CPT-11 (SN-38) Chemotherapy may be Selectively Applicable to Biliary Tract Cancer with Low hMLH1 Expression

KEN SATO, YOSHIHIKO KITAJIMA, NAOHIKO KOHYA, YASUO KOGA, KAZUMA OHTAKA and KOHJI MIYAZAKI

Department of Surgery, Saga University Faculty of Medicine, Nabeshima 5-1-1, Saga 849-8501, Japan

Abstract. Biliary tract cancer is of highly malignancy with a poor 5-year survival. However, established chemotherapeutic regimens have not yet been established. Previously, we have reported that hMLH1, a mismatch repair (MMR) gene was frequently (57%) found to be lacking in surgically resected biliary tract carcinomas and the patients lacking the expression of hMLH1 revealed a poorer prognosis than those patients who possessed it. The MMR gene has been considered to be associated with sensitivity to various chemotherapeutic agents that act on DNA. A loss of MMR expression has been reported to increase sensitivity to topoisomerase inhibitors such as etoposide (ETP) or camptothecins (CPT). In the present study, whether or not hMLH1 deficiency resulted in a higher sensitivity to irinotecan (CPT-11) active form (SN-38) was investigated using a short interfering (Si)RNA system. A quantitative reverse transcription-polymerase chain reaction (RT-PCR) was conducted to measure the levels of hMLH1 expression in seven cancer cell lines, and this was compared with the drug sensitivity (IC50) to SN-38. The hMLH1 expression was correlated with the IC50 for SN-38, although the relationship was not statistically significant (R=0.717, p=0.0715). SiRNA double strand RNA (dsRNA) was transiently transfected into KMG-C (gallbladder cancer) cells. hMLH1 mRNA expression was repressed by hMLH1 dsRNA in a dose-dependent manner in comparison to the control dsRNA. The cell growth of the hMLH1 dsRNA transfected group was decreased by approximately 50% by SN-38 exposure. Flow cytometry was also carried out to examine the effect of the SN-38 treatment on the cell cycle. Following hMLH1 dsRNA transfection, the subG1 fraction was increased in comparison with the control in a dose-dependent manner. In conclusion, a low expression of hMLH1 in biliary tract cancer may aid in predicting its responsiveness to CPT-11(SN38).

Biliary tract cancer is of highly malignancy, leading to a poor 5-year survival rate (1). Thus effective chemotherapy is important in order to prolong the survival of cancer patients, especially in non-resectable or non-curable resection cases. However, the effective chemotherapeutic regimens for biliary tract cancer have not been optimally established. Recently, single or combination usage of 5-FU, cisplatin and gemcitabine has been suggested for biliary tract cancer, however the therapeutic effect is limited, the response rate ranging from approximately 15–35% (2-5).

Recently, it has been reported that the mismatch repair (MMR) gene is associated with sensitivity to various chemotherapeutic agents that act on DNA (6-7). In fact, MMR deficient cells have shown resistance to monofunctional alkylating agents such as N-methyl-N-nitrosourea (MNU), dacarbazine (DTIC) and temozolomide (TMZ) or platinum-based drugs such as cisplatin (8-12). The MMR components have been shown to participate in the recognition of DNA adducts brought about by these drugs (9, 11-13). The recognition of the damaged bases by MMR initiates a signal transduction pathway that can activate cell-cycle checkpoints and trigger apoptosis (11, 14-16). Using biliary tract cancer cells, we previously demonstrated that the expression of a deficient DNA repair gene (MGMT) and a proficient mismatch repair gene (hMLH1) are related to sensitivity against the alkylating agents MNU and DTIC (8). A loss of MMR expression has also been reported to demonstrate sensitivity to topoisomerase inhibitors such as etoposide (ETP) or camptothecins (CPT ) (17-19).

The camptothecins (CPT) are broad-spectrum anticancer drugs that specifically target DNA topoisomerase I (Topo I) (20). The CPT interfere with the catalytic cycle of topoisomerases by stabilizing the covalent complex formed by the topoisomerase and cleaved DNA, referred to as the cleavage complex (21). Irinotecan (CPT-11) is a semisynthetic analog of camptothecin, originally isolated from the Chinese/Tibetan ornamental tree Canptotheca acuminata. CPT-11 has been studied for its effects against esophageal, gastric, lung and especially colon carcinomas.

