Decreased expression of hMLH1 correlates with reduced 5-fluorouracil-mediated apoptosis in colon cancer cells

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Abstract. Patients with sporadic microsatellite instable colorectal cancers, in most of which the function of the hMLH1 mismatch repair gene is impaired, do not gain a survival benefit from 5-fluorouracil (5-FU)-based chemotherapy. However, the effect of hMLH1 on the cytotoxicity induced by 5-FU has not yet been sufficiently confirmed. In this study, we assessed the effect of hMLH1 on cytotoxicity and apoptosis induced by 5-FU using newly developed cell lines. We constructed two cell lines: SW480 (originally hMLH1-proficient), in which the expression of hMLH1 was reduced using a small interfering RNA (siRNA) technique, and HCT116 (originally hMLH1-deficient), in which the expression of hMLH1 can be regulated by doxycycline. Using these cell lines, a clonogenic survival assay, 4',6-diamidino-2-phenylindole (DAPI) staining and an Annexin-V assay were performed. Moreover, the incorporation of 5-FU into DNA was determined using tritium-labeled 5-FU. In both of our two cell lines, hMLH1-deficient cells exhibited approximately 2.4-fold clonal surviving fraction compared to hMLH1-proficient cells for 10 days after the administration of 5-FU. Additionally, hMLH1-deficient cells treated with 5-FU exhibited 34-45% less apoptosis than hMLH1-proficient cells according to the results of DAPI staining and Annexin-V assay. Furthermore, hMLH1-deficient cells treated with 5-FU exhibited an approximately 2-fold greater incorporation of 5-FU into DNA than control cells, suggesting that the recognition of 5-FU-incorporated DNA is impaired in hMLH1-deficient cells, resulting in reduced apoptosis. Our conclusions were that decreased expression of hMLH1 in colon cancer cells reduced the apoptosis induced by 5-FU, suggesting that hMLH1 is a key determinant of 5-FU chemosensitivity.

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Introduction

Colorectal cancer (CRC) is one of the most common malignancies worldwide. The biological development of CRC involves genetic instability. Approximately 80-85% of CRCs develop through the chromosomal instability [also known as the microsatellite stable (MSS)] pathway. Meanwhile, another form of genetic instability correlated with the development of CRC is microsatellite instability (MSI), which is caused by the inactivation of a DNA mismatch-repair (MMR) gene, such as hMLH1, hMSH2, hMSH6, hPMS2 or hMSH3. Most sporadic CRCs with MSI are implicated in the epigenetic silencing of hMLH1 (1,2). In these tumors, the loss of the expression of hMLH1 due to methylation of the promoter region allows the occurrence of MSI (3-5).

MMR deficiency causes multiple errors in repetitive DNA sequences, such as mono- and dinucleotide repeats (6,7). In cases of MMR-deficient status, all nucleotide repeat sequences, including those in the coding region of the genome, are at risk of mutation. In particular, mutations in genes which encode for transforming growth factor (TGF)-ß receptor type II, the insulin-like growth factor (IGF)-II receptor and the Bax gene have been reported to play a key role in the development of CRC with MSI (8-10). However, the functional significance of these genes is not always clear because of the very high mutation rate at the repetitive sequences in CRCs with MSI.

Previous studies have reported that improved survival was observed in patients with MSS tumors who received 5-fluorouracil (5-FU)-based chemotherapy (11-16), while such therapy did not extend the lives of patients with MSI tumors (17-19). This clinical result suggests that the microsatellite stable or unstable status of CRCs is an important determinant of 5-FU-based chemosensitivity. Alternatively, the loss of hMLH1 expression, and not of microsatellite status, may be responsible for reduced chemosensitivity.

In vitro, the role of MMR in drug cytotoxicity has been explored using methodologies with genetically matched cells possessing an altered MMR status. One commonly used system applies the hMLH1-deficient HCT116 human colon cancer cell line and a corrected clone (HCT116 3-6) into which a normal human chromosome 3 (which contains the hMLH1 gene) was introduced by microcell fusion (20). However, that system is problematic because the restored chromosome 3 also contains several vital oncogenes and tumor suppressor genes, such as RASSF1, TGF-ß type II receptor and the von

Hippel-Lindau (VHL) tumor suppressor gene. In that system, 1 or some of such pivotal genes may have affected the results of experiments with HCT116 and HCT116 3-6, and the function of hMLH1 may not have been properly evaluated. For example, the RASSF1A isoform of RASSF1 overexpression is known to reduce *in vitro* colony formation and the tumorigenicity of cancer cell lines *in vivo* (21). On the other hand, VHL gene deletion frequently occurs in sporadic colon carcinoma. Because this deletion is not present in adenomas, the VHL gene may play a role in colonic carcinogenesis and represents a relatively late event in colonic neoplasia progression (22,23). Additionally, the loss of TGF-ß-mediated growth inhibition is one of the *in vivo* mechanisms through which TGF-ß type II receptor inactivation contributes to the formation of colon cancer (24).

One study demonstrated that treatment with a demethylating agent, 5-aza-2'-deoxycytidine of hMLH-methylated SW48 cells, induced higher sensitivity to 5-FU (25). Even in that system, however, recovery of the expression of methylated gene products other than hMLH1 may have also occurred, and the results may have been affected. Thus, previous reports have failed to rigorously confirm the effect of hMLH1 protein on drug cytotoxicity.

Therefore, in order to precisely evaluate the function of the hMLH1 gene on drug cytotoxicity, we developed 2 cell lines in which only the expression of hMLH1 was regulated via small interfering RNA (siRNA) and the TetOff system. In the present study, we strictly assessed the effect of hMLH1 status on the cytotoxicity and apoptosis induced by 5-FU administration.

Materials and Methods

Plasmid construction. A plasmid vector encoding siRNA targeting hMLH1 (pSilencer-siMLH1) was constructed by annealing oligonucleotides 5'-GATCCGGTTCACTACTAG TAAACTTTCAAGAGAAGTTTACTAGTAGTGAACCTT TTTTGGAAA-3' and 5'-AGCTTTTCCAAAAAAGGTTCA CTACTAGTAAACTTCTCTTGAAAGTTTACTAGTAGT GAACCG-3', which were designed using online software provided by Ambion (Austin, TX). Duplex oligonucleotides were subcloned into the BamHI-HindIII site of pSilencer 2.1-U6 neo vector (Ambion), following the manufacturer's instructions. As a negative control plasmid, we used pSilencer neo Negative Control plasmid, which lacks significant homology to the genome, supplied with the pSilencer 2.1-U6 neo vector kit.

