Thymidylate synthase and methylenetetrahydrofolate reductase gene polymorphisms: relationships with 5-fluorouracil sensitivity

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The relationship of thymidylate synthase (TS) and methylenetetrahydrofolate reductase (MTHFR) gene polymorphisms on 5-fluorouracil (FU) sensitivity was tested on 19 human cancer cell lines (head and neck, breast, digestive tract) in the absence and presence of folinic acid (FA) supplementation. Thymidylate synthase polymorphisms in the 5′ promoter region (double or triple tandem repeats) and 3′ untranslated region (6-bp deletion) were analysed by PCR. The C677T and A1298C MTHFR polymorphisms were determined by melting curve analyses (LightCycler). Thymidylate synthase activity and intracellular concentration of the reduced folate 5-10 methylenetetrahydrofolate (CH₂FH₄) were measured (biochemical assays). Thymidylate synthase activity was significantly different according to 5′ TS genotype, heterozygous cell lines (2R/3R) exhibiting higher TS activities than homozygous ones (P = 0.05). However, whether in the absence or presence of FA, FU sensitivity was not statistically associated with either 5′ or 3′ TS polymorphism. Basal CH₂FH₄ cellular concentrations were lowest in C677T homozygous wild-type (wt) (C/C) cell lines. FU sensitivity was not linked to C677T polymorphism. In contrast, there was a marked trend for a greater FU efficacy in mutated A1298C variants (C/C + A/C) as compared to wt homozygous cell lines (A/A) (P = 0.055 and 0.085 without and with FA supplementation, respectively). This results suggest for the first time a potential role of A1298C MTHFR polymorphism on fluoropyrimidine sensitivity.

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5-Fluorouracil (FU) and fluoropyrimidine prodrugs remain the drugs of choice for the treatment of colorectal (Wils et al., 2001), breast (Fumoleau et al., 2003) and head and neck cancers (Posner et al., 2000). The conversion of FU into fluorodeoxyuridine monophosphate (FdUMP) leads to the inhibition of thymidylate synthase (TS, EC 2.1.1.45, the key enzyme of de novo deoxythymidine 5′-monophosphate synthesis, Figure 1) and subsequent DNA synthesis arrest. The presence of FdUMP induces the formation of an inactive ternary complex between TS, FdUMP, and the methyl donor-reduced folate 5-10 methylenetetrahydrofolate (CH₂FH₄). Experimental studies have clearly established that the stabilisation of the ternary complex, and thus optimal TS inhibition, requires elevated cellular concentrations of CH₂FH₄ (Frosst et al., 1995). The C677T (exon 4) and A1298C (exon 7) single-nucleotide polymorphisms (SNPs) are the two most commonly linked with altered phenotypes, both associated with lower enzyme activity (Frosst et al., 1995; Weisberg et al., 1998). The frequency of the mutated 677 TT genotype is around 10–15% in Caucasians, and only a few percent in Afro-Americans (Ueland et al., 2001). The 677C>T mutation enhances the thermolability of the enzyme (Frosst et al., 1995). The mutated
677 TT genotype is associated with elevated plasma homocysteine concentrations, as well as DNA hypomethylation that is involved in carcinogenesis processes. The influence of C677T polymorphism on disease risk is closely related to the nutritional folate status. In case of folate intake deficiency, 677 TT genotype is related to increased risk of congenital neural tube defect and to colorectal cancer (Ueland et al., 2001). The A1298C genotype has been less extensively studied (Van der Put et al., 1998; Weisberg et al., 1998) and the frequency of the mutated 1298 CC genotype reported in Japanese patients is around 3% (Uran et al., 2002). Interestingly, Uran et al. (2002) recently reported that the mutated 677 TT genotype was associated with greater methotrexate toxicity, whereas the mutated 1298 CC genotype was linked with better methotrexate efficacy. Since MTHFR enzymatic deficiency may theoretically favour thymidine synthesis via an increase of CH2FH4, one can hypothesise that tumours exhibiting mutated MTHFR genotypes linked to enzymatic deficiency may be more sensitive to FU cytotoxicity than wild-type (wt) MTHFR genotype tumours. Such a possible impact of MTHFR genotype may also affect normal host tissues. So far, the influence of C677T and A1298C polymorphisms on fluorouracil sensitivity and/or toxicity remains unknown.

