ROLE OF hMLH1 PROMOTER HYPERMETHYLATION IN DRUG RESISTANCE TO 5-FLUOROURACIL IN COLORECTAL CANCER CELL LINES

Christian N. ARNOLD¹, Ajay GOEL¹ and C. Richard BOLAND¹,2*
¹Department of Medicine and Cancer Center, University of California at San Diego, San Diego, CA, USA
²Veterans Affairs Medical Center, La Jolla, CA, USA

Loss of DNA mismatch repair (MMR) occurs in 10–15% of sporadic colorectal cancer, is usually caused by hMLH1 hypermethylation, and has been shown to confer resistance to various chemotherapeutic reagents, including 5-fluorouracil (5-FU). We tested the hypothesis that demethylation of the hMLH1 promoter in hypermethylated colorectal cancer cells would restore MMR proficiency and drug sensitivity to 5-FU. We used the MMR-deficient cell lines SW48, HCT116, HCT116+chr2 and the proficient cell line HCT116+chr3. After treatment with the demethylating agent 5-Aza-2’-deoxycytidine (5 aza-dC), hMLH1 mRNA and protein expression were determined by RT-PCR and immunoblots. The methylation status for hMLH1 was investigated by methylation-specific PCR. Cells were subsequently treated with 5-FU and the in vitro growth characteristics ascertained by clonogenic assays. hMLH1 hypermethylation was reverted in SW48 cells 24 hr after treatment with 5 aza-dC and was accompanied by hMLH1 mRNA and protein reexpression. While 5 aza-dC alone did not affect the growth of SW48 cells, all other cell lines responded with a pronounced growth inhibition. 5-FU treatment strongly reduced the colony formation of HCT116+chr3 cells. These effects were significantly less in the MMR-deficient cells. Combined treatment of SW48 cells resulted in a similar growth pattern as seen in 5-FU only treated HCT116+chr3 cells. We demonstrate that in vitro resistance to 5-FU can be overcome by reexpression of hMLH1 protein through 5 aza-dC-induced demethylation in hypermethylated cell lines. Induction of the expression of methylated tumor suppressor or MMR genes could have a significant impact on the development of future chemotherapy strategies.

Key words: mismatch repair; microsatellite instability; DNA methylation; 5-FU resistance

Colorectal cancer (CRC) is one of the most common malignancies worldwide. The majority of CRC are sporadic, whereas the familial cancer syndromes hereditary nonpolyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP) comprise about 2–3% and < 1% of all CRC, respectively. Of the sporadic CRC, 12–15% are characterized by high-grade microsatellite instability (MSI-H) as a result of uncorrected nucleotide base pair mismatches and slippage mistakes at simple repetitive sequences termed microsatellites. These errors are usually corrected by the DNA MMR system. A defective MMR system permits insertion or deletion mutations in coding microsatellites of key tumor suppressor and growth-regulatory genes.

The human MMR system functions through the interaction of several proteins, of which the most important are hMLH1 and hMSH2. hMLH1 forms complexes with either hPMS2 or hMLH3, while hMSH2 heterodimerizes with either hMSH3 or hMSH6. Germline mutations of several members of the MMR system⁴–¹⁰ have been linked with HNPCC.¹¹,¹² The defect of the MMR system in sporadic CRC is principally caused by aberrant hMLH1 promoter methylation, which in turn results in transcriptional silencing, leading to microsatellite instability (MSI).¹³–¹⁵

The MMR system is a replication fidelity complex that recognizes and repairs mismatched base pairs that occur during DNA replication, chemical mutagenesis and other processes. It has been previously shown that cells deficient in MMR are resistant to some cytotoxic agents that act through DNA damage. The alkylating agent N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) produces O⁶-methylguanine as its principal adduct, which in turn is recognized by the proficient MMR system, leading to cell cycle arrest and death.¹⁶ A similar phenomenon is seen after treatment of cells with 6-thioguanine (6-TG), which leads to DNA adduct formation.¹⁷ An intact MMR system recognizes the adduct formation and causes cell cycle arrest, thereby preventing the propagation of mutations in daughter cells. Similarly, platinum-based chemotherapeutic results in DNA crosslinking, which is also recognized by a proficient MMR system.¹⁸

