The Impact of Thymidylate Synthase and Methylenetetrahydrofolate Reductase Genotypes on Sensitivity to 5-Fluorouracil Treatment in Colorectal Cancer Cells

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Abstract- 5-fluorouracil (5-FU) is one of the major components of many standard regimens for chemotherapy of colorectal cancer (CRC) and some other malignancies. Given the known relationship between thymidylate synthase (TS) and methylenetetrahydrofolate reductase (MTHFR) activity and 5-FU metabolism, this study investigated the impact of selected functional polymorphisms of the TS and MTHFR genes on chemotherapy resistance in 5 human CRC cell lines. HCT116, SW1116, HT29/219, LS180, and Caco-2 CRC cells were cultured as monolayer and their chemosensitivity to 5-FU, oxaliplatin, and irinotecan was determined by MTT assay. Genomic DNA was extracted from the cultured cells, and a 6-bp insertion or deletion (6-bp ins/del) polymorphism in 3´-UTR of the TS gene was determined by the PCR-RFLP method. Genotyping of MTHFR 677 C/T and 1298A/C single nucleotide polymorphism (SNP) was also performed by MS-PCR and PCR-RFLP, respectively. Caco-2 with the homozygous TS 6-bp ins/ins and MTHFR 677 T/T and 1298 C/C genotype, was the most 5-FU resistant cell line. HCT116 with the homozygous TS 6-bp del/del and MTHFR 1298 A/A and heterozygous MTHFR 677 C/T genotype was the least 5-FU resistant cell. LS180, the second most 5-FU resistant cell line, was heterozygous for all three polymorphic sits. HT29/219 and SW1116 cells with homozygous TS 6-bp ins/ins and heterozygous MTHFR 677 C/T and 1298 A/C genotypes had intermediate 5-FU sensitivity. The results indicate that TS 3´-UTR 6-bp insertion and MTHFR 677T and 1298C alleles increase 5-FU resistance in CRC cells. No relationship was observed between TS and MTHFR genotypes and oxaliplatin or irinotecan sensitivity in these cells.

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Introduction

Colorectal cancer (CRC) is the second most common cause of cancer death in the developed countries and a leading cause of cancer-related mortality worldwide (1). The main problems for treatment of CRC patients are the development of resistance to chemotherapy and progression to metastatic disease. The first choice of therapy for CRC patients is surgery. CRC patients at stage I are usually treated by curative surgery alone, but the treatment of postoperative high-risk stage II and stage III cancers are followed by chemotherapy to reduce the risk of tumor recurrence (2).

The antimetabolite 5-fluorouracil (5-FU) in combination with leucovorin plus oxaliplatin (FOLFOX) or irinotecan (FOLFIRI) are standard treatments in the current adjuvant therapy of advanced CRC (3). More recently, targeted therapies of metastatic CRC with a number of drugs against the VEGF or EGFR has also been developed (4). Tumors resistance in the course of treatment is a common problem that limits the efficiency of cancer therapy. In fact, response rate around 50 % to the combined chemotherapy has been reported in metastatic CRC patients, and less than 10 % of patients might survive more than 2 years (4).

Many mechanisms have been suggested to contribute to drug resistance including mechanisms implicated in drug influx and effluxes, processing of drug-induced damage and drugs inactivation (5). Heritable genetic variants and activity of several enzymes including