Numerous experimental and clinical studies have previously shown that elevated tumoral TS activity or expression is related to FU resistance (Beck et al., 1994; Peters et al., 2002). The TS gene is located on chromosome 18p. A genetic polymorphism has been reported in the 5' regulatory region (cis-acting enhancer element) of the TS promoter that presents either double or triple tandem repeats of a 28 bp sequence in Caucasian and Asian populations (Horie et al., 1995; Marsh et al., 1999). In an expression assay, Horie et al. (1995) first reported that expression of the gene with triple repeat was higher than that of the gene with double repeat. Clinical studies have reported that triple repeat homozygous tumors (3R/3R) exhibit either higher TS mRNA or TS protein levels as compared to double repeat homozygous (2R/2R) (Kawakami et al., 2001b; Pullarkat et al., 2001). Recent clinical studies performed on small sets of patients have suggested that 5′ TS polymorphism may influence fluoropyrimidine sensitivity, with lower response rate in homozygous 3R/3R patients as compared to others (Marsh et al., 2001; Pullarkat et al., 2001; Villafranca et al., 2001; Park et al., 2002). We recently analysed 5′ TS genotype on a large set of metastatic colorectal cancer patients receiving FU-based therapy and found similar response rates in 3R/3R, 3R/2R and 2R/2R patients (Etienne et al., 2002). A second TS polymorphism

**Table 1** Cell line characteristics and FU sensitivity (mean ± s.e. from three separate experiments)

| Tumour type | Name          | Origin      | FU IC50 (μM) | Opt FU IC50 (μM) | Basal CH2FH4 (pmol mg−1 protein−1) |
|-------------|---------------|-------------|--------------|------------------|-----------------------------------|
| Breast      | MCF7          | Pr Rochefort| 6.3 ± 2.0    | 1.3 ± 0.3        | 0.9 ± 0.3                         |
|             | T47D          | Pr Rochefort| 10.2 ± 1.1   | 1.8 ± 0.2        | ND                                |
|             | CAL51         | CAL         | 4.1 ± 0.1    | 4.1 ± 0.1        | ND                                |
|             | ZR75          | Pr Rochefort| 1.0 ± 0.3    | 0.2 ± 0.0        | 0.5 ± 0.1                         |
|             | CAL85-2       | CAL         | 2.0 ± 0.1    | 0.6 ± 0.1        | ND                                |
|             | CAL120        | CAL         | 7.0 ± 1.3    | 2.1 ± 0.2        | ND                                |
| Colon       | CAL14         | CAL         | 2.9 ± 0.6    | 1.5 ± 0.2        | ND                                |
|             | WIDR          | EORTC       | 3.6 ± 0.2    | 0.9 ± 0.1        | 0.4 ± 0.0                         |
|             | COLO205       | ATCC (CCL222)| 0.8 ± 0.1     | 0.2 ± 0.0        | 3.3 ± 2.1                         |
|             | SW620         | ATCC (CCL227)| 13.8 ± 2.7   | 7.3 ± 0.3        | 0.4 ± 0.1                         |
|             | SW403         | ATCC (CCL230)| 0.6 ± 0.1    | 0.2 ± 0.3        | ND                                |
|             | CAL124        | CAL         | 0.3 ± 0.1    | 0.1 ± 0.4        | ND                                |
| Intestine   | HUTU80        | ATCC (HTB40)| 9.7 ± 0.9    | 9.7 ± 0.1        | ND                                |
| Pancreas    | HS766T        | ATCC (HTB134)| 16.5 ± 2.4   | 8.3 ± 0.3        | ND                                |
| Head and neck| CAL33        | CAL         | 0.6 ± 0.1    | 0.2 ± 0.2        | ND                                |
|             | CAL27         | CAL         | 1.8 ± 0.2    | 0.4 ± 0.3        | 0.8 ± 0.3                         |
|             | Hep2          | ATCC (CCL23)| 25.4 ± 3.5   | 8.6 ± 0.1        | ND                                |
|             | KB            | ATCC (CCL17)| 7.3 ± 1.1    | 1.6 ± 0.2        | 1.3 ± 0.1                         |
|             | Detroit 562   | ATCC (CCL138)| 2.9 ± 1.0    | 1.0 ± 0.3        | 0.3 ± 0.0                         |

Opt FU IC50 = optimal FU IC50 obtained with FA supplementation (see Material and Methods); ND = not detectable; CH2FH4 = 5-methyltetrahydrofolate. Cell line origins: CAL cell lines come from our institute; Pr Rochefort is from INSERM U 184, Montpellier, France; EORTC, European Organisation for Research and Treatment of Cancer; ATCC, American Type Culture Collection (Rockeville, MD, USA).
Experimental Therapeutics

5-Fluorouracil sensitivity and genetic polymorphisms

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Consisting of a 6 bp deletion at bp 1494 in the 3’ untranslated region has recently been reported (Ulrich et al., 2000). The deleted 3’ variant is associated with decreased TS mRNA levels in colorectal tumours (Ulrich et al., 2000). No study has been reported on the relationship between 3’ TS polymorphism and fluoropyrimidine sensitivity.