It has been demonstrated that a number of MMR-deficient cell lines of several tumor types are resistant to the effects of cisplatin, carboplatin and its amine analogues.¹⁹ Recently, different studies have proved that the complementation of a defective MMR system is able to correct drug resistance to various clinically used chemotherapeutic agents. Complementation was initially performed by chromosome transfer containing a wild-type MMR gene locus.¹⁶,²⁰,²¹ Several reports showed that chromosome 3-based replacement of hMLH1 renders DNA MMR-defective cell lines competent in this activity, which restores drug sensitivity to a variety of drugs.¹⁶,²⁰ The same response has been reported for hMSH2-defective cell lines in which MMR function was restored by chromosome 2 transfer.²¹

Recently, a number of studies have reported restoration of the functionality of genes that had been transcriptionally silenced due to aberrant promoter methylation. It has been shown that treatment of MMR-deficient colorectal and ovarian cells with 5 aza-dC resulted in MMR restoration due to demethylation of the hMLH1 gene.²² There is an emerging body of literature demonstrating the demethylation effects of 5 aza-dC on a variety of tumor cell lines, which permits the reexpression of a number of crucial tumor suppressor genes.²³–²⁵ 5 aza-dC can lead to the functional restoration of MMR function by the reexpression of hMLH1. After restoration of the MMR function by 5 aza-dC treatment, cisplatin-resistant cell lines are rendered sensitive to platinum-based chemo therapeutics.

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Dr. Arnold’s current address is: Department of Gastroenterology, Hepatology and Endocrinology, University of Freiburg, Freiburg, Germany.

The first two authors contributed equally to this paper.

*Correspondence to: 2 Hoblitzelle, 3500 Gaston Avenue, Baylor University Medical Center, Dallas, TX 75246. Fax: 214-818-9292.
E-mail: rickbo@baylorhealth.edu

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drugs. Sensitization occurred in cells that were deficient in MMR due to hMLH1 promoter hypermethylation, but not in those with mutational defects in hMLH1.

5 aza-dC has been used clinically in the treatment of hematologic neoplasias. The clinical use of 5 aza-dC in the treatment of solid cancers resistant to common anticancer drugs due to hypermethylation of DNA repair genes or other tumor suppressor genes could potentially open a novel approach to therapy by restoring gene expression and increasing the sensitivity of cells to common therapeutic regimens.

In this study, we intended to examine the molecular effects of 5 aza-dC on the MMR-deficient hMLH1 hypermethylated cell line SW-48 in comparison to the cell line HCT116, which is MMR-deficient on the basis of homozygous hMLH1 mutations. Following 5 aza-dC exposure, we treated these cell lines with 5-FU, which is used in most CRC treatment regimens. Our results strongly suggest that 5 aza-dC treatment demethylates the hMLH1 promoter, restores its expression, and thus sensitizes drug-resistant SW-48 cells to 5-FU treatment, providing the same effect as has been demonstrated for chromosome 3-based hMLH1 transfer in HCT116 cells.

MATERIAL AND METHODS

Reagents

5 aza-dC was obtained from Sigma Chemical (St. Louis, MO) and dissolved in phosphate-buffered saline (PBS) at a stock concentration of 1 mg/ml and maintained at 4°C. 5-FU was purchased from Sigma and dissolved in Iscove’s modified Dulbecco’s media (IMDM) at a concentration of 1 mM and stored at 4°C.

Cell lines and culture

The human CRC cell lines SW48 and HCT116 were obtained from American Type Culture Collection (Rockville, MD) and maintained in growth medium (IMDM) containing 10% fetal bovine serum (FBS). The HCT116 cell lines containing human chromosome 2 (HCT116/ch2) and chromosome 3 (HCT116+chr3) were grown as described previously. They were maintained in growth medium containing 10% PBS and 400 μg/ml G418 (Gibco-BRL, Gaithersburg, MD).

Microsatellite analysis

Genomic DNA was extracted from various cell lines with the Qiagen tissue DNA extraction kit (Qiagen, Valencia, CA). MSI status was determined with 4 consensus mononucleotide markers (BAT25, BAT26, TGFRII, BAX) for high-grade microsatellite instability (MSI-H). As a microsatellite stable (MSS) control we used autoradiography. Band shifts were judged according to the logic neoplasias. The clinical use of 5 aza-dC in the treatment of solid cancers resistant to common anticancer drugs due to hypermethylation of DNA repair genes or other tumor suppressor genes could potentially open a novel approach to therapy by restoring gene expression and increasing the sensitivity of cells to common therapeutic regimens.

