DNA methylation and sensitivity to antimetabolites in cancer cell lines

SHIN SASAKI[1,2], TAKASHI KOBUNAI[3], JOJI KITAYAMA[1] and HIROKAZU NAGAWA[1]

[1]Department of Surgical Oncology, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655;
[2]Department of Surgery, Kanto Rosai Hospital, 1-1 Kizukisumiyoshi-cyo, Nakahara-ku, Kawasaki 211-8510;
[3]Taiho Pharmaceutical Co., Ltd., 224-2, Hiraishi-Ebisuno, Tokushima 771-9194, Japan

Abstract. The prediction of the cellular direction of metabolic pathways toward either DNA synthesis or DNA methylation is crucial for determining the susceptibility of cancers to antimetabolites such as fluorouracil (5-FU). We genotyped the methylenetetrahydrofolate reductase (*MTHFR*) gene in NCI-60 cancer cell lines, and identified the methylation status of 24 tumor suppressor genes using methylation-specific multiplex ligation-dependent probe amplification. The susceptibility of the cancer cell lines to seven antimetabolites was then determined. Cells homozygous for CC at MTHFR-A1298C were significantly more sensitive to cyclocytidine, cytarabine (AraC) and floxuridine than those with AA or AC (p=0.0215, p=0.0166, and p=0.0323, respectively), and carried more methylated tumor suppressor genes (p=0.0313). Among the 12 tumor suppressor genes which were methylated in >25% of cancer cell lines, the methylation status of *TIMP3*, *APC* and *IGSF4* significantly correlated with sensitivity to pyrimidine synthesis inhibitors. In particular, cells with methylated *TIMP3* had reduced mRNA levels and were significantly more sensitive to aphidicolin-glycinate, AraC and 5-FU than cells with unmethylated *TIMP3*. We speculate that MTHFR-A1298C homozygous CC might direct the methylation rather than the synthesis of DNA, and result in the methylation of several tumor suppressor genes such as *TIMP3*. These genes could be useful biological markers for predicting the efficacy of antimetabolites.

Introduction

Thymidylate synthase (TS) is a key enzyme involved in the reductive methylation of deoxyuridine-5'-monophosphate (dUMP) to deoxythymidine-5'-monophosphate (dTMP), leading to pyrimidine *de novo* deoxynucleotide biosynthesis. Hence, regulation of this enzyme is important for DNA synthesis, and it has been accepted as an excellent target for chemotherapy (1-4). The co-substrate for TS is 5,10-methylenetetrahydrofolate (5,10-methylene THF, or CH2THF), which contributes to the formation of a ternary complex with TS and fluorodeoxyuridine monophosphate (FdUMP), a metabolite converted from 5-fluorouracil (5-FU) (5).

5,10-Methylene THF stabilizes the complex (6,7) and its intracellular concentration is mainly regulated by methylenetetrahydrofolate reductase (MTHFR), an enzyme that irreversibly converts 5,10-methylene THF into 5-methyltetrahydrofolate (5-methyl THF) (8,9). 5-Methyl THF is a substrate for the conversion of homocysteine to methionine, and subsequently to S-adenosylmethionine (SAdoMet), which is a methyl group donor that contributes to intracellular methylation, including hypermethylation of the promoter regions of several tumor suppressor genes (8-10). Predicting the direction of the metabolic pathway of reductive folates, whether for DNA synthesis or methylation, is crucial for determining the susceptibility of cancers to antimetabolites such as 5-FU. Two single nucleotide polymorphisms (SNPs) of *MTHFR*, C677T and A1298C, alter the activity of the methylenetetrahydrofolate reductase enzyme, and have been intensively evaluated for their association with 5-FU efficacy (11-13). However, there are many discrepancies between the results.

Recently methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) has been developed, which utilizes the methylation-sensitive restriction enzyme *Hha*I. This enables the promoter methylation status and copy number changes of up to 40 selected sequences to be identified (14,15). This simple and reliable technique has been used in several clinical investigations (16-19).

Correspondence to: Dr Shin Sasaki, Department of Surgery, Kanto Rosai Hospital, 1-1 Kizukisumiyoshi-cyo, Nakahara-ku, Kawasaki 211-8510, Japan
E-mail: sasakishin@kantoh.rofuku.go.jp

The aim of this study was to evaluate the association between cytodirection for DNA synthesis/methylation and sensitivity to antimetabolites. This was achieved by genotyping the *MTHFR* gene, and determining the methylation status of tumor suppressor genes and the susceptibility of well-characterized NCI-60 cancer cell lines to antimetabolites.