The purpose of the present study was to analyse the relationship between major TS and MTHFR polymorphisms on FU sensitivity. To this end, we investigated a large panel of 19 human cancer cell lines representative of fluoropyrimidine-treated tumours (digestive, breast, head and neck), and expressing spontaneous sensitivity to FU. Special attention was paid to the reduced folate status, and FU sensitivity was evaluated in the absence of FA supplementation in order to take into account the different fluoropyrimidine-based protocols administered to patients. In addition to the possible relationships between studied polymorphisms and FU cytotoxicity, we analysed the relationship between TS activity and polymorphisms, as well as links between CH₂FH₄ basal concentrations and MTHFR polymorphisms.

MATERIAL AND METHODS

Primers and probes

Primers and probes were all synthesised by Prologos (Paris, France). For TS polymorphisms, forward and reverse primers were GTGCGCTCTCGTTTCCCCC and GCTGCGAGGCCGCCAGGGGA-CA, respectively, for 5’ TS polymorphism, and GAGGAAATGCA-GAACACCTTC and AATCTGAGGGAGCTGAGTAAC, respectively, for 3’ TS polymorphism.

For MTHFR polymorphisms, anchor probes were labelled on the 3’ extremity with fluorescein. Mutated specific probes were labelled on the 5’ extremity to avoid extension by PCR.

Primers for C677T variants were: forward 5’-TGG CAG GTT ACC CCA AAG G-3’; reverse 5’-TGA TGC CCA TGT CCG TGC-3’. Labelled probes for C677T variants were: anchor 5’-TGG CAG GCC TGT GCA CCT GAA GCA CTT GAA GGA GAA GGT GTC T-3’; C variant (wild type) 5’-Red 640-CGG GAG CCG ATT TCA TCA T-3’ phos; T variant (mutated) 5’-Red 640-CGG GAG CCG ATT TCA TCA T-3’ phos. Primers for A1298C variants were: forward 5’-TTG TGG CAG ATT CCG CCA CTA CTA C-3’; reverse 5’-CTT CAC TTT GCC ATT CCT GGT TG-3’. Labelled probes for A1298C variants were: anchor 5’-AAG GAG GAG CTG CTT AAG ATG TGG TGG GAG GAG GCT-3’; A variant (wild type) 5’-Red 705-ACC AGT GAA AGT GTC TTT GA-3’; T variant (mutated) 5’-Red 705-ACC AGT GCA GAA AGT GTC TTT GA-3’ phos.

Cell lines

This study was conducted on a panel of 19 human cancer cell lines (six breast, seven digestive tract, five head and neck, one pancreas) expressing spontaneous sensitivity to FU (not previously exposed to FU), with IC₅₀ ranging from 0.3 to 25 μM (Table 1). Cell doubling time ranged from 1.3 to 6.3 days (mean 2.7; median 2.2).

Biochemical investigations

Of the 19 cell line panel, 14 had been previously investigated for FU sensitivity with or without FA supplementation, intracellular reduced folate content, and TS activity (Chéraudame et al., 1997a), as briefly described below. In order to match the physiological circulating folate concentration in humans, cells were grown in a folate-controlled medium for 10 days before experiments were started (folate-free DMEM medium supplemented with 40 nm of all-5-methyltetrahydrofolate, 0.1 mM of l-ascorbic acid for folate stabilisation, 10% FBS, 2 mM glutamine, 50 000 U l⁻¹ penicillin and 80 μM streptomycin) and all experiments have been subsequently performed in this folate-controlled medium.

Cytotoxicity experiments

Cells were exposed for 5 days to various FU (14 concentrations ranging from 0.01 to 500 μM), FA (6 concentrations ranging from 0.01 to 300 μM of pure l-FA) or FU + FA concentrations (sextuplicates in 96-well microtitration plates). Growth inhibition was assessed by the MTT test (Carmichael et al., 1987) and the dose–effect curves were analysed on GraphPad software (ISI, USA).