MSP of hMLH1, p16 and HIC1

The methylation status of the hMLH1, p16 and HIC1 promoters was determined by a methylation-specific polymerase chain reaction (MSP) according to the method of Herman et al. Cell line DNA was extracted using the Qiagen tissue DNA extraction kit according to the manufacturer’s protocol. DNA concentration was determined by spectrophotometry. The DNA methylation pattern in the CpG islands of the respective promoters was determined by chemical modification of unmethylated but not the methylated cytosines to uracil and subsequent PCR using specific primers for either methylated or the modified unmethylated DNA. DNA (1 μg) in a volume of 50 μl was denatured by NaOH for 10 min at 37°C. Thirty μl of 10 mM hydroquinone (Sigma) and 520 μl of 3 M sodium bisulfite (Sigma) at pH 5.5 were added, and the samples were incubated at 50°C for 16–18 hr. DNA was purified using the Wizard DNA purification resin (Promega, Madison, WI) according to the manufacturer’s manual and eluted in 50 μl of purified water. DNA was further desulfonated with NaOH for 10 min at room temperature followed by ethanol precipitation at −80°C for 1 hr. The modified DNA was eluted in water and stored at −20°C. The specific primers for the methylated and unmethylated MSP and their respective location from the transcriptional start site (TSS) were as follows: hMLH1-M 5‘-ACGTAGACGTTTTATTTATTGAGGTTCG-3’ (forward), 5‘-CCTCATGTAATACCCCGGC-3’ (backward); −717 from TSS); hMLH1-U 5‘-TTTGATGTAGATTGTATTATTAGGTTGTGTT-3’ (forward), 5‘-ACCACCTCATCATAAATCCACCA-3’ (backward); −721 from TSS); p16-M 5‘-TTATTAGGGGTTGGGCACCGT-3’ (forward), 5‘-GACCCCCGACCCGACCGTAA-3’ (backward); −167 from TSS); p16-U 5‘-TTATTAGGGGTTGGGCACCGT-3’ (forward), 5‘-CACCAACCAACACACCTAA-3’ (backward); −167 from TSS); HIC1-M 5‘-TCGTTTTTCGCGTTTTGTGCTC-3’ (forward), 5‘-AACCAGAAACCTATCACCCTTCTG-3’ (backward); −617 from TSS); HIC1-U 5‘-TTTGTGTGTTTTGTTTGTGTTTGTT-3’ (forward), 5‘-CACCCCTAACACACCCCTAAC-3’ (backward); −617 from TSS). The PCR mixture contained 1 × PCR buffer 

RT-PCR

Total cellular RNA was isolated from cell lines with TRIZol (Life Technologies) following the manufacturer’s protocol. RNA concentration was determined by spectrophotometry. cDNA was synthesized after initial denaturation at 80°C for 5 min at 37°C for 60 min from 2.5 μg of total cellular RNA by reverse transcription in a 20 μl reaction containing 25 μg/ml random hexamers (Roche Molecular Biochemicals, Indianapolis, IN), 0.5 mM dNTPs, 1 × RT buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol), 2,000 U/ml RNase inhibitor and 10,000 U/ml of Moloney murine leukemia virus reverse transcriptase. The
reaction was terminated by 95°C denaturation for 5 min. An MJ Research PTC 200 DNA Engine (Watertown, MA) was used to amplify a 288 bp product of hMLH1 exon 1. The primer sequence for the forward primer was 5'-CAGGTTTACATTTCAACAGTG-3' and for the backward primer 5'-TTACCTTCA-CATCCAGCAGTGGC-3'. PCR products were separated on a 1% agarose gel, stained with 0.5 µg/ml ethidium bromide and visualized under UV illumination.