Materials and methods

Cancer cell lines and preparation of genomic DNA. Twenty-seven cancer cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD). These were part of the 59 cancer cell lines intensively characterized by the National Cancer Institute (NCI), to include cytotoxicity data for 70,000 compounds (http://dtp.nci.nih.gov). The cells were maintained in recommended culture medium and grown in 10-cm culture dishes. Genomic DNA was isolated using the Wizard genomic DNA purification kit (Promega, Madison, WI) according to the manufacturer's protocol.

MTHFR and CBS genotyping. Genotyping of *MTHFR* and cystathionine ß-synthase (*CBS*), genes involved in the metabolic pathway of reductive folates, was performed using previously described PCR/RFLP methods (20) with some modifications. Briefly, 15 μl PCR reactions contained 20 ng genomic DNA, 7.5 μl 2X HotStar Taq Master mix (Qiagen, Tokyo, Japan), and 5 pmol of each primer. The primers for MTHFR-C677T were as follows: forward 5'-TGA AGG AGA AGG TGT CTG CGG GA-3'; reverse 5'-AGG ACG GTG CGG TGA GAG TG-3'. Primers for MTHFR-A1298C were as follows: forward 5'-GCA AGT CCC CCA AGG AGG-3'; reverse 5'-GGT CCC CAC TTC CAG CAT C-3'.

MTHFR-C677T amplification was for 35 cycles at 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 60 sec. MTHFR-A1298C was amplified for 35 cycles at 94˚C for 30 sec, 57˚C for 30 sec and 72˚C for 30 sec. PCR products were digested overnight at 37˚C with *Hinf*I or *Mbo*II for MTHFR-C677T and A1298C, respectively.

For CBS 844ins68bp, the primers were as follows: forward 5'-CTG GCC TTG AGC CCT GAA-3'; reverse 5'-GGC CGG GCT CTG GAC TC-3'. The PCR conditions were the same as for MTHFR C677T. All PCR products were resolved on 3% agarose gels which differentiate bands produced by wild and variant alleles: at 198 bp and at 175 and 23 bp for MTHFR-C677T; at 79, 37, and 29 bp and at 108 and 37 bp for MTHFR-A1298C; and at 184 bp and 252 bp for CBS 844ins68bp.

MS-MLPA. Normal genomic DNA (Promega) and CpGenome™ Universal methylated DNA (Chemicon International Inc., Temecula, CA) were used as controls. Control DNA and genomic DNA (50 ng) isolated from the 27 cancer cell lines were subjected to MS-MLPA analysis using a SALSA MS-MLPA kit ME001 tumor suppressor (FALCO biosystems, Kyoto, Japan) according to the manufacturer's protocol. The MLPA PCR reaction was analyzed by ABI-310 genetic analyzer and GeneMapper software v3.5 (Applied Biosystems, Foster City, CA).

Preparation of total RNA and quantitative real-time PCR. Total RNA was isolated from cells grown in 10-cm culture
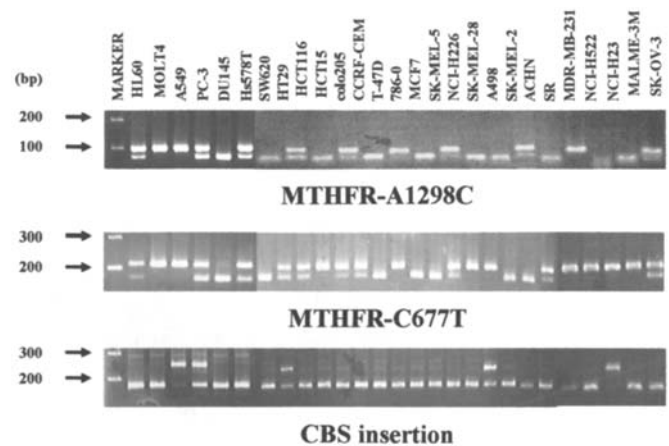

Figure 1. Genotyping of *MTHFR* and *CBS*. Genotyping of *MTHFR* and *CBS* in 27 cancer cell lines using PCR/RFLP. The expected band sizes are described in the Materials and methods.