A plasmid vector that expresses hMLH1 under the control of doxycycline administration with the help of the TetOff system was constructed as follows. Total-RNA was extracted from HT29 cells using TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was obtained by reverse transcription-polymerase chain reaction (RT-PCR) using random primers and a SuperScript II Reverse Transcriptase Kit (Invitrogen). We subcloned the hMLH1 gene using forward primer 5'-GGCGC CAAAATGTCGTTCGTG-3' and reverse primer 5'-ACATC CCACAGTGCATAAATAAC-3'. The yielded fragment was ligated into the pCRII vector (Invitrogen) via the TA cloning method, and was recloned into expression vector pCEP4 (Invitrogen) to confirm the expression of hMLH1. pCEP4-hMLH1 plasmid was digested with *Not*I and the isolated fragment was inserted into pTRE2-hyg (Clontech, Heidelberg, Germany) that had been digested with *Not*I(pTRE2-hyg-hMLH1).

Cell culture and transfection. SW480 and HCT116 human colon cancer cell lines were obtained from American Type Cell Culture (Manassas, VA). The cells were maintained in RPMI1640 (SW480) (Sigma, St. Louis, MO) or McCoy's 5A (HCT116) medium (Invitrogen) supplemented with 10% fetal bovine serum. To establish SW480 cell lines stably expressing the siRNA targeting hMLH1 (SW480-siMLH1), SW480 cells were transfected with pSilencer-siMLH1 using a Gene Pulser Xcell electroporation system (Bio-Rad, San Diego, CA), following the manufacturer's instructions. During the electroporation procedure, SW480 cells were suspended with 1 ml of phosphate-buffered saline (PBS) in 4-mm-long cuvettes and electroporated with the following settings: 260 V, 1050 μF and ∞ ohm. The transfected cells were then maintained with a medium containing 1500 μg/ml of G418. Approximately 3 weeks after beginning the selection, >20 colonies were isolated and their extracts were screened by Western blotting using an antibody against hMLH1. We also generated negative control cells (SW480-siControl) in a similar manner using pSilencer neo Negative Control.

Next, we generated cell lines in which hMLH1 expression was regulated by a doxycycline-induced system (HCT116-MLH1-TetOff). HCT116 cells were transfected with pTetOff-Neo (Clontech) using Effectene Transfection Reagent (Qiagen, Hilden, Germany). The selection for obtaining stable clones was initiated 2 days after transfection by adding 150 μg/ml G418 to the medium. Approximately 3 weeks after beginning the selection, >50 colonies were isolated and screened by transient transfection with pTRE2-Luc (Clontech) for the expression of luciferase in induced and non-induced cells (with or without 2 μg/ml of doxycycline). The clone with the lowest background and the highest induction of luciferase was then transfected with pTRE2-Hyg-hMLH1. The selection of stable clones was initiated 2 days after transfection by adding 200 μg/ml hygromycin to the medium. Approximately 3 weeks after beginning the selection, >50 colonies were isolated and their extracts were screened by Western blotting using the antibody against hMLH1.

Western blot analysis. The cells were seeded into 6-well plates and grown to near confluence. Cultured cells were washed twice with ice-cold PBS and lysed with lysis buffer [0.1 M Tris-HCl, 4% sodium dodecyl sulfate (SDS), 10% glycerol, 0.004% bromophenol blue and 10% 2-mercaptoethanol]. The lysates were collected and boiled for 5 min. Cell lysates were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membranes were blocked using Tris-buffered saline with Tween 20 (Sigma) (TBS-T) buffer containing 5% bovine serum albumin for 1 h. The membranes were then incubated with anti-hMLH1 antibody (clone G168-15; Pharmingen, San Diego, CA) or anti-hPMS2 antibody (clone A16-4; Pharmingen) overnight at 4˚C. The membranes were then washed 3 times with TBS-T and the target proteins were

visualized by probing with the alkaline phosphatase-conjugated secondary antibody followed by development using a colorimetric method (Promega, Madison, WI). The expression levels of hMLH1 and hPMS2 were determined by densitometry using Scion Image, which is distributed by Scion Corporation (Frederick, MD), and reported as a ratio to actin, which was chosen as a housekeeping gene.

Reporter gene analysis of MSI. A detailed description of the procedures for reporter gene analysis of MSI was previously given by Wheeler *et al* (7). pCAR-OF reporter plasmid, a gift from Dr Bert Vogelstein (The Johns Hopkins University, Baltimore, MD), carries a hygromycin resistance gene as well as a ß-galactosidase (ß-gal) gene containing a 58-base pair out-of-frame poly(CA) tract at the 5' end of its coding region. Therefore, this reporter would not generate ß-gal activity unless a frame-restoring mutation (i.e., insertion or deletion) arose following transfection. For the purposes of the present analysis, the SW480-siMLH1 cells and SW480-siControl cells were transfected with pCAR-OF plasmid by electroporation as described above. Cells then were selected with hygromycin for 6 weeks at 300 μg/ml. For ß-gal staining, near confluent cultured cells were fixed with 0.5% glutaraldehyde in PBS for 10 min, rinsed twice at 37˚C for 15 min in PBS containing 1 mM $MgCl_2$ and then stained at 37˚C for ~6 h with a freshly prepared solution of PBS containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-Gal) (Sigma), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$ and 1 mM $MgCl_2$. ß-gal was expressed in blue staining cells and the expression was consistent with the MSI of transfected cells with the occurrence of frame slippage in the pCAR-OF vector. For image analysis, randomly selected images of optical fields were acquired with a charge-coupled device camera (Olympus, Tokyo, Japan) attached to an Olympus invertoscope.

Clonogenic survival assay. A clonogenic survival assay was performed by seeding $5x10^2$ cells/well into 20-mm 6-well plates. After 24 h, the growth medium was removed and a medium containing 5-FU was added at a concentration of 0, 1, 2.5 or 5 μM. The cells were then continuously treated at 37˚C with 5% CO_2 for 10 days for colony formation, with the medium being changed every 3 days. After incubation, the colonies were washed in PBS, fixed with methanol for 15 min and washed again in PBS. The colonies were then stained with 0.5% crystal violet (Sigma) for 10 min and rinsed briefly with water. The colonies were counted using the low-power objective of the microscope. The number of colonies treated and untreated with 5-FU was compared and the ratios of the surviving colonies were determined.

4',6-diamidino-2-phenylindole (DAPI) staining. The cells were treated with 5 μM of 5-FU for 96 h on 2-well glass slides (Nalge Nunc International, Rochester, NY). They were then washed twice with PBS and stained with 10 μg/ml DAPI, followed by incubation for 1 min in the dark. DAPI-labeled nuclei were visualized using a fluorescence microscope (Olympus). Three fields were randomly selected from each area under x100 magnification, underwent nuclear staining with DAPI of >100 cells in each field, and were independently captured and counted.

