이학석사 학위논문

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2013년 8월

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이 논문을 이학석사 학위논문으로 제출함

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Correlation between Promoter Methylation Status of *ABCG2* and Drug Sensitivity in Colorectal Cancer Cell Lines

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Abstract

Colorectal cancer (CRC) is the second most commonly diagnosed cancer in worldwide. Although colon cancer is possible to cure by surgery, chemotherapy, and radiotherapy, however, the incidence rate of recurrence and metastasis are still high. One of the reasons is that resistance of chemotherapy.

ATP-binding cassette protein G2 (ABCG2) as a drug efflux pump contains a 655-amino-acid polypeptide transporter with six transmembrane domains forms a homodimer on plasma membranes. Therapeutic drugs for colorectal cancer patients such as 5-FU, irinotecan and oxaliplatin have been identified as substrates of *ABCG2*. DNA methylation of the *ABCG2* promoter site, which consists of many CpG islands, could play a role in the epigenetic regulation of gene expression.

In this study, I investigated whether the methylation status of *ABCG2* promoter was related to drug sensitivity. I studied expression patterns of *ABCG2* and methylation status of the promoter. First, I analyzed the mRNA expression levels of *ABCG2* by conducting RT-PCR and qRT-PCR. MS-PCR and bisulfite sequencing analysis were performed to identify methylation status of the *ABCG2* promoter site. According to the results, the *ABCG2* mRNA level was shown low in 13 cell lines (SNU-283, SNU-769B, SNU-1040, SNU-1047, SNU-C4, Colo201, Colo320, HCT 15, Ls174T, NCI-H716, SW403, SW480 and SW1116). Promoter methylated CRC cell lines, SNU-C4, Ls174T and NCI-H716 were selected and the cell proliferation assay for anticancer drugs was performed after 5-aza-2'-deoxycytidine (5-aza) treatment. As results, the *ABCG2* mRNA expression level was increased when 5-

aza was treated with the cell lines, in addition, demethylation was induced by 5-aza which enhanced the *ABCG2* expression in the cell lines, and the cell viability recovered in the cell lines with re-expressed *ABCG2* and this recovery was inversely correlated to drug sensitivity. Taken together, I found that *ABCG2* gene expression, regulated by promoter methylation, changes drug sensitivity in some CRC cell lines.

Thus, this study suggests that DNA methylation of *ABCG2* promoter sites can be a chemotherapeutic resistance marker for colorectal cancer patients who have resistance to anticancer drugs.

Key words: Colorectal cancer (CRC), chemotherapy, drug sensitivity, ABCG2, DNA methylation, 5-aza-2'-deoxycytidine

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Introduction

Colorectal cancer (CRC) is the second most commonly diagnosed cancer in women and the third in men worldwide, with over 1.2 million new cancer cases and 608,700 deaths estimated to have occurred in 2008 [21]. Although it is possible to cure colon cancer by surgery, the cure rate is moderate to poor depending on the stage of cancer determined by how many lymph nodes are involved and how deep the penetration is through the bowel wall: stage III, node-positive disease and stage II, node-negative disease [3, 28, 41]. The patients with stage II and stage III colorectal cancers remain at high risk for tumor recurrence after curative resection. Therefore, they may benefit from additional treatment including adjuvant therapy [1]. Chemotherapy and radiotherapy have been mainly used as initial treatment to shrink any cancer and then usually surgery done to remove any tumors [9, 16]. During the chemotherapy, a significant obstacle for the successful treatment of colorectal cancer patients is intrinsic or acquired drug resistance in patients who respond to chemotherapy [17]. Many mechanisms of resistance such as amplification or mutation of drug target genes, hypoxia, heterogeneity of cell subpopulations, and drug defect transport or overexpression of p170 (protein of multidrug resistance), have been identified and studied using principally tumor cell lines [10, 42]. A major mechanism of drug resistance *in vitro* is the over-expression of an energy-dependent drug efflux pumps known as the ABC (ATP binding cassette) superfamily including P-glycoprotein (MDR1), the multidrug resistance protein (MRP), and ABCG2 [18]. They transport various compounds such as lipids,

bile acids, xenobiotics and peptides for antigen presentation. Over-expression of *MDR1* and *MRP* could be caused by gene amplification or transcriptional activation, or posttranscriptional events. In some human lung cancer cells with a low level of drug resistance, *MRP* mRNA was increased up to 3-fold because of transcriptional activation not the absence of gene amplification [6, 13].

ABCG2, otherwise known as breast cancer resistance protein (BCRP) and mitoxantrone resistance-associated gene (MXR), was identified in high mitoxantrone resistant MCF-7/AdrVp and human colon cancer cell line, S1-M1-80. According to some studies, ABCG2 contains a 655-amino-acid polypeptide transporter with six transmembrane domains and forms a homodimer. Additionally, the ABCG2 protein was reported to be a 72 kDa protein. As a half transporter, two nucleotide binding protein is required to perform as a drug efflux pump [26, 44]. *ABCG2* is generally expressed in the human body and controls the absorption, distribution, and elimination of various toxins to protect normal cells from malignant changes. In cancer cells, the roles of *ABCG2* are that it limits the attainment of therapeutic intracellular concentrations of drugs, shields cancer cells from hypoxia and extrudes anticancer drugs from cancer stem cells [6, 31, 33, 34, 37]. *ABCG2* expression is regulated by a TATA-less promoter which contains several SP1, AP1 and AP2 sites and putative CpG islands. It has been noted that the potential CpG islands in the promoter site may be regulated by methylation [6]. Furthermore, the 5' region upstream of the basal promoter was revealed as both a positive and negative regulatory domain [6, 39]. *ABCG2* expression has been shown to be up-regulated in some renal clear cell carcinomas and lung cancer, breast cancer and multiple myeloma cell lines after treating them with 5-aza-2'-

deoxycytidine (5-aza), a DNA demethylating agent [6, 27, 29, 37, 38]. Therefore, this observation suggested that the DNA methylation of the *ABCG2* promoter site, which consists of many CpG islands, could play a role in the epigenetic regulation of gene expression [39].

Epigenetic processes are crucial for development and differentiation, but it undoubtedly occurs in mature humans and mice due to random changes or environmental influences. There are two molecular mechanisms that mediate epigenetic phenomena: DNA methylation and histone modification [20]. In mammals, DNA methylation might be responsible for the inhibition of gene expression, X chromosome inactivation, genomic imprinting, chromatin modification, and silencing of endogenous retroviruses [7, 20, 25, 32]. DNA methylation suppresses gene expression through two steps: first, modified cytosine bases inhibit the association of several DNA-binding factors as well as transcription factors because the methylation of CpG dinucleotides is located within the DNA recognition sites of these factors [43]; and second, Methyl-CpG-binding proteins (MBPs) that interact with methyl-CpG can elicit repressive transcription from methylated DNA [19]. Operating with transcriptional co-repressors, MBPs lead to the silencing of transcription and the modification of surrounding chromatins and these factors suggest that there is a link between DNA methylation and chromatin modification [23, 36].

ABCG2 causes certain chemotherapeutic drug resistance such as mitoxantrone, doxorubicin, and daunorubicin in breast cancers by releasing its substrates which include topoisomerase I and II inhibitors [12]. Furthermore, over-expression of *ABCG2* was found in drug-selected cell lines from breast, colon, gastric, lung and

ovary cancers [2]. In a study using NSCLS (non-small cell lung cancer), the chemotherapeutic response rate in patients was found to be correlated with *ABCG2* expression [45]. Fluorouracil and leucovorin have been established as the standard adjuvant chemotherapy following resection among patients with stage III colon cancer. Irinotecan, oxaliplatin and bevacizumab are currently used as treatment with the long-time standard chemotherapy [4, 5, 40]. Although 5-fluorouracil (5-FU) has been developed over 40 years ago for the treatment of solid tumors in colorectal cancer, a clinical response was obtained in < 40% of patients with advanced disease, and these phenomena were followed unavoidably by the development of resistance [14]. There was a study using HCT116 p53 wild-type and null isogenic colorectal cancer cell lines resistant to antimetabolite 5-FU, topoisomerase I inhibitor irinotecan (CPT-11) and DNA damaging agent oxaliplatin in order to identify the characteristics of these resistant cell lines. In the oxaliplatin-resistant cell line, the mRNA level was increased for a couple of genes such as nucleotide excision repair gene *ERCC1* and ATP-binding cassette transporter breast cancer resistance protein *ABCG2*. In the CPT-11-resistant cell line, only *ABCG2* was over-expressed [8]. In addition, 5-FU resistance was increased in the *ABCG2* transfected MDCKII cells [22]. Thus, drug resistance might be induced by the regulation of *ABCG2* expression in 5-FU, irinotecan and oxaliplatin resistant cell lines. To investigate whether the *ABCG2* expression level and methylation status of the promoter affect drug sensitivity in colorectal cancer, I investigated the expression pattern of *ABCG2* and the methylation status of the *ABCG2* promoter.