Protein isolation and western blot analysis

Cells were washed in PBS and lysed by pulse sonication. Protein concentration was determined with the BCA protein assay kit (Pierce, Rockford, IL) following the manufacturer’s instructions and proteins were stored at −80°C. Fifty µg of total proteins from each sample were mixed with an equal amount of Laemmeli gel loading buffer and denatured for 5 min at 95°C. Proteins were resolved by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) along with molecular weight markers (Amersham Pharmacia Biotech, Piscataway, NJ). Proteins on one gel were stained with Coomassie blue R250 (Sigma) and Ponceau red (Sigma) to verify equal protein loading, and the proteins on the second gel were electroblotted onto Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech). Blots were blocked in PBST (0.1% Tween 20 in phosphate-buffered saline) containing 2% nonfat dry milk for 1 hr at room temperature. The primary purified mouse antihuman hMLH1 monoclonal antibody (clone 6168-15; Pharmingen, San Diego, CA) was used at a concentration of 1:250 at 4°C overnight. After washing with PBST, the blots were subsequently incubated with the secondary antibody (horse-radish peroxidase-conjugated antimouse IgG; Amersham Pharmacia Biotech) at a dilution of 1:5,000 for 45 min. Blots were washed with PBST and the hMLH1 protein-antibody complexes were detected with an enhanced chemiluminescent system (Amersham Pharmacia Biotech) at a dilution of 1:5,000 for 45 min. Blots were washed with PBST and the hMLH1 protein-antibody complexes were detected with an enhanced chemiluminescent system (Amersham Pharmacia Biotech).

RESULTS

Microsatellite analysis

As described before, all cell lines except HT29 were MSI-H. SW48, HCT116, HCT116+ch2, HCT116+chr3 and HCA7 cells had deletion mutations at BAT 25, BAT 26, TGFβRII and BAX. As a consequence of chromosome 3 transfer and corrected MMR function, the initially unstable cell line HCT116+chr3 acquired and maintained a wild-type allele (A10) for TGFβRII, which is also located on chromosome 3.

5 aza-dC demethylates the hMLH1 promoter

DNA from SW48 cells was subjected to bisulfite modification. Subsequently, MSP was performed to determine the promoter methylation status of hMLH1. As early as 24 hr after initial treatment with 5 µM 5 aza-dC, unmethylated hMLH1 was detectable by MSP (Fig. 1). Promoter demethylation was not complete but remained detectable for 16 days after treatment.

5 aza-dC transcriptionally activates hMLH1

Previous studies have demonstrated a strong correlation between hypermethylation of the hMLH1 promoter and transcriptional silencing of gene expression. We first intended to find the lowest 5 aza-dC dose to induce and maintain hMLH1 reexpression with minimal toxicity. SW48 cells were treated with 5 and 10 µM doses of 5 aza-dC. Figure 2a shows that as soon as 24 hr after treatment with 5 µM 5 aza-dC, cells started to form transcripts. Doubling the dose to 10 µM did not change the intensity or duration of expression; lower doses than 5 µM were not able to reexpress hMLH1 transcripts reliably (not shown). Transcripts remained detectable for 16 days after treatment.

Transcriptional activation is followed by protein expression of hMLH1 after 5 aza-dC treatment

We next determined the extent of protein expression after 5 aza-dC-induced transcriptional activation of the hMLH1 gene in SW48 cells. We were able to detect protein expression 48 hr after 5 aza-dC treatment and 24 hr after the first mRNA detection. The delay of protein expression might be explained by additional posttranscriptional modifications or intervening mechanisms prior to translation. Proteins remained detectable for 13 days after initial treatment (Fig. 2b). These experiments proved that demethylation of the hMLH1 promoter by 5 aza-dC treatment was followed by the expression and maintenance of mRNA and protein.

p16 and HIC1 methylation profile

It has been shown that hypermethylated cell lines are not only methylated at the hMLH1 promoter, but also at a variety of different tumor suppressor genes, including p16. We investigated the effect of 5 aza-dC on p16 methylation status in SW48 cells and detected promoter demethylation 1 day after 5 aza-dC treatment, which lasted for 16 days, similar to hMLH1 gene demethylation (Fig. 3a). Similar as for the hMLH1 promoter, methylation was not complete. This might be due to the missing intrinsic demethylating activity of 5 aza-dC. Its effect in reverting gene silencing is due to the inhibition of DNA methyltransferase 1 (DNMT1) that complements the methylation of hemimethylated DNA after replication. Unlike SW48 cells, HCT116 cells have 1 unmethylated, but mutated, p16 allele; the second allele is methylated.32 It has been shown that HCT116 cells do not express p16, and thus the methylation of the second allele is functionally important. Despite 5 aza-dC treatment, we were not able to detect a difference of the...
promoter methylation status for p16 before or after treatment. It is possible that the methylated allele was partly demethylated after 5aza-dC treatment, but the demethylation is not sufficiently complete to permit gene expression, which could be why MSP with specific primers for the methylated DNA remained positive. Alternatively, there could be other abnormalities in this allele, which were beyond the scope of our analysis.

