DNA methylation and sensitivity to antimetabolites in cancer cell lines

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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 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 HhaI. 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).
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 \textit{MTHFR} gene, and determining the methylation status of tumor suppressor genes and the susceptibility of well-characterized NCI-60 cancer cell lines to antimetabolites.

\textbf{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 cytoxicity 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.

\textit{MTHFR} and \textit{CBS} genotyping. Genotyping of \textit{MTHFR} and cystathionine \(\beta\)-synthase (\textit{CBS}), genes involved in the metabolic pathway of reductive folates, was performed using previously described PCR/RFLP methods (20) with some modifications. Briefly, 15 \(\mu\)l PCR reactions contained 20 ng genomic DNA, 7.5 \(\mu\)l HotStar Taq Master mix (Qiagen, Tokyo, Japan), and 5 pmol of each primer. The primers for \textit{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 \textit{MTHFR-A1298C} were as follows: forward 5'-GCA AGT CCC CCA AGG AGG-3'; reverse 5'-GGT CCC CAC TTC CAG CAT C-3'.

\textit{MTHFR-C677T} amplification was for 35 cycles at 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 30 sec. \textit{MTHFR-A1298C} was amplified for 35 cycles at 94˚C for 30 sec, 57˚C for 30 sec, 55˚C for 30 sec and 72˚C for 60 sec. \textit{MTHFR-C677T} and \textit{A1298C} were digested overnight at 37˚C with \textit{HinfI} or \textit{MboII} for \textit{MTHFR-C677T} and \textit{A1298C}, respectively.

For CBS \(844\text{ins68}\) bp, the primers were as follows: forward 5'-CTG GCC TTG AGC CCT GAA-3'; reverse 5'-GGC CGG TGA GAG TG-3'. Primers for \textit{CBS} were as follows: for \textit{CBS 844ins68}: forward 5'-TGA AGG AGA AGG TGT CTG CGG GA-3'; reverse 5'-AGG ACG GTG CGG TGA GAG TG-3'. The PCR conditions were the same as for \textit{MTHFR} and \textit{CBS} genotyping.

\textit{MS-MLPA}. Normal genomic DNA (Promega) and CpGenome\textsuperscript{TM} 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 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 Biosystems) using TaqMan EZ RT-PCR core reagents, primers and TaqMan probes for \textit{TIMP3} (Hs00165949_m1) and \textit{gyceraldehyde-3-phosphate dehydrogenase} (GAPDH; Hs99999905_m1) from Assay-on-Demand sets (Applied Biosystems). PCR reaction mixtures (25 \(\mu\)l) contained 20 ng total RNA, 5 \(\mu\)l 5X TaqMan EZ buffer, 3 mM manganese acetate, 300 \(\mu\)M dATP, dCTP, dGTP, and dUTP, 2.5 U rTth DNA polymerase, 0.25 U AmpErase UNG, and 1.25 \(\mu\)l 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 \textit{TIMP3} expression value was obtained by dividing the \textit{TIMP3} value by the \textit{GAPDH} value. Experiments were performed in triplicate and the mean of each relative \textit{TIMP3} expression value was calculated.

\textbf{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.
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 (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).

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 HhaI sites was detected in normal control DNA. By contrast, all HhaI 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 cytotoxicity 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.
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

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

<table>
<thead>
<tr>
<th>Genomic variants</th>
<th>Aphidicolin</th>
<th>Cyclocytidine</th>
<th>AraC</th>
<th>FUDR</th>
<th>5-FU</th>
<th>Florafur</th>
<th>6MP</th>
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<td>MTHFR-A1298C</td>
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<td>0.0166</td>
<td>0.0323</td>
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<td>N.S.</td>
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<td>N.S.</td>
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<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
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<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
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<td>N.S.</td>
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<tr>
<td>Methylation status</td>
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<td>0.0253</td>
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<td>N.S.</td>
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<td>N.S.</td>
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<td>0.0012</td>
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<tr>
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<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

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.
sensitivity has not been well defined (11-13), we first analyzed their relationship and that of genetic variations of CBS with sensitivity to seven antimitobolites 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 sensitivity 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 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 antimitobolites, 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).

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

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