Correspondence to: Kohji Miyazaki, Department of Surgery, Saga University Faculty of Medicine, Nabeshima 5-1-1, Saga 849-8501, Japan. e-mail: miyazak2@cc.saga-u.ac.jp

Key Words: hMLH1, SN38, biliary tract cancer, SiRNA.
(22). However, no study has reported whether or not CPT-11 is effective against biliary tract cancer.

The toxicity of CPT is primarily caused by the conversion of a single-strand break (SSB) to a double-strand break (DSB) during the S-phase where the replication fork collides with the cleavage complexes formed between topoisomerase I-DNA and CPT (23). Some of the MMR components participate in various DNA repair processes including DNA repair and recombination (24).

We recently reported that hMLH1 expression was frequently (57%) found to be lacking in surgically resected biliary tract cancer and the patients lacking the expression of hMLH1 revealed a poorer prognosis than those patients who possessed it (25). The hMLH1 expression might thus be a molecular marker in biliary tract cancer possessing high malignant potential.

The aim of the present study was to investigate whether hMLH1 deficiency resulted in a higher sensitivity to CPT-11 (SN-38). In the human body, CPT-11 is converted into its active form, SN-38, by carboxylesterases that are abundantly present in the liver (26). SN-38 is widely used for in vitro experiments for assessing the toxicity of CPT-11.

A quantitative reverse transcription-polymerase chain reaction (RT-PCR) was conducted to measure the levels of hMLH1 expression in seven cancer cell lines and this was compared with drug sensitivity (IC50) to SN-38. Furthermore, short interfering (Si) RNA of the hMLH1 gene was introduced into the gallbladder cancer cell line (KMG-C) and the effect of down-regulation of hMLH1 expression on sensitivity to SN-38 was observed. Finally, flow cytometry was carried out to examine the effect of SN-38 treatment on the cell cycle.

Materials and Methods

Cell lines and culture. Six biliary tract cancer cell lines (KMG-C, G-415, GB-d1, HBDC, HAG-1, TFK-1) and SW-48 (a colon cancer cell line) were used in this study. KMG-C, G-415 and GB-d1 were obtained as described previously (8). HBDC was established in our laboratory (Iiao Wan) (27) and HAG-1 was kindly provided by Dr. Nakano (Department of Internal medicine, Kyushu University, Kyushu, Japan). TFK-1 was obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Japan). The first five cell lines were cultured in Williams' medium E (W/E, ICM Biomedicals INC, Costa Mesa, CA, USA), while TFK-1 and SW-48 were cultured in RPMI-1640 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% inactivated fetal bovine serum (FBS ) (JRH Biosciences, Lenexa, Kansas, USA), 2 mM glutamine, 100 µg/ml kanamycin and 20 µg/ml tetracycline hydrochloride. All cell lines were incubated at 37°C in a humidified atmosphere of 5% CO2 in air.

Drug. SN-38 was supplied from Yakult, Co. Ltd. (Tokyo, Japan) and 10 mM of the stock solution was prepared in DMSO and diluted in medium at the approximate dose immediately before use.

MTT assay. Drug sensitivity to SN-38 was analyzed by the MTT (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay using a Cell Titer96 Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, WI, USA). Each of the cell lines were seeded in 96-well culture plates at a density ranging from 8~12x103 cells/well. After 24 h, the cells were exposed to SN-38 for 72 h, at a concentration ranging from 10 ~20 µM. The cells were washed in phosphate-buffered saline (PBS) and resuspended in fresh medium and then 20 µl of MTT was added to the cultures. After 4 h incubation, the reaction was stopped by the addition of a solubilization/stop solution. Absorbance at 530 nm was measured using a multiwell plate reader (CS9300UPC, Shimadzu Co. Kyoto, Japan). Every experiment was done in triplicate and data are presented as a mean±S.D value of the three individual experiments.

