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Antisense Inhibition of hMLH1 Is Not Sufficient for Loss of DNA Mismatch Repair Function in the HCT116+Chromosome 3 Cell Line

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Abstract

We have reported that transfer of chromosome 3 (Chr3) containing a single wild-type copy of the hMLH1 gene into HCT116 colon cancer cells, a cell line deficient in DNA mismatch repair (MMR) activity attributable to inactivating hMLH1 mutations, corrects all of the aspects of the MMR repair-deficient phenotype. We inhibited the expression of the wild-type hMLH1 gene using antisense RNA in HCT116+Chr3 cells to determine if this would result in reversion to the MMR-deficient phenotype. Despite profound inhibition of hMLH1 expression, DNA MMR activity and alkylatenon sensitivity were not impaired in the antisense-transfected HCT116+Chr3 cells. Additionally, arrest of the cell cycle at the G2 phase with alkylatenon damage occurs in these cells, a phenotype associated with MMR proficiency. These results indicate that even with a reduction in the expression of hMLH1 protein below the limits of detection by Western blotting, DNA MMR activity remained fully functional (by direct DNA MMR activity assay). We would speculate that hMLH1 is expressed in substantially greater abundance than would be minimally necessary for DNA MMR and that minor reductions in the expression of this protein would not be sufficient to permit DNA MMR dysfunction. Alternatively, Chr3 may contain a second hMLH1 homologue that might overlap with the function of hMLH1.

Introduction

HNPCC is a familial cancer syndrome (1) that accounts for a small portion of the total colon cancer incidence in the Western world but is one of the commonest type of familial susceptibility to cancer (2). Germ line mutations in the DNA MMR genes have been demonstrated in a large number of families (3). At least six human DNA MMR genes have been cloned and characterized: hMSH2, hMSH3, and hMSH6 (GTBP) are human homologues of the Escherichia coli MutS genes; hMLH1, hPMS1, and hPMS2 are human homologues of the MutL genes. DNA MMR activity is inactivated in nearly all of the HNPCC tumors (4–9). However, in as many as 50% HNPCC kindreds, germ line mutations cannot be found in any of the known DNA MMR genes, suggesting that other genes or epigenetic phenomena might be responsible for this disease.

The HCT116 human colon cancer cell line is known to have homozygous inactivating mutations in the DNA MMR repair gene hMLH1 on Chr3 (7), exhibits MSI, and is defective in MMR (10). Our laboratory has used this cell line as the recipient for the transfer of an additional whole copy of human Chr3 to study the impact of restoration of hMLH1 on the biology of the cells. Whole chromosome transfer ensured the restoration of a singly, properly regulated copy of hMLH1. The transfer of Chr3 was compatible with growth of the cell line in vitro and in nude mice (11), restored MMR activity, corrected MSI, and sensitized the cells to MNNG (10). This cell line has been useful for studying MMR, because it is one of the only cell lines that is fully tumorigenic, yet lacks both MSI and chromosomal instability (3). We also developed a revertant mutant of HCT116+Chr3 (called the M2 clone) in which hMLH1 expression was again inactivated by mutagen exposure, which led to a loss of its MMR activity, and the cells again became resistant to MNNG (12). The chromosome transfer experiment indicated that restoration of hMLH1 was necessary to reconstitute DNA MMR activity to HCT116 cells. However, Chr3 contains several other genes that may have participated in the DNA MMR function. The development of the M2 clone indicated that inactivation of the gene was sufficient for reversion to the MMR-deficient phenotype. However, the M2 clone was isolated after exposure to MNNG, and other mutations may have occurred in addition to the inactivation of hMLH1. To explore this issue, we tested the hypothesis that wild-type hMLH1 on Chr3 is exclusively responsible for the restoration of DNA MMR function in HCT116+Chr3. We stably transfected an antisense construct of hMLH1 into HCT116+Chr3 cells to determine whether inhibition of the hMLH1 gene would reverse the MMR-proficient characteristics of HCT116+Chr3 cells. We found

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3 The abbreviations used are: HNPCC, hereditary nonpolyposis colorectal cancer; MMR, mismatch repair; Chr3, chromosome 3; MSI, microsatellite instability; MNNG, N-methyl-N′-nitro-N-nitrosoguanidine.

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that profound inhibition of hMLH1 expression did not abrogate DNA MMR activity.

Materials and Methods

**Cell Culture.** The human colon tumor cell line HCT116 was purchased from the American Type Culture Collection (Rockville, MD) and maintained in Iscove’s modified Dulbecco’s medium (IMDM) containing 10% fetal bovine serum. HCT116+Chr3 was established in our laboratory as described elsewhere (10) and maintained in IMDM containing 10% fetal bovine serum and 400 μg/ml Genetin (G418, Life Technologies, Inc., Rockville, MD).

