Irinotecan Inactivation Is Modulated by Epigenetic Silencing of *UGT1A1* in Colon Cancer

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Abstract

Purpose: Irinotecan is used in the first-line treatment of metastatic colorectal cancer. The UGT1A1-metabolizing enzyme, expressed in liver and colon, is primarily involved in the inactivation of its active metabolite 7-ethyl-10-hydroxycamptothecin (SN-38). Herein, we explored the role of DNA methylation in the silencing of *UGT1A1* gene expression in colon cancer and its influence on cellular SN-38 detoxification.

Experimental Design and Results: *UGT1A1* mRNA was repressed in most primary tumors (41 of 50; 82%) and in three colon cancer cell lines (HCT-116, HCT-15, and COLO-320DM). Bisulfite sequencing of the *UGT1A1* gene revealed the aberrant methylation of specific CpG islands in UGT1A1-negative cells. Conversely, hypomethylation was observed in HT-29, HT-115, and LOVO cells that overexpress UGT1A1. Direct methylation of the *UGT1A1* promoter resulted in the complete repression of transcriptional activity. Treatment with demethylating and histone deacetylase inhibitor agents had the capacity to reverse aberrant hypermethylation and to restore UGT1A1 expression in hypermethylated UGT1A1-negative cells but not in hypomethylated cells. Loss of *UGT1A1* methylation was further associated with an increase in UGT1A1 protein content and with an enhanced inactivation of SN-38 by 300% in HCT-116 cells.

Conclusions: We conclude that DNA methylation represses *UGT1A1* expression in colon cancer and that this process may contribute to the level of tumoral inactivation of the anticancer agent SN-38 and potentially influence clinical response.

Colorectal cancer is the third leading cause of cancer-related deaths worldwide. Of patients suffering from colorectal cancer, up to 60% already have overt metastases at the time of diagnosis (1). Irinotecan is the anticancer drug used in first-line treatment of metastatic colorectal cancer (2–5). However, there is marked variability in response to therapy as well as frequency and severity of toxicities. Its active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38), is primarily inactivated through glucuronidation, mediated mainly by the UDP-glucuronosyltransferase UGT1A1 enzyme (6–8). The *UGT1A1* gene encodes an enzyme that acts as a drug detoxifier by catalyzing conjugation reactions with glucuronic acid. Interindividual differences in the inactivating pathway of SN-38 caused by common genetic polymorphisms in the glucuronidation pathway have been shown as major determinants for irinotecan toxicity and clinical outcome (9–13). In particular, the *UGT1A1*28* promoter allele, leading to a lower expression of the gene, was identified as clinically valuable for predicting severe irinotecan toxicity in cancer patients (9, 10, 12).

Another factor that needs to be considered when regarding a patient's response to an anticancer drug is the intrinsic susceptibility of cancer cells defined by the expression of an array of genes that determine local tumor drug concentration. This includes activities of metabolic enzymes, such as UGT1A1, which likely establish local tissue drug concentration. It has been shown that the metabolic capacities of tumor cells influence response outcome, such as the local formation of SN-38 from CPT-11 by carboxylesterases (14). Recently, glucuronidation has been identified as a mechanism of intrinsic drug resistance to SN-38 in human colon cancer cells (15–17). In addition, clinical studies support the hypothesis that the individual glucuronidation capacity contributes to tumoral response, likely through the modulation of both plasma and tumoral concentrations of SN-38 (11, 13). UGT1A1 is expressed with a large variability in primary colon tumors and during colon tumorigenesis, suggesting that the level of UGT1A1 expression may contribute to the differential chemosensitivity of colon tumors (18–20). Although the mechanisms involved in the variability of UGT1A1 expression in colon tumors remain unknown, recent observations support that mechanisms other than genetic polymorphisms may contribute to the differential expression of the UGT1A1 metabolic pathway in colon tumors.

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Table 1. Primers sequences and probes

Name	Primer sequence*
Constructs primers	
Common primer (R)	5′-TGGCAGGAGCAAAGGCGCCGCTCGAGCGG-3′
P-269bp (F)	5′-CGAGCTCGGAGGTTCTGGAAGTACTTGGC-3′
P-540bp (F)	5′-CGACGCGTGTGCTGGACTCAATAAATATTG-3′
P-1316bp (F)	5′-CGACGCGTGGATCTTGGGCCAGTGGAATG-3′
P-2218bp (F)	5′-CGACGCGTGGCCTAGTTCCTAGCATAGTG-3′
Bisulfite sequencing	
1 (F)	5′-ATTAGGGAGTTATAGTTTTTGG-3′
1 (R)	5′-CAACTAACCCTTTACTTACTACA-3′
2 (R)	5′-GGGGAAGATTTTTGTTTATATG-3′
2 (F)	5′-AATACCCACTCCACAACTCC-3′
3 (F)	5′-TGTGAGTTTGGTTTATTTTATGG-3′
3 (R)	5′-TACTTCCAAAACCTCAAAATCC-3′
4 (F)	5′-TTGGTGTAT(CT)GATTGGTTTTTG-3′
4 (R)	5′-CAATAACTACCATCCACTAAAATC-3′
5 (F)	5′-GGTTTTGGAAGTATTTTGTTGTG-3′
5 (R)	5′-CAACCATAAC(AG)CCTTTACTCCTA-3′
6 (F)	5′-GGGTTTAGTGGTGTTTTATGTT-3′
6 (R)	5′-ATAACATCAAAACTACTTTCTACC-3′
7 (F)	5′-GATTTTGTTATGTTTTTGTTTGG-3′
7 (R)	5′-CAAAACTCAATAAATCCTAAACA-3′
8 (F)	5′-GTAGGTTTTAGTTATTTGTTTGAA-3′
8 (R)	5′-CAATCCCAAAAACACTACATC-3′
Primer for automatic sequencing	
1 (F)	5′-GTAATGAAGGTGAGTTTTATAG-3′
1 (R)	5′-CTAAATATCCTAAAAACCTATAC-3′
2 (R)	5′-ATACTACCTACTCACTTATATC-3′
3 (F)	5′-TGTGAGTTTGGTTTATTTTATGG-3′
4 (R)	5′-CAATAACTACCATCCACTAAAATC-3′
5 (F)	5′-GGTTTTGGAAGTATTTTGTTGTG-3′
6 (F)	5′-GGGTTTAGTGGTGTTTTATGTT-3′
7 (R)	5′-CAAAACTCAATAAATCCTAAACA-3′
8 (R)	5′-CAATCCCAAAAACACTACATC-3′
QRT-PCR *UGT1A1*	
QRT-PCR (F)	5′-GACTGTCCAGGACCTATTGAGCTC-3′
QRT-PCR (R)	5′-CATTAATGTAGGCTTCAAATTCCTGG-3′
QRT-PCR (P)	5′-(FAM)ATCATGCCCAATATGGTT(MGB)-3′

