The Effect of O\textsuperscript{6}-methylguanine-DNA Methyltransferase (MGMT) and Mismatch Repair Gene (hMLH1) Status on the Sensitivity to Alkylating Agent 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea (ACNU) in Gallbladder Carcinoma Cells

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Abstract. The aim of this study was to correlate O\textsuperscript{6}-methylguanine-DNA methyltransferase (MGMT) and mismatch repair gene (hMLH1) expression in gallbladder carcinoma cells with drug sensitivity to the bifunctional alkylating agent 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea (ACNU). Using 5 gallbladder carcinoma cell lines and 1 colon carcinoma cell line (SW48), the MGMT and hMLH1 status was assessed both by RT-PCR and Western blot analyses. Sensitivity to ACNU was estimated by the MTT assay. MGMT\textsuperscript{+}/hMLH1\textsuperscript{+} status was revealed in 2 gallbladder carcinoma cells, MGMT\textsuperscript{+}/hMLH1\textsuperscript{+} in another 3 gallbladder carcinomas and MGMT\textsuperscript{–}/hMLH1\textsuperscript{+} in SW48. MGMT\textsuperscript{–}/hMLH1\textsuperscript{+} and MGMT\textsuperscript{–}/hMLH1\textsuperscript{–} cells were more sensitive to ACNU compared with MGMT\textsuperscript{+}/hMLH1\textsuperscript{+} cells. These results indicate that MGMT, but not hMLH1, expression is an important determinant for drug sensitivity to ACNU in gallbladder carcinoma cells. The drug effect of ACNU, which depends on the MGMT status, was verified using xenograft tumors grown in nude mice. Furthermore, the apoptotic index of MGMT\textsuperscript{+}/GB-d1 xenografts was significantly increased by ACNU treatment, compared with that of MGMT\textsuperscript{+}/KMG-C. In conclusion, the sensitivity to ACNU was not associated with hMLH1 status, but was found to depend only on the MGMT status. ACNU might be a useful chemotherapeutic agent for MGMT\textsuperscript{–} gallbladder carcinoma, which is associated with poor patient outcome.

Gallbladder carcinoma is an uncommon but highly malignant tumor with a poor 5-year survival rate (1). Although radical resection is the only potentially curative therapy for patients with gallbladder carcinoma, complete resection is occasionally impossible because of the malignant behavior, including cancer metastasis and invasion. The discovery of molecular markers predicting tumor malignancy or sensitivity to chemotherapy is thought to improve patient outcome with gallbladder carcinoma. Previously, we reported frequent loss of O\textsuperscript{6}-methylguanine methyltransferase (MGMT) expression in surgically-resected gallbladder carcinoma specimens, and that the prognosis of patients with MGMT\textsuperscript{–} tumors was poorer than of those with MGMT\textsuperscript{+} tumors (2). Furthermore, the combined loss of MGMT and mismatch repair genes, such as hMLH1 and hMSH2, was a more significant prognostic marker than single loss of MGMT.

Alkylating agents are known to exert cytotoxic effects on cells, providing the basic rationale for the use of these agents in cancer chemotherapy (3). Alkylating agents are divided into several groups, according to their preferred sites of action and abilities to form interstrand crosslinks in DNA. Dacarbazine (DTIC) or Temozolomide (TMZ) are classified as monofunctional alkylating agents, whereas 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea (ACNU) and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) are the bifunctional chloroethylnitrosoureas, while cyclophosphamide is in the nitrogen mustard class of compounds (4-6).

MGMT is a well known enzyme that repairs O\textsuperscript{6}-methylguanine, introduced by monofunctional alkylating agents (7-11). MGMT also removes O\textsuperscript{6}-chloroethylguanine residues induced by bifunctional chloroethylnitrosoureas, thereby averting the formation of lethal cross-links (12, 13). Thus, tumor cells with high MGMT levels exert higher resistance to alkylating agents, compared with MGMT-deficient tumors (14, 15).