To show that *ABCG2* expression is regulated by promoter methylation in colorectal cancer cell lines, I analyzed the mRNA expression of *ABCG2* and methylation status of *ABCG2* promoter by conducting reverse transcriptase-PCR (RT-PCR), quantitative real-time PCR (qRT-PCR), methylation specific-PCR (MS-PCR) and bisulfite sequencing analysis in 32 colorectal cancer cell lines. Afterwards, 5-aza was used to treat cell lines methylated at *ABCG2* promoter site that had been pretreated with the anticancer drugs 5-FU, irinotecan and oxaliplatin, and the cell lines were examined for variations in drug sensitivity using the cell proliferation assay, WST-1 assay. Because drug sensitivity increased in some demethylated colorectal cancer cell lines, the results of this study suggest that DNA methylation of *ABCG2* can be a drug resistance marker for colorectal cancer patients who have resistance to chemotherapeutic drugs.

Materials and Methods

Cell culture

The 32 colorectal cancer cell lines were provided by the Korean Cell Line Bank (KCLB., Seoul, Korea). All cell lines were cultured in RPMI1640 medium except for Caco2 and WiDr. Caco2 was maintained in Minimum Essential Medium and Dulbecco's Modified Eagle's Medium was used for WiDr. Each medium was supplemented with 10 % fetal bovine serum and 1.1 % penicillin / streptomycin. Cells were incubated in humidified incubators at 37 ˚C with 5 % CO_2 and 95 % air.

Genomic DNA extraction

Genomic DNA was extracted from the 32 colorectal cancer cell lines using the G-DEX^{TM} IIc genomic DNA extraction kit (Intron biotechnology., Gyeonggi, Korea) following the manufacturer's instructions. Cells treated with trypsin were collected then suspended in cell lysis buffer. RNase A solution was added to the cell lysates and they were incubated at 37 ˚C. The protein precipitation step was carried out by adding PPT buffer, vortexing and then centrifuging the samples. The supernatant, which included the DNA, was collected and inverted with 2-propanol and then, the mixture was centrifuged at 13,000 rpm. The DNA pellet was dissolved in DNA Rehydration buffer after washing with 70 % ethanol.

RNA isolation and cDNA synthesis

Cells were collected with trypsinization and suspended in easy-BLUE™ (Intron biotechnology). Total RNA was isolated according to the manufacturer's instructions. For cDNA synthesis, QuantiTect Reverse Transcription kit (Qiagen., Venlo, Netherlands) was used. The mixture was composed 1 μg of total RNA, 2 μl gDNA wipe buffer and DEPC water to make a mixture volume up to 14 μl. After incubation at 42 °C for 2 min, 4 μl of RT buffer, 1 μl of RT primer mix and 1 μl of RTase were mixed together and incubated at 42 °C for 45 min. The final reaction mixture was held at 95 °C for 2 min.

Bisulfite modification of genomic DNA

For bisulfite modification, 2 μg of genomic DNA from the 32 colorectal cancer cell lines were required. Bisulfite modification was processed by using EZ DNA Methylation™ Kit (Zymo Research, Orange, CA, USA) following manufacturer's instructions.

Reverse transcriptase-PCR (RT-PCR)

To analyze the *ABCG2* mRNA expression level, 1 μl of synthesized cDNA was amplified in a 14 μl PCR mixture that contains 10 X PCR buffer (with $MgCl_2$), dNTPs, forward and reverse primers (10 pmol / ul) (Table 2), distilled water and i-Taq DNA polymerase (Intron biotechnology). The RT-PCR conditions were 5 min at 94 °C for an initial denaturation, followed by 35 cycles of 94 °C for 30 sec, 65 °C for 1 min, and 72 °C for 30 sec and a final elongation of 7 min at 72 °C. The reaction was done using a

programmable thermal cycler (PCR System 9700, Applied Biosystems., Foster City, CA, USA). The PCR products were fractionated on a 1.5 % agarose gel containing ethidium bromide.

Quantitative real-time PCR (qRT-PCR)

Quantitative real-time PCR was performed in 386 well PCR plate containing the SYBR green Master Mix (Applied Biosystems), distilled water, 10 ng cDNA templates, 900 nM of *ABCG2* forward and reverse primer (Table 2) [24]. qRT-PCR analysis was performed with the 7900HT Fast Real-Time PCR System (Life Technologies Co., Carlsbad, CA, USA). The results were normalized to the housekeeping gene, *β-actin*, and the cycle threshold (Ct) values were extracted.

Methylation specific-PCR (MS-PCR)

The PCR reactions were performed at 94 °C for 5 min, and then 45 cycles of 94 °C for 30 sec, 53 °C for 1 min for the methylated region and 54 °C for 1 min for the unmethylated region, and 72 °C for 30 sec, and finally 72 °C for 7 min for both PCR reactions. To analyze the methylation of the *ABCG2* promoter region, 1 μl bisulfite modified DNA was amplified in a PCR mixture that contained 10 X PCR buffer, dNTPs, forward and reverse primers for methylated or unmethylated DNA (10 pmol / μl) (Table 2), 5 X Q solution, distilled water and *Taq* DNA polymerase (Qiagen).

Bisulfite sequencing analysis

The specific primers for the bisulfite sequencing analysis were designed using the MethPrimer software (http://www.urogene.org/methprimer/index1.html) (Table 2). The PCR reaction was done at 94 °C for 5 min, with 40 amplification cycles of 94 °C for 30 sec, 52 °C for 1 min and 72 °C 30 sec with a final extension step at 72 °C for 7 min. The amplicons from the bisulfite sequencing primers were inserted into the pGEM-T Easy vector (Promega., Madison, WI, USA) for TA-cloning. Sequences from five individual colonies for each colorectal cancer cell line were seqeunced using universal pUC/M13 primers and each sequence was analyzed using a Taq dideoxy terminator cycle sequencing kit on an ABI 3730 DNA sequencer (Applied Biosystems).

Protein extraction and Western blot analysis

The collected cells were lysed in PRO-PREP Protein extraction solution (Intron biotechnology) and placed on ice for 20 min. The supernatant was collected after the lysates were centrifuged at 13,000 rpm for 20 min at 4 ℃. The concentrated proteins determined by SMART micro BCA protein assay kit (Intron biotechnology) were mixed with 4 X SDS sample buffer and boiled at 95 ℃ for 5 min. The proteins were loaded on a 4-12 % Bis-Tris gel (Invitrogen., CA, USA) at 100-150 volt for about 2 hr and then transferred to a PVDF membrane (Invitrogen) by electro-blotting at 270 mAmp constant current for 1 hr 20 min. The transferred membrane was blocked with 2.0 % non-fat dry milk containing 0.5 % Tween 20-TBS buffer and 1 mM of $MgCl_2$ for 1 hr at room temperature. Primary antibodies against ABCG2 (Sigma-Aldrich, St. Louis, MO, USA) (1:500) and β-actin (Applied Biological Materials Inc., Richmond, Canada) (1:5000) were introduced

to the membrane and incubated at room temperature for 1 hr. Peroxidase conjugated mouse IgG antibody (Jackson Immunoresearch, Baltimore, USA) (1:10000) was used as a secondary antibody and incubated with the membrane at room temperature for 1 hr. Finally, the membrane, which was treated with chemiluminescent working solution WESTZOL (Intron biotechnology), was exposed to Fuji RX film (Fujifilm, Tokyo, Japan) for 1 to 15 min.

5-aza-2'-deoxycytidine (5-aza) treatment

For treatment with 5-aza-2'-deoxycytidine, 2×10^5 cells / ml were seeded in two 75 cm^2 culture flasks. On the next day, one of the flasks was treated with 3 μM of 5-aza (Sigma-Aldrich) and the other flask received the same volume of DMSO as an untreated group. After 48 hr incubation time, the cells were harvested for RNA.

WST-1 assay

Cells were seeded on a 96-well plate at 2×10^4 cells / well and incubated overnight at 37 °C in 5 % CO_2 and 95 % air. Next day, anticancer drugs including 5-FU (Sigma-Aldrich), Irinotecan (Sigma-Aldrich) and Oxaliplatin (Sigma-Aldrich) were added separately into the well at 48 hr after 5-aza treatment. Cell proliferation reagent WST-1 (Roche., NSW, Australia) was added to each well after 72 hr incubation time from the addition of the anticancer drugs. Then, the plates were incubated at 37 °C for 4 hr and the absorbance was measured with a MULTISKAN FC Microplate Photometer (Thermo Scientific Inc., Bremen, Germany) at 450 nm.