Abbreviations: F, forward; R, reverse; P, probe; FAM, carboxyfluorescein; MGB, minor groove binder.
*Accession no. for *UGT1A1* reference sequence: AF297093.

It is well documented that colon cancer arises from a series of genetic alterations that includes a variety of mechanisms of gene silencing, such as point mutations, loss of heterozygosity, and homozygous deletions. In addition to these genetic abnormalities, promoter DNA hypermethylation is an additional mechanism that plays a critical role in the progression of colorectal cancer. It has been shown that sporadic colorectal cancers are often associated with an abnormal methylation of CpG-rich sites (CpG islands) in promoter regions of multiple loci, including genes involved in the cell cycle, growth regulation, DNA repair, metabolism, and apoptosis, whose silencing is a common and early event in human colon neoplasia mediated by epigenetic occurrences (21–24). Cytosine methylation results in transcriptional repression either by interfering with transcription factor binding or by inducing a repressive chromatin structure (25). While exploring the mechanisms involved in the variable expression of UGT1A1 in colon tumors, we hypothesized that, in addition to genetic polymorphisms, such as the common *UGT1A1*28* allele, silencing of the glucuronidation pathway by epigenetic mechanisms could have the potential to induce various effects at the systemic and tissue levels, including an enhanced intratumoral exposure to SN-38. Previous studies support that therapeutic efficacy of anticancer drugs can be predicted by epigenetic modifications (26–29). A notable example is the *MGMT* gene, responsible for repairing DNA damage caused by alkylating chemotherapeutic drugs (30). The extent of promoter methylation of the *MGMT* gene predicts a favorable response to antineoplastic therapy in cancer patients (31).

The mechanisms underlying the inactivation of *UGT1A1* expression in a large proportion of colon tumors and the extent

of its role in determining tumoral response to anticancer drugs remain unknown (18, 19, 32–34). Our study was thus designed to assess if promoter hypermethylation arises as a mechanism that modulates UGT1A1 expression in colon cancer cells and, consequently, that could contribute to determine SN-38 inactivation in colon tumors. Results indicate that epigenetic modifications in the *UGT1A1* gene occur in colon tumor cell lines and that the consequence of UGT1A1 repression is a modulation of anticancer drug exposure in cancer cells.

Materials and Methods

Colon cell lines and primary colon tumor samples. Colon cancer cells HT-29, HCT-116, COLO-320DM, HCT-15, and LOVO were obtained from American Type Culture Collection (Manassas, VA). HT-115 cells were obtained from European Collection of Cell Culture (Salisbury, United Kingdom). All cell lines were growth in the medium recommended by American Type Culture Collection and European Collection of Cell Culture. HCT-116 and HT-29 cells were kept in McCoy's 5A medium with 1.0 mmol/L sodium pyruvate (Sigma, Oakville, Ontario, Canada). HT-115 cells were kept in DMEM with 2 mmol/L L-glutamine (all from Wisent, St-Bruno, Quebec, Canada). LOVO cells were kept in F-12K medium with 2 mmol/L L-glutamine. COLO-320DM and HCT-15 were kept in a modified RPMI 1640 to obtain a final concentration of 2 mmol/L L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mmol/L HEPES, and 1.0 mmol/L sodium pyruvate. All medium were added with 100 IU/mL penicillin, 50 μg/mL streptomycin, and 10% fetal bovine serum, except for the HT-115 cell line, which required 15% fetal bovine serum (Wisent). All cells were incubated at 37°C in a humidified incubator with 5% CO_2 as specified by the supplier.

Primary tumor specimens were obtained from 50 patients who underwent surgery at Hôtel-Dieu de Québec. All patients provided an informed consent and the project was approved by the institutional human research review board. All specimens were quick frozen in liquid nitrogen and stored at −80°C until processing.

Reexpression of UGT1A1 by 2′-deoxy-5-azacytidine and trichostatin A treatment of colon carcinoma cell lines. 2′-Deoxy-5-azacytidine (5-Aza-dC; Sigma) and trichostatin A (New England Biolabs, Beverly, MA) were resuspended in sterile 1× PBS and 100% DMSO, respectively. Treatment with both reagents resulted in final concentrations of 0.1% DMSO or PBS in the culture medium. The treatment groups consisted of trichostatin A alone, 5-Aza-dC alone, or combined trichostatin A and 5-Aza-dC. The control group consisted of medium with 0.1% PBS for the groups treated with 5-Aza-dC alone and medium with 0.1% DMSO for groups treated with trichostatin A alone or in combination with 5-Aza-dC. Cells were seeded 24 hours before treatment in 100-mm^2 dishes to obtain 95% confluence of the control cells after 4 days of culture. For the 5-Aza-dC-treated groups, cells were cultured with 5 μmol/L 5-Aza-dC for 72 hours, whereas trichostatin A–treated groups were incubated for 72 hours in culture medium before adding 300 nmol/L trichostatin A for 16 hours. The groups treated with both agents were incubated with 5 μmol/L 5-Aza-dC for 72 hours followed by trichostatin A treatment (300 nmol/L) for 16 hours. All treatments with 5-Aza-dC and trichostatin A were done as described previously (35).