Flow cytometric analysis by Annexin-V-fluorescein isothiocyanate (FITC) and propidium iodide staining. Apoptosis was also assayed by the detection of membrane externalization of phosphatidylserine with Annexin-V-FITC conjugate using an Annexin-V assay kit following the manufacturer's protocol (Medical & Biological Laboratories, Nagoya, Japan). Briefly, after incubation with 5-FU (5 μM) for 96 h, adherent cells were detached with 0.5% trypsin-ethylenediamine tetra-acetic acid (EDTA) (Invitrogen) at 37˚C. The cells were then washed once with PBS and suspended in 85 μl Annexin-V-FITC binding buffer containing 5 μl propidium iodide and 10 μl Annexin-V-FITC. After incubation for 15 min at room temperature, the suspended cells were diluted with an additional 400 μl of Annexin-V-FITC binding buffer. The fluorescence intensity of the diluted solutions was measured using a FACS Caliber flow cytometer (Becton Dickinson, San Jose, CA) within 1 h.

Quantification of 5-FU incorporated into DNA. 5-FU incorporated into DNA was quantified by measuring the radioactivity of tritium-labeled 5-FU. The cells were treated with 1 μM of tritium-labeled 5-FU spiked with 20 μCi. Three days later, the cells were washed 2 times with ice-cold PBS followed by incubation in 10% trichloroacetic acid for 30 min at 4˚C. They were then washed in trichloroacetic acid and solubilized in 5% NaOH. Radioactivity was measured using a liquid scintillation counter (Beckman Coulter, Fullerton, CA). Radioactivities were adjusted based on the DNA content of cells which were similarly treated with non-labeled 5-FU.

Results

Construction of cell lines targeting hMLH1 expression control. In order to investigate the precise function of hMLH1 in the cytotoxic effects induced by 5-FU, we generated hMLH1 knockdown derivatives of SW480 colon cancer cells using the siRNA technique. The original SW480 cells were hMLH1-proficient MSS cancer cells. In contrast, the hMLH1 expression of the constructed derivatives, designated as SW480-siMLH1, was reduced to 1/20th of that of the control cells (SW480-siControl) (Fig. 1A). We also examined hPMS2 protein expression in our cell lines, because hMLH1 generally interacts with hPMS2 to form a heterodimer, hMutLα, and it has been reported that hPMS2 is unstable in the absence of its cognate partner (26). hPMS2 expression in SW480-siMLH1 cells was diminished to 1/4th of that of the control cells (Fig. 1A).

Next, we constructed hMLH1 protein-inducible HCT116-MLH1-TetOff cells using the TetOff expression system. The original HCT116 cells lacked chromosome 3, including the hMLH1 gene, and subsequently exhibited the MSI phenotype. The HCT116-MLH1-TetOff cells expressed hMLH1 protein in the absence of doxycycline in medium, while the addition of 2 μg/ml doxycycline to the medium reduced the protein expression to 1/10th (Fig. 1B). Additionally, in HCT116-MLH1-TetOff cells, hPMS2 expression was diminished in the presence of doxycycline (1/10th compared to the cells in an absence of doxycycline) (Fig. 1B).

In order to confirm whether the decreased expression of hMLH1 in SW480-siMLH1 cells could eliminate the function