Statistical analysis

Numerical data for all graphs were expressed as the mean ± standard deviation (S.D). A $p < 0.05$ was considered as significant and statistical analysis was done with SPSS software version 20.0.

Table 1. List of the transcriptional regulation sites and genomic region in *ABCG2*. Nucleotides positions of the sites in *ABCG2* genome are designated with the transcriptional start site (+1) determined in the prior study [6].

Potential site	Genomic position
Promoter site	-36 to -266
XBBF	-363 to -378
CpG island	-249 to -402
SP1 site	-210 to -222 -178 to -187 -151 to -160 -116 to -127 -37 to -49
AP1 site	-349 to -360 +124 to +136
CCAAT box	-275 to -280
AP2 site	-38 to -50 +107 to +118
Exon 1	+1 to +532

Table 2. Primer sequences for RT-PCR, qRT-PCR, MS-PCR and Bisulfite sequencing PCR

Name	Sequences	Size (bp)	References
MXR RT F	5'-GTTTATCCGTGGTGTGTCTGG-3'	652	
MXR RT R	5'-CTGAGCTATAGAGGCCTGGG-3'		
ABCG2 qRT F	5'-CAGGTCTGTTGGTCAATCTCACA-3'	76	[24]
ABCG2 qRT R	5'-TCCATATCGTGGAATGCTGAAG-3'		
ABCG2 M F	5'-TATTTATTTAATTTGTTTTGGGTGC-3'	141	*MethPrimer software (www.urogene.org/methprimer/index1.html)*
ABCG2 M R	5'-TCATTAAACTAATCAATACCTCGTC-3'		
ABCG2 U F	5'-TTTATTTAATTTGTTTTGGGTGTGA-3'	139	
ABCG2 U F	5'-TCATTAAACTAATCAATACCTCATC-3'		
ABCG2 BS F	5'-AAATTATTTATTTAATTTGTTTTGG-3'	282	
ABCG2 BS R	5'-CCAACAAAACTAATACCACC-3'		

F: Forward, R: Reverse, RT: RT-PCR specific primer, qRT: qRT-PCR specific primer, M: Methylation specific primer, U: Unmethylation specific primer, BS: Bisulfite sequencing specific primer

Table 3. Correlation between the promoter methylation status and *ABCG2* expression. -: PCR bands were not detected; +: PCR bands were detected; % Methylation: level of methylated CpGs ((# methylated CpG / # total CpG) * 100); % expression: Relative expression level of *ABCG2* as the ratio to the *β-actin* expression level ((*ABCG2* level / *β-actin* level) * 100).

Cell lines	Methylation	Unmethylation	% of Methylation	% of expression
SNU-61	+	-	1.9	8.7
SNU-81	+	+	0.0	0.4
SNU-175	+	+	3.8	7.2
SNU-283	-	-	14.3	0.1
SNU-407	+	+	2.9	0.5
SNU-503	+	+	2.9	33.5
SNU-769A	+	+	57.1	1.0
SNU-769B	+	+	0.0	0.2
SNU-1033	+	-	0.0	4.3
SNU-1040	-	-	0.0	0.3
SNU-1047	+	+	0.0	0.2
SNU-1197	+	+	0.0	0.7
SNU-C1	+	-	7.6	3.8
SNU-C2A	+	+	5.7	3.3
SNU-C4	+	+	28.6	0.0
SNU-C5	+	-	1.9	1.8
Caco2	+	-	0.0	9.6
Colo201	+	-	2.9	0.0
Colo205	+	-	19.0	0.7
Colo320	+	-	17.1	2.0
DLD1	+	-	0.0	1.6
HCT 8	+	+	0.0	2.0
HCT 15	+	+	1.0	0.6
HCT 116	+	+	1.0	3.9
HT 29	+	-	0.0	5.3
LoVo	+	-	30.5	3.6
Ls174T	+	+	24.8	0.0
NCI-H716	+	+	45.7	0.1
SW403	+	-	1.0	0.2
SW480	+	+	4.8	0.0
SW1116	+	-	4.8	0.1
WiDr	+	-	0.0	8.9

Results

Expression of *ABCG2* in colorectal cancer cell lines

Colorectal cancer cell lines were examined by RT-PCR and qRT-PCR to identify the mRNA expression level of *ABCG2*. After gel electrophoresis, the obtained RT-PCR bands were processed by ImageJ (http://rsbweb.nih.gov/ij/) as the rate of *ABCG2* expression (*ABCG2* expression = (amplified *ABCG2* / amplified *β-actin*) × 100). The *ABCG2* mRNA band was detected in 23 cell lines (range of expression rate from 2.4 to 116.7, data not shown) but it was not observed in 9 cell lines (Fig. 2A and Table 3). Additionally, I classified the groups into high (> 1) and low (< 1) groups according to the relative expression level shown by the qRT-PCR (Fig. 2B) (Relative expression level = (*ABCG2* expression level / *β-actin* expression) level × 100, Table 3). SNU-61, SNU-175, SNU-503, SNU-769A, SNU-1033, SNU-C1, SNU-C2A, SNU-C5, Caco2, DLD1, Colo320, HCT 8, HCT 116, HT 29, LoVo and WiDr showed a higher mRNA expression level of *ABCG2* and SNU-81, SNU-283, SNU-407, SNU-769B, SNU-1047, SNU-1197, SNU-C4, Colo201, Colo205, HCT 15, Ls174T, NCI-H716, SW403, SW480 and SW1116 belonged to the lower group. The relative expression level was not detected in 4 cell lines, SNU-C4, Colo201, Ls174T and SW480. Taken together, there were 8 cell lines that had low or no mRNA expression for *ABCG2* in RT-PCR and qRT-PCR analysis, SNU-283, SNU-769B, SNU-C4, Colo201, Ls174T, NCI-H716, SW403 and SW480.

Figure 1. Schemes of CpG island in the promoter region of *ABCG2*.

A. Schemes of *ABCG2* gene 5' upstream region. CpG island (-402 to -249), AP1 (black arrows), AP2 (grey arrow) SP1 (white arrows) and CCAAT box are included in this region [6, 37].

B. Genomic map of the CpG dinucleotide sites. Horizontal bars indicate introns and vertical bars indicate the distribution of the *ABCG2* CpG dinucleotides (-592 to +358).

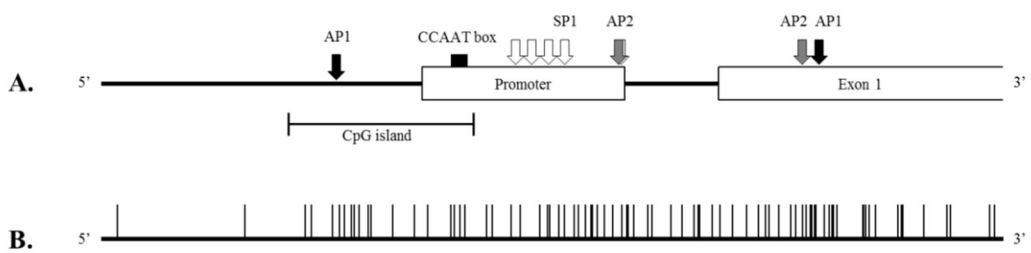
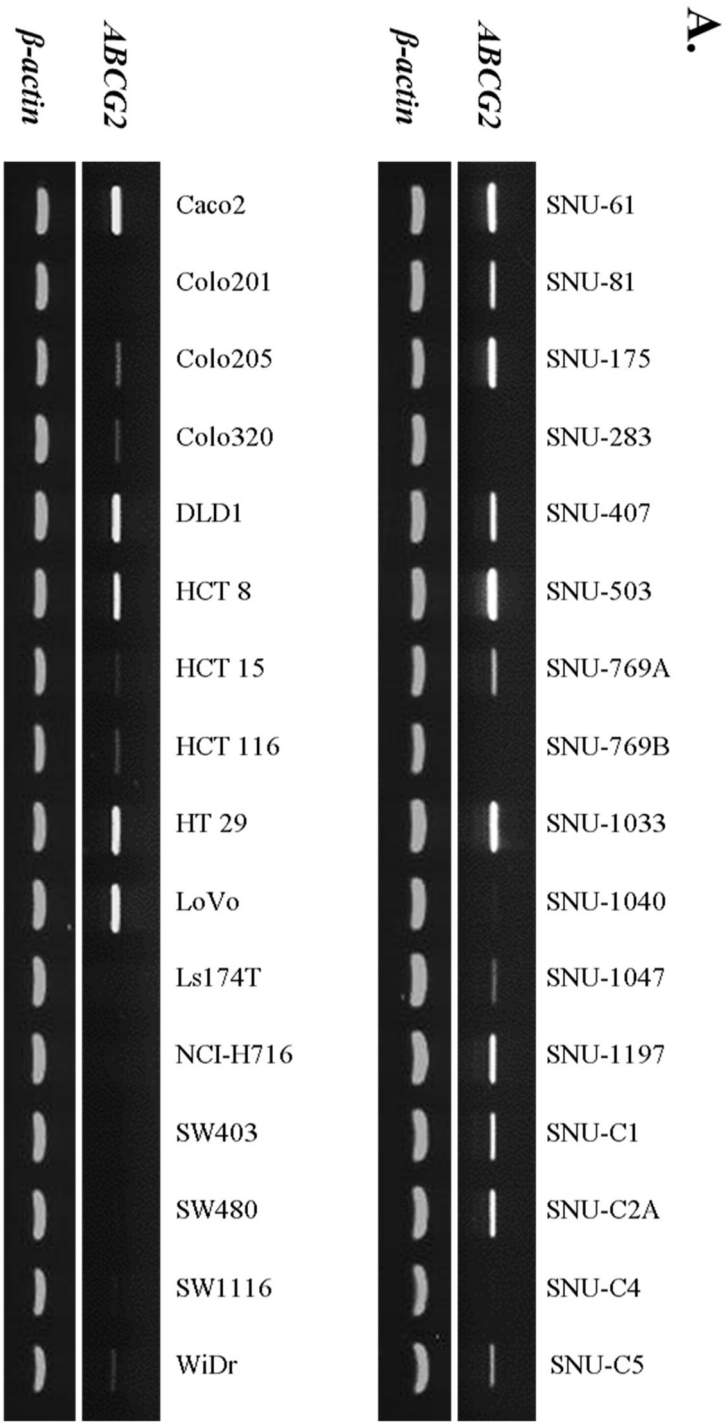