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thymidine phosphorylase, thymidylate synthase (TS) and MTHFR enzyme, CYP450, GST, and UGT-mediated phase I and II metabolism; as well as the ABC and SLC transport pathways have been shown to correlate with tumor sensitivity phenotype in CRC patients treated with 5-FU combined with irinotecan, oxaliplatin or targeted biological agents (6,7). TS expression and activity in tumor cells has been previously shown to correlate with 5-FU response (8,9). Several studies have also suggested that genetic variants of TS gene can affect the sensitivity to 5-FU treatment (10). A 6-bp insertion or deletion polymorphism in 3´-UTR of the TS gene is associated with TS gene expression in CRC tumor tissues (11,12). In clinical studies, this polymorphism has been suggested to correlate with the response to the 5-FU-based treatment. Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in folate metabolism, and its activity is related to the cellular levels of 5, 10-methylene-tetrahydrofolate, which is used for conversion of dUMP to dTMP by the enzyme TS. There are several genetic variants demonstrated to correlate with MTHFR activities. The MTHFR C677T and A1298C polymorphisms result in an accumulation of 5,10-methylene-THF, which increases the sensitivity to 5-FU based treatment (13-17). The previous study on CRC patients suggested that response to FOLFOX therapy in CRC patients may be driven by MTHFR germinal polymorphisms (18). Optimal inhibition of TS by 5-FU requires an elevated level of MTHF, which is regulated by MTHFR enzyme. Therefore, both TS and MTHFR activities are key determinants of clinical response to 5-FU based chemotherapy.

For adjuvant therapy of advanced CRC, 5-FU is more frequently used in combination with other chemotherapeutic agents with different mechanism of action such as oxaliplatin (OXA) or irinotecan (19). Given the interplay between MTHFR and TS in mediating 5-FU-based chemotherapy, the present study has been carried out to evaluate the impact of selected TS and MTHFR polymorphisms on chemotherapy resistance in human CRC cells. The identification of predictive molecular markers of tumor response would provide a valuable tool for predicting treatment outcomes and identification of subgroups of patients who are most likely to benefit from the specific treatment.

Materials and Methods

Chemicals

Unless otherwise specified, all chemicals were purchased from Millipore Sigma (New Delhi, India) and were prepared and stored according to the manufacturers' instructions. All media and cell culture reagents were obtained from Gibco-Invitrogen.

Cell culture and drug treatments

The cell lines used in this study were obtained from the National Cell Bank of Iran (NCBI, Pasteur Institute, Tehran). The human colon carcinoma cell lines HT29/219, Caco-2, HCT116, SW1116, and LS180 were grown in either RPMI 1640 or in DMEM supplemented with 10 % fetal bovine serum as previously described (20).

For drug cytotoxicity assays, a triplicate aliquots of cells were seeded in 96-well microtiter plates (3000-5000 cells per well). Twenty-four hours after seeding, the sub-confluent cells were exposed to increasing concentrations of either 5-FU (20), oxaliplatin (25, 50, 100, 200, 300 µg/ml), or Irinotecan (10, 20, 30, 40, 50 µg/ml) and incubated in a humidified 5% CO_2 atmosphere at 37° C for 48 h. Cell viability was determined by MTT assay as previously described (20). The drug concentrations resulting in 50% of growth inhibition (IC_{50}) was determined from the dose-response curves. The Software GraphPad PRISM Version 5.00 (GraphPad Software, San Diego, CA) was used to analyze the dose-response graphs.

DNA extraction and genotyping of the TS and MTHFR genes

DNA was extracted from the cultured CRC cells using the standard phenol/chloroform method. Genotyping for the TS 3′-UTR 6 bp ins/del polymorphism was carried out according to the PCR-RFLP method described by Ulrich *et al*., (21). Briefly, genomic DNA from the samples was used as a template in PCR reactions using specific primers listed in Table 1. The 152-bp PCR product was subjected to DraI enzyme (MBI Fermentas, Vilnius, Lithuania) digestion overnight at 37° C. The digested products were then analyzed by electrophoresis in 3% agarose gels, stained with GelRed™ (Biotium, CA, USA), and visualized under UV illumination.

Genotyping of MTHFR at codon 677 of DNA from CRC cell lines was performed by the mutagenically separated-PCR (MS-PCR) method as previously described (22). PCR products were separated by electrophoresis on 3% agarose gel and visualized as described above. Genotyping of MTHFR at codon 1298 was examined by PCR-RFLP of DNA samples using the

enzyme MboII (MBI Fermentas, Lithuania) and analyzed as previously described (23).