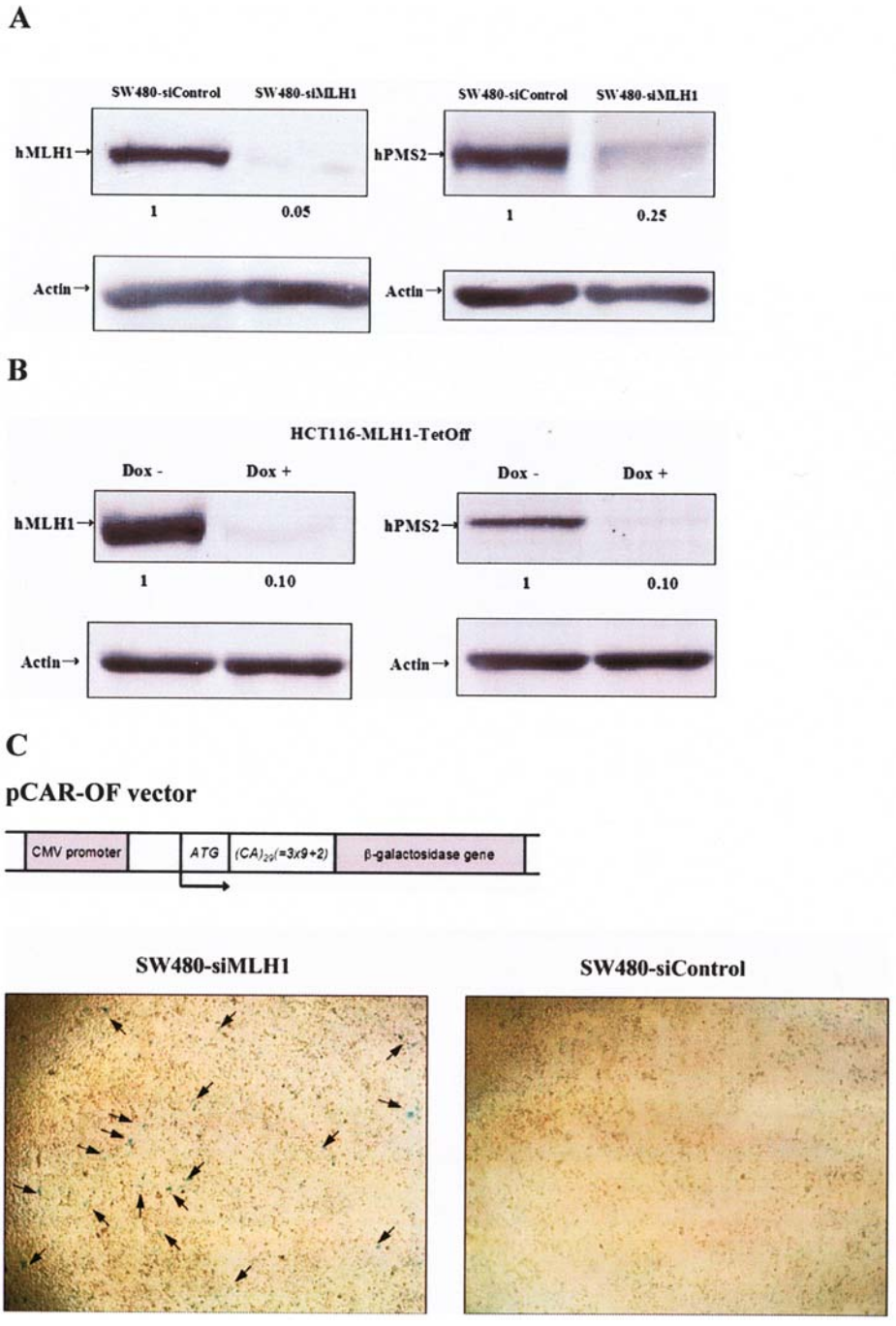

Figure 1. Construction of cell lines in which hMLH1 expression is regulated. Western blot analysis of SW480 cells in which siRNA targeting hMLH1 was stably expressed (SW480-siMLH1) and cells transfected with the vector expressing scrambled RNA (SW480-siControl) (A). The expression of hMLH1 in SW480-siMLH1 was reduced to 1/20th of that in SW480-siControl (left). The expression of hPMS2, the cognate partner of hMLH1, in SW480-siMLH1 was reduced to 1/4th of that of the control cells (right). Western blot analysis of HCT116-MLH1-TetOff cells cultured in the absence (-) or presence (+) of 2μg/ml doxycycline (Dox) (B). The expression of hMLH1 in HCT116-MLH1-TetOff cells in the presence of doxycycline was reduced to 1/10 of that in the absence of doxycycline (left). The expression of hPMS2 in HCT116-MLH1-TetOff cells in the presence of doxycycline was also reduced to 1/10 of that in the absence of doxycycline (right). The schema of the construct of the pCAR-OF vector (C, upper part) . The vector contains the ß-galactosidase (ß-gal) reporter gene with out-of-frame poly(CA) tract. SW480-siMLH1 cells and control cells were transfected with pCAR-OF. After 6 weeks at 300 μg/ml of hygromycin selection, an *in situ* ß-gal assay was performed (lower part). In some SW480-siMLH1 cells, ß-gal activity was observed (left, arrow), while none of the control cells exhibited ß-gal activity (right).

of MMR, a reporter assay was performed by transfecting pCAR-OF plasmid containing an out-of-frame ß-gal coding region. The results indicated that ß-gal was expressed in SW480-siMLH1 cells, suggesting that hMLH1 function had been lost and that MSI does occur in those cells. In contrast, none of the SW480-siControl cells expressed ß-gal (Fig. 1C).

Cytotoxic effect of 5-FU based on hMLH1 expression. To examine the effect of hMLH1 protein expression on the cytotoxicity of 5-FU, a clonogenic survival assay was performed. As shown in Fig. 2A, SW480-siMLH1 cells were more resistant to 5-FU than the control cells. SW480-siMLH1 cells exhibited a 2.4-fold clonal survival fraction compared to

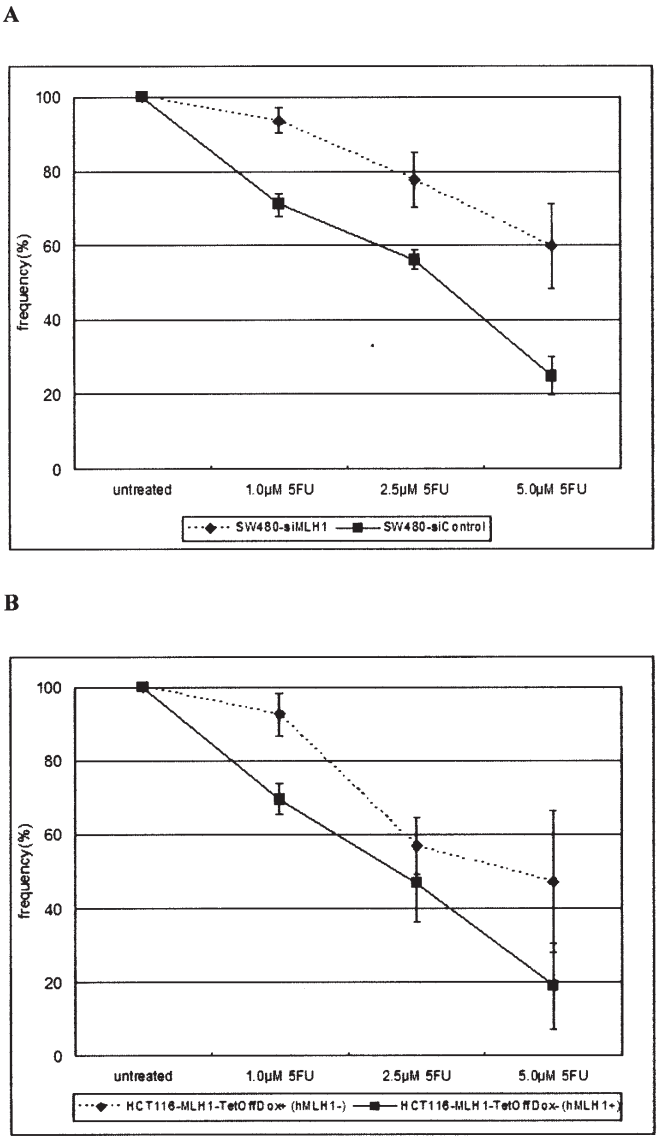

Figure 2. Clonogenic survival assays of SW480-siMLH1 and SW480-siControl cells (A) and HCT116-MLH1-TetOff cells (B) in the presence and absence of doxycycline. Cells were incubated with each concentration of 5-FU for 10 days. The relative frequencies of the surviving fraction compared to that of the untreated cells are indicated. Shown are the means of 3 independent experiments with standard error.

SW480-siControl cells in 5 μM of 5-FU. Nevertheless, these results could not rule out the possibility that other genes, including microsatellite regions, may have been mutated by impaired MMR thus affecting the cell survival results. Therefore, we also performed a similar assay using HCT116-MLH1-TetOff cells. Since HCT116 are originally cells with MSI, the comparison between HCT116-MLH1-TetOff cells in the absence and presence of doxycycline would highlight the effect of hMLH1 under conditions in which the effects of other genes would be completely eliminated. In these cells, the restoration of hMLH1 expression increased cytotoxicity by 5-FU administration (Fig. 2B). In other words, the loss of hMLH1 expression reduced the cytotoxicity, as happened in the case of SW480-siMLH1 cells. These results suggest that the impairment of hMLH1 function, and not other genes affected by impaired MMR, is mainly responsible for reduced cytotoxicity by 5-FU.