**RNA Isolation.** RNA from HCT116, HCT116+Ch3, and HCT116+Ch3 with antisense construct to hMLH1 was purified by a modification (13) of the procedure developed by Glisin et al. (14). The HCT116 and HCT116+Ch3 cell lines with or without the antisense construct were grown to 60–70% confluency. The cells were washed with PBS and lysed with 4 M guanidine isothiocyanate/0.1 M Tris-HCl (pH 7.5)/1% 2-mercaptoethanol solution prepared as described previously (15). The lysate was passed six times through a 21-gauge needle syringe to shear DNA and decrease the viscosity of the solution, and then 10% sodium sarkosyl (N-lauroylsarcosine, sodium salt) was added to a final concentration of 0.5%. The lysates were then gently layered over 6.1 M CsCl/25 mM NaAc (pH 5.2)/10 mM EDTA solution, and the RNA was collected by centrifugation in a Beckman ultracentrifuge at 20°C at 110,000 × g overnight. The RNA pellet was dissolved in 0.5 ml autoclaved DNase-free water and precipitated with 10% sodium sarkosyl and dissolved in 10 mM sodium phosphate buffer (pH 7.0).

**Plasmid Construction.** The hMLH1 cDNA was reverse transcribed from HCT116+Chr3 RNA using a random hexamer primer and MMLV transcriptase. A 1217-bp 5' end of the reverse transcribed hMLH1 cDNA was amplified using PCR. This 1217-bp PCR product was subcloned into the mammalian expression vector pDR2 using BamHI and XhoI restriction sites. Forty micrograms of a pDR2-AS (a 1217-bp antisense cDNA subcloned in a pDR2 vector) construct were transfected into HCT116+Chr3 colon cancer cells using the calcium phosphate method (16). Briefly, the pDR2-AS construct was precipitated with calcium phosphate, and the resultant precipitates were incubated overnight with HCT116+Ch3 cells. The cells were then washed with PBS to remove excess precipitates and grown in selective medium with G418 (400 μg/ml) and hygromycin B (300 μg/ml). After 2 weeks of incubation in selective medium, several independent resistant clones were selected, and the successful transfection of the antisense construct into HCT116+Ch3 was confirmed by reverse transcription PCR and Northern blot analysis. The antisense clones were named AS1 (HCT116+Ch3+AS1), AS2 (HCT116+Ch3+AS2), AS3 (HCT116+Ch3+AS3), AS4 (HCT116+Ch3+AS4), and AS5 (HCT116+Ch3+AS5). In this study, the AS1, AS3, and AS4 antisense clones were studied in detail.

**Western Blot Analysis.** Levels of hMLH1 protein were measured as described previously (17). Briefly, total protein concentrations were measured by the method of Lowry et al. (18), and 50 μg of total protein from each sample were resolved by 7.5% SDS-PAGE. The proteins were electrophoresed onto polyvinylidene difluoride membranes (Millipore, Marlborough, MA) using a Semiphor TE 70 ( Hoefer, San Francisco, CA). The blots were blocked in 2% nonfat dry milk for 2 h at room temperature and incubated with anti-hMLH1 (Ab-1) monoclonal antibody (Oncogene Science, Cambridge, MA) for 1 h at room temperature, followed by horseradish peroxidase-conjugated secondary antibody for 30 min at room temperature. The hMLH1 protein-antibody complex was detected using an enhanced chemiluminescence kit (ECL kit, Amersham) according to the manufacturer’s manual.

**Mismatch Repair Assay.** Cell-free extracts were prepared from HCT116, HCT116+Chr3, and HCT116+Chr3 containing anti-hMLH1 construct as described previously (10, 17) and MMR activity was measured as described (10, 17, 19, 20). Briefly, an M13mp2 DNA heteroduplex, containing a G*G base mispair at position 88 in the coding sequence of the lac Z α complementation gene, was obtained by hybridizing a single-stranded viral (+) DNA and a (−) strand of RF DNA. The reaction mixture contained 30 mM HEPES (pH 7.8); 7 mM MgCl₂; 4 mM ATP; 200 μM CTP, GTP, and UTP; 100 μM dATP, dGTP, dTTP, and dCTP; 40 mM creatine phosphate; 100 mg/ml of creatine phosphokinase; 15 mM sodium phosphate (pH 7.5); 1 fmol of the indicated heteroduplex DNA; and 50 μg of cell extract protein. The incubation was carried out at 37°C for 30 min. The repair was directed to the (−) strand of M13mp2 by the presence of a nick. The DNA heteroduplex was then purified and introduced by electroporation into E. coli NR1962 (mutS strain), which were plated on minimal medium in a soft-agar layer containing 0.5 ml of a log-phase culture of CSH50 (the α-complementation strain), 0.5 mg of isopropyl-β-D-thiogalactopyranoside, and 2 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. The plates were incubated at 37°C for 16–24 h. After incubation the plaques were assigned to one of the following phenotypes: blue, colorless, or mixed. If no repair occurred, mixed plaques were observed containing both blue and colorless progeny. Repair of the substrate in a cell extract reduced the percentage of mixed plaques and increased the percentage of pure-color plaques. As the nick directs repair to the (−) strand, the (+) phenotype increases and the (−) phenotype decreases, i.e., producing more colorless plaques in this instance. Repair efficiency is expressed in percentage as 100 × (1 – the ratio of the percentages of mixed bursts obtained from extract-treated and mock-treated samples) (20).