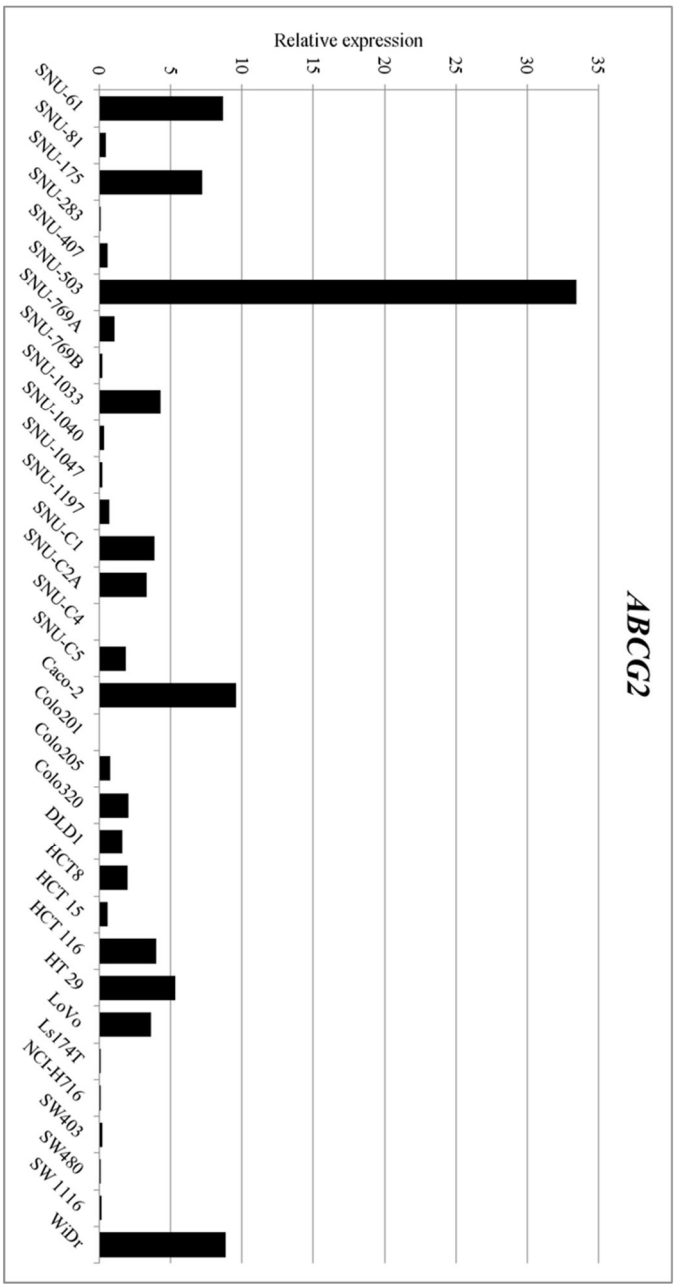
Figure 2. Expression analysis of *ABCG2* gene was performed in 32 colorectal cancer cell lines by RT-PCR and qRT-PCR.

A. RT-PCR analysis for screening the *ABCG2* mRNA expression level in 32 colorectal cancer cell lines. *ABCG2* expression was shown in 23 cell lines (SNU-61, SNU-81, SNU-175, SNU-407, SNU-503, SNU-769A, SNU-1033, SNU-1047, SNU-1197, SNU-C1, SNU-C2A, SNU-C5, Caco-2, Colo205, Colo320, DLD1, HCT 8, HCT 15, HCT 116, HT 29, LoVo, SW1116 and WiDr) but not in 9 cell lines (SNU-283, SNU-769B, SNU-1040, SNU-C4, Colo201, Ls174T, NCI-H716, SW403 and SW480).

B. Quantitative differences of the *ABCG2* mRNA expression by qRT-PCR analysis


A.
β-actin
ABCG2
Caco2
Colo201
Colo205
Colo320
DLD1
HCT 8
HCT 15
HCT 116
HT 29
LoVo
Ls174T
NCI-H716
SW403
SW480
SW1116
WiDr
β-actin
ABCG2
SNU-61
SNU-81
SNU-175
SNU-283
SNU-407
SNU-503
SNU-769A
SNU-769B
SNU-1033
SNU-1040
SNU-1047
SNU-1197
SNU-C1
SNU-C2A
SNU-C4
SNU-C5

B.


ABCG2
Relative expression
0
5
10
15
20
25
30
35
SNU-61
SNU-81
SNU-175
SNU-283
SNU-407
SNU-503
SNU-769A
SNU-769B
SNU-1033
SNU-1040
SNU-1047
SNU-1197
SNU-C1
SNU-C2A
SNU-C4
SNU-C5
Caco-2
Colo201
Colo205
Colo320
DLD1
HCT8
HCT 15
HCT 116
HT-29
LoVo
Ls174T
NCI-H716
SW403
SW480
SW 1116
WiDr

Evaluation of the promoter methylation status of *ABCG2* gene by methylation specific-PCR and bisulfite sequencing analysis

To determine whether *ABCG2* expression is related with epigenetic changes such as CpG methylation of the promoter site, I investigated the methylation status of the *ABCG2* promoter site in 32 colorectal cancer cell lines with MS-PCR and bisulfite sequencing analysis. Genomic DNA, which was modified with sodium bisulfite, had all unmethylated cytosines converted uracils but methylated cytosines remained unchanged. The specific designed primers (Table 2) for MS-PCR amplified the unmethylated and methylated sequences located from -273 to -414 which contained 21 CpG islands (Fig. 1 and Table 1). Methylated DNAs were detected in all cell lines except for SNU-283, and there was a weak methylated band in SNU-1040 (Fig. 3A). Unmethylated DNAs were amplified weakly in most of the cell lines except for SNU-283 and SNU-1040 which did not show any methylated DNA bands. There were 10 cell lines (SNU-769B, SNU-1047, SNU-C4, Colo201, HCT 15, Ls174T, NCI-H716, SW403, SW480 and SW1116) that had low or no expression of *ABCG2* mRNA and methylated DNAs. The expression levels of both *ABCG2* mRNA and amplified methylated DNAs were observed in the other 20 cell lines. In SNU-C4, Colo201, Ls174T and SW480, methylated bands were present but *ABCG2* gene expression was not detected in RT-PCR and qRT-PCR (Fig. 2) at the