Table I. Result of genotyping of MTHFR and genomic variants of CBS 844ins68bp.

MTHFR-A1298C	AA	AC	CC
	13 (48.2%)	7 (25.9%)	7 (25.9%)
MTHFR-C677T	CC	CT	TT
	10 (37.0%)	10 (37.0%)	7 (26.0%)
CBS 844ins68	NN	NI	II
	22 (81.5%)	3 (11.1%)	2 (7.4%)

NN, homozygous for the absence of insertion; NI, heterozygous for the insertion; II, homozygous for the insertion.

dishes using Isogen (Nippongene, Tokyo, Japan), according to the manufacturer's protocol. Quantitative real-time PCR was performed on a 7300 real-time PCR system (Applied Biosytems) using TaqMan EZ RT-PCR core reagents, primers and TaqMan probes for TIMP3 (Hs00165949_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Hs99999905_m1) from Assay-on-Demand sets (Applied Biosystems). PCR reaction mixtures (25 μl) contained 20 ng total RNA, 5 μl 5X TaqMan EZ buffer, 3 mM manganese acetate, 300 μM dATP, dCTP, dGTP, and dUTP, 2.5 U rTth DNA polymerase, 0.25 U AmpErase UNG, and 1.25 μl each primer and TaqMan probe mix. Thermal cycling conditions were 50˚C for 2 min and 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. Quantification was performed using the relative standard curve method. A relative TIMP3 expression value was obtained by dividing the TIMP3 value by the GAPDH value. Experiments were performed in triplicate and the mean of each relative TIMP3 expression value was calculated.

Statistical analysis. Correlations were analyzed using the Student's t-test with JMP software (SAS Institute Inc., Cary, NC). Probability (p) values <0.05 were considered statistically significant.
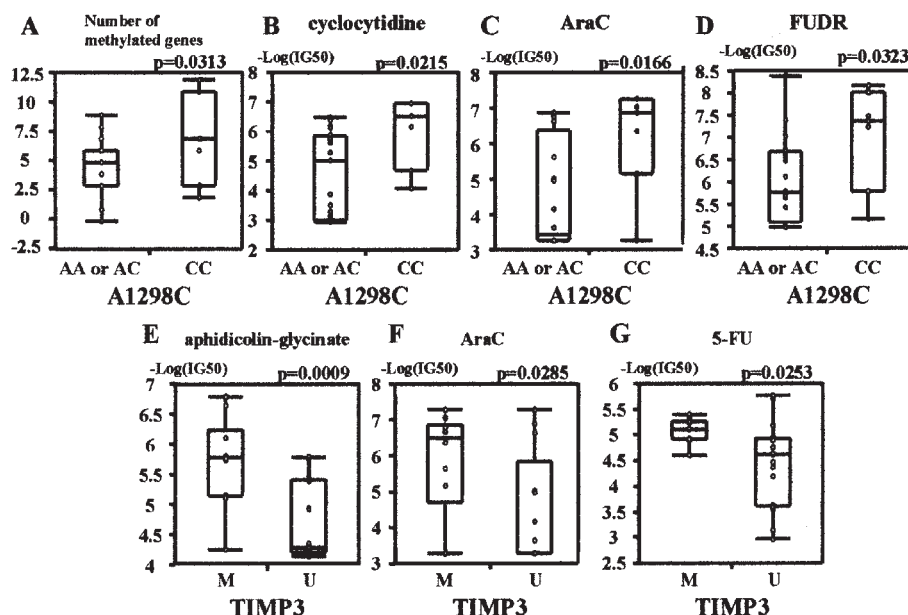
Figure 2. (A) Analysis of association between MTHFR-A1298C genotyping and number of methylated tumor suppressor genes. The vertical lines indicate the 25th and 75th quantiles, and the line across the middle of the box indicates the median sample value. Analysis of association between MTHFR-A1298C genotyping and chemosensitivities to cyclocytidine (B), AraC (C) and FUDR (D). MTHFR-A1298C homozygous CC cells were significantly more sensitive than those with AA or AC (p=0.0215, p=0.0166, p=0.0323, respectively). IG50 represents the concentrations required to inhibit growth by 50%. Analysis of association between methylation status of *TIMP3* and chemosensitivities to aphidicolin-glycinate (E), AraC (F), and 5-FU (G). Cells with methylated *TIMP3* were significantly more sensitive to chemodrugs than those with unmethylated *TIMP3* (p=0.0009, p=0.0285, p=0.0253, respectively).