Table 1. Nucleotide sequences of the primer sets

Primer name	Forward	Reverse
TS 3´-UTR	5'-CAAATCTGAGGGAGCTGAGT-3'	5'-CAGATAAGTGGCAGT ACAGA-3'
MTHFR 1298	5'-CTTTGGGGAGCTGAAGGACTACTAC-3'	5'-CACTTTGTGACCATTCCG GTTTG-3'
MTHFR 677	Normal: 5'-GCTTTGAGGCTGACCTGAAGACCTTGAAGGA GAAGGTGTCTGCGGCAGC-3' Mutant: 5´-CACTTGAAGGAGAAGGTGTCTGCGGGACT-3´	5'-TCACCTGGATGGGAAAG ATC-3'

Statistical analysis

The results of cytotoxicity experiments were obtained from 3 independent experiments each done in triplicate. The mean values from different excremental groups were compared via one-way ANOVA and differences with $P \leq 0.05$ considered statistically significant.

Results

Genotyping of the TS and MTHFR gene variants in CRC cell lines

The genomic DNA was extracted from CRC cells, and the genotype of TS (3´-UTR 6-bp ins/del) was identified by PCR-RFLP. The expected fragment sizes are 70 bp and 88 bp for the presence of the 6-bp insertion allele; the variant 6-bp deletion allele lacks the DraI restriction site and therefore produces a single 152 bp band. As shown in figure 1A, the genotype of HT29/219, SW1116, and Caco-2 cells is 6-bp ins/ins homozygous, LS180 is heterozygous, and HCT116 is 6-bp del/del homozygous.

MTHFR 677 C/T and 1298 A/C genotypes in 5 CRC cell lines were also determined. As shown in figure 1B, the genotype of Caco-2 at position 677 is TT homozygous, whereas the rest of the cell lines are heterozygous for this allele. The HCT116 and Caco-2 cells are homozygous for MTHFR 1298 A and C alleles, respectively (Figure 1C). Other cell lines including HT29/219, LS180, and SW1116 have heterozygous genotype at this position.

Relationship between genotypes and 5-FU sensitivity of CRC cells

The cell lines investigated in this study have previously been characterized by 5-FU sensitivity (20). Therefore, this allowed us to examine the influence of TS and MTHFR genotypes on 5-FU response of these cell lines in the present study. A comparison of IC_{50} values revealed that the Caco-2 was the most resistant cell line (IC_{50}, 15.7 µM) ($P<0.05$), followed by LS180 (IC_{50}, 9.64 µM) and HT29/219 (IC_{50}, 8.67 µM), whereas SW1116 (IC_{50}, 1.74 µM) and HCT116 (IC_{50}, 0.66) cells showed the highest sensitivity (Figure 2). Data are shown as mean±SD of three independent experiments. Summary of all IC_{50}s for all cell lines is shown in Table 2.

To investigate whether the genotypes of TS and MTHFR contributes to drug resistance, the genotypes of 5 CRC cell lines were compared in relation to 5-FU sensitivity. As shown in Table 2, Caco-2 with homozygous wild-type TS (6-bp ins/ins) and biallelic mutation for both MTHFR 677 T/T and MTHFR 1298 C/C genotype was the most 5-FU resistant cell line. However, the homozygous TS mutant (6-bp del/del) cell line, HCT116, with wild-type MTHFR 1298 AA and heterozygous 677 CT genotype was the least resistant cell line. LS180, the second most 5-FU resistant cell line, was heterozygous for all three polymorphic positions. In addition, HT29/219 and SW1116 cell lines presenting homozygous TS ins/ins and heterozygous for both MTHFR 677 C/T and 1298 A/C genotypes had intermediate 5-FU sensitivity. These results show that the genetic variation of TS and MTHFR could influence sensitivity to 5-FU treatment and that there is a coordinated effect between TS 3´-UTR 6-bp insertion and MTHFR 677T and 1298 C alleles in increasing 5-FU chemoresistance.