The mismatch repair (MMR) gene hMLH1 is also involved in apoptosis, step-induced by monofunctional alkylating...
agents, including MNU and TMZ (16, 17). However, sensitivity to BCNU, a bifunctional chloroethylnitrosourea that forms O\textsubscript{6}-chloroethylyguanine-DNA adducts, seems not to be affected by the MMR status of the cells (18, 19).

These findings suggest that assessment of MGMT as well as of MMR expression helps to determine the drug sensitivity to alkylating agents. However, there has been no report, to our knowledge, that correlates both MGMT and hMLH1 expression with sensitivity to alkylating agents in gallbladder carcinoma. In the present study, we analyzed MGMT and hMLH1 expression in 5 gallbladder carcinoma cell lines and assessed whether or not drug sensitivity to ACNU was affected by the expression status of these two genes. Using gallbladder carcinoma xenografts with different MGMT and hMLH1 status, we also investigated in vivo growth and apoptosis affected by ACNU treatment.

Materials and Methods

Cell lines and culture. Five gallbladder carcinoma cell lines (KMG-C, G-415, GBK-1, GB-d1 and TGBC-2TKB) and 1 colon cancer cell line (SW48) were used. KMG-C was provided by Dr. H. Yano (Department of Pathology, Kurume University, Kurume, Japan), GBK-1 was provided by Dr. H. Egami (Department of Surgery, Kumamoto University, Kumamoto, Japan), G-415 was provided by Dr. S. Koyama (Department of Internal Medicine, Tsukuba University, Tsukuba, Japan). GB-d1 was provided by Dr. Date (Department of Surgery, Kyusu University, Fukuoka, Japan). TGBC-2TKB and SW48 were obtained from the RIKEN Cell Bank (Ibaraki, Japan). The 5 gallbladder carcinoma cell lines were cultured in Williams' medium E (W/E, ICN Biomedicals Inc., Costa Mesa, California, USA), and SW48 was cultured in RPMI1640 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% inactivated FBS (JRH Biosciences, Lenexa, Kansas, USA), 2 mM glutamine, 100 Ìg/ml kanamycin and 20 Ìg/ml tetracycline hydrochloride; all 6 lines were incubated at 37°C in a humidified atmosphere of 5% CO\textsubscript{2} in air.

Drugs. ACNU was kindly supplied by Sankyo Co. (Tokyo, Japan), and was dissolved in distilled water and diluted in W/E for all 5 gallbladder carcinoma cell lines, or in RPMI medium for SW48.

Reverse transcriptase (RT) –PCR. Total RNA was isolated from each cell line using ISOGEN (Nippongene, Toyama, Japan). The MGMT and hMLH1 mRNA expressions were analyzed by RT-PCR using the RNA LA PCR™Kit (AMV) Ver.1.1 (Takara Biomedicals, Tokyo, Japan). For each cell line, a 1-µg sample of total RNA was used for first-strand cDNA synthesis. The resulting cDNA was subsequently amplified by PCR, with 35 cycles of 94°C for 1 min, 60°C for 30 sec and 72°C for 90 sec. The primers for MGMT, hMLH1 and GAPDH were as follows: MGMT forward, 5'-CAC GAA ATA AAG CTC CTG GG-3'; MGMT reverse, 5'-CTG CCA GGG CTG CTA ATT GC-3'; hMLH1 forward, 5'-GGC CAG CTA ATG CTA TCA AA-3'; hMLH1 reverse, 5'-ATG CCT GCA TTG TGT ACT GA-3'; GAPDH forward, 5'-TGG TAT CCT GGA AGG ACT CAT GAC-3'; GAPDH reverse, 5'-ATG CCA GTG AGC TTC CCG TTC AGC-3'. The proposed sizes of the PCR products were 282 bp for MGMT and 492 bp for hMLH1.