Expression analysis of* UGT1A1 *by quantitative real-time PCR. RNA from primary tumor samples and cancer cell lines was extracted with Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH) as described in the manufacturer's protocol. RNA was resuspended in RNAsecure water (Ambion, Austin, TX) and concentrations were determined by spectrophotometric reading. RNA (1 μg) was converted to cDNA with SuperScript II RNase H^- (Invitrogen, Burlington, Ontario, Canada) using the manufacturer's protocol. Relative expression of *UGT1A1* was measured by quantitative real-time PCR (QRT-PCR) using the ABI Prism 7000 Sequence Detector. Analyses were done in 25 μL reaction volumes using 1 μL of cDNA template, 12.5 μL of 2× Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 850 nmol/L of each primer, and 200 nmol/L *UGT1A1* probe. The primers and probes for *UGT1A1* were designed with the Primer Express Software (Applied Biosystems). All primer sequences are shown in Table 1 and were designed to be exon-exon so that none amplified genomic DNA (data not shown). PCR amplification began with a 95°C, 10-minute step to activate the AmpliTaq Gold DNA polymerase followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The housekeeping genes used were eukaryotic endogenous 18S rRNA and human endogenous β_2-microglobulin control (Applied Biosystems) quantified according to the manufacturer's protocols. Relative gene expression data were calculated using the Δ(Δ)Ct method described previously by K. Livak (PE-ABI; Sequence Detector User Bulletin 2). This method uses a single sample, termed calibrator sample, for comparison with samples of unknown gene expression level. The calibrator sample is analyzed on every assay plate with the unknown samples of interest. Results are obtained with the following formula: fold induction = $2^{-[\Delta(\Delta)Ct]}$; Δ(Δ)Ct is defined as [Ct g_i (unknown sample) − Ct β_2-microglobulin (unknown sample)] − [Ct g_i (calibrator sample) − Ct β_2-microglobulin (calibrator sample)], where g_i is the gene of interest. The calibrator sample can be any sample chosen to represent 1-fold expression of the gene of interest (in this study, untreated cells).

Transient transfection of* UGT1A1 *promoter constructs in colon cell lines. Variable-length sequences of the *UGT1A1* promoter region (269, 540, 1,316, and 2,218 bp) were amplified from genomic DNA using the primers listed in Table 1 and were directionally cloned into the *Mlu*I/*Xho*I sites for 540-, 1,316-, and 2,218-bp constructs and the *Sac*I/*Xho*I sites for 269-bp constructs of the pGL3 basic vector (Promega, Madison, WI). All constructs were sequenced before functional analysis. Each plasmid was methylated and mock methylated (without *S*-adenosylmethionine) with *Sss*I methylase (New England Biolabs) using the manufacturer's protocol. Confirmation of complete methylation was assessed by digestion with the *Hpa*II restriction enzyme that cuts only the unmethylated CCGG restriction sites in the plasmid. Migration on agarose gel confirmed that methylated plasmids remain undigested after *Hpa*II digestion in contrast to unmethylated plasmids that were digested in multiple fragments depending on the length of the promoter regions. This method has been successfully done in previous studies (36–38). DNA was purified with the Qiaex II kit (Qiagen, Hilden, Germany) and resuspended in Tris-HCl (pH 7.9) for subsequent transfection. Cells were plated in 24-well microplates at 70% confluence in growth medium. For each well, 0.7 μg reporter construct was cotransfected with 0.1 μg *Renilla* luciferase plasmid and 3 μL Exgen-500 (Promega) into HCT-116, HCT-15, COLO-320DM, LOVO, and HT-115 cells using Exgen-500 transfection reagent (MBI Fermentas, Burlington, Ontario, Canada) according to the manufacturer's protocol. For the cell line HT-29, transfections were done with Lipofectamine 2000 (Invitrogen) as described by the manufacturer. Briefly, cells were plated in 24-well microplates at 90% confluence in growth medium. Reporter construct (0.6 μg) was cotransfected with 0.2 μg *Renilla* luciferase plasmid (Promega) using 3 μL Lipofectamine 2000 per well. Cells were harvested 24 hours after transfection and assayed for promoter activity by use of the Dual-Luciferase Reporter Assay System according to the manufacturer's recommendations (Promega). Luciferase activity was measured by using 40 μL of cell lysate in a 96-well plate on an LB96V microplate luminometer (EG&G Berthold, Bad Wildbad, Germany).