We next examined the methylation status of HIC1 to determine whether 5aza-dC also exerts demethylating effects on HCT116 cells. HIC1 is a putative tumor suppressor gene on 17p13.3 activated by p53 and is hypermethylated in about 50% of CRC.33 HIC1 is fully methylated in HCT116 cells (data not shown). We treated HCT116 cells with various doses of 5aza-dC and detected a PCR product after MSP with primers specific for unmethylated DNA 3 days after 5aza-dC treatment (Fig. 3b). Of particular interest is that a 5aza-dC concentration of 0.1 μM was able to exert the demethylating effect, while higher doses increased toxicity in this cell line. This was in contrast to SW48 cells, which did not show signs of demethylation at low doses and tolerated higher doses of 5aza-dC.

**FIGURE 2—RT-PCR of hMLH1 transcripts and Western blots from SW48 cells.** (a) SW48 cells were treated with 5 μM 5aza-dC for 24 hr at day 0. SW480 cells served as a positive control. HCA7 was used as a negative control. Brisk expression of hMLH1 was observed at day 1, was maximal at days 2 and 3 and was still faintly detected at day 16. In the lower panel, β-actin expression as an internal control is shown. The experiment was repeated to confirm reproducibility of the results. (b) SW48 cells were treated with 5 μM 5aza-dC as described. Western blots show weak (+) protein expression 2 days after treatment followed by stronger expression on days 4–13. SW480 cells used as an hMLH1-expressing, MMR-proficient positive control showed a strong signal, compared with the hMLH1-deficient negative control, HCT116. Experiments were repeated to document reproducibility of the results. The upper band is the appropriate MW for hMLH1, and the lower band is nonspecific, typically obtained with this antibody.

**MMR-corrected SW48 cells are sensitive to 5-FU**

We treated cells with 5aza-dC to restore MMR proficiency in the hMLH1-deficient cell line SW48. We had previously demonstrated that correction of MMR deficiency by chromosome 3 transfer in HCT116 cells rendered the cells susceptible to pharmacologically relevant doses of 5-FU.34 We hypothesized that 5aza-dC treatment of SW48 cells would produce a similar result and compared 5aza-dC-treated SW48 cells with HCT116+chr3 cells for their response to 5-FU treatment. We used HCT116 and HCT116+chr2 as MMR-deficient controls. HCT116+chr2 was used to determine if there were any differences between MMR-deficient cells with or without chromosome transfer and to control for the selection effect of G418 (required for the maintenance of trisomy 2).
5 aza-dC treatment alone did not cause a significant decrease in the growth of SW48 cells as determined by clonogenic assays (Fig. 4a). On the contrary, 5 aza-dC caused a significant decrease in colony formation of HCT116 cells, which was even stronger in the chromosome-transferred derivatives HCT116(chr2) and HCT116(chr3) (p < 0.001). SW48 cells tolerated 5 aza-dC concentrations up to 10 μM and responded with negligible decreases in colony formation. For HCT116(chr2) and HCT116(chr3) cells, treatment with 0.1 μM 5 aza-dC reduced colony formation to 34% and 56%, respectively. Concentrations of more than 0.5 μM were also highly toxic to HCT116 cells; 1 μM reduced colony formation to less than 20% compared to untreated cells.

The difference in response by these cell lines is not likely to be explained by the reexpression of other growth-regulatory genes, as HCT116 cells have been shown not to be characterized by the CpG island methylator phenotype at tumor suppressor genes known to be involved in colorectal carcinogenesis. However, we have no data at this time to explain how the transfer of an additional chromosome renders cells more vulnerable to 5 aza-dC treatment. The toxicity of 5 aza-dC and similar derivatives is thought to be caused by large adduct formation leading to chromosomal breaks and apoptosis.18 We assume that HCT116 cells are more susceptible to DNA adduct formation and thus tolerate only minimal doses of demethylating agents such as 5 aza-dC.