Apoptosis induced by 5-FU based on hMLH1 expression. We examined the relationship between apoptosis induced by 5-FU and hMLH1 expression. Apoptosis was assessed by 2 different assays. First, DAPI staining was performed (Fig. 3A). After 96 h of incubation in 5 μM of 5-FU, the typical morphological changes which indicate apoptosis were observed less frequently in SW480-siMLH1 cells than in control cells (average 5.6 vs. 10%) (Fig. 3B). In addition, HCT116-MLH1-TetOff cells without hMLH1 expression were more resistant to apoptosis than cells with hMLH1 expression (average 12.2 vs. 21.5%) (Fig. 3C).

To confirm this result, an Annexin-V apoptosis assay was also performed (Fig. 4A and B). In the present analysis, apoptotic cells were evaluated at 5 μM of 5-FU for 96 h. SW480-siMLH1 cells were less frequently sorted into early apoptotic fraction than the control cells (average 12.4 vs. 18.5%) (Fig. 4C). Similar results were obtained in the HCT116-MLH1-TetOff cells without and with hMLH1 expression (average 17.7 vs. 28%) (Fig. 4D). These results suggest that the loss of hMLH1 expression definitely induces resistance to apoptosis induced by 5-FU.

Difference in 5-FU incorporated into DNA according to hMLH1 status. One of the major mechanisms of 5-FU-mediated cytotoxicity is the incorporation of 5-FU metabolites into cellular DNA (27). To determine the effect of hMLH1 status on the incorporation of 5-FU metabolites, we measured the radioactivity of cells treated with tritium-labeled 5-FU using SW480-siMLH1 and SW480-siControl cells. In this experiment, SW480-siMLH1 cells exhibited 2-fold greater radioactivity than SW480-siControl cells, suggesting that the hMLH1-mediated MMR system recognizes 5-FU metabolites, resulting in the prevention of incorporation or the elimination of 5-FU metabolites.

Discussion

Clinical investigations have revealed that CRCs with MSI are more resistant to 5-FU-based chemotherapy than those with MSS (11-16). However, it remains unclear why MSI cancer is resistant to 5-FU, despite the improved survival rate it confers. There are a number of challenges in elucidating the biological characteristics of MSI cancer. However, although most MSI cancers are caused by the loss of expression of hMLH1 protein, all of the *in vitro* systems used to date have failed to distinguish whether hMLH1 is truly responsible or whether other mechanisms, affected by MMR deficiency, may be at work. The commonly used HCT116 3-6 cells have failed to rule out the effect of other genes on chromosome 3 (28-30) and the results recently reported in a study using a demethylating agent may have also been affected by demethylated genes besides hMLH1 (25).

In the present study, we strictly assessed the effect of hMLH1 status on cytotoxicity and apoptosis induced by 5-FU administration by using two newly developed cell lines: SW480 (originally hMLH1-proficient), in which the expression of the siRNA targeted to hMLH1 reduced the expression of hMLH1, and HCT116 (originally hMLH1-deficient), in which the expression of hMLH1 can be tightly regulated by doxycycline with the help of the TetOff system.

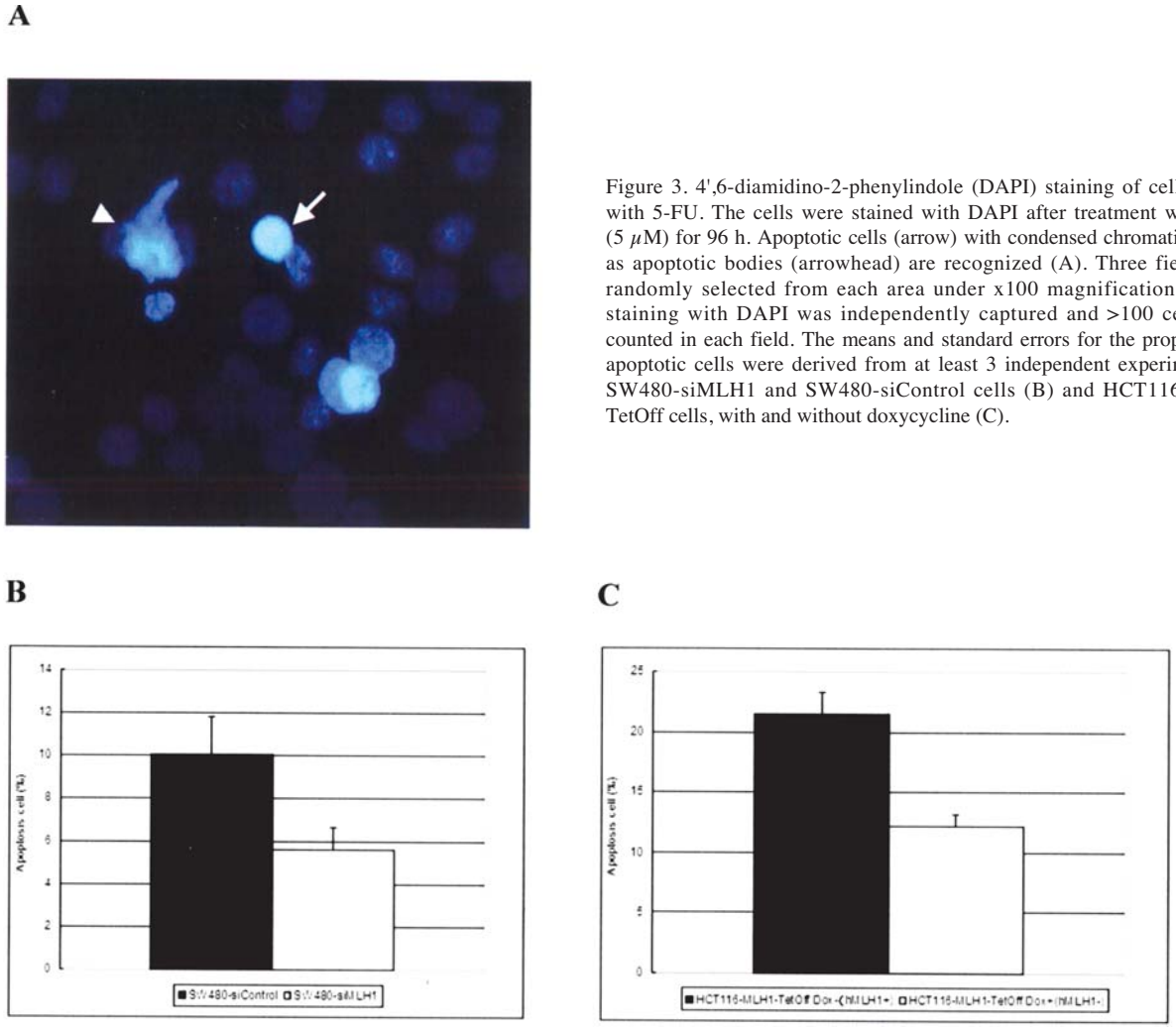

Figure 3. 4',6-diamidino-2-phenylindole (DAPI) staining of cells treated with 5-FU. The cells were stained with DAPI after treatment with 5-FU (5 μM) for 96 h. Apoptotic cells (arrow) with condensed chromatin as well as apoptotic bodies (arrowhead) are recognized (A). Three fields were randomly selected from each area under x100 magnification, nuclear staining with DAPI was independently captured and >100 cells were counted in each field. The means and standard errors for the proportion of apoptotic cells were derived from at least 3 independent experiments for SW480-siMLH1 and SW480-siControl cells (B) and HCT116-MLH1-TetOff cells, with and without doxycycline (C).