Sodium bisulfite genomic DNA modification and DNA sequencing. To analyze CpG methylation of the *UGT1A1* 5′ region, genomic DNA was extracted from colon cell lines with Tri-Reagent as described in the manufacturer's protocol. The bisulfite reaction, converting all unmethylated but not methylated cytosines to uracil, was done as follows. DNA (1 μg) was modified by sodium bisulfite using the CpGenome DNA Modification kit (Intergen, Purchase, NY) according to the

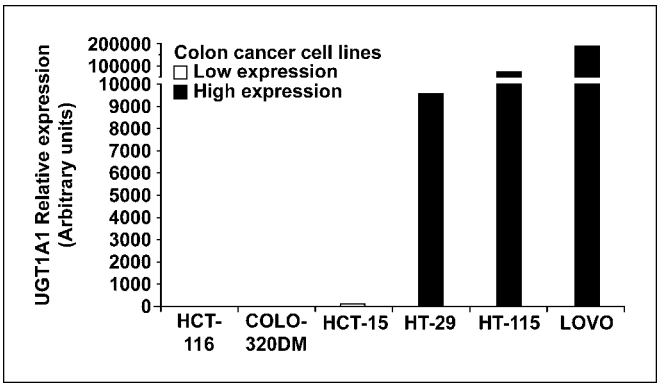

Fig. 1. Relative expression of *UGT1A1* mRNA in colon cancer cell lines. *Columns,* mean of relative expression of *UGT1A1* (arbitrary units) determined by QRT-PCR. The housekeeping gene was used as an endogenous control and showed a coefficient of variation of 15%.

manufacturer's instructions. Amplification primers used for bisulfite sequencing are described in Table 1. All primers used were designed with the demo version of the Primer Premiere software (Premier Biosoft, Palo Alto, CA), which predicts primer hairpins, dimers, and false priming and consequently allows very high primer quality. Nested primers were used for sequencing whenever amplification primers did not allow good readings. Sequences obtained using such primers had excellent quality and low background. Modified DNA (100 ng) was amplified with the following conditions for each primer pairs: 95°C, 10-minute step to activate the AmpliTaq Gold DNA polymerase followed by 40 cycles at 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute. PCR products were purified with the QIAquick PCR Purification kit (Qiagen) and eluted in 30 μL nuclease-free water. DNA cycle sequencing was carried out in 20 μL reactions on a Perkin-Elmer GenAmp PCR System 9700 (Perkin-Elmer, Foster City, CA) using 6 μL purified PCR product, 3.2 pmol primer, and the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer) as described by the manufacturer. The methylation percentage corresponds to the fluorescence peak area values of cytosine divided by the addition of the fluorescence peak area values of cytosine and thymidine. Methylation status was calculated as the mean of the methylation percentages of CpG for each CpG region and includes all CpG in that region. The same procedure was used in previous studies (39, 40).

Western blot analyses. Western blot analyses of UGT1A1 protein were done on microsomal preparations isolated from the HCT-116 cell line after treatment with 5-Aza-dC alone or in combination with trichostatin A as described previously (41). Commercial microsomal preparations of liver and ileum (n = 5 subjects pools; Tissue Transformation Technologies, Edison, NJ), in addition to a preparation of HEK293-UGT1A1 (BD Gentest, Woburn, MA), were used as comparative models. Briefly, 90 μg of microsomal proteins were separated by 10% SDS-PAGE. The separated proteins were transferred onto nitrocellulose membranes and probed with a specific anti-human UGT1A1 antiserum (1:2,500 dilution) as described previously (42).

SN-38 glucuronidation assays. SN-38 was generated from irinotecan HCl (McKesson, ON, CA) as follows: 1.25 mL NaOH (6 mol/L) was added to 2.5 mL irinotecan HCl (20 mg/mL) and incubated overnight at 55°C in the dark. The solution was neutralized with 1.25 mL HCl (6 mol/L), and 150 μL HCl (6 mol/L) was added to obtain pH ≤3. The solution was incubated for 3 hours at room temperature in the dark. The pH was readjusted to 3 with 1 mol/L NaOH and the solution was centrifuged at 3,000 rpm for 10 minutes. The supernatant was removed and the pellet was washed twice with 2.5 mL ultrapure water. The product was frozen in a bath of dry ice/ethanol and placed in a lyophilizator. The product was certified by high-performance liquid chromatography with mass spectrometry and UV detection analysis for purity ≥97%. SN-38 glucuronide was produced from enzymatic assays with human liver microsomes, isolated, and quantified on a SN-38 calibration curve. The enzymatic assays with human colon cancer cell lines were done as described previously (41). Briefly, microsomal fractions (120 μg) were added to the reaction mixture containing alamethicin (60 μg/mg protein) and 10 μmol/L SN-38 in a final volume of 100 μL. The assays were incubated at 37°C in a shaking water bath for 4 hours. The reaction was stopped in 200 μL methanol plus 1% HCl (2 N) followed by centrifugation at 14,000 × g for 10 minutes. The supernatant was evaporated under nitrogen at 35°C and resuspended in a solution of methanol and water (1:1) and 0.5% HCl (2.0 mol/L). Detection of SN-38 and SN-38 glucuronide was done by high-performance liquid chromatography coupled with fluorescence detection as described previously (41).

Statistical analyses. All statistical analyses were done using the JMP V4.0.2 software (SAS Institute, Cary, NC). All P values shown were obtained with rank sums of the Wilcoxon/Krustal-Wallis test.

Results

UGT1A1 ***expression in primary colon tumors and colon cancer cell lines.*** To confirm previous reports of variable expression of *UGT1A1* in colon tumor samples, its expression was analyzed by QRT-PCR in 50 primary colon tumor samples. Results showed a coefficient of variability of 350% with two groups of baseline levels of *UGT1A1* mRNA; tumors with high expression levels (18%; up to 12,112 arbitrary units) and those with undetectable or very low UGT1A1 mRNA content [82%; expression level <400 arbitrary units (1-382); data not shown]. With the objective to study cellular models that represent this array of *UGT1A1* expression, we analyzed six human colon carcinoma cell lines. Of the cell lines investigated, half did not express *UGT1A1*. At baseline, expression of the *UGT1A1* mRNA transcript was undetectable in HCT-116 and COLO-320DM (1 relative unit) and very low in HCT-15 (108 relative units). In contrast, HT-29, LOVO, and HT-115 cell lines were associated with high baseline expression of *UGT1A1* (Fig. 1). The role of methylation in the silencing of *UGT1A1* gene expression in cell lines was then investigated.