**Cloning Efficiency.** Exponentially growing cells (5 × 10⁵) were suspended in 1 ml serum-free RPMI 1640 and treated with various concentrations (0, 1.0, 2.5, and 5 μM) of MNNG at 37°C for 45 min. After treatment, the cells were washed once with serum-free medium; resuspended in fresh growth medium, diluted, and plated into duplicate wells at 10⁴, 10³, 10², 10, and 1 cell/well as described previously (10, 21). Fresh serum-containing media was replaced every 3 days. Ten days later, the cells were washed with PBS, fixed with methanol, and rewashed with PBS. The cells were stained with 3% Giemsa stain (Sigma Chemical Co., St. Louis, MO) for 15 min and rinsed with water. The number of colonies consisting of >50 cells were counted. The relative surviving fraction for each cell
line was expressed as a ratio of the plating efficiency in treated cultures to that observed in the controls.

Cell Cycle Analysis. Cell cycle analysis was performed using flow cytometry as described previously (21). Briefly, the cells (1 × 10^5) were plated in 6-cm plates and treated with 5 μM MNNG at 37°C for 45 min in serum-free RPMI 1640. After treatment, the cells were washed in serum-free medium and then resuspended in fresh growth medium. The cells were harvested every 24 h for 5 days. Nuclei were isolated by resuspending 1 × 10^6 cells/ml in a nuclear lysis buffer containing 10 mM Tris-HCl (pH 7.5), 0.32 m sucrose, 3 mM MgCl_2, 2 mM CaCl_2, and 0.2% (v/v) NP40 (Sigma Chemical Co.) and incubating on ice for 10 min. The suspension was centrifuged at 2000 × g for 10 min and resuspended in nuclear lysis buffer without NP40 at a density of 4 × 10^6 cells/ml. The cells were spun at 2000 × g for 10 min and resuspended in lysis buffer [0.1 M Tris-HCl (pH 7.4), 0.15 M NaCl, 1 mM CaCl_2, 0.5 mM MgCl_2, 0.01% NP40, 0.1% RNase A] and 2 μg/ml propidium iodide and processed for flow cytometric analysis as described previously (21).

Results

To determine whether the lack of expression of the transferred hMLH1 gene would reverse the corrected MMR phenotype, an antisense construct of the hMLH1 gene was used to inhibit expression of the hMLH1 gene present in HCT116 1 Chr3 colon cancer cells. We did not observe any morphological differences between the antisense-containing HCT116 1 Chr3 cells and the HCT116 1 Chr3 cells. The protein levels of hMLH1 were decreased significantly in antisense-transfected cells compared with those in non-transfected HCT116 1 Chr3 colon cancer cells. The protein levels of hMLH1 were decreased significantly in antisense-transfected cells compared with those in non-transfected HCT116 1 Chr3 colon cancer cells (Fig. 1). The AS1 clone showed a 50% reduction in hMLH1 protein levels, whereas no protein was detectable in the AS3 and AS4 clones.

DNA MMR activity of HeLa (MMR-proficient control), HCT116 1 Chr3, and antisense clones is shown in Fig. 2. Antisense clones, AS1, AS3, and AS4, had DNA MMR activity comparable to proficient cells (HCT116 1 Chr3 and HeLa; Fig. 2). It is evident from Fig. 2 that
inhibition of hMLH1 protein in antisense clones AS1, AS3, and AS4 did not decrease the activity of DNA MMR.

HCT116 colon cancer cells are resistant to MNNG, whereas HCT116+Chr3 cells are sensitive to MNNG treatment as shown previously (10, 21). To determine whether the antisense construct-containing cells remained sensitive to MNNG treatment, the cloning efficiency of these cells was measured. Fig. 3 depicts the cloning efficiency of HCT116 and HCT116+Chr3, with or without transfection of antisense construct. Antisense construct containing clones showed no significant change in cloning efficiency compared with HCT116+Chr3 cells.