Western blot analysis. Whole cell lysate from the cultured cells was prepared using lysis buffer (1.0% Triton X-100, 20 mM HEPES-NaOH, pH 7.2). Briefly, cells were incubated on culture plates in 1.2 ml of lysis buffer with gentle shaking for 30 min at 37°C. The supernatant was subsequently collected by centrifugation at 15,000 rpm for 30 min. Fifty Ìg of the cell lysates were separated by gel electrophoresis: 16% Tris-Glycine gel (Invitrogen, CA, USA) for MGMT, 10% Tris-Glycine gel for hMLH1. These electrophoretically separated extracts were transferred onto Hybond™ ECL™ nitrocellulose membrane (Amersham Biosciences, Freiburg, Germany). The membrane was blocked with 1xTBS containing 0.1% Tween20 and 1% BSA, and the blots were probed with polyclonal antibody specific for MGMT (NOVUS Biologicals, Littleton, CO, USA) and monoclonal antibody specific for hMLH1 (BD Pharmingen, San Jose, CA, USA). The signals were developed using an enhanced chemiluminescent (ECL) detection kit (Amersham Pharmacia Biotech).

MTT assay. Drug sensitivity to the clinically-used alkylating agent ACNU was analyzed by the MTT assay using the Cell Titer96 Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison,WI, USA). Cells were seeded on 96-well culture plates at a density ranging from 8 – 12x10\textsuperscript{3} cells/well in triplicate. After 24 h, the cells were exposed to 100 Ìg ACNU for 2 h. The culture medium with ACNU was then removed and replaced with fresh medium. After 1 – 4 day, 20 µl of (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)(MTT) was mixed for 4 h, and the reaction was stopped by adding solubilization/stop solution. Absorbance at 570 nm was measured using a multiwell plate reader (CS9300PC, Shimadzu Co., Kyoto, Japan). The data are presented as means±S.D of triplicate measurements. The experiments were independently performed three times.

ACNU treatment against xenograft tumor in nude mice. Female athymic BALB/cA Jcl mice (nu/nu, 5 weeks old) were obtained from Nihon Crea Co. (Osaka, Japan). They were kept under specific-pathogen-free conditions, and were given Á-irradiated food and autoclaved water. Approximately 1x10\textsuperscript{6} cells of GB-d1, KMG-C or SW48 were injected subcutaneously into both flanks of the mice. Eight mice (16 tumors) were evaluated per cell line. When the tumor xenografts became palpable, the mice were weighed, and sizes of tumors were measured in 2 perpendicular dimensions with a caliper twice a week. Tumor volume (V) was determined by the formula (19) V=0.5 x a x b\textsuperscript{2}, where a is the longer of the 2 dimensions of the tumor (mm), and b is the shorter of the 2 dimensions. When the xenograft volume reached 150 to 200 mm\textsuperscript{3}, the tumors were measured in triplicate measurements. The experiments were independently performed three times.

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Acute toxicity of ACNU was estimated from weight loss of the mice.

TUNEL assay. Xenografts derived from nude mice were fixed in 10% formalin overnight and embedded in paraffin. The TUNEL assay was performed using ApopTagR peroxidase kits (Intergen Company, NY, USA). Paraffin-embedded tissue sections (thickness, 4 μm) were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. The sections were washed in PBS (50 mM sodium phosphate, pH 7.4, 200 mM NaCl) and incubated in 0.3% hydrogen peroxide for 5 min to block endogenous peroxidase activity. The sections were rinsed twice with PBS for 5 min and then treated with proteinase K (20 μg/ml) for 15 min. After equilibration buffer had been applied, the sections were immersed in working-strength TdT enzyme and incubated at 37°C for 1 h. The reaction was terminated by adding stop/wash buffer for 10 min at room temperature. The sections were washed 3 times in PBS for 1 min, and incubated for 30 min at room temperature with anti-digoxigenin peroxidase conjugate. After washing in PBS for 2 min, the color reaction was developed in peroxidase substrate for 5 min. The sections were finally counterstained by 0.5% methyl green. The percentage of TUNEL-positive cancer cells was calculated by scoring approximately 800 cells in each specimen (5 visual fields x 200 fields). The apoptotic index was estimated as the ratio of apoptotic cell number to total cell number.