To assess the growth response of cells to 5-FU, we treated cell lines with increasing doses of 5-FU (Fig. 4b). It has been shown previously that the optimal dose for 5-FU treatment is in a range from 1 to 5 μM,34 a concentration that is commonly achieved in tissue after i.v. drug administration. Treatment with 1 μM 5-FU did not affect colony formation in any cell line. After administration of 2.5 μM 5-FU, HCT116+chr3 cells responded with a 40% decrease in colony formation compared to 11% in HCT116, 17% in HCT116+chr2 and 15% in SW48 cells, respectively. HCT116+chr3 cells showed an 82% reduction in the clonal surviving fraction after 5 μM treatment (p = 0.0031), compared to a 33% reduction in HCT116 (p = 0.0031), 38% in HCT116+chr2 (p = 0.0007) and 44% in SW48 cells (p = 0.0004), respectively. We thus confirmed and extended the in vitro resistance of MMR-deficient cell lines to 5-FU.

We finally examined the response to combined 5 aza-dC and 5-FU treatment. Combined treatment showed synergistic cytotoxicity in all cell lines (Fig. 4c). Because of the limited tolerance of HCT116 cells to 5 aza-dC, cell lines were pretreated with only 0.1 μM 5 aza-dC. The additional administration of various 5-FU doses together with 5 aza-dC produced more pronounced decreases in colony formation than with 5-FU treatment alone. As expected, low-dose 5 aza-dC treatment combined with 5-FU did not potentiate a decrease in colony-forming ability in SW48 cells. However, growth curves were identical for SW48 and HCT116+chr3 cells after pretreatment with 5 μM 5 aza-dC and subsequent 5-FU treatment for SW48 cells and 5-FU treatment only for HCT116+chr3 cells (Fig. 4d). The growth decrease was significant compared to HCT116 cells (p = 0.0052) and HCT116+chr2 cells (p = 0.0082).

With this experiment, we demonstrated that reexpression of hMLH1 by promoter demethylation will sensitize SW48 cells to 5-FU chemotherapy in vitro, in the same way as chromosome 3

**FIGURE 3** – Demethylation of the p16 and HIC1 promoters by 5 aza-dC. (a) SW48 cells were treated with 5 μM 5 aza-dC. One day after treatment, p16 promoter demethylation was detected by PCR with specific primers for unmethylated DNA (u). The PCR with specific primers for methylated DNA (m) remained positive throughout the duration of the experiment, suggesting incomplete demethylation of the promoter. Experiments were repeated twice to confirm reproducibility of the results. (b) HCT116 cells were subjected to 0.1 μM 5 aza-dC treatment. The HIC1 methylation status was examined before and after 5 aza-dC treatment. A band for the unmethylated promoter became visible 3 days after treatment. There were no differences in methylation status detectable among HCT116, HCT116+chr2 and HCT116+chr3 cells (not shown).
transfer renders HCT116+chr3 cells susceptible to 5-FU treatment.

**DISCUSSION**

The human MMR system has been extensively studied over the past years and it is well accepted that it is responsible for the S-phase correction of nucleotide mismatches, certain types of errors caused by DNA polymerase, and for the recognition of certain adducts formed by DNA damaging agents. Defects of the MMR system render cells more susceptible to neoplastic transformation and result in the mutator phenotype of CRC.35,36 Furthermore, it has been demonstrated that a nonfunctioning MMR system caused by a defect of one of its major constituents permits tolerance in cancer cells to drugs that are usually toxic to tumor cells.22

Carethers et al.34 demonstrated that cells deficient in MMR activity are significantly more tolerant to 5-FU than the near isogenic, MMR-proficient HCT116+chr3 cells. This phenomenon has been proved in response to a variety of chemotherapeutic agents for a number of cell lines defective in MMR.37– 40 Our data show that cells with a nonfunctioning MMR system may be sensitized to 5-FU treatment regardless of the mechanism underlying the MMR defect. HCT116 cells, which are characterized by homozygous mutations in the coding region of the hMLH1 gene, are resistant to 5-FU treatment, as opposed to their chromosomally complemented counterpart HCT116+chr3, which is MMR-proficient and has been shown to be sensitive to 5-FU treatment in vitro. Here we show for the first time that by reexpression of hMLH1 in SW48 cells by demethylation of the aberrantly methylated hMLH1 promoter, resistance to 5-FU treatment can be overcome; consequently, SW48 cells respond in the same way as HCT116+chr3 cells to 5-FU treatment.