Intracellular reduced folate measurement

After 5 days of growth (175 cm² plates), cells were harvested, washed three times in phosphate buffer saline at +4°C and cell pellets containing approximately 50 × 10⁶ cells were stored in liquid nitrogen. 5-10 Methylentetrahydrofolate was measured on a 15 000 g cytosol, as previously described (Chéraudame et al., 1997a), based on the entrapment assay initially developed by Bunni et al. (1988). This assay is based on the stoichiometric formation of a stable ternary complex between CH₂FH₄, excess purified TS (0.225 μM final concentration), and excess 3H-FdUMP (0.35 μM final concentration). Recovery calculated from controls containing known CH₂FH₄ concentrations was 90% on average. Sensitivity limit was 0.3 pmol mg⁻¹ protein. Intra- and interassay reproducibility were 9.4 and 25.0%, respectively.

Measurement of TS activity

Thymidine synthase activity was measured according to the tritium-release assay initially described by Spears and Gustavsson (1988) and modified by us (Etienne et al., 2002). Cells (4 × 10⁶ cells ml⁻¹ in 50 ml Tris HCl buffer pH 7.3 containing 2 mM dithiothreitol) were sonicated on ice bed (three times at 10 s intervals) and centrifuged at 100 000 g (× 4°C). The assay consisted in incubating 25 μl of cytosol with excess 3H-dUMP (1 μM final concentration) and CH₂FH₄ (0.62 mM final concentration) in a total volume of 55 μl (in the previous buffer). After 0, 10, 20 and 30 min of incubation at +37°C, the reaction was stopped on ice bed. The excess of 3H-dUMP was removed by adding 300 μl of activated charcoal (15%) containing 4% trichloroacetic acid (5 min centrifugation at 14 000 g, room temperature). The 3H₂O formed during the incubation was then measured in an aliquot of the above supernatant. Results were expressed as fmols of 3H₂O formed per min per mg of protein, based on the linear regression obtained from the incubation times.

Cytosolic proteins were determined by the Bradford colorimetric assay (Protein Assay Reagent, Biorad Laboratories, Germany) with human serum albumin as standard. The sensitivity limit was 10 fmol min⁻¹ mg⁻¹ protein. The CV for interassay reproducibility (N = 7) was 15%.

Gene polymorphisms

Thymidine synthase and MTHFR genetic polymorphisms were analysed on DNA extracted from cell pellets stored in liquid nitrogen.

Determination of 5’ and 3’ TS polymorphisms

For the 5’ polymorphism, a fragment containing the 28-bp repeats was amplified (expected fragment size was 220 bp for 2R and 248 bp for 3R). For the 3’ polymorphism, a fragment containing the 6 bp deletion was amplified (expected fragment sizes were 110 bp for the wild type and 104 bp for the variant allele). In each case, PCR were run on a GeneAmp® PCR system 9700 (Applied Biosystems, Courtabeuf, France) in a 25 μl final volume containing 50 ng of genomic DNA, 1 mM MgCl₂, 2.5 μl of buffer 10 x, 1.25 mM of dNTPs, 0.15 μM of each specific forward and reverse primer and 0.05 U μl⁻¹ of Taq polymerase Cetus (Perkin Elmer, Courtabeuf, France).
France). After 30 cycles of amplification (denaturation at 94°C for 30 s, annealing at 62°C for 60 s, and extension at 72°C for 90 s), amplification products were electrophoresed on acrylamide gel at 8%. For 5’ polymorphism, products of 220 bp (2R/2R), 248 bp (3R/3R) or both (2R/3R) were observed. For 3’ polymorphism, products of 110 bp (6bp/6bp), 104 bp (0bp/0bp) or both (6bp/0bp) were observed.