Statistical analysis. The data were analyzed for significance using the Student’s t-test.

Results

MGMT and hMLH1 status in gallbladder carcinoma cell lines. In RT-PCR analysis, MGMT mRNA was expressed in KMG-C and G-415, but not in GBK-1, GB-d1, TGBC-2TKB or SW48. On the other hand, expression of hMLH1 mRNA was detected in all 5 gallbladder carcinoma cell lines, but not in the colorectal cancer cell line SW48 (Figure 1). Western blot analysis revealed that the expression of MGMT and hMLH1 protein in the 6 cells was consistent with the findings in RT-PCR (Figure 1).

Sensitivity of cell lines to ACNU in vitro. The sensitivities of the 6 cell lines to ACNU are depicted in Figure 2A, and inhibition rates at day 4 by ACNU are shown in Figure 2B. Cell proliferations of GBK-1, GB-d1 and TGBC-2TKB with MGMT+/hMLH1+ and SW48 with MGMT+/hMLH1+ were gradually inhibited by ACNU treatment from day 2 to day 4. In contrast, those of KMG-C and G-415 with MGMT+/hMLH1+ were not particularly suppressed. The inhibition rates (IR) of 3 MGMT–/hMLH1+ cell lines and MGMT+/hMLH1+ were not particularly suppressed. The inhibition rates (IR) of 3 MGMT+/hMLH1+ cell lines and MGMT+/hMLH1+ were not particularly suppressed. The inhibition rates (IR) of 3 MGMT+/hMLH1+ cell lines and MGMT+/hMLH1+ were not particularly suppressed.

ACNU sensitivity of tumor xenografts in vivo. The growth curves of GB-d1, KMG-C and SW48 tumors in nude mice after treatment with ACNU (50 mg/kg) are provided in Figure 3. The KMG-C xenografts were not affected by ACNU treatment. In sharp contrast, the growth of the GB-d1 and SW48 xenografts was significantly suppressed by ACNU, and the IR of GB-d1 and SW48 tumors on day 21 after the first administration ACNU were 88.3% and 72.9%, respectively. These results indicate that MGMT+/hMLH1+...
Figure 2. Cell proliferation of 6 cell lines under ACNU treatment was analyzed with the MTT assay (A) and inhibition rates on day 4 were calculated (B). Six cell lines were treated with 100 μM ACNU for 24 h. After 1 – 4 days, absorbance at 570 nm in live cells was measured. The data are shown as the mean±S.D of triplicate measurements.
and MGMT⁻/hMLH¹⁻ tumors are much more sensitive to ACNU than MGMT⁺/hMLH¹⁺ tumors.

In assessment of acute toxicity to ACNU, there was no significant difference in body weight between the cell lines (data not shown). No mice died of drug toxicity within the 21-day study period.

Apoptotic index of xenografts derived from nude mice under ACNU treatment. In KMG-C xenografts, the number of apoptotic cells was not affected by ACNU treatment, compared with the controls (Figures 4, 5). In GB-d1, the number of apoptotic cells significantly increased upon ACNU treatment, compared with the controls. In the 2 xenografts, the apoptotic index was 1.053 and 5.336, respectively, thus, demonstrating that ACNU significantly induced apoptosis in GB-d1 xenografts, but not in KMG-C xenografts.

Discussion

Gallbladder carcinoma is the most common among biliary tract carcinomas. Despite improved diagnostic capabilities, overall survival still remains poor because of the aggressive tumor biology (21). Thus, effective chemotherapy for this malignancy is necessary to improve the patient survival. Phase II trials using single-agent chemotherapy, such as MMC, 5-FU and CDDP or 5-FU-based combination chemotherapy have been performed, however they have not yet improved response rates or prolonged survival (22-26). If chemotherapeutic drugs are determined according to some sensitive marker, patient survival with gallbladder carcinoma might be improved.

Recently, a notable study using knockout mice has demonstrated that deficient MGMT and proficient hMLH1 expression is implicated in determining apoptosis in bone marrow under MNU treatment (27). This report prompted us to consider selective chemotherapy using an alkylating agent, when drug sensitivity is predicted by hMLH1 as well as by MGMT expression in gallbladder carcinoma.

In the present study, we used ACNU as a chemotherapeutic drug. ACNU is a water-soluble derivative of chloroethylnitrosourea, commonly used to treat cancer patients in Japan, especially those with brain tumor (28, 29).
ACNU primarily induces O\textsuperscript{6}-chloroethylguanine DNA adducts, and these adducts can be repaired by MGMT, similarly to the repair of O\textsuperscript{6}-methylguanine (30). Using gallbladder carcinoma cells, we demonstrated that 3 MGMT\textsuperscript{–}/hMLH1\textsuperscript{+} cells and MGMT\textsuperscript{–}/hMLH1\textsuperscript{–} SW48 cells were more sensitive to ACNU, compared with 2 MGMT\textsuperscript{+}/hMLH1\textsuperscript{+} cells. The tumor growths of MGMT\textsuperscript{–}/hMLH1\textsuperscript{–} GB-d1 and MGMT\textsuperscript{–}/hMLH1\textsuperscript{–} SW48 were markedly inhibited by ACNU administration in nude mice, however that of MGMT\textsuperscript{+}/hMLH1\textsuperscript{–} KMG-C was hardly affected. These results indicate that drug sensitivity to ACNU was not affected by hMLH1, but rather by MGMT status in the gallbladder carcinoma cell lines. These findings are in contrast with previous reports that both MGMT and hMLH1 were crucial for determining sensitivity to monofunctional alkylating agents, such as MNU or TMZ (16). O\textsuperscript{6}-methylguanine, induced by these monofunctional alkylating agents, was recognized by MMR including hMLH1. The recognition of O\textsuperscript{6}-methylguanine by MMR initiates a signal transduction, resulting in activation of cell-cycle checkpoint and apoptosis (31-33). In contrast, ACNU is a bifunctional alkylating agent, which induces O\textsuperscript{6}-chloroethylguanine in cellular DNA. If the O\textsuperscript{6}-chloroethylguanine is not repaired, the DNA adduct eventually forms interstrand DNA cross-links (30, 34) and disrupts DNA synthesis, leading to cell death (35, 36). It was previously reported that the cross-links induced by bifunctional alkylating agents were not recognized by MMR, including hMLH1 (18, 19). Thus, sensitivity to ACNU possibly depends on the MGMT status and is not associated with the hMLH1 status.

The data obtained from the TUNEL assay, using xenografts in nude mice, also support this notion that the apoptotic index of GB-d1 xenografts with MGMT\textsuperscript{–} was higher compared with that of KMG-C xenografts with MGMT\textsuperscript{+} by ACNU treatment.

Previously, we found loss of MGMT expression in 23 out of 39 (59%) surgically resected gallbladder carcinoma specimens, and confirmed that the prognosis of patients with MGMT\textsuperscript{–} tumors was poorer than of those with MGMT\textsuperscript{+} tumors (2). These results indicated that MGMT\textsuperscript{–} gallbladder...
carcinoma with poor prognosis is a more suitable candidate for ACNU treatment, compared with MGMT+ carcinoma. The present study demonstrated that MGMT- gallbladder carcinoma were clearly sensitive to the bifunctional alkylating agent ACNU, \textit{in vitro} and \textit{in vivo}. ACNU may be useful as a chemotherapeutic agent against MGMT- gallbladder carcinoma.

References


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