Quantitative RT-PCR. For a quantitative estimate of the hMLH1 mRNA expression in the six biliary tract cancer cell lines and the SW-48 cells, real-time PCR was performed using the primer pairs as follows: hMLH1 forward, 5’-CCACAAGTATCCAGTATT-3’, hMLH1 reverse, 5’-GAACCTTCATACATCATATT-3’, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5’-TGG TAT CTT GGA AGG ACT CAT GAC-3’; and GAPDH reverse, 5’-ATG CCA GTG AGC TTC CCG TTC AGC-3’. After reverse transcription, PCR was carried out for each cDNA. PCR amplification was carried out using a Light-Cycler™ system and the Light-Cycler-FastStart™ DNA master SYBR green I kit (both Roche, Mannheim, Germany). The amplifications were performed in a mixture of 1 µl of cDNA suspension, 20 pmol of primer, 4 mM of MgCl2 and 1 µl of Light-Cycler-FastStart™ DNA master SYBR green. After the cDNA was denatured at 95°C for 3 min, a PCR of 30 cycles composed of denaturation for 15 sec at 95°C, annealing for 5 sec at 60°C, and extension for 10 sec at 72°C was completed. The melting curves were then described according to the protocol with the following conditions: 0 sec denaturation period at 95°C, starting temperature of 65°C, ending temperature of 95°C, and a rate of temperature increase of 0.1°C/sec. The quantitative hMLH1 values for each cell line were estimated by dividing by the levels of GAPDH expression. The experiment for each sample was performed in triplicate and the mean was then calculated.

Transient transfection of hMLH1 SiRNA (dsRNA) and protein extraction. 5x105 cells of KMG-C were seeded onto 6-well plates and incubated overnight. Twenty-one base pairs (bp) of double-strand (ds) RNA for hMLH1, which were constructed by iGene, Co.Ltd (Tsukuba, Japan), was transiently transfected into KMG-C cells for 4 h using a OligofectAMINE (Life Technology, Inc, Japan) at a concentration ranging from 5-40 nM. Randomly constructed dsRNA with a 21 bp length was used as a control transfection. At 48 h or 72 h after transfection, the protein was extracted from each cell using a lysis buffer (1% Triton X-100, 50 mM Tris, 150 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)). The lysate was sonicated and the supernatant was collected by centrifugation at 15000 rpm for 30 min.

Western blot analysis. Forty µg of cell extracts from each cell line were separated by gel electrophoresis on a 10% Bis-Tris gel (Invitrogen CA, USA). These electrophoretically separated extracts were transferred onto a Hybond™ ECL™ nitrocellulose membrane (Amersham Biosciences, NJ, USA). The membrane was blocked with 1 x Tris-buffered saline (TBS) containing 0.1% Tween20 and 1%
bovine serum albumin (BSA), and blots were probed with monoclonal antibodies specific for hMLH1 (BD Pharmingen, San Jose, CA, USA). The signals were developed using an ECL™ western blotting detection reagents (Amersham Biosciences, NJ, USA).

For quantitative analysis, band intensities were evaluated densitometrically using the LAS 1000 plus system (Fujifilm, Tokyo, Japan).

Cell growth assay. Ten thousand KMG-C cells were seeded onto 6 well plates. Ds RNA for hMLH1 was transfected in triplicate at a final concentration of 20 or 40 nM. At 48 h after transfection, the cells were exposed to SN-38 at a concentration of 50 or 100 nM for 48 h. The cells were collected and the mean number for each well was calculated. The effects of hMLH1 dsRNA were estimated by dividing the cell number of hMLH1 dsRNA transfectants from that of control dsRNA transfectants.

Flow cytometry. The cell preparation was the same as described for the cell growth assay. Cells were harvested using 0.25% tripsin-0.02% EDTA and were fixed in 70% ethanol at 4°C. Later, the ethanol was removed, and the cells were stained with propidium iodide solution (50 ìg/ml in PBS with 100 U/ml RNaseA) for 30 min at room temperature in the dark. All samples were analyzed using FACScalibur (counting 10,000 cells per sample) and Cell Quest software Version 2.0 (both Becton Dickinson, NJ, USA).