Results

Genotyping of MTHFR and CBS genomic variants. For the *MTHFR* polymorphism C677T, 10 of 27 cell lines (37.0%) were CC homozygous, 10 (37.0%) were CT heterozygous and seven (26.0%) were TT homozygous. For the *MTHFR* polymorphism A1298C, 13 cell lines (48.2%) were AA homozygous, seven (25.9%) were AC heterozygous and seven (25.9%) were CC homozygous.

Genomic variants of CBS 844ins68bp were identified in 27 human cancer cell lines (Fig. 1): 22 of 27 cell lines (81.5%) were homozygous for the absence of insertion (NN), 3 (11.1%) were heterozygous for the insertion (NI) and 2 (7.4%) were homozygous for the insertion (II) (Table I).

Correlations between MTHFR or CBS genomic variant genotypes and sensitivity to antimetabolites. Cytotoxicity data for the seven antimetabolites aphidicolin, cyclocytidine, cytarabine (AraC), floxuridine (FUDR), fluorouracil (5-FU), ftorafur and thiopurine (6MP) were available from the NCI web site. We analyzed the correlation between these data and the *MTHFR* or *CBS* genomic variant genotypes in the 27 cancer cell lines. The Student's t-test revealed that sensitivity to cyclocytidine, AraC and FUDR was significantly higher in cells homozygous for CC at MTHFR-A1298C compared with AA or AC cells (p=0.0215, p=0.0166, p=0.0323, respectively). There was no association between MTHFR-C677T genotype or *CBS* variant and sensitivity to antimetabolites (Fig. 2, Table II).

Identification of 24 tumor suppressor gene promoter methylation by MS-MLPA. We evaluated the methylation status of the promoter regions of 24 tumor suppressor genes in the 27 cancer cell lines using MS-MLPA. No amplification of the 26 *Hha*I sites was detected in normal control DNA. By contrast, all *Hha*I sites were amplified and detected as peaks in methylated DNA (Fig. 3), indicating that the method is a reliable detector of methylation status. Representative MS-MLPA results are shown in Fig. 3. The number of methylated tumor suppressor genes was extremely varied (Fig. 4); the number of methylated genes in cell lines cells homozygous for CC in the region MTHFR-A1298C was significantly higher than in cells with AA or AC (p=0.0313) (Fig. 2).

Correlations between the methylation status of tumor suppressor genes and sensitivity to antimetabolites. We analyzed the correlation between the methylation status of 12 tumor suppressor genes whose methylations were detected in more than seven of the 27 cancer cell lines and the sensitivity to antimetabolites. As shown in Table II, the methylation status of *TIMP3, APC* and *IGSF4* was significantly correlated with the cytotoxicity of >3 pyrimidine synthesis inhibitors. By contrast, there was no significant association between methylation status and cytotoxocity of purine synthesis inhibitors, 6MP.

TIMP3 mRNA expression and sensitivity to pyrimidine synthesis inhibitors. The Student's t-test revealed that methylation of *TIMP3* was significantly correlated with sensitivity to aphidicolin-glycinate, AraC and 5-FU (p=0.0009, p=0.0285, p=0.0253, respectively) (Fig. 2, Table II). To determine whether *TIMP3* methylation would down-regulate *TIMP3* mRNA expression, we used quantitative real-time PCR. As shown in Fig. 5, the relative *TIMP3* expression value was lower in cell lines in which the *TIMP3* promoter region was methylated according to MS-MLPA, compared with cell lines carrying unmethylated *TIMP3* promoter regions.