Sensitivity of CRC cell lines to growth inhibition by oxaliplatin and irinotecan

We next examined the relationship between TS and MTHFR genotypes and resistance to oxaliplatin and

irinotecan, two other commonly used drugs for 5-FU-based adjuvant treatment of cancer, in 3 CRC cell lines. The three human CRC cell lines, HT29/219, HCT116, and LS180 were seeded in 96-well plates and 24 h later, oxaliplatin and irinotecan were added at the concentrations of 25-300 µg/ml and 10-50 µg/ml, respectively, for 48 h as described in the "Materials and Methods" section. There was no association between TS or MTHFR genotypes and chemosensitivity to oxaliplatin or irinotecan in these cell lines. As shown in figure 2, HT29/219 cell line (IC_{50}, 166±0.39 µg/ml) with wild-type TS genotype (Table 2) was significantly more sensitive to oxaliplatin than HCT116 (IC_{50}, 119±0.65) (*P*=0.00) and LS180 (IC_{50}, 0.86±0.44) (*P*=0.00) cells. On the contrary, HCT116 with mutant TS genotype was more resistant to irinotecan (IC_{50}, 27.5±0.58 µg/ml) than HT29/219 (IC_{50}, 25.5±0.64 µg/ml) (*P*<0.05) and LS180 (IC_{50}, 16.1±0.49 µg/ml) (*P*=0.00). The difference in sensitivity to irinotecan between HT29/219 and LS180 cells was also statically significant (*P*=0.00). The results show that oxaliplatin and irinotecan sensitivity of these CRC cells cannot be predicted from TS and MTHFR genotypes and requires information from other influential markers.