Our results indicate the precise effect of hMLH on 5-FU cytotoxicity and help to clarify the mechanism of resistance of this agent in MSI cancers.

One of the two cell lines, SW480-siMLH1 cells, was derived from MMR-proficient SW480 cells. The induction of 5-FU resistance by the reduction of hMLH1 expression in these cells directly indicated that the loss of hMLH1 was responsible for 5-FU resistance. In these cells, however, the microsatellite phenotype may have been changed to MMR-deficient, as indicated by the reporter gene assay. Therefore, other genes affected by MMR-deficient status (e.g., genes including a microsatellite region) may have affected the results. Another cell line, HCT116-MLH1-TetOff cells, was of great use in solving this problem. HCT116 cells were originally MMR-deficient and the recovery of MMR by the induction of hMLH1 expression would not affect the function of genes other than those with which hMLH1 directly interacts. Therefore, the fact that similar results were obtained in both of these 2 cell lines confirms that the loss of hMLH1 expression, but not other genes affected by MMR-deficiency, is responsible for resistance to 5-FU.

Moreover, we showed that apoptosis is less likely to occur in hMLH-deficient cells. A previous report indicated that there were no differences in the apoptotic effects of fluorodeoxyuridine, a 5-FU derivative metabolized by thymidine phosphorylase, between HCT116 and HCT116 3-6 cells (29). However, as indicated above, the system used in that study was problematic in its evaluation of hMLH1 status. Our 2 different assays indicated that the loss of hMLH1 expression reduced the apoptotic effects of 5-FU, suggesting that the enhanced cytotoxic effect of 5-FU brought about by the expression of hMLH1 is largely due to an increase in apoptosis. These results are consistent with a previous study, which showed that overexpression of hMLH1 induced apoptosis (31). In the apoptotic pathway induced by 5-FU, hMLH1 is likely to be involved in the recognition of misincorporated DNA because it has been proven that a heterodimeric complex of MutL related proteins (hMLH1/hPMS2) interacts with the MutS (hMSH2/hMSH6 or hMSH2/ hMSH3)-related proteins that have already bonded to mispaired bases (7). With respect to this point, our final experiment is informative (Fig. 5). The loss of hMLH1 may have induced the impairment of the recognition of 5-FU metabolites incorporated into DNA, the impossibility of inducing apoptosis and the subsequent accumulation of 5-FU metabolites in living cells. Thus, our results indicate that the loss of hMLH1 expression is a key determinant in impaired misincorporated DNA recognition and reduced apoptosis in treatment with 5-FU, resulting in insensitivity to 5-FU-based chemotherapy.

The p53 tumor suppressor plays an important role in the apoptosis pathway in the treatment of DNA-damaging agents. p53 has been shown to directly regulate the expression levels