Table II. Correlations between genomic variants or methylation status of tumor suppressor genes and sensitivity to antimetabolites.

|	Aphidicolin	Cyclocytidine	AraC	FUDR	5-FU	Ftorafur	6MP
Genomic variants							
MTHFR-A1298C	N.S.	0.0215	0.0166	0.0323	N.S.	N.S.	N.S.
MTHFR-C677T	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
CBS 844ins68	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Methylation status							
RASSF1	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
ESR1	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
GDH13	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
RARB	N.S.	N.S.	N.S.	N.S.	N.S.	0.0152	
TIMP3	0.0009	N.S.	0.0285	N.S.	0.0253	N.S.	N.S.
TP73	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
APC	N.S.	0.0222	0.0261	0.0012	N.S.	N.S.	N.S.
CHFR	N.S.	N.S.	N.S.	N.S.	0.0451	N.S.	N.S.
IGSF4	0.0021	0.0308	0.0082	0.0347	N.S.	N.S.	N.S.
DAPK1	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
GSTP1	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
CASP8	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

N.S., not significant.
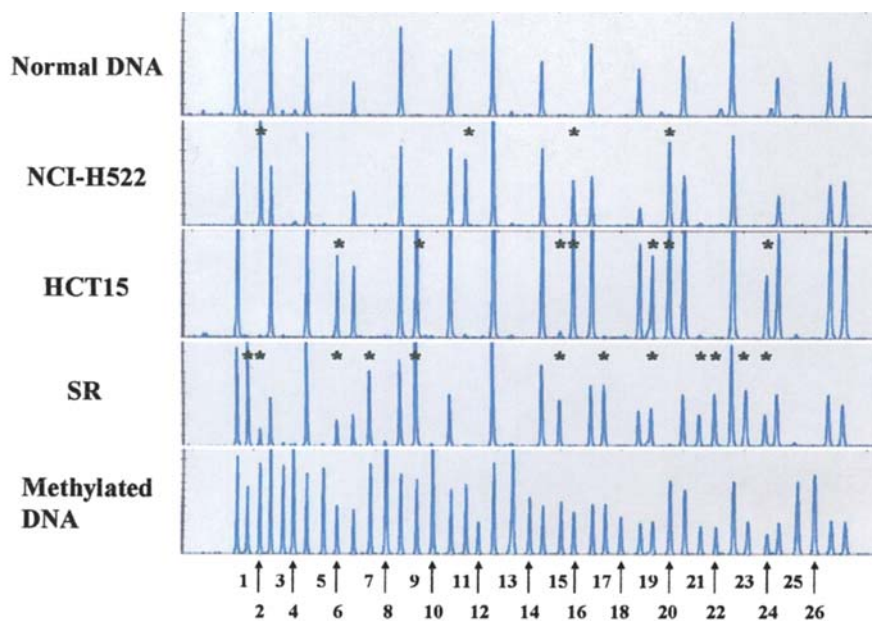
Figure 3. Analysis of methylation status of the promoter regions of 24 tumor suppressor genes in 27 cancer cell lines using MS-MLPA with 26 different probes. *Methylated. 1, TIMP3; 2, APC; 3, CDKN2A; 4, MLH1; 5, ATM; 6, RARB; 7, CDKN2B; 8, HIC1; 9, CHFR; 10, BRCA1; 11, CASP8; 12, CDKN1B; 13, PTEN; 14, BRCA2; 15, CD44; 16, RASSF1; 17, DAPK1; 18, VHL; 19, ESR1; 20, RASSF1; 21, TP73; 22, FHIT; 23, IGSF4; 24, CDH13; 25, GSTP1; 26, MLH1.

Discussion

5-FU and the fluoropyrimidine prodrugs are chemotherapeutic agents widely used for a variety of cancers, and particularly for colorectal cancers. The prediction of cellular susceptibility to such antimetabolites is therefore of crucial importance for colorectal cancer patients. 5-FU efficacy is dependent, in part, on the direction of the cellular metabolic pathway toward either DNA synthesis or DNA methylation.