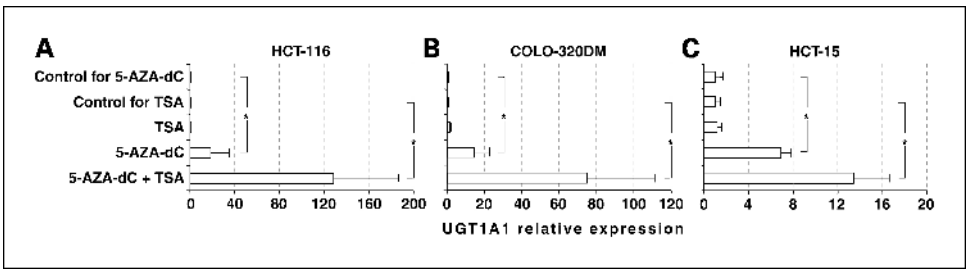

Fig. 2. *UGT1A1* expression following 5-Aza-dC (5 μmol/L) and/or trichostatin A (*TSA*; 300 nmol/L) treatment in colon cancer cell lines. Results for all three low *UGT1A1* mRNA expression cell lines: (*A*) HCT-116, (*B*) COLO-320DM, and (*C*) HCT-15. All expression results were assessed by QRT-PCR using β_2-microglobulin as an endogenous control. DMSO and PBS did not modify *UGT1A1* mRNA expression. *Columns,* mean of two separated experiments done in triplicate; *bars,* SD. *, $P < 0.05$.

Table 2. Relative *UGT1A1* expression compared with the control group for each cell line

Colon adenocarcinoma cell lines	Control for 5-Aza-dC (PBS)	Control for TSA (DMSO)	5-Aza-dC (fold increased)	TSA (fold increased)	5-Aza-dC + TSA (fold increased)
HCT-116	1	1	19 (19)	1 (1)	128(128)
COLO-320DM	1	1	15 (15)	1 (1)	75 (75)
HCT-15	309	244	2,132 (7)	293 (1)	4,075 (13)

NOTE: Cells were treated as described in Materials and Methods with 5-Aza-dC alone (5 μmol/L) or in combination with trichostatin A (300 nmol/L).

Effects of 5-Aza-dC and trichostatin A treatment on* UGT1A1 *expression in colon cancer cell lines. To study the potential role of methylation in the silencing of *UGT1A1* gene expression, cell lines were treated with 5-Aza-dC and/or trichostatin A, demethylating and histone deacetylase inhibitor agents, respectively. QRT-PCR analysis revealed, on treatment with 5-Aza-dC alone, a significant increase in *UGT1A1* mRNA (up to 19-fold) for cells with a very low expression of the gene (HCT-116, COLO-320DM, and HCT-15; Fig. 2, Table 2). The combination of 5-Aza-dC and trichostatin A treatment increased the expression of *UGT1A1* by 13-fold for the HCT-15 cell line and up to 75- to 128-fold for the COLO-320DM and HCT-116 cell lines, respectively (Fig. 2), compared with a modest increase (up to 3-fold) for cells with high basal *UGT1A1* expression (data not shown). For all cell lines, trichostatin A alone failed to increase significantly *UGT1A1* expression. These results suggest that demethylation by 5-Aza-dC restored the expression of the *UGT1A1* gene, supporting that methylation is a mechanism affecting the transcription of *UGT1A1* mRNA in various colon cancer cell lines with very low basal expression of this gene.

In vitro *methylation of the* UGT1A1 *promoter represses transcriptional activity.* To assess if the methylation of CpG in the promoter region of *UGT1A1* affects directly the transcriptional activity of the gene, we studied various lengths of methylated and unmethylated *UGT1A1* promoters. Four promoter constructions were analyzed for luciferase activity after *in vitro* methylation (Fig. 3*A*). For methylated promoter constructions, a plasmid digestion was done with the *Hpa*II restriction enzyme to ensure that methylation was complete. *Hpa*II cuts only unmethylated CCGG sites (Fig. 3*B*). Luciferase expression of unmethylated plasmids (Fig. 3*C-E*) varied from 5- to 25-fold over the pGL3 basic vector and *UGT1A1* expression was almost completely abolished for the methylated plasmids. Significant promoter activity in cell lines expressing undetectable or very low *UGT1A1* mRNA levels indicated the presence of transcription factors required for *UGT1A1* expression. These results show that methylation of the *UGT1A1* promoter directly affects the transcriptional level of the gene.

Methylation profiles of colon cancer cell lines correlate with* UGT1A1 *expression. The promoter of *UGT1A1* encompasses a region of at least 3.3 kb upstream of the start codon involved in the constitutive and inducible expression of the gene (43). We therefore studied at least 5 kb of the 5′ untranslated region for the presence of CpG islands and included sequences of the first exon.