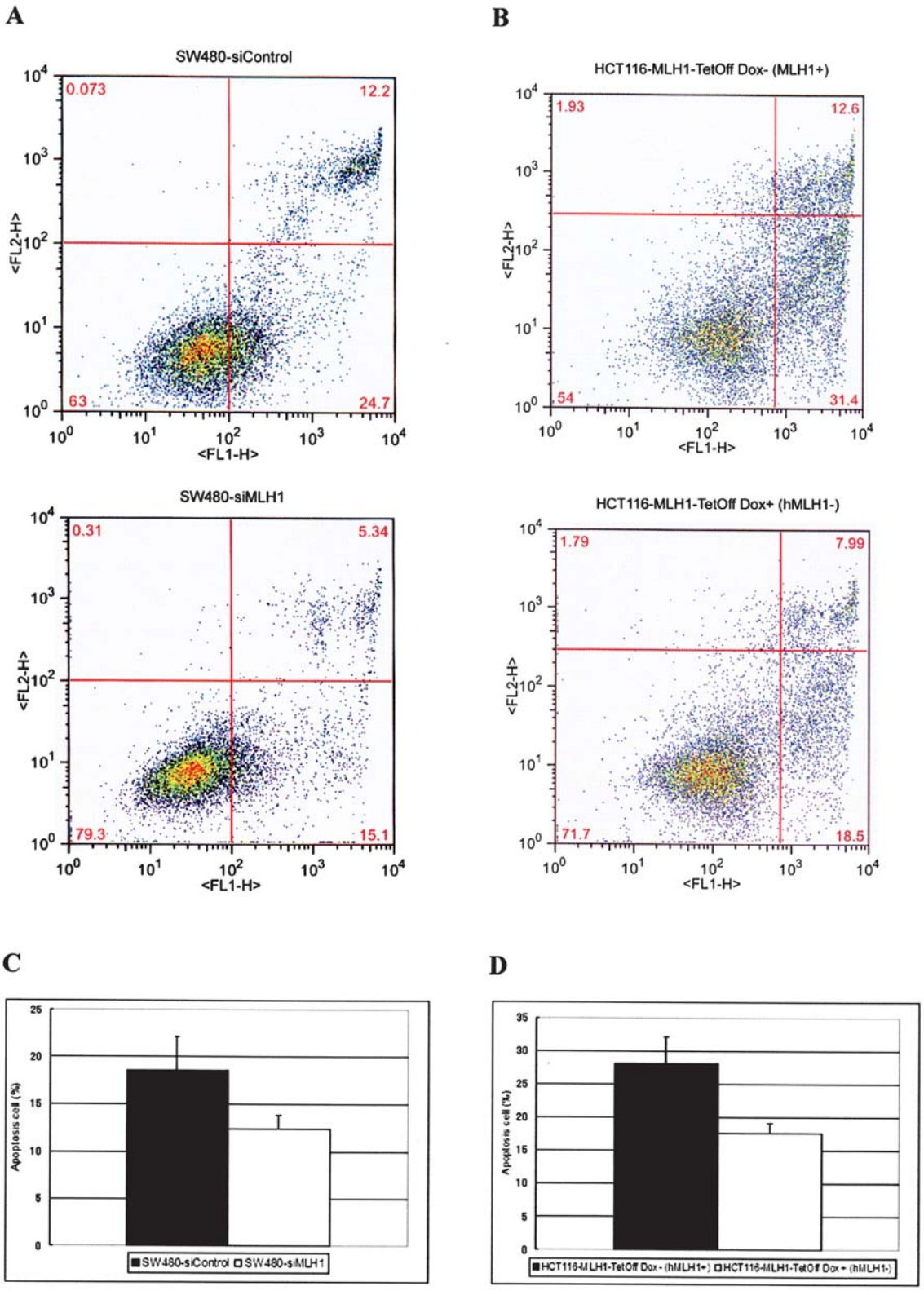

Figure 4. Annexin-V apoptosis assay. After incubation with 5-FU (5 μM) for 96 h, cells were washed and suspended in 85 μl Annexin-V-FITC binding buffer containing 5 μl propidium iodide (PI) and 10 μl Annexin-V-FITC. Fifteen min after incubation at room temperature in the dark, the suspension was diluted in 400 μl Annexin-V-FITC binding buffer. Fluorescence was measured using a FACS Caliber flow cytometer within 1 h for SW480-siMLH1 and SW480-siControl cells (A) or HCT116-MLH1-TetOff cells with and without doxycycline (B). The cells sorted at the Annexin-V+/PI- fraction (bottom right quadrant) were consistent with those with early apoptotic status. The means and standard errors for the proportion of early apoptotic cells were derived from at least 3 independent experiments (C, D).

of Bax (32), and both p53 and Bax have been shown to be important determinants of the cellular response to chemotherapeutic agents (33). *In vitro* analysis using colon cancer cells has revealed that disruption of p53 induces resistance to 5-FU (33-35). Additionally, clinical investigations have shown that the overexpression of p53 in colon cancer is correlated with resistance to 5-FU (36-38). However, this observation seems to contradict our results and those of previous reports

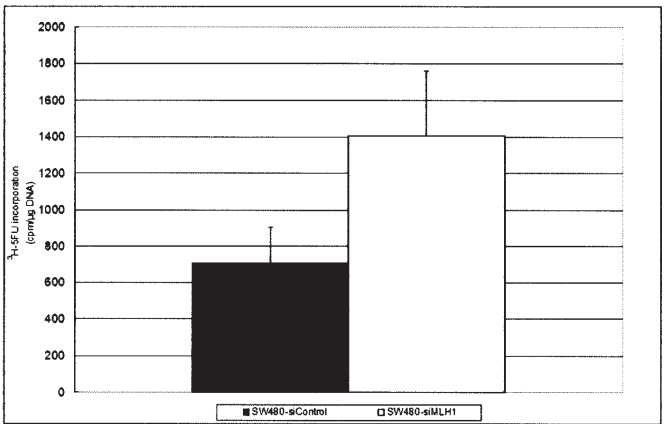

Figure 5. 5-FU incorporated into cellular DNA. SW480-siControl and SW480-siMLH1 cells were incubated with 20 μCi of tritium-labeled 5-FU (1 μM) for 96 h. The cells were washed in trichloroacetic acid and were solubilized in 5% NaOH. Radioactivity was measured using a liquid scintillation counter (Beckman Coulter, Fullerton, CA). Radioactivity was corrected based on the amount of DNA in the cells obtained from an experiment using non-labeled 5-FU. The means and standard errors for the radioactivities of incorporated tritium-labeled 5-FU (cpm/μg of DNA) were derived from at least three independent experiments.

regarding hMLH1 expression and 5-FU resistance (17-19, 28-30) because most MSI cancers, which are usually accompanied by a loss of hMLH1, carry wild-type p53 while MSS cancers (i.e., those having intact hMLH1) usually show mutated p53. The present results indicate that both cells with wild-type p53 (HCT116) and mutated p53 (SW480) show a similar alteration of 5-FU sensitivity according to the change in hMLH1 expression status. Since clinical investigations have indicated that patients with MSS cancer are more sensitive to 5-FU than those with MSI cancer (17-19), hMLH1 seems to correlate more with 5-FU sensitivity than with p53 status. This result suggests that hMLH1 may be a suitable target for new anti-cancer drugs and future gene therapies. For example, demethylating agents could be a candidate for drugs against MSI cancers with methylated hMLH1.

The present results, that cells without hMLH1 expression are more resistant to 5-FU than cells with hMLH1 expression, are consistent with the results of previous molecular and clinical studies (17-19,28-30). Specifically, we have clearly elucidated that hMLH1 expression, and not other genes in chromosome 3 or other genes affected by MMR status, is truly responsible for 5-FU sensitivity. However, further investigation is required to clarify how hMLH1 is involved in the apoptosis induced by 5-FU and, particularly, in the recognition of 5-FU incorporated into DNA. Knocking out other MMR genes, such as hMSH2, hMSH6, hPMS2 and hMSH3, or proliferating cell nuclear antigen, which interacts with factors that participate in mismatch recognition (39), may be useful in elucidating this problem.

In conclusion, we constructed cell lines in which only hMLH1 protein expression was strictly regulated and, using them, examined the differences in cytotoxicity and apoptosis according to hMLH1 expression status. Our results suggest that hMLH1 is a key determinant of 5-FU chemosensitivity and may be more important than p53. Moreover, the induction of resistance to 5-FU through the loss of hMLH1 expression may be related to the impairment of recognition of 5-FU-incorporated DNA. In clinical settings, hMLH1 could serve not only as a biomarker that predicts sensitivity to 5-FU-based chemotherapy, but also as a molecular target in future anti-cancer therapies.