The metabolic pathway of reductive folates involves the participation of several factors such as *MTHFR* (8-10). As the effect of *MTHFR* SNPs C677T and A1298C on 5-FU
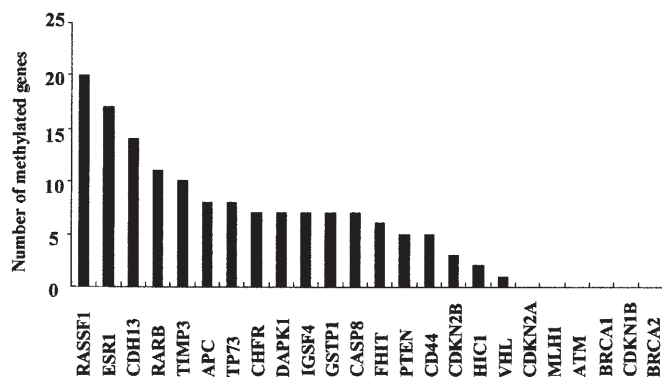
Figure 4. The number of methylated cell lines in 24 tumor suppressor genes examined. There were differences in susceptibility of tumor suppressor genes to methylation.
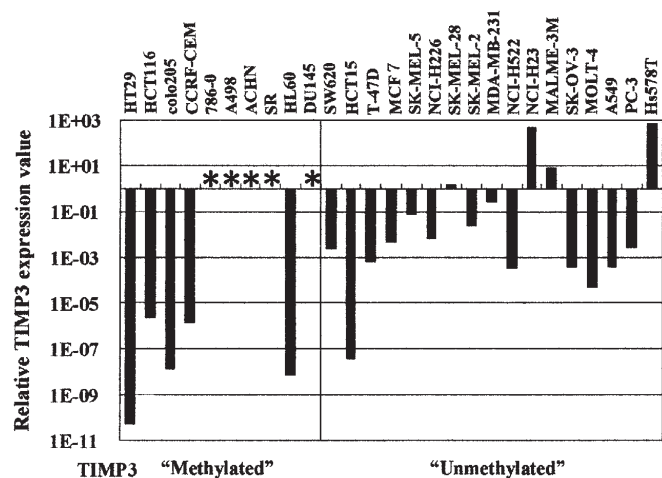

Figure 5. Quantitative real-time PCR showing that the relative expression ratio of TIMP3/GAPDH in cell lines with methylated TIMP3 was less than in cell lines with unmethylated TIMP3. *The threshold cycle for TIMP3 was undetectable.

We next analyzed the methylation status of the promoter regions of 24 tumor suppressor genes in 27 cancer cell lines. MS-MLPA used 26 different probes after first confirming the sensitivity and specificity of the method using normal and methylated DNA as controls. As shown in Fig. 3, the methylated 'peaks' were obvious in most cases, however, there were some indefinite bands such as CD44 in HCT-15. To resolve this problem we prepared four more control DNAs and calculated the means and standard deviations (SD) for the height of the bands at each site (data not shown). We then regarded bands whose height was below the mean + 2SD as negative (unmethylated) and above the mean + 2SD as positive (methylated). Fig. 4 shows that the number of cell lines in which methylation was detected in tumor suppressor genes was extremely varied. Although the method is undoubtedly limited because of the restricted number of sites available to detect methylation status, it is sufficient to detect cellular susceptibility to methylation. Cells homozygous for CC at MTHFR-A1298C carried more methylated genes than those with AA or AC (Fig. 2).

To investigate the association between gene methylation and sensitivity to antimetabolites, we selected 12 tumor suppressor genes that were methylated in >25% of cancer cell lines examined. The methylation status of *TIMP3, APC* and *IGSF4* was significantly correlated with sensitivity to more than three pyrimidine synthesis inhibitors. By contrast, there was no significant association between methylation status and sensitivity to the purine synthesis inhibitor, 6MP, as expected.

Methylation-associated inactivation of *TIMP3* is common in many human cancers, including colorectal cancers (24), and *TIMP3* methylation has also been associated with a more malignant potential and poorer prognosis (25-27). We therefore focused on *TIMP3* methylation and showed that cell lines with methylated *TIMP3* were significantly more sensitive to aphidicolin-glycinate, AraC and 5-FU than those with unmethylated *TIMP3* (p=0.0009, p=0.0285, p=0.0253, respectively; Fig. 2).

Although a significant correlation between methylation and reduced mRNA/protein expression of TIMP3 has previously been reported (28), we evaluated these associations by quantitative real-time PCR to confirm the accuracy of the MS-MLPA method applied in this study. The relative expression of TIMP3 in cell lines with methylated *TIMP3* was reduced in comparison with cell lines with unmethylated *TIMP3* (Fig. 5), assuring the accuracy of this method in the detection of methylation.