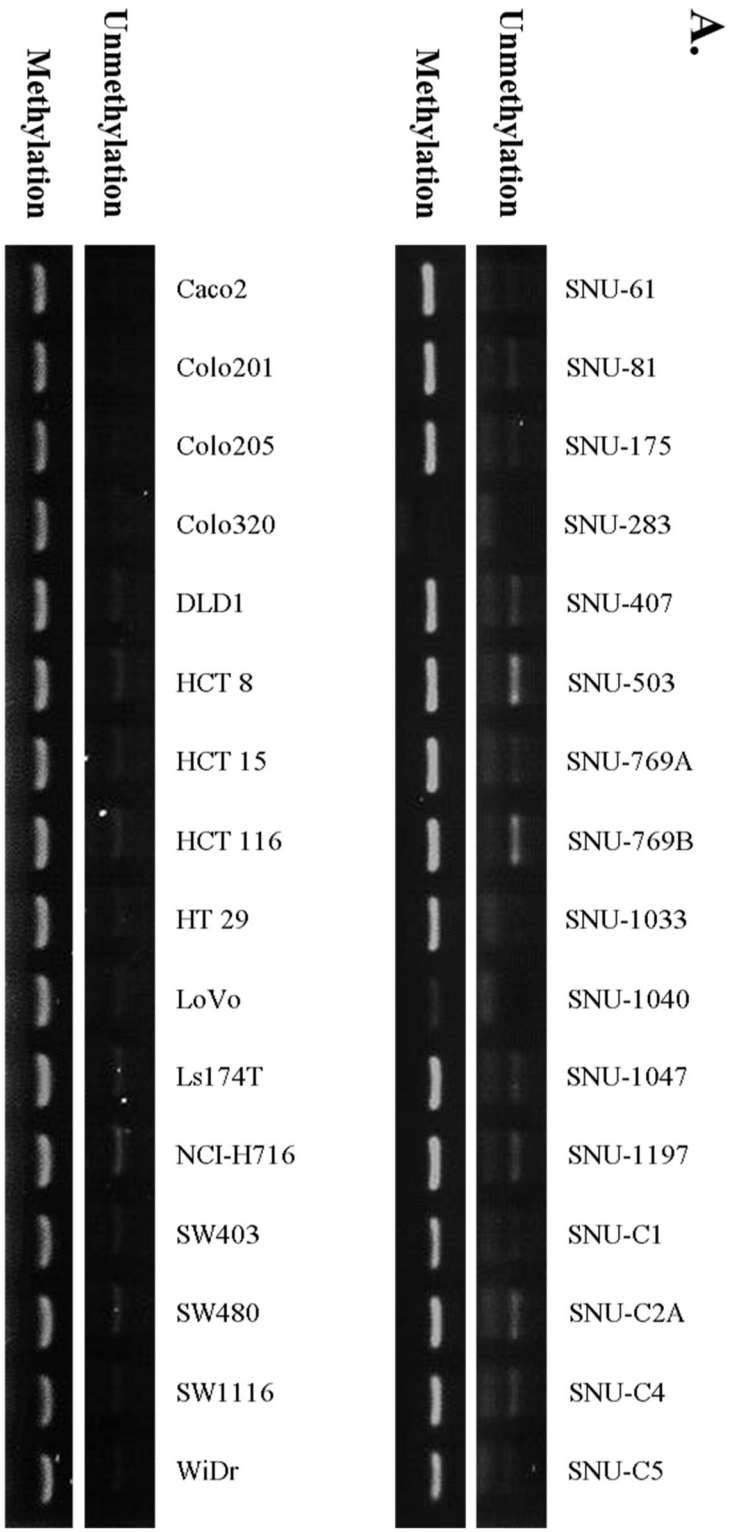
same time. The CpG island region (-136 to -417) that contains 21 CpG dinucleotide sites (Fig. 1 and Table 1) and part of the promoter for the *ABCG2* gene was amplified with a bisulfite sequencing specific primer set (Table 2). Part of the CpG island sequence was determined in Fig. 3B. SNU-769A represented the methylated CpG dinucleotides sequence and Caco2 represented the unmethylated sequence around the seven CpG islands. The methylation status of the CpG islands in the *ABCG2* promoter was shown in Fig. 3C. To compare with the *ABCG2* mRNA and methylation status of the promoter, the percentage of methylation was analyzed (Table 3). The percentage of promoter methylation of *ABCG2* in 8 cell lines was more than 10 % and SNU-769A (57.1 %), NCI-H716 (45.7 %) and SNU-C4 (28.6 %) were verified as having a hypermethylated *ABCG2* promoter. SNU-C4, Ls174T and NCI-H716 had more than 20 % methylation in the promoter and simultaneously detected low or no *ABCG2* gene expression (Fig. 2).

Figure 3. Methylation status of *ABCG2* gene was shown in the 32 colorectal cancer cell lines by MS-PCR and bisulfite sequencing analysis. gDNA of the examined cell lines was processed by bisulfite modification before each analysis.

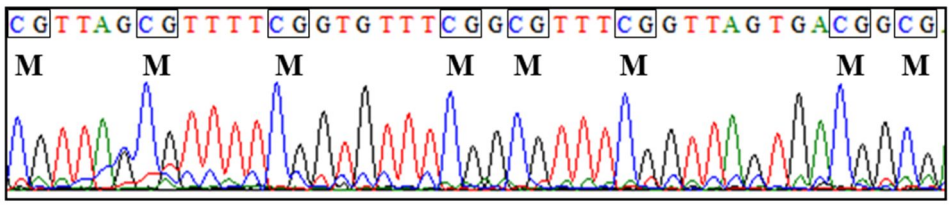
A. Unmethylated and Methylated products were amplified by primers that recognize methylated and unmethylated sequences.

B. Reperesentative sequence diagrams of methylated DNA sequencing analysis in SNU-769A (methylated) and Caco2 (unmethylated) cell lines. Boxes indicate CpG dinucleotide sites in 21 CpG islands. M: Methylated site; U: Unmethylated site

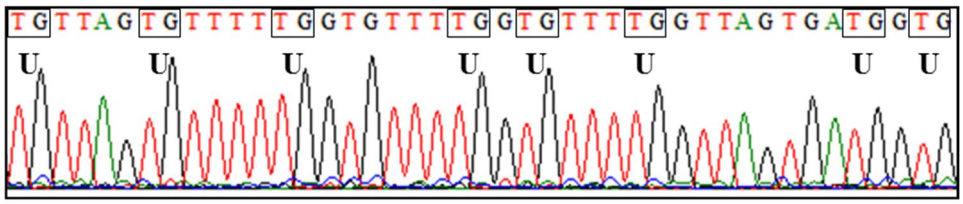
C. Bisulfite sequencing analysis of 21 CpG dinucleotides. All circles represent CpG dinucleotides. Open circles are unmethylated CpGs and closed circles are methylated CpG dinucleotides.


A.
Methylation
Unmethylation
Caco2
Colo201
Colo205
Colo320
DLD1
HCT 8
HCT 15
HCT 116
HT 29
LoVo
Ls174T
NCI-H716
SW403
SW480
SW1116
WiDr
Methylation
Unmethylation
SNU-61
SNU-81
SNU-175
SNU-283
SNU-407
SNU-503
SNU-769A
SNU-769B
SNU-1033
SNU-1040
SNU-1047
SNU-1197
SNU-C1
SNU-C2A
SNU-C4
SNU-C5

B.