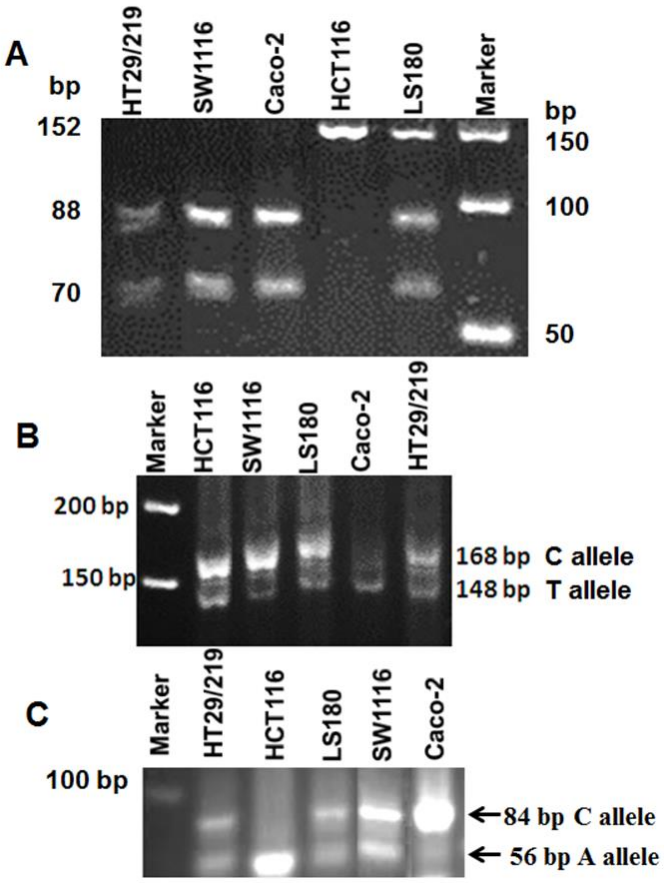

Figure 1. Genotyping of TS 3´-UTR 6-bp ins/del, MTHFR 677C/T, and MTHFR 1298 A/C polymorphisms in human CRC cell lines. **A.** PCR-RFLP assay for genotyping of TS 3´-UTR 6-bp ins/del polymorphisms. Digestion of the 152-bp PCR product by DraI enzyme produces 70 bp and 88 bp fragments for the presence of the 6-bp insertion allele, whereas the variant 6-bp deletion allele lacks the DraI restriction site and therefore remains undigested (152 bp). **B.** MS-PCR assay for genotyping of MTHFR 677C/T SNP produces a 168 bp band for the presence of C allele and a 148 bp for T allele. **C.** PCR-RFLP assay for genotyping of MTHFR 1298A/C polymorphisms. Digestion of the 163 bp PCR product by MboII enzyme of the 1298A allele yields five fragments of 56, 31, 30, 28, and 18 base pairs, whereas the 1298C allele results in four PCR bands of 84, 31, 30, and 18 base pairs. The three possible genotypes are discernible by detection of the 84- and 56-bp fragments. The digested PCR products were separated by electrophoresis on a 3% agarose gel

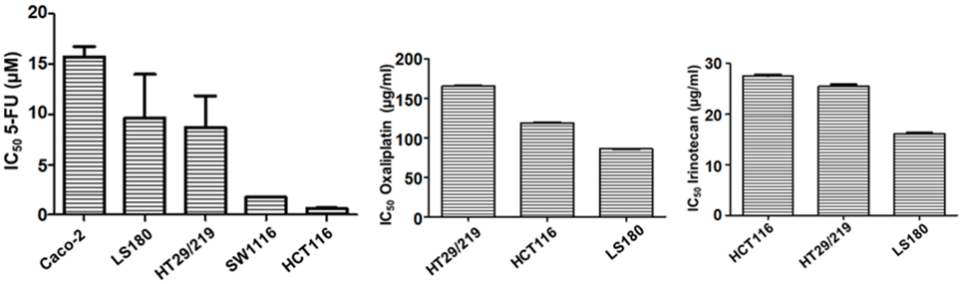

Figure 2. Differential cytotoxicity of 5-FU, oxaliplatin, and irinotecan in human CRC cell lines

Table 2. Characteristics of human CRC cell lines

	Caco-2	LS180	HT29/219	SW1116	HCT116
TS 3´-UTR 6bp ins/del genotype	+/+	+/-	+/+	+/+	-/-
MTHFR 677 C/T genotype	TT	CT	CT	CT	CT
MTHFR 1298 A/C genotype	CC	AC	AC	AC	AA
IC_{50} (5-FU) (µM)	15.7 ± 1.01	9.64 ± 4.33	8.67 ± 3.17	1.74±0.05	0.66±0.03
IC_{50} (Oxaliplatin) (µg/ml)	ND	86 ± 0.44	166 ± 0.39	ND	119 ± 0.65
IC_{50} (Irinotecan) (µg/ml)	ND	16.1 ± 0.49	25.5 ± 0.64	ND	27.5 ±0.58

ND: not determined
IC_{50} value for each cell line is presented from three independent assays

Discussion

5-FU remains the most widely used reagent for chemotherapy of CRC and a number of other common malignancies (24). The presence or development of resistance to 5-FU is a major obstacle to successful cancer therapy. In advanced colorectal cancer, 5-FU produces response rates of only 20 to 25%. The main mechanism of 5-FU action is inhibition of TS enzyme via the formation of a covalent ternary complex among its active metabolite 5-FdUMP, TS and 5, 10-methylenetetrahydrofolate (MTHF) (25). The stability of the complex depends on the cellular MTHF pools, which is regulated by the enzyme MTHFR. Therefore, 5-FU clinical response is presumed to be influenced by both TS and MTHFR activities. Genetic variants with functional impact on the activity and/or expression of both enzymes have been described (17,26).

