cancer (1–5,8). Although mutations in *MSH2* and *MLH1* are apparently the major cause for HNPCC and certain sporadic tumors, defects in other MutS and MutL homolog genes (*MSH6*, *PMS1* and *PMS2*, *MLH3*) are also responsible for a small portion of these tumors (45–47). This is also true for tumor cell lines that have been identified as defective in MMR (11,25,32,48–50). These findings indicate that most of these MMR-deficient tumor cells are

Figure 5. Restoration of MMR to 5637 extracts by a partially purified HeLa nuclear component. HeLa nuclear extract was fractionated in a phosphocellulose column as described (25,26). Samples (2 μ l) of each fraction were tested for their ability to restore MMR to 5637 nuclear extract (50 μ g) on a 5' G-T substrate. Mismatch repair (fml) is shown by the solid line with closed circles; protein concentration (mg/ml) is indicated by the solid line with open circles; and KCl concentration (M) is represented by the dotted line with open squares.

associated with defects in MutS or MutL homologs. However, it is noteworthy that a significant portion (~30%) of HNPCC cases contain no detectable mutations in any of the known MutS and MutL homolog genes (51), suggesting that mutations in additional genes (either MMR genes or genes unrelated to MMR) are responsible for these HNPCC families. Recently, Exo1 has been shown to be involved in MMR (52–55) and germ-line mutations of the gene have indeed been identified in HNPCC (44).

Human MMR is homologous to the *Escherichia coli* methyl-directed, MutHLS-dependent system. The two systems have comparable substrate specificities and repair mechanisms (reviewed in 1). In fact, the identification of the known human MutS and MutL homolog genes was based on their protein sequence homology to the *E.coli* MutS and MutL proteins (reviewed in 1,7). It has been well documented that, although MutS and MutL proteins are the key components in the *E.coli* repair reaction, other activities, e.g. MutH, helicase II and exonucleases, are absolutely required as well (56). Given the similarities between the human and the *E.coli* systems, there is little doubt that other activities, beside the known components (hMutS and hMutL homologs, RPA, Exo1 and PCNA), must be involved in the human MMR reaction. Therefore, it is likely that the genes responsible for the predisposition of the HNPCC families with no mutations in the known MMR genes code for as yet unidentified MMR components.

Our results clearly show that the bladder tumor cell line 5637 is defective in strand-specific MMR. Mapping of the excision intermediates indicates that repair in 5637 cells is almost completely blocked either before or at the step of excision. In view of the homology between the human and the bacterial systems, this finding suggests that the bladder cell line may be defective in an activity that is functionally homologous to

bacterial MutS, MutL, MutH, DNA helicase II or exonuclease activities, which are required in initiation of mismatch-provoked excision (56,57). Although hMutH and helicase have not been identified, hMutS α , hMutS β , hMutL α , Exo1 and PCNA have been shown to be required for repair initiation in human cells (25,26,29,37,38,55). Also, two additional hMutL heterodimers have recently been identified (58–60), but their involvement in the MMR reaction has not been established. DNA polymerases δ and ϵ have been shown to participate in MMR not only at the step of resynthesis, but also at the step of excision (61,62). Recently, studies by Tran *et al.* (62) have suggested an involvement of the editing function of pol δ/ϵ in MMR excision. Our complementation experiments have indicated that hMutS α , hMutS β , hMutL α , RPA or PCNA is unable to restore MMR to the 5637 extract (see Fig. 3). However, the mutant extract can be complemented *in vitro* by a partially purified HeLa nuclear fraction derived from phosphocellulose chromatography (Fig. 5). The complementing activity, if it is indeed an MMR component, could be an exonuclease (including Exo1 and the editing function of DNA polymerases), helicase or MutH-like protein. Alternatively, the defect in 5637 cells could be due to imbalanced expression of MMR genes, which is responsible for a partial MMR defect in a bladder tumor cell line (44).

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