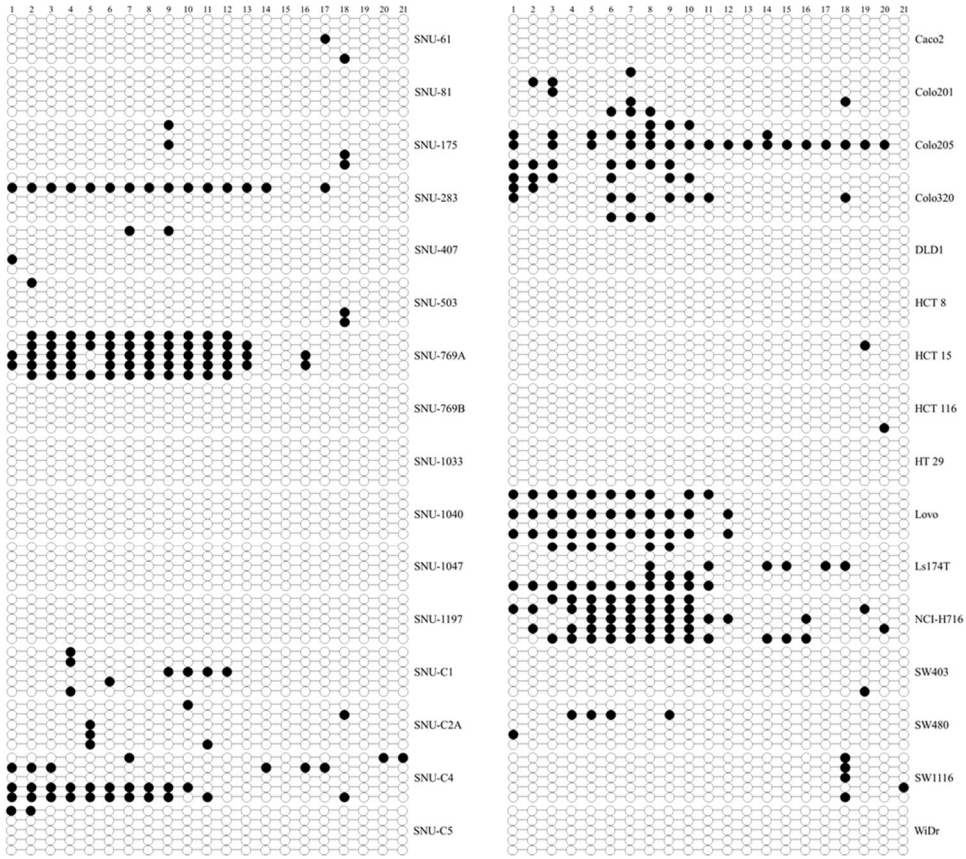


SNU-769A



Caco2

C.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
SNU-61
SNU-81
SNU-175
SNU-283
SNU-407
SNU-503
SNU-769A
SNU-769B
SNU-1033
SNU-1040
SNU-1047
SNU-1197
SNU-C1
SNU-C2A
SNU-C4
SNU-C5
Caco2
Colo201
Colo205
Colo320
DLD1
HCT 8
HCT 15
HCT 116
HT 29
Lovo
Ls174T
NCI-H716
SW403
SW480
SW1116
WiDr

Recovery of *ABCG2* mRNA expression after treatment with 5-aza-2'-deoxycytidine

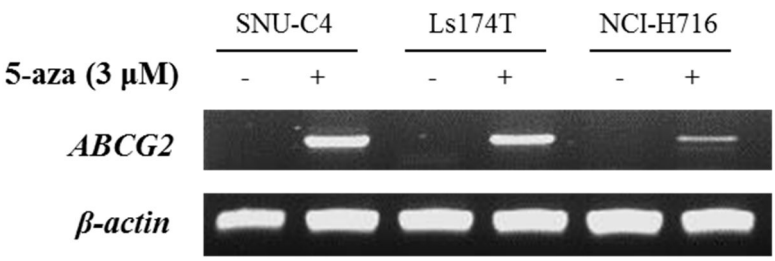
To determine whether DNA methylation affects *ABCG2* expression, three colorectal cancer cell lines (SNU-C4, Ls174T and NCI-H716) that showed methylated DNAs in the MS-PCR and over 20 % methylated CpG dinucleotides in the bisulfite sequencing analysis and weak or no *ABCG2* mRNA expression were treated with 5-aza. In all three cell lines, *ABCG2* mRNA expression recovered when the cell lines were cultured with 3 μM of 5-aza for 48 hr (Fig. 5A and B). Furthermore, there was no significant re-expression of *ABCG2* when the Ls174T and NCI-H716 cell lines were treated with Trichostatin A (TSA, histone deacetylase inhibitor) (Data not shown). Therefore, re-expression of *ABCG2* mRNA resulted from demethylation mediated by 5-aza not acetylation.

Figure 4. A comparison of *ABCG2* mRNA expression level between 5-aza treated and non-treated cell lines. I observed whether re-expression of *ABCG2* occurred in 3 cell lines after treatment with or without 3 μM of 5-aza.

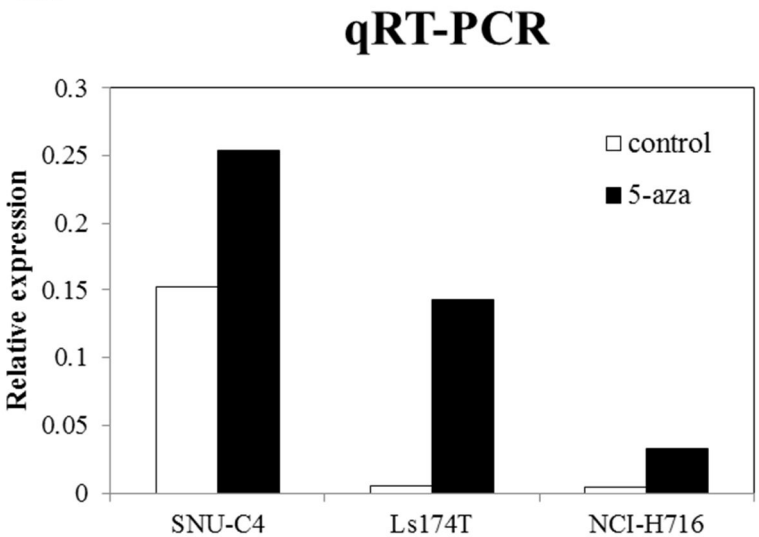
A. RT-PCR

B. qRT-PCR

A.


SNU-C4
Ls174T
NCI-H716
5-aza (3 μM)
- + - + - +
ABCG2
β-actin

B.


qRT-PCR
Relative expression
0.3
0.25
0.2
0.15
0.1
0.05
0
control
5-aza
SNU-C4
Ls174T
NCI-H716

Drug sensitivity can be reversed by 5-aza treatment in some colorectal cancer cell lines.

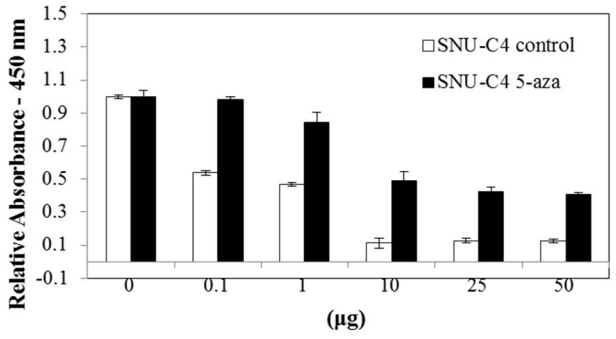
To determine whether mRNA re-expression by demethylation affects anticancer drug sensitivity, I performed the WST-1 assay using 5-aza treated colorectal cancer cell lines which expressed a low mRNA level under relative expression level 1 and had over 20 % methylation of the promoter (Table 3). Selected cell lines, SNU-C4, Ls174T and NCI-H716, were treated with chemotherapeutic drugs in a dose dependent manner used to treat CRC patients known as *ABCG2* substrates: 5-FU, irinotecan and oxaliplatin. Drug sensitivity was measured inversely by cell viability depending on the absorbance at 450 nm. In the SNU-C4, Ls174T and NCI-H716 cell lines with 5-aza, the cell viability significantly increased in the presence of 5-FU, irinotecan and oxaliplatin at all drug concentrations (Fig. 5A, B and C).

5-aza potentiated the cell viability with 5-FU (1.83-fold to 4.33-fold increase with 10 μg), irinotecan (1.52-fold to 2.43-fold increase with 200 μM) and oxaliplatin (1.48-fold to 1.62-fold increase with 50 and 100 μM) in SNU-C4. In Ls174T with 5-aza, cell viability was maximally increased at 50 μg of 5-FU (2.67-fold), 200 μM of irinotecan (2.45-fold) and 25 μM of oxaliplatin (2.18-fold). The cell viability of 5-aza treated NCI-H716 reached the greatest level at 50 μg of 5-FU (1.40-fold), 200 μM of irinotecan (1.35-fold) and 50 μM of oxaliplatin (1.40-fold). SNU-C4 (2.92-fold increase with 5-FU) and Ls174T (2.22-fold increase with irinotecan and 2.08-fold increase with oxaliplatin) showed a maximum increase in cell viability for each anticancer drug, and a minimal increase was detected in NCI-H716 (1.33-fold increase with 5-FU, 1.25-fold increase with irinotecan and 1.32-fold increase with oxaliplatin) according to the average for cell viability. Additionally,

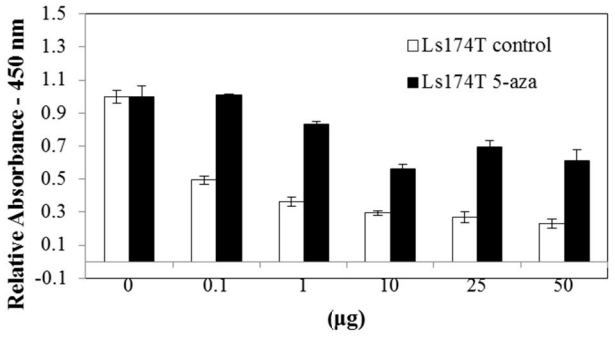
increments of cell viability were observed at the greatest level when the cell lines were treated 5-FU (2.18-fold) and oxaliplatin (1.65-fold) had the lowest level for the average enhanced cell viability. Taken together, 5-aza treatment inducing demethylation of *ABCG2* in some colorectal cell lines can have an effect on the decrease of drug sensitivity.