The methylation profiles of all six colon cancer cell lines were determined for CpG +15 to −21 in the proximal promoter and first exon and for CpG −65 to −49 in the distal promoter (Fig. 4*A*). Correlation analyses were then done between gene expression and methylation levels. Five CpG regions covering the promoter and the first exon of the *UGT1A1* gene showed significant differences between high and low *UGT1A1* expression cell lines (Fig. 4*B*). Regions, including CpG −16 to −13 (region D), −5 to −1 (region B), and +3 to +6 (region A), exhibited the most significant correlations with *UGT1A1* mRNA expression ($P < 0.01$). Two other regions, including CpG −21 to −18 (region E) and −9 to −7 (region C), were also

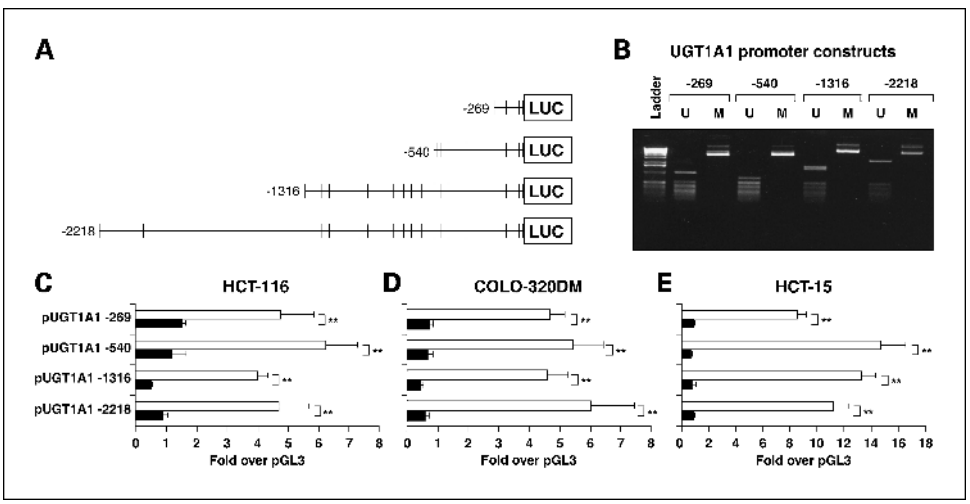

Fig. 3. Repression of *UGT1A1* promoter activity by DNA methylation in luciferase constructs. *A, UGT1A1* promoter constructs of variable lengths and their associated CpG. *B,* digestion of plasmid DNA with the methylation-sensitive restriction enzyme *Hpa*II, which cuts CCGG but not C^{m}CGG sites. Luciferase activity in fully methylated (*black*) and unmethylated (*white*) promoter constructs for all three low *UGT1A1* mRNA expression cell lines: (*C*) HCT-116, (*D*) COLO-320DM, and (*E*) HCT-15. *Columns,* mean of two separate experiments done in triplicate; *bars,* SD. *LUC,* luciferase; *M,* methylated; *U,* unmethylated. **, $P < 0.01$.

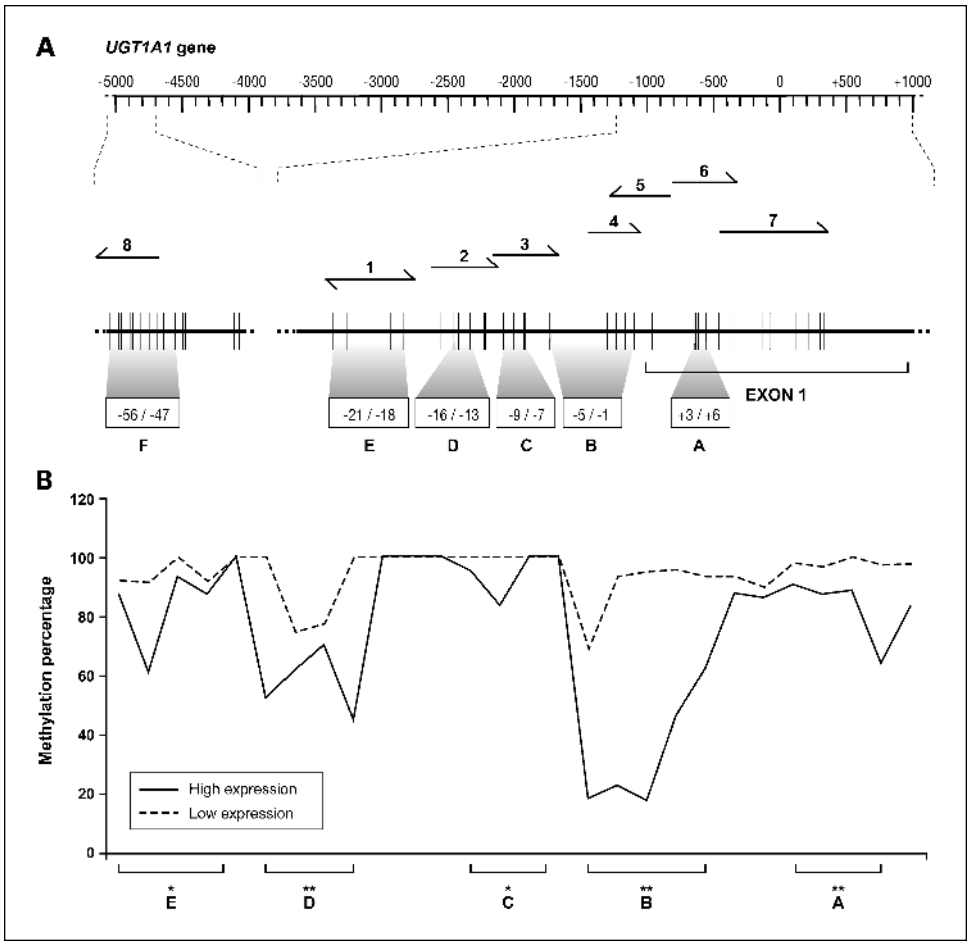

Fig. 4. Schematic representation of the *UGT1A1* gene methylation profile. *A,* schematic map of the CpG-rich regions (*regions A-F*) in *UGT1A1. Vertical ticks,* CpG sites. Amplicons 1 to 8 produced by PCR for bisulfite sequencing are also indicated. *Arrow,* direction of the bisulfite sequencing (for each amplicon). Nucleotide position of the C moiety of CpG regions relative to the ATG are as follows: F (−5,000 to −4,885), E (−1,157 to −892), D (−704 to −642), C (−513 to −432), B (−330 to −4), and A (+21 to +233) based on the *UGT1A1* reference sequence AF297093. *B,* methylation profiles of colon cancer cell lines in regions A to E. Methylation status is presented as the mean of the methylation percentages of CpG for the indicated region and includes all CpG in that region. Comparison was done between percentages for a specific region in UGT1A1-negative and UGT1A1-positive cell lines. For significantly different methylation percentages between low-expressing and high-expressing cell lines, regions were reamplified and resequenced multiple times (at least thrice) for confirmation. *, $P < 0.05$; **, $P < 0.01$.