We next screened *MTHFR* SNPs and determined the methylation status of the promoter regions of tumor suppressor genes. A comparison of these results with the sensitivity to antimetabolites in 27 cancer cell lines revealed that the variant allele C at MTHFR-A1298C correlated with sensitivity to pyrimidine synthesis inhibitors. As homozygous CC cells have many methylated genes, and cells with methylated *TIMP3* were significantly more sensitive to antimetabolites, we speculate that MTHFR-A1298C homozygous CC might direct metabolic pathways toward methylation rather than DNA synthesis. Moreover, the methylation of several tumor suppressor genes is a possible mechanism by which MTHFR-A1298C homozygous CC provides potent biological malignancy against the

sensitivity has not been well defined (11-13), we first analyzed their relationship and that of genetic variations of *CBS* with sensitivity to seven antimetabolites in 27 cancer cell lines. *CBS* catalyzes the conversion of homocysteine to cystathionine, and the most thoroughly investigated variant of *CBS* is 844ins68bp (21,22). The Student's t-test revealed that sensitivity to cyclocytidine, AraC and FUDR was significantly higher in cells homozygous for CC in the MTHFR-A1298C region than in cells with AA or AC (p=0.0215, p=0.0166, p=0.0323, respectively). By contrast, genotyping of MTHFR-C677T and *CBS* variants yielded no correlation with antimetabolite sensitivities.

These results support previous data that mutated *MTHFR* variants at position 1298 were associated with 5-FU efficacy (11,13). Etienne *et al* also showed that SNPs at *MTHFR*-A1298C were unrelated to the outcome of 5-FU-based chemotherapy in the clinical setting, but were significantly linked to survival, with homozygous mutated (CC) patients having the worst prognosis (12). This also supported previous findings (23).

sensitivity to chemodrugs. Furthermore, MTHFR-A1298C homozygous CC and/or TIMP3 methylation in cancer cells could be a useful biological marker for the prediction of anti-metabolite efficacy. Further studies with a large number of clinical samples are required to confirm this hypothesis.