Statistical analysis. The relationships with IC50 for SN-38 with the six biliary tract cancer cell lines and SW48 were assessed statistically by Pearson’s correlation using the Statview software program (SAS Institute Cary, NC, USA).

Results

Sensitivity to SN-38 in the six biliary tract cancer cell lines and SW48. Drug sensitivity to SN-38 was assessed by the MTT assay. The IC50 of SN-38 for each cell line is shown in Figure 1. SW-48 was particularly sensitive to SN-38, whereas TFK-1 was the most resistant among the seven cancer cell lines. The IC50 for TFK-1 was 22.5-fold higher than that of SW-48.

hMLH1 mRNA expression in the six biliary tract cancer cell lines and SW-48. The expression of hMLH1 mRNA in each cell line was quantitatively assessed by real-time PCR and compared with the IC50 for SN-38. SW-48 was used as a control because the hMLH1 expression in SW-48 was deficient by conventional RT-PCR (8). In fact, the hMLH1 mRNA level in SW-48 by real-time PCR was extremely low and below the threshold level of this experiment. The relationship between hMLH1 mRNA expression and the IC50 for SN-38 is shown in Figure 2. A positive correlation was found between these two factors, although the relationship was not statistically significant (R=0.717, p=0.0715).

Down-regulation of hMLH1 expression by hMLH1 SiRNA transfection. In order to down-regulate hMLH1 expression in the KMG-C cells, SiRNA (dsRNA) was transiently transfected into KMG-C cells. As shown in Figure 3A, 48 h after transfection, hMLH1 mRNA expression was repressed by hMLH1 dsRNA in a dose-dependent manner in comparison with the control dsRNA. At 72 h after transfection, hMLH1 mRNA expressions were at lower levels than those at 48 h. The band intensity (%) of each group, which is estimated by setting the control as 100% is shown in Figure 3B. In the case of 40 nM of hMLH1 dsRNA transfection, hMLH1 mRNA expressions were down-regulated to 34.3% and 24.4% at 48 h and 72 h, respectively.
Figure 2. The relationship hMLH1 expression and IC\textsubscript{50} for SN-38 in six biliary tract cancer cell lines and SW48. hMLH1 expression in each cell line was measured by real-time RT-PCR and the quantitative hMLH1 value in each cell line was estimated by dividing them with the GAPDH expression values. A positive correlation between hMLH1 expression and IC\textsubscript{50} for SN-38 was observed (R=0.717, p=0.0715).

Figure 3. A) Western blot analysis of hMLH1 expression after 5, 20 or 40 nM hMLH1 SiRNA(dsRNA) transfection (a: 48 h after transfection), (b: 72 h after transfection). NC: negative control, ds: double strand, transfected with hMLH1 SiRNA(dsRNA). B) The band intensity of each group after hMLH1 SiRNA transfection. Each bar graph demonstrates the relative intensity (%) compared with control (100%).
Increased sensitivity to SN-38 after hMLH1 dsRNA transfection. The results of the cell growth assay is shown in Figure 4. To examine the influence of transfection, hMLH1 dsRNA at 20 nM (A) or 40 nM (B) was transfected into KMG-C cells without SN-38. Forty eight hours later, KMG-C cells were exposed to SN-38 at 50 nM or 100 nM concentrations for an additional 48 h. Cell growth was simply inhibited by approximately 50% with SN-38 exposure. The cell growth of the hMLH1 dsRNA 20 nM transfected group was further inhibited by approximately 70% of that of the negative control (Figure 4A). The cell number of the hMLH1 dsRNA 40 nM transfected group was repressed to 55.6% and 52.6% of that of the negative control by SN-38 at 50nM and 100nM concentration, respectively.

Flow cytometry. The cell fractions of the KMG-C cells which were transfected by a negative control or by hMLH1 dsRNA with or without SN-38 treatment are shown in Figure 5. The subG1 fraction slightly increased in comparison to the KMG-C control in each transfection group. In the negative control dsRNA transfection (NC) group, the G2-M fraction of the KMG-C cells increased in comparison to the no treatment group with the SN-38 treatment in a dose-dependent manner. In the hMLH1 dsRNA transfection group, the subG1 fraction predominantly increased with treatment by SN-38 in a dose-dependent manner.