Our study on a panel of 5 CRC cell lines showed a 23.8-fold variation in 5-FU response between cells (Table 2). Based on the TS and MTHFR genotyping results, it was found that the wild-type TS 3´-UTR 6-bp insertion and two MTHFR polymorphisms, C677T and A1298C are positively associated with 5-FU resistance in CRC cell lines (Table 2, Figure 2). 5-FU resistance was markedly higher in Caco-2 cell line with the homozygous TS 6-bp ins/ins and MTHFR 677TT and 1298 CC genotype than the cell lines with other genotypes.

The lack of response to 5-FU has been previously shown to correlate with an increased expression of the target TS enzyme, by circumventing the inhibition that results from the binding of the 5-FU metabolite, FdUMP, to the enzyme (27). Presence of the deleted allele (6-bp del) has been reported to result in TS mRNA instability *in vitro* and decreased TS mRNA levels in CRC tumors (11). Therefore, CRC cells with the 6-bp deleted allele would be expected to show increased sensitivity towards 5-FU.

The three human CRC cell lines, LS180, HT29/219, and SW1116, share the same MTHFR genotypes (677 CT and 1298 AC). In contrast to what would be expected, LS180 cells with the heterozygote TS 6-bp ins/del genotype displayed higher resistance to 5-FU than HT29/219 and SW1116 cells which are homozygous for 6-bp ins variant (Table 2, Figure 2). It has been previously reported that carcinoembryonic antigen (CEA) expression protect CRC cells against the cytotoxic effects of 5-FU (20,28). Our previous study has determined that LS180 cells express a much higher level of CEA protein than other cell types used in the current study (20). Therefore, the high CEA expression level may be also involved in 5-FU resistance in this cell line.

A number of clinical studies have evaluated the role of the TS and MTHFR gene variants in predicting response in CRC patients treated with 5-FU-based chemotherapy, but the results were inconsistent (15,29-31). Our result is consistent with a previous experimental study on 19 other human cancer cell lines that reported a greater 5-FU efficacy in cell lines homozygous for the MTHFR 1298 C allele compared

with cells homozygous for the 1298A allele (32). Also, another study on human cancer cell lines transfected with 677C or 677T MTHFR cDNA demonstrated a significantly higher sensitivity to 5-FU in the 677T cells compared to the 677C cells (14).

To examine the specificity of the association between genetic markers and drug response, we compared the sensitivity of three CRC cell lines, HT29/219, HCT116, and LS180 to oxaliplatin and irinotecan. No clear relation was observed between either TS 3´-UTR polymorphism or between the MTHFR 677C/T and 1298 A/C variants and response to these two drugs (Figure 2). The mechanisms of action of oxaliplatin and irinotecan are different from that of 5-fluorouracil. Irinotecan exerts its antitumor activity through its active metabolite, SN38, which irreversibly binds topoisomerase I enzyme to DNA, thereby triggering genomic damage and apoptosis (33).

The main mechanism of action of oxaliplatin is mediated through the formation of DNA adducts, leading to DNA damage-induced apoptosis (34).

Other mechanisms have been also proposed to contribute to 5-FU resistance, including a high expression of dihydropyrimidine dehydrogenase and a low expression of enzymes responsible for 5-FU activation, orotate phosphoribosyltransferase and uridine monophosphate kinase (35). It has been also shown that microsatellite instability (MSI) and mismatch repair deficiency are predictive markers of poor response to 5-FU treatment in CRC (36,37).

In conclusion, the findings of this study suggest that TS 3´-UTR 6-bp insertion and MTHFR 677T and 1298 C alleles increase 5-FU resistance in CRC tumors. Therefore, the genotyping of CRC patients for these alleles may be useful in predicting which cancer patients will best respond to 5-FU-based chemotherapy. The identification of molecular markers that are involved in chemotherapy resistance is of major interest for developing cancer treatment strategies. Although the use of these factors as individual predictive markers has produced somewhat conflicting results, however, the use of markers in combination could reliably predict the treatment response (38).

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