Acknowledgments

References

 1. Wang TL, Diaz LA Jr, Romans K, *et al*: Digital karyotyping identifies thymidylate synthase amplification as a mechanism of resistance to 5-fluorouracil in metastatic colorectal cancer patients. Proc Natl Acad Sci USA 101: 3089-3094, 2004.
 2. Van Triest B and Peters GJ: Thymidylate synthase: a target for combination therapy and determinant of chemotherapeutic response in colorectal cancer. Oncology 57: 179-194, 1999.
 3. Plasencia C, Rooney PH, Taron M, Martinez-Balibrea E, McLeod HL and Abad A: Chromosomal imbalance maps of human 5FU-resistant colorectal cancer cell lines: Implications in the analysis of 5FU-acquired resistance mechanisms. Int J Oncol 22: 945-953, 2003.
 4. Peters GJ, Backus HH, Freemantle S, *et al*: Induction of thymidylate synthase as a 5-fluorouracil resistance mechanism. Biochim Biophys Acta 1587: 194-205, 2002.
 5. Kuhn JG: Fluorouracil and the new oral fluorinated pyrimidines. Ann Pharmacother 35: 217-227, 2001.
 6. Yin MB, Zakrzewski SF and Hakala MT: Relationship of cellular folate cofactor pools to the activity of 5-fluorouracil. Mol Pharmacol 23: 190-197, 1983.
 7. Houghton JA, Torrance PM, Radparvar S, Williams LG and Houghton PJ: Binding of 5-fluorodeoxyuridylate to thymidylate synthase in human colon adenocarcinoma xenografts. Eur J Cancer Clin Oncol 22: 505-510, 1986.
 8. Goyette P, Sumner JS, Milos R, Duncan AM, Rosenblatt DS, Matthews RG and Rozen R: Human methylenetetrahydrofolate reductase: isolation of cDNA mapping and mutation identification. Nat Genet 7: 551, 1994.
 9. Frosst P, Blom HJ, Milos R, *et al*: A candidate genetic risk factor for vascular disease: a common mutation in methylene-tetrahydrofolate reductase. Nat Genet 10: 111-113, 1995.
10. Miller JW, Nadeau MR, Smith J, Smith D and Selhub J: Folate-deficiency-induced homocysteinaemia in rats: disruption of S-adenosylmethionine's co-ordinate regulation of homocysteine metabolism. Biochem J 298: 415-419, 1994.
11. Etienne MC, Ilc K, Formento JL, *et al*: Thymidylate synthase and methylenetetrahydrofolate reductase gene polymorphisms: relationships with 5-fluorouracil sensitivity. Br J Cancer 90: 526-534, 2004.
12. Etienne MC, Formento JL, Chazal M, *et al*: Methylenetetra-hydrofolate reductase gene polymorphisms and response to fluorouracil-based treatment in advanced colorectal cancer patients. Pharmacogenetics 14: 785-792, 2004.
13. Sohn KJ, Croxford R, Yates Z, Lucock M and Kim YI: Effect of the methylenetetrahydrofolate reductase C677T polymorphism on chemosensitivity of colon and breast cancer cells to 5-fluorouracil and methotrexate. J Natl Cancer Inst 96: 134-144, 2004.
14. Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F and Pals G: Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res 30: e57, 2002.
15. Nygren AO, Ameziane N, Duarte HM, *et al*: Methylation-specific MLPA (MS-MLPA): simultaneous detection of CpG methylation and copy number changes of up to 40 sequences. Nucleic Acids Res 33: e128, 2005.
16. Bunyan DJ, Eccles DM, Sillibourne J, *et al*: Dosage analysis of cancer predisposition genes by multiplex ligation-dependent probe amplification. Br J Cancer 91: 1155-1159, 2004.
17. Renkonen ET, Nieminen P, Abdel-Rahman WM, *et al*: Adenomatous polyposis families that screen APC mutation-negative by conventional methods are genetically heterogeneous. J Clin Oncol 23: 5651-5659, 2005.
18. Hogervorst FB, Nederlof PM, Gille JJ, *et al*: Large genomic deletions and duplications in the BRCA1 gene identified by a novel quantitative method. Cancer Res 63: 1449-1453, 2003.
19. Langerak P, Nygren AO, Schouten JP and Jacobs H: Rapid and quantitative detection of homologous and non-homologous recombination events using three oligonucleotide MLPA. Nucleic Acids Res 33: e188, 2005.
20. Paz MF, Avila S, Fraga MF, *et al*: Germ-line variants in methyl-group metabolism genes and susceptibility to DNA methylation in normal tissues and human primary tumors. Cancer Res 62: 4519-4524, 2002.
21. Sebastio G, Sperandeo MP, Panico M, de Franchis R, Kraus JP and Andria G: The molecular basis of homocystinuria due to cystathionine beta-synthase deficiency in Italian families, and report of four novel mutations. Am J Hum Genet 56: 1324-1333, 1995.
22. Tsai MY, Bignell M, Schwichtenberg K and Hanson NQ: High prevalence of a mutation in the cystathionine beta-synthase gene. Am J Hum Genet 59: 1262-1267, 1996.
23. Nuckel H, Frey UH, Durig J, Duhrsen U and Siffert W: Methylenetetrahydrofolate reductase (MTHFR) gene 677C>T and 1298A>C polymorphisms are associated with differential apoptosis of leukemic B cells *in vitro* and disease progression in chronic lymphocytic leukemia. Leukemia 18: 1816-1823, 2004.
24. Esteller M, Corn PG, Baylin SB and Herman JG: A gene hypermethylation profile of human cancer. Cancer Res 61: 3225-3229, 2001.
25. Hamilton JP, Sato F, Greenwald BD, *et al*: Promoter methylation and response to chemotherapy and radiation in esophageal cancer. Clin Gastroenterol Hepatol 4: 701-708, 2006.
26. Darnton SJ, Hardie LJ, Muc RS, Wild CP and Casson AG: Tissue inhibitor of metalloproteinase-3 (TIMP-3) gene is methylated in the development of esophageal adenocarcinoma: loss of expression correlates with poor prognosis. Int J Cancer 115: 351-358, 2005.
27. Kang SH, Choi HH, Kim SG, Jong HS, Kim NK, Kim SJ and Bang YJ: Transcriptional inactivation of the tissue inhibitor of metalloproteinase-3 gene by dna hypermethylation of the 5'-CpG island in human gastric cancer cell lines. Int J Cancer 86: 632-635, 2000.
28. Xue WC, Chan KY, Feng HC, Chiu PM, Ngan HY, Tsao SW and Cheung AN: Promoter hypermethylation of multiple genes in hydatidiform mole and choriocarcinoma. J Mol Diagn 6: 326-334, 2004.