Figure 5. A comparison of cell viability for anticancer drugs in the colorectal cancer cell lines with or without 5-aza treatment. (a) SNU-C4, (b) Ls174T and (c) NCI-H716 were treated with A. 5-FU (0, 0.1, 1, 10, 25 and 50 μg), B. irinotecan (0, 5, 30, 50, 100 and 200 μM) and C. oxaliplatin (0, 5, 10, 25, 50 and 100 μM) for 72 hr after demethylation by 5-aza and cell viability was determined using WST-1 assay.

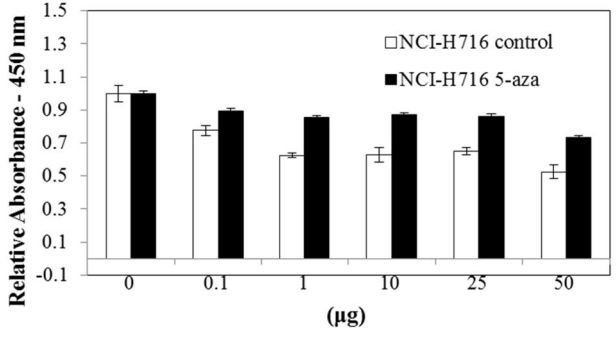
A. (a)


5-FU
Relative Absorbance - 450 nm
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
0
0.1
1
10
25
50
(μg)
SNU-C4 control
SNU-C4 5-aza

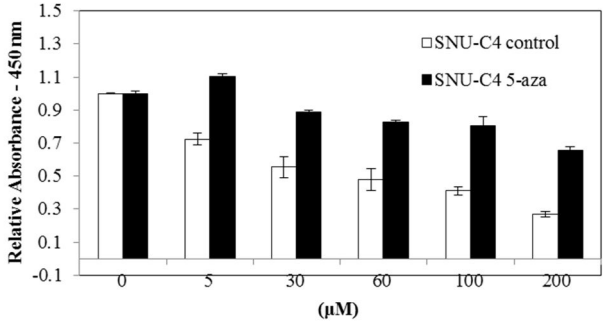
(b)


5-FU
Relative Absorbance - 450 nm
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
0
0.1
1
10
25
50
(μg)
Ls174T control
Ls174T 5-aza

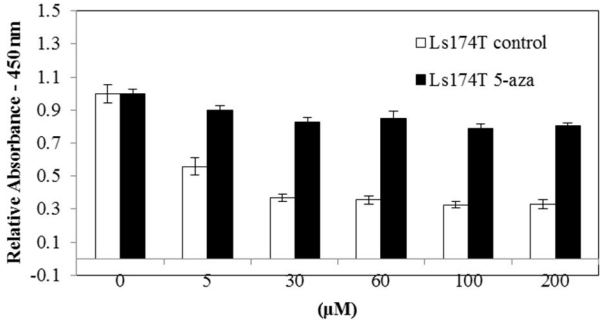
(c)


5-FU
Relative Absorbance - 450 nm
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
0
0.1
1
10
25
50
(μg)
NCI-H716 control
NCI-H716 5-aza

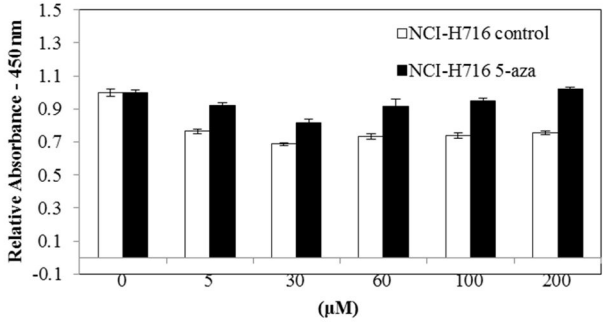
B. (a)


Irinotecan
Relative Absorbance - 450 nm
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
0
5
30
60
100
200
(μM)
SNU-C4 control
SNU-C4 5-aza

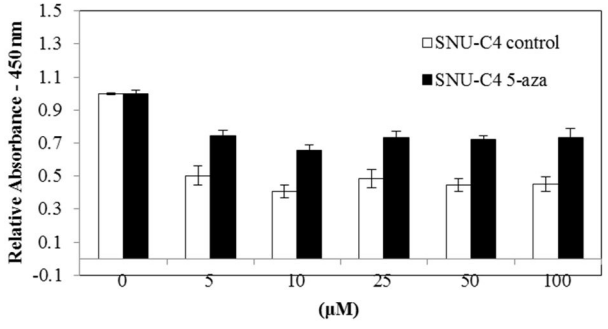
(b)


Irinotecan
Relative Absorbance - 450 nm
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
0
5
30
60
100
200
(μM)
Ls174T control
Ls174T 5-aza

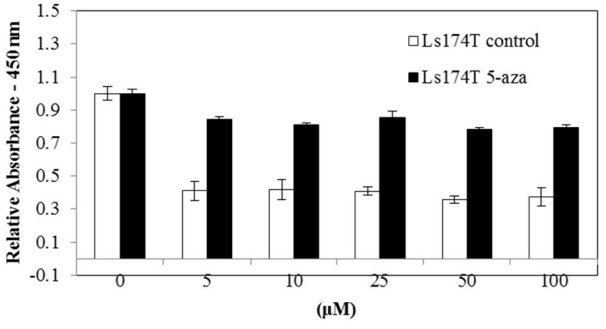
(c)


Irinotecan
Relative Absorbance - 450 nm
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
0
5
30
60
100
200
(μM)
NCI-H716 control
NCI-H716 5-aza

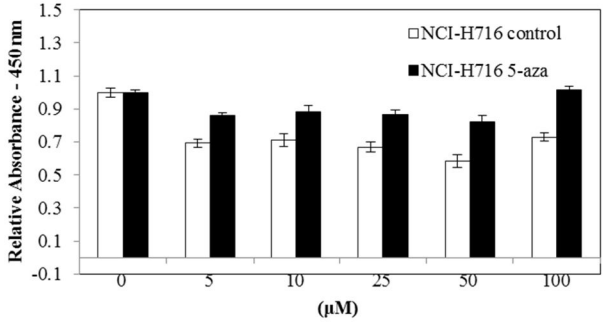
C. (a)


Oxaliplatin
Relative Absorbance - 450 nm
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
0
5
10
25
50
100
(μM)
SNU-C4 control
SNU-C4 5-aza

(b)


Oxaliplatin
Relative Absorbance - 450 nm
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
0
5
10
25
50
100
(μM)
Ls174T control
Ls174T 5-aza

(c)


Oxaliplatin
Relative Absorbance - 450 nm
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
0
5
10
25
50
100
(μM)
NCI-H716 control
NCI-H716 5-aza

Discussion

Over-expressed *ABCG2* is associated with anticancer drug resistance by mediating drug efflux. MCF-7/AdrVp cells are a multidrug-resistant human breast cancer subline which does not express P-gp or MRP1 known multidrug resistance transporters but does express *ABCG2*. In this cell line, the multidrug resistance phenotype is acquired by *ABCG2* overexpression [12]. DNA methylation has been known to be responsible for inhibiting gene expression. Methylation of the transcriptional regulatory region including the transcriptional binding sites induces the transcriptional repression of several genes [19, 43]. In a prior study on lung cancer cells, it was discovered that methylation of the *ABCG2* promoter is inversely correlated with its expression [30]. There was a study that showed DNA methylation-dependent formation like the association of methylated lysine 9 on histone H3 in the CpG islands of the *ABCG2* gene might induce the inactivation of *ABCG2*, and this has been shown by treatment with 5-aza-2'-deoxycytidine. Therefore, this observation suggested that DNA methylation of the *ABCG2* promoter site, which consists of many CpG islands, could play a role in the epigenetic regulation of gene expression [37].