Discussion

MMR plays an important role in the cellular response to various DNA damaging agents. The cells that are defective in MMR are tolerant to alkylating agents, such as platinum in cisplatin (9-12, 28), and 5FU (29). In sharp contrast, the colorectal cancer cells defective in MMR have been reported to exhibit higher sensitivity to camptothecin (17). In the present study, the hMLH1 expression was correlated with the IC50 for SN-38 using six biliary tract cancer cell lines. Moreover, for the first time, evidence that the reduction of hMLH1 expression by SiRNA raised sensitivity to SN-38 has been shown. Furthermore, apoptosis was induced more strongly in the hMLH1 dsRNA transfected cells in comparison to the control transfected cells under SN-38 exposure.

Jacob et al. (17) recently reported that colorectal cancer cell lines defective in DNA MMR exhibited high sensitivity to topoisomerase inhibitors. Resistance to the drugs was achieved by the functional complement of hMLH1 transfection (17). In contrast, using the SiRNA system, we have verified the restoration of drug sensitivity to SN-38 in biliary tract cancer cells.

SN-38 interacts with the cellular Topo I-DNA complex (cleavage complex). The response of this drug appears to correspond to the level of the cleavage complexes (30). Stabilization of the cleavage complexes after CPT-11 (SN-38) treatment is accompanied by G2-M arrest and apoptosis.
Figure 5. Flow cytometry cell cycle fractions of KMG-C cells transfected by negative control (NC) or hMLH1 dsRNA (hMLH1) with or without SN-38 treatment. The top panel shows the fraction of KMG-C without transfection or SN-38.
Furthermore, the repression of hMLH1 expression by in the KMG-C cells transfected with a control SiRNA.

MMR participates in the induction of apoptosis in response to a variety of DNA lesions (14, 33). MMR also recognizes and repairs base-base mispairs, as well as small insertion or deletion loops arising during DNA replication (33-35). In addition to mutation avoidance, some of the MMR components participate in various DNA repair processes including DSB repair and DNA recombination (24).

In our study, the SN-38 treatment induced a G2-M arrest in the KMG-C cells transfected with a control SiRNA. Furthermore, the repression of hMLH1 expression by hMLH1-SiRNA increased the apoptotic rather than the G2-M fraction with SN-38 treatment. These data may indicate that topo I-DNA complexes are more stabilized in the down-regulation of hMLH1, which could possibly have led to a higher level of apoptosis with drug treatment. However, we cannot define the precise mechanism at present.

Several authors have reported the implication of the p53 gene and MMR system in the efficacy of CPT-11. Magrini et al. reported the effects of the expression of these two genes on CPT-11 on colon cancer cell lines. They showed that apoptosis was most induced by CPT-11 exposure in the p53+/−/hMLH1−/− group, compared with the p53+/+ hMLH1−/− and the p53+/+ hMLH1+/+ group (36). In the present study, apoptosis following SN-38 treatment was more strongly induced in the hMLH1 cells down-regulated by hMLH1-SiRNA, in comparison to the control SiRNA transfection. We discovered a deletion in the p53 gene of the KMG-C cells by DNA sequencing (data not shown). Therefore, our data partially support their data that a double loss of p53 and hMLH1 induces apoptosis more strongly than loss of hMLH1 alone. In addition, in more than half of biliary tract carcinomas, mutation in the p53 gene was detected in our previous study (37).

In conclusion, we demonstrated that hMLH1 expression is associated with sensitivity to SN-38 using real-time RT-PCR and a SiRNA system. The hMLH1 expression was frequently lost in surgically resected biliary tract cancer specimens and the patients with negative hMLH1 expression had a poor prognosis (25).

Thus, evaluation of hMLH1 expression in biliary tract cancer may aid in predicting responsiveness to CPT-11 (SN38). The clinical use of CPT-11 based on hMLH1 expression may lead to an improved prognosis for patients with biliary tract cancer.

References