found in the promoter ($P < 0.05$). All other CpG islands analyzed (CpG +8 to +15 and CpG −65 to −49) were found to be fully methylated in all cell lines (data not shown). Three of these regions were predicted as CpG islands by the CpGPlot program (http://www.ebi.ac.uk/emboss/cpgplot/) and include CpG regions A, D, and F. Regions A and D exhibited the highest differences in methylation profiles between high and low *UGT1A1* expression cell lines. In contrast, the third region (F) predicted by CpGPlot (from −47 to −56) was fully methylated for all CpG sites. These results suggest that the extent of methylation of specific CpG regions in the *UGT1A1* gene predicts its expression in colon cancer cell lines.

Demethylating treatment increases the inactivation of the anticancer agent SN-38 in HCT-116 cells. Compared with the SN-38-sensitive HCT-116 cell line, HT-29 cells were reported previously to exhibit intrinsic resistance to SN-38, owing to enhanced drug clearance via glucuronidation (15, 17). UGT1A1 is undetectable in HCT-116 cells but is abundant in HT-29 cells. These data suggest that the extent of promoter methylation could determine *UGT1A1* expression and, ultimately, local SN-38 tumor concentrations. To ascertain this hypothesis, we assessed the capacity of 5-Aza-dC and trichostatin A treatment to modulate UGT1A1 protein levels and SN-38 glucuronidation in HCT-116 cells. Western blotting and SN-38 glucuronidation activities were done with microsomal subcellular fractions, where UGTs are localized. In treated cells, UGT1A1 protein levels were significantly higher compared with control cells (Fig. 5*A*). In parallel, SN-38 glucuronide formation was increased by 300% in treated HCT-116 cells compared with untreated cells (Fig. 5*B*). The analysis of the *UGT1A1* genomic regions by bisulfite treatment followed by sequencing confirmed the absence of methylated CpG in treated cells. These results show that the absence of a high percentage of methylation in the *UGT1A1* promoter is associated with a significantly higher SN-38 glucuronidation capacity of tumor cells.

Discussion

Transcriptional silencing by CpG island hypermethylation is a crucial event in colon carcinogenesis. This epigenetic mechanism has also been identified as a predictor of therapeutic efficiency for anticancer drugs used to treat various neoplasia (26, 27, 29, 44). In this study, we showed that DNA methylation governs the expression of the *UGT1A1*-metabolizing gene in colon cancer and that this mechanism may contribute to the differential chemosensitivity of colon tumors to irinotecan by influencing cellular inactivation of its active metabolite SN-38.

In this study, of the 50 primary colon tumors investigated for *UGT1A1* expression, >82% showed undetectable or extremely low levels of *UGT1A1* mRNA content. Previous studies showed that *UGT1A1* is expressed in normal colon, but our results clearly indicate that its expression is lost in most colon tumors (32–34). On the other hand, >18% of tumors overexpressed

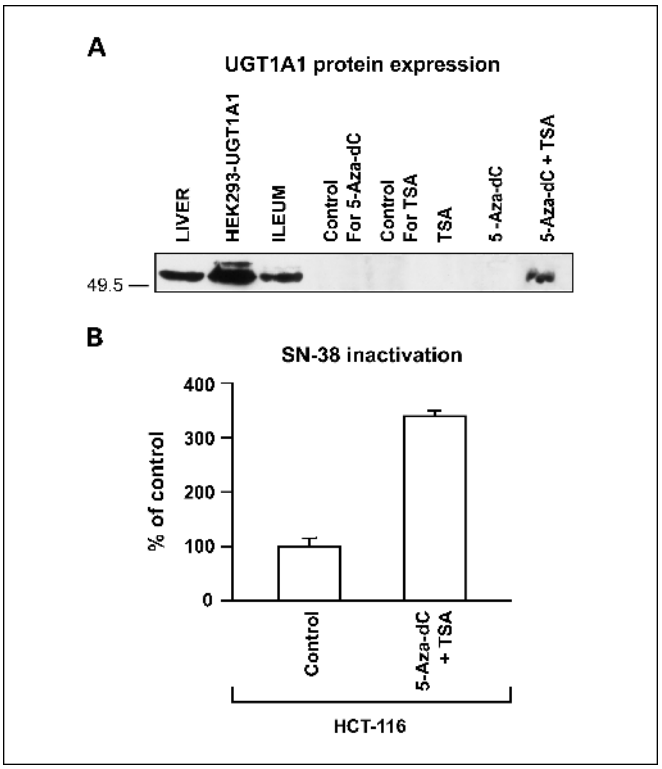

Fig. 5. Demethylation treatment increases protein levels and inactivation of SN-38 through glucuronidation. *A,* Western blot analysis of UGT1A1 protein after treatment with 5-Aza-dC alone or in combination with trichostatin A for the HCT-116 cell line. *B,* enzymatic assays for SN-38 glucuronidation with the isolated microsomal preparations from cells treated with both 5-Aza-dC and trichostatin A. Percentage of SN-38 glucuronide formation.