References

1. Thibodeau SN, French AJ, Cunningham JM, *et al*: Microsatellite instability in colorectal cancer: different mutator phenotypes and the principal involvement of hMLH1. Cancer Res 58: 1713-1718, 1998.
2. Veigl ML, Kasturi L, Olechnowicz J, *et al*: Biallelic inactivation of hMLH1 by epigenetic gene silencing, a novel mechanism causing human MSI cancers. Proc Natl Acad Sci USA 95: 8698-8702, 1998.
3. Cunningham JM, Christensen ER, Tester DJ, *et al*: Hypermethylation of the hMLH1 promoter in colon cancer with microsatellite instability. Cancer Res 58: 3455-3460, 1998.
4. Kane MF, Loda M, Gaida GM, *et al*: Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. Cancer Res 57: 808-811, 1997.
5. Herman JG, Umar A, Polyak K, *et al*: Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. Proc Natl Acad Sci USA 95: 6870-6875, 1998.
6. Thibodeau SN, Bren G, Schaid D: Microsatellite instability in cancer of the proximal colon. Science 260: 816-819, 1993.
7. Wheeler JM, Bodmer WF, Mortensen NJ: DNA mismatch repair genes and colorectal cancer. Gut 47: 148-153, 2000.
8. Markowitz S, Wang J, Myeroff L, *et al*: Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. Science 268: 1336-1338, 1995.
9. Souza RF, Appel R, Yin J, *et al*: Microsatellite instability in the insulin-like growth factor II receptor gene in gastrointestinal tumours. Nat Genet 14: 255-257, 1996.
10. Rampino N, Yamamoto H, Ionov Y, *et al*: Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. Science 275: 967-969, 1997.
11. Lothe RA, Peltomaki P, Meling GI, *et al*: Genomic instability in colorectal cancer: relationship to clinicopathological variables and family history. Cancer Res 53: 5849-5852, 1993.
12. Gryfe R, Kim H, Hsieh ET, *et al*: Tumor microsatellite instability and clinical outcome in young patients with colorectal cancer. N Engl J Med 342: 69-77, 2000.
13. Halling KC, French AJ, McDonnell SK, *et al*: Microsatellite instability and 8p allelic imbalance in stage B2 and C colorectal cancers. J Natl Cancer Inst 91: 1295-1303, 1999.
14. Lukish JR, Muro K, DeNobile J, *et al*: Prognostic significance of DNA replication errors in young patients with colorectal cancer. Ann Surg 227: 51-56, 1998.
15. Bubb VJ, Curtis LJ, Cunningham C, *et al*: Microsatellite instability and the role of hMSH2 in sporadic colorectal cancer. Oncogene 12: 2641-2649, 1996.
16. Wright CM, Dent OF, Barker M, *et al*: Prognostic significance of extensive microsatellite instability in sporadic clinicopathological stage C colorectal cancer. Br J Surg 87: 1197-1202, 2000.
17. Ribic CM, Sargent DJ, Moore MJ, *et al*: Tumor microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. N Engl J Med 349: 247-257, 2003.
18. Carethers JM, Smith EJ, Behling CA, *et al*: Use of 5-fluorouracil and survival in patients with microsatellite-unstable colorectal cancer. Gastroenterology 126: 394-401, 2004.
19. Jover R, Zapater P, Castells A, *et al*: Mismatch repair status in the prediction of benefit from adjuvant fluorouracil chemotherapy in colorectal cancer. Gut 55: 848-855, 2006.
20. Koi M, Umar A, Chauhan DP, *et al*: Human chromosome 3 corrects mismatch repair deficiency and microsatellite instability and reduces N-methyl-N'-nitro-N-nitrosoguanidine tolerance in colon tumor cells with homozygous hMLH1 mutation. Cancer Res 54: 4308-4312, 1994.
21. Van der Weyden L, Tachibana KK, Gonzalez MA, *et al*: The RASSF1A isoform of RASSF1 promotes microtubule stability and suppresses tumorigenesis. Mol Cell Biol 25: 8356-8367, 2005.
22. Zhuang Z, Emmert-Buck MR, Roth MJ, *et al*: Von Hippel-Lindau disease gene deletion detected in microdissected sporadic human colon carcinoma specimens. Hum Pathol 27: 152-156, 1996.

23. Giles RH, Lolkema MP, Snijckers CM, *et al*: Interplay between VHL/HIF1alpha and Wnt/beta-catenin pathways during colorectal tumorigenesis. Oncogene 25: 3065-3070, 2006.
24. Biswas S, Chytil A, Washington K, *et al*: Transforming growth factor beta receptor type II inactivation promotes the establishment and progression of colon cancer. Cancer Res 64: 4687-4692, 2004.
25. Arnold CN, Goel A, Boland CR: Role of hMLH1 promoter hypermethylation in drug resistance to 5-fluorouracil in colorectal cancer cell lines. Int J Cancer 106: 66-73, 2003.
26. Raschle M, Marra G, Nystrom-Lahti M, Schar P, Jiricny J: Identification of hMutLbeta, a heterodimer of hMLH1 and hPMS1. J Biol Chem 274: 32368-32375, 1999.
27. Meyers M, Hwang A, Wagner MW, *et al*: A role for DNA mismatch repair in sensing and responding to fluoropyrimidine damage. Oncogene 22: 7376-7388, 2003.
28. Carethers JM, Chauhan DP, Fink D, *et al*: Mismatch repair proficiency and *in vitro* response to 5-fluorouracil. Gastroenterology 117: 123-131, 1999.
29. Meyers M, Wagner MW, Hwang HS, Kinsella TJ, Boothman DA: Role of the hMLH1 DNA mismatch repair protein in fluoropyrimidine-mediated cell death and cell cycle responses. Cancer Res 61: 5193-5201, 2001.
30. Meyers M, Wagner MW, Mazurek A, Schmutte C, Fishel R, Boothman DA: DNA mismatch repair-dependent response to fluoropyrimidine-generated damage. J Biol Chem 280: 5516-5526, 2005.
31. Zhang H, Richards B, Wilson T, *et al*: Apoptosis induced by overexpression of hMSH2 or hMLH1. Cancer Res 59: 3021-3027, 1999.
32. Miyashita T and Reed JC: Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 80: 293-299, 1995.
33. Bunz F, Hwang PM, Torrance C, *et al*: Disruption of p53 in human cancer cells alters the responses to therapeutic agents. J Clin Invest 104: 263-269, 1999.
34. Longley DB, Boyer J, Allen WL, *et al*: The role of thymidylate synthase induction in modulating p53-regulated gene expression in response to 5-fluorouracil and antifolates. Cancer Res 62: 2644-2649, 2002.
35. Boyer J, McLean EG, Aroori S, *et al*: Characterization of p53 wild-type and null isogenic colorectal cancer cell lines resistant to 5-fluorouracil, oxaliplatin, and irinotecan. Clin Cancer Res 10: 2158-2167, 2004.
36. Liang JT, Huang KC, Cheng YM, *et al*: P53 overexpression predicts poor chemosensitivity to high-dose 5-fluorouracil plus leucovorin chemotherapy for stage IV colorectal cancers after palliative bowel resection. Int J Cancer 97: 451-457, 2002.
37. Elsaleh H, Powell B, McCaul K, *et al*: P53 alteration and microsatellite instability have predictive value for survival benefit from chemotherapy in stage III colorectal carcinoma. Clin Cancer Res 7: 1343-1349, 2001.
38. Ahnen DJ, Feigl P, Quan G, *et al*: Ki-ras mutation and p53 overexpression predict the clinical behavior of colorectal cancer: a Southwest Oncology Group study. Cancer Res 58: 1149-1158, 1998.
39. Umar A, Buermeyer AB, Simon JA, *et al*: Requirement for PCNA in DNA mismatch repair at a step preceding DNA resynthesis. Cell 87: 65-73, 1996.