**Determination of MTHFR polymorphisms** The C677T (Ala→Val) and A1298C (Glu→Ala) variants were analysed simultaneously by means of melting curve analyses on LightCycler (Roche), based on the fluorescence resonance energy transfer (FRET) principle (see Figure 2 for a typical example of analysis). We used a method derived from that initially described by Nakamura et al (2002). A duplex PCR amplification was first run in 20 μl final volume containing 80 ng genomic DNA (2 μl), 2 μl of ready-to-use Hotstart PCR mixture (LightCycler Faststart DNA Master Hybridization Probes kit, Roche Diagnostic, France), 3 mM MgCl2, 0.2 μM of each primer, 0.2 μM of each specific anchor fluorescein-labelled probe, and 0.4 μM of each specific wt variant (C 677 or A 1298 LC-red-labelled probe), in PCR grade water. After DNA denaturation and enzyme activation (8 min at 95°C), DNA was amplified for 45 cycles (15 s at 95°C, 10 s at 55°C and 10 s at 72°C). At the end of PCR, melting curves of the DNA/probes complexes synthesised were performed by increasing temperature gradually (0.1°C s⁻¹) up to 95°C. Methylenetetrahydrofolate reductase variant identification was based on the fact that the melting temperature of the DNA/probe complex is lower in the case of DNA/probe T/C mismatch at nucleotide 677 or DNA/probe C/A mismatch at nucleotide 1298. The C677T genotype was monitored at 640 nm and the melting curve showed a single peak at 64°C for C/C samples, a single peak at 55°C for T/T samples, and two peaks for heterozygous cell lines. The A1298C genotype was monitored up to 95°C and the melting curve showed a single peak at 64°C for C/C samples, a single peak at 55°C for T/T samples, and two peaks for heterozygous cell lines. The A1298C genotype was monitored at 705 nm and the melting curve showed a single peak at 63°C for T/T samples, and two peaks for heterozygous cell lines. The A1298C genotype was monitored at 705 nm and the melting curve showed a single peak at 63°C for T/T samples, and two peaks for heterozygous cell lines.

**Data analysis and statistics**

All experiments were performed in triplicate. 5-Fluorouracil IC₅₀ was defined as the concentration causing 50% growth inhibition as LC-red-labelled probes, in order to confirm the presence of the specific analysed mutation.

| Table 2 | Description of TS activity, TS and MTHF polymorphisms |
|---------|------------------------------------------------------|
| **Tumour type** | **Cell lines** | **TS activity** (pmol min⁻¹ mg⁻¹ protein) | **TS polymorphisms** | **MTHFR polymorphisms** |
| Breast | MCF7 | 18.1 ± 3.0 | 3R/3R | C677T genotype |
| | T47D | 29.1 ± 1.0 | 3R/3R | A1298C genotype |
| | CAL51 | 48.6 ± 5.6 | 2R/2R | homozygous mut types (TT). |
| | ZR75 | 14.4 ± 0.9 | 2R/3R | homozygous wt (CC), 64°C and 55°C for heterozygous (CT) and 55°C for homozygous mut types (TT). |
| | CAL85-2 | 41.6 ± 0.9 | 2R/3R | homozygous mut types (TT). |
| | CAL120 | 17.1 ± 1.5 | 2R/3R | homozygous wt (CC), 64°C and 55°C for heterozygous (CT) and 55°C for homozygous mut types (TT). |
| Colon | CAL14 | 11.6 ± 2.1 | 2R/3R | homozygous mut types (TT). |
| | WIDR | 9.5 ± 3.0 | 2R/3R | homozygous wt (CC), 64°C and 55°C for heterozygous (CT) and 55°C for homozygous mut types (TT). |
| | COLO205 | 19.9 ± 3.8 | 2R/3R | homozygous mut types (TT). |
| | SW620 | 24.6 ± 2.4 | 2R/3R | homozygous mut types (TT). |
| | SW403 | 6.7 ± 0.2 | 2R/3R | homozygous mut types (TT). |
| | CAL124 | 7.2 | 2R/3R | homozygous mut types (TT). |
| Intestine | HUTU80 | 82.8 ± 10.0 | 2R/3R | homozygous mut types (TT). |
| Pancreas | HS766T | 172.2 ± 0.3 | 2R/3R | homozygous mut types (TT). |
| Head and neck | CAL33 | 34.3 ± 0.9 | 2R/3R | homozygous mut types (TT). |
| | CAL27 | 38.4 ± 5.8 | 2R/3R | homozygous mut types (TT). |
| | Hep2 | 58.3 ± 1.9 | 2R/3R | homozygous mut types (TT). |
| | KB | 9.7 ± 1.9 | 2R/3R | homozygous mut types (TT). |
| | Detroit 562 | 18.6 | 2R/3R | homozygous mut types (TT). |

TS = thymidylate synthase; MTHFR = methylenetetrahydrofolate reductase.
compared to control cells. In the conditions with FA supplementation, for each tested FA concentration we computed a potentiation factor (F) equal to the FU IC₅₀ without FA divided by the