UGT1A1, a characteristic recently associated with a resistance phenotype to SN-38 in colon cancer cells (15).

Transcriptional regulation of drug-metabolizing genes could occur through a variety of mechanisms, and DNA methylation constitutes a well-documented process for regulating gene transcription, especially in cancer (21–24). The role of epigenetics has never been reported previously for *UGT* genes. To investigate on the role of methylation in silencing *UGT1A1* gene expression, *UGT1A1*-negative adenocarcinoma colon cell lines were selected as *in vitro* models representing a large proportion of primary colon tumor samples that do not express *UGT1A1*. In these colon cancer cells lacking basal *UGT1A1* expression, hypermethylation of CpG islands was observed. Treatment of *UGT1A1*-negative colon cell lines with the DNA methyltransferase inhibitor 5-Aza-dC restored *UGT1A1* expression, supporting that this gene is regulated by methylation. Besides, the drastic repression of reporter gene expression in the presence of methylated *UGT1A1* promoters compared with unmethylated constructs in colon cell lines is consistent with a direct effect of DNA methylation on *UGT1A1* gene expression. Additionally, the significant luciferase activity induced by unmethylated *UGT1A1* promoter in colon cell lines that do not express basal *UGT1A1* mRNA indicates that the loss of *UGT1A1* expression in these cells is not caused by a lack of transcription factors required for *UGT1A1* expression. Furthermore, recent studies have established a link between two important epigenetic modifications, DNA methylation and histone acetylation, in the regulation of gene expression and silencing. In addition to DNA methylation, histone acetylation mediates changes in the nucleosomal and chromatin structure of promoters, presumably affecting the accessibility of transcription factors to their *cis*-regulatory elements (45). In our experiments, a remarkable synergic increase of *UGT1A1* expression was shown for the combined treatment of 5-Aza-dC and trichostatin A. Results support that this combined treatment is more active on methylation status and chromatin conformation to increase *UGT1A1* mRNA expression as observed for other hypermethylated genes (46, 47). Altogether, results designate DNA hypermethylation as one of the mechanisms responsible for *UGT1A1* repression in colon cancer cell lines.

Analyses of *UGT1A1* promoter methylation status in cell lines revealed that the extent of methylation in specific regions of the promoter predicts *UGT1A1* gene expression. Several dinucleotide CpG sequences are located within the core motif of putative sites for transcription factors. *In vitro* experiments with variable lengths of *UGT1A1* promoters were also valuable in defining the potential CpG sites involved in transcriptional silencing. Data suggest that *UGT1A1* is silenced as a result of CpG hypermethylation within a minimal region encompassing 260 bp upstream of the start codon. A previous study reported that *UGT1A1* is regulated by HNF1α and HNF1β through a putative HNF1 site in the proximal promoter (48). The localization of CpG islands in this study reveals that the HNF1 site is located in the methylated region B, between CpG −1 and −4. Additional analyses are needed to determine if methylation prevents the binding of HNF1α and HNF1β or other transcriptional factors on these putative binding sites in colon tissues.

The exact role of UGT1A1 in colon carcinogenesis remains unknown, but there are potential consequences to the repression of this metabolic pathway in relation to cancer therapy. In our study, treatment with 5-Aza-dC and trichostatin A had the capacity to restore UGT1A1 protein expression in HCT-116 cells, lacking basal *UGT1A1* expression, and resulted in cells that efficiently inactivate SN-38 through glucuronidation. These results support that exposure to SN-38 and the intrinsic susceptibility of cancer cells may be partially defined by *UGT1A1* promoter methylation status and expression. The large family of UGT enzymes, broadly expressed in human tissues, belongs to the phase II drug-metabolizing enzymes involved in ~35% of phase II drug-metabolizing reactions for therapeutic drugs, including several anticancer drugs (49). Similar to other cytotoxic drugs, response to irinotecan in colon cancer patients may be determined by alterations in the metabolism of active metabolites. SN-38 is largely metabolized in human by glucuronidation and mostly by UGT1A1 (6–8). An important consequence associated with positive *UGT1A1* methylation, and subsequent repression of the UGT1A1-mediated metabolic pathway, is the prospect that colon tumors associated with lower rates of SN-38 glucuronidation would retain higher levels of the compound. This could lead to a higher sensitivity to irinotecan. Conversely, tumor cells that overexpress *UGT1A1* have the ability to inactivate a large proportion of the active drug that reaches the tumor. The presence of high levels of UGT activity and expression was identified as a characteristic recently associated with a resistance phenotype to SN-38 in colon cancer cells (15).

Several studies recently exposed the clinical importance of variable UGT1A1 activity governed by genetic polymorphisms in response to irinotecan-based chemotherapy (9–13). These investigations were focused on the role of constitutive variations of the *UGT1A1* gene and showed that UGT1A1-deficient patients are at higher risk for severe hematologic and gastrointestinal toxicities. However, another important finding is the link between glucuronidation genotypes and variable response of cancer patients to irinotecan (11, 13). These observations further support that impaired glucuronidation may determine chemotherapeutic response by influencing drug concentration in tumors. Consequently, a contribution of the epigenetic silencing of UGT1A1 to irinotecan sensitivity of colon tumors is expected. In addition, silencing of UGT1A1 activity would affect other molecules metabolized primarily by this enzyme.

In conclusion, the present findings support the notion that methylation of *UGT1A1*, in addition to common genetic polymorphisms, may contribute to define tumors likely to respond to irinotecan in opposition to tumors that overexpress *UGT1A1*. The effect on irinotecan therapy outcome of aberrant *UGT1A1* hypermethylation in colon cancer deserves further attention.

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