Furthermore, the reexpression of hMLH1 occurred at a transcriptional level after 5 aza-dC treatment followed by the expression and maintenance of hMLH1 protein. Sensitization occurred specifically in the hMLH1-methylated cell line (SW48) but not in cells with mutations in the coding sequences of the hMLH1 gene (i.e., HCT116 and HCT116+chr2). However, the data of our study have to be confirmed in other cell lines hypermethylated at the hMLH1 promoter.

All cell lines treated with 5 aza-dC in this study showed promoter hypermethylation at p16, an important growth-regulatory gene that controls the G1/S-phase cell cycle transition.35 Other studies have previously demonstrated the induction of p16 expression after 5 aza-dC treatment in hypermethylated cell lines.23

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**Figure 4** – Clonogenic assays. (a) SW48, HCT116, HCT116+chr2 and HCT116+chr3 cells were cultivated in growth medium or exposed to 5 aza-dC for 24 hr. Medium was exchanged and cells were allowed to form colonies over a period of 10–12 days. Shown are the means of 3 independently performed experiments with the respective standard deviations. Results are expressed as the relative surviving fraction as described in the text. (b) Cells were continuously exposed to increasing concentrations of 5-FU over 10–12 days. Cells were washed, fixed, stained and colonies were counted. Results are expressed as the frequency in percent of the relative surviving fraction. Shown are the means of 3 independently performed experiments with the respective standard deviations. (c) Cells were pretreated with 0.1 µM 5 aza-dC for 24 hr and then continuously exposed to various concentrations of 5-FU. Cells were allowed to form colonies for 10–12 days. Experiments were repeated 3 times to ensure reproducibility. (d) SW48 cells were pretreated with 5 µM 5 aza-dC for 24 hr and then exposed to different doses of 5-FU (1, 2.5 and 5 µM). HCT116, HCT116+chr2 and HCT116+chr3 cells were only treated with 5-FU. Cells were allowed to form colonies for 10–12 days. Shown are the means of 3 independently performed experiments with the respective standard deviations.
Reexpression of p16 is a possible explanation for the synergistic effect of low-dose 5aza-dC and 5-FU treatment in HCT116, HCT116 + chr2 and HCT116 + chr3 cells. 5aza-dC at a dose higher than 0.5 μM was highly cytotoxic to the HCT116 cell line family, which was less pronounced at lower doses. Low-dose 5aza-dC had a significant impact on colony formation, which was further decreased after the addition of 5-FU. An alternative mechanism for the substantial decrease in colony formation could be the induction of additional, unidentified growth-regulatory genes by promoter demethylation, or the cytotoxic activity of the drug itself. It is well known that 5aza-dC promotes the formation of toxic DNA adducts. It is possible that DNA was already damaged as a result of 5aza-dC exposure before 5-FU treatment. 5-FU administration might have further increased DNA damage and induced cell death without influencing p16 expression. However, preliminary data from our laboratory show that 5aza-dC-treated cells are not arrested at G1/S, which might be expected after p16 reexpression. Thus, we assume that p16 expression might not have played a significant role in the observed growth arrest.

hMLH1 induction in cancers with aberrantly methylated hMLH1 promoters is a possible treatment option for CRC with the MSI-H phenotype. Most of the tumors with the MSI-H phenotype (with the notable exception of tumors in HNPCC) have hMLH1 promoter methylation and are thus suitable for treatment with a demethylating agent. Low or absent hMLH1 expression has been observed in a variety of tumors. Methylation studies have shown that de novo promoter methylation in breast and ovarian cancer may be induced by chemotherapy. These tumors generally have a worse outcome in terms of disease-free and overall survival. It is reasonable to speculate that such tumors might also regain sensitivity to standard chemotherapy by the addition of 5aza-dC-induced promoter demethylation, which would appear to improve the response to treatment.

Patients with MSI-H CRC have been observed to experience better long-term survival when compared to MSS or microsatellite low (MSI-L) cancers, regardless of 5-FU chemotherapy. However, it is not known if it is the intrinsic biology of the tumor or the treatment that is responsible for the difference in survival. Our data suggest that pharmacologically feasible doses of 5-FU might not affect tumor growth in patients with MSI-H tumors. It will be of clinical importance to study further the biology of MSI-H tumors, specifically the cell cycle regulation during 5aza-dC exposure in vivo xenograft models to gain further evidence for the suitability of this drug as an option for future CRC treatment regimens.

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