HCT116 cells, which are deficient in MMR activity, escape from G2 arrest on exposure to MNNG, whereas proficient cells such as HCT116+Chr3 undergo G2-M arrest in response to MNNG (21). The antisense construct containing clones AS1, AS3, and AS4 exhibited cell cycle arrest at the G2 checkpoint after MNNG treatment similar to that of HCT116+Chr3 cells (Fig. 4).

Discussion

The hMLH1 gene product is generally accepted to be necessary for DNA MMR activity in human cells, and inactivation of this gene clearly leads to MMR deficiency and MSI in HNPCC patients and sporadic MSI colon tumors. We have shown previously that transfer of a single wild-type copy of Chr3 to the HCT116 colon cancer cell line, a cell line deficient in MMR activity, is sufficient to correct MMR deficiency (10). It has been a reasonable conclusion that the hMLH1 gene is responsible for restoring the MMR activity in HCT116+Chr3 cells, but the large number of genes involved in the transfer of Chr3 could allow for more complex interpretations.

One hypothesis for the retention of the MMR-proficient phenotype in the anti-hMLH1-transfected HCT116+Chr3 cells is that the low levels of protein observed in antisense clones are sufficient to retain the MMR activity (>50% reduction in protein levels in AS1, and in AS3 and AS4 protein levels not detectable by Western blot). It is also possible that another MutL homologue is present on Chr3 that can restore MMR activity in the absence of hMLH1; however, it is also generally accepted that hMLH1 is an obligatory component of the human DNA MMR system (22). The stability of the hMutL complex depends on hMLH1 protein (23). It is generally thought that inhibition of hMLH1 will completely abrogate DNA MMR activity and can be measured using a G^*G mismatch substrate. Alterations in the expression of hPMS1, hPMS2, or hMLH3 might be expected to result in malfunctions of the MMR specificity for different types of DNA mismatches, but the loss of hMLH1 would be expected to disrupt all of the types of DNA MMR. The fact that inhibition of this MutL homologue on Chr3 does not reverse the characteristics of the MMR phenotype suggests either that another uncharacterized MutL homologue with hMLH1-like function is located on this Chr3 or that hMLH1 is expressed in great abundance compared with that required for measurable DNA MMR activity.

To date, two other human MLH homologues (MED1 and MLH3) have been identified (24–26). MED1 is an endonuclease that interacts with hMLH1 and is located on chromosome 3q21–22 (24). This gene has been shown to be mutated in some human carcinomas with MSI (25). Another MutL homologue located on Chr3 might have similar redundancy. However, MED1 has not yet been demonstrated as a substitute for hMLH1 nor has it been shown to heterodimerize with hPMS2 or hPMS1, the partners of hMLH1 (24). MLH3 is located on chromosome 14q24.3, and its functional deficiency has not yet been shown to be associated with mammalian MSI (26).

In yeast, the MSH3 and MSH6 proteins share overlapping functions as they complex with MSH2 to form two distinct heterodimers that recognize different varieties of DNA mismatches during the S phase of the cell cycle (27). The functional redundancy of different MMR protein complexes may influence the biological phenotype when a mutation in one MMR gene occurs. In humans, germ line mutations in hMSH6 produce a variant phenotype of HNPCC, and hMSH2 germ line mutations have never been found in HNPCC. The redundancy between these two genes means that the loss of either one results in a less penetrant form of HNPCC, which suggests a less severe impact on MMR activity than does inactivation of hMSH2 (27, 28).

It is possible that even minute levels of hMLH1 (i.e., those not detectable by Western blot analysis) might be sufficient to produce the MMR-proficient phenotype in the antisense-transfected cells. The human DNA MMR system is incompletely understood at the cellular level. It has been suggested that the MutS homologues may interact with DNA as a sliding clamp and that the MutL homologues act as a signal transduction complex when the MutS homologues encounter DNA damage (29). This model would accommodate the speculation that very
low levels of hMLH1 may be sufficient for DNA MMR activity and that even profound inhibition of this protein may not result in DNA MMR dysfunction.

In summary, inhibition and complete absence of detectable hMLH1 protein in a cell line corrected for the DNA MMR-deficient phenotype did not reverse DNA MMR proficiency. We speculate that either a second mutL homologue on Chr3 may have redundant hMLH1 function or that minute levels of hMLH1 is satisfactory for competent DNA MMR function in HCT116+Chr3 cells.

References


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