To study the correlation between the methylation patterns of the *ABCG2* promoter region and gene expression in colorectal cancer cell lines, I performed MS-PCR and bisulfite sequencing analysis. The *ABCG2* mRNA levels were examined by RT-PCR and quantitative real-time PCR. First, I classified the CRC cell lines into high or low *ABCG2* expression groups according to the relative expression level

shown by the qRT-PCR (Fig. 2B). Then, I selected cell lines which had a hypermethylated promoter site identified by MS-PCR (Fig. 3) and bisulfite sequencing analysis (Fig. 4). As a result, SNU-C4, Ls174T and NCI-H716 were selected because they expressed a low *ABCG2* gene under relative expression level 1 and had more than 20 % methylated CpG dinucleotides in the promoter site (Table 3). The three cell lines were treated with demethylating agent 5-aza to determine whether DNA demethylation increases *ABCG2* mRNA expression. After treating the cell lines including SNU-C4, Ls174T and NCI-H716 with 3 μM 5-aza for 48 hr *ABCG2* mRNA was expression in all cell lines (Fig. 5). Consequently, demethylation of the CpG dinucleotides in the *ABCG2* promoter up-regulated *ABCG2* gene expression, in other words, the promoter is regulated negatively by DNA methylation in some colorectal cancer cell lines. However, I did find that SNU-769A moderately expressed *ABCG2* gene and had hypermethylation of the promoter CpG islands (Table 3). As referred to earlier in the study, 1 allele of the chromosome was methylated but another allele was not methylated in the moderate *ABCG2*-expressing cells (NCI-H460, NCI-H441 and NCI-H358 cell lines) [30]. Therefore, there is a possibility that 1 allele might be methylated in SNU-769A but to make sure of this speculation, additional DNA sequencing required to analyze both alleles of SNU-769A.

Various epigenetic modification types affect the regulation of genes such as acetylation at Lys and methylation at Arg and Lys. When the Ls174T and NCI-H716 cell lines were treated with TSA, there was no significant re-expression of the *ABCG2* gene (Data not shown). Taken together, these results suggest that methylation of the *ABCG2* promoter region might have an influence on *ABCG2*

expression but acetylation might not be related to the regulation of the gene in some colorectal cancer cell lines. However, it is necessary to perform additional experiments such as the ChIP assay to determine whether other mechanisms or proteins are involved in the regulation steps of *ABCG2* expression because methylation is not the only mechanism of epigenetic regulation.

In a prior study, it was shown that the development of drug resistance is not dependent on P-gp or MRP but is related to the upregulated protein expression of ABCG2 in mitoxantrone-resistant HT 29 colon carcinoma cell line [35]. Likewise, *ABCG2* was over-expressed in irinotecan and oxaliplatin resistant cell lines and 5-FU resistance was increased in *ABCG2* transfected MDCKII cells [8, 22]. 5-FU, irinotecan and oxaliplatin are substrates for *ABCG2* [46]. In summary, these reports suggested that over-expressed *ABCG2* contributes to drug resistance in cancer cells.

After I confirmed that demethylation can enhance *ABCG2* gene expression in SNU-C4, Ls174T and NCI-H716, I investigated whether drug sensitivity can be affected by the increased *ABCG2* gene expression from the 5-aza-induced demethylation. Cell viability was measured by WST-1 assay and inversely indicates drug sensitivity. SNU-C4, Ls174T and NCI-H716 were treated with 5-FU, irinotecan and oxaliplatin for 72 hr after 48 hr treatment with 5-aza. The reversible effects of drug sensitivity appeared significantly in all cell lines treated with 5-aza (Fig. 5). 5-aza maximally potentiated the cell viability of 5-FU (4.33-fold at 10 μg) in SNU-C4, irinotecan (2.45-fold at 200 μM) in Ls174T and oxaliplatin (2.18 -fold at 50 μM) in Ls174T. In NCI-H716, a minimal increase was measured according to the average increased cell viability (1.33-fold at 5-FU, 1.25-fold at irinotecan and 1.32-fold at oxaliplatin). Because inverse cell viability is considered equivalent to

drug sensitivity, I concluded that drug sensitivity was decreased in the 5-aza treated CRC cell lines despite the differences in the increased levels of cell viability. The reason why there were differences in the increased levels of cell viability is thought to be because of distinctions in the expression level of the *ABCG2* mRNA in each cell line. Actually, the increments for the ratio of *ABCG2* expression in the 5-aza treated cell lines were 1.66-fold in SNU-C4, 25.16-fold in Ls174T and 6.89-fold in NCI-H716. Taken together, I found that 5-aza induced demethylation of the promoter site in some colorectal cell lines might have an effect on the decrease in drug sensitivity through the positive regulation of *ABCG2* mRNA expression based on various tests. There are several combination chemotherapies for CRC patients such as FOLFIRI (5-FU, irinotecan and leucovorin), FOLFOX (5-FU, oxaliplatin and leucovorin) and FOLFOXIRI (5-FU, irinotecan, oxaliplatin and leucovorin) [5, 11, 15]. According to the results, over-expression of the *ABCG2* gene as well as the *ABCG2* methylation status may be useful as a marker of drug resistance in CRC patients regarding those regimens, and it is possible to understand individual specific drug sensitivity for each CRC patient. Thus, this study is meaningful in terms of anticancer treatment because appropriate therapy could be achieved in colorectal cancer patients.

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국문 초록

대장암은 전세계적으로 여성에게서 두 번째, 남성에게서 세 번째로 많이 진단되는 질환이다. 수술을 통한 암세포의 제거에도 불구하고, 환자의 생존율은 암의 주기가 증가할수록 낮아지는 경향을 보인다. 생존율을 높이기 위해, 3 기 또는 4 기 직장암 환자의 경우 수술 이외에 화학적, 방사선적 요법이 추가적으로 적용되기도 한다. 화학적 치료가 실시되는 도중에 중대한 문제점 중 하나는 환자에게서 발견되는 약물에 대한 자연 내성과 획득 내성이다. 항암제 내성이 획득되는 주요한 경로 중 하나는 에너지 의존적 약물 방출 펌프인 *MDR1*, *MRP* 그리고 *ABCG2* 유전자의 과발현에 기인한다. 유전자 발현 조절 장치 중 하나인 DNA 메틸화 기작을 통해 *ABCG2* 의 발현이 억제되는 현상이 여러 논문에서 관찰되었다. 또한 대장암 치료제인 5-FU, irinotecan, oxaliplatin 은 *ABCG2* 의 기질임과 동시에 *ABCG2* 발현이 증가되었을 때, 그 내성이 증가한다는 사실이 알려져 있다. 이 사실을 바탕으로 본 연구에서는 32 개 직장암 세포주에서 *ABCG2* 의 프로모터 부위의 메틸화 양상을 MS-PCR, bisulfite sequencing analysis 를 통해 관찰하였고, 역전사 PCR 과 실시간 PCR 을 통한 mRNA 발현

양상과 비교하였다. 그 결과, 32 개 직장암 세포주 중 13 개 세포주에서 mRNA 발현이 감소되어 있거나 억제되어 있었고, 이 중 3 개 세포주에서 20% 이상의 *ABCG2* 촉진 유전자의 CpG 섬 부위의 메틸화가 관찰되었다. 위 3 개 세포주 (SNU-C4, Ls174T, NCI-H716)에서 *ABCG2* 유전자가 DNA 메틸화에 의한 발현 억제 조절을 받고 있다는 사실을 확인하기 위해, 탈메틸화 약제인 5-aza-2'-deoxycytidine 을 처리 한 뒤 발현 회복 양상을 관찰하였다. 역전사 PCR 과 실시간 PCR 에서 위 세가지 세포주의 *ABCG2* 메신저 RNA 의 발현 회복이 관찰되었다. 메틸화 제거 기작에 의한 *ABCG2* 유전자 재발현 현상이 항암제 감수성이 미치는 영향을 알아보기 위해 5-aza-2'-deoxycytidine 를 처리한 SNU-C4, Ls174T, NCI-H716 세포주에 5-FU, irinotecan, oxaliplatin 을 적용하고, WST-1 검사법을 이용해 450 nm 에서 측정한 흡광도로 세포 생존도를 분석하였다. 5-aza 와 5-FU 또는 oxaliplatin 이 함께 처리된 SNU-C4 세포주에서 약제 감수성이 감소하였고, Ls174T 세포주에서는 모든 약제에 대한 감수성이 유의하게 감소된 반면에, NCI-H716 세포주에서는 유의한 약제 감수성의 변화가 관찰되지 않았다. 결론적으로, 일부 대장암 세포주에서 *ABCG2* 프로모터의 CpG 섬 부위의 탈메틸화는 *ABCG2* 유전자 발현을 증가시키며, *ABCG2* 유전자 발현이 증가함으로 인해 대장암 치료제인 5-FU, irinotecan

그리고 oxaliplatin 의 약제 감수성이 감소되는데 영향을 미치는 것으로 보인다. 이로써, *ABCG2* 의 과발현은 항암제 내성을 가지는 대장암 환자에게서 약제 내성을 판별할 수 있는 marker 로 이용 될 가능성이 있으며, 프로모터 부위의 메틸화 양상을 조사하여 특정 약물에 대한 감수성을 파악할 수 있게 된다면 화학적 요법의 치료 효율을 높일 수 있을 것이라 전망된다.

주요어: 대장암, 화학적 치료법, 약제내성, *ABCG2*, DNA 메틸화, 5-aza-2'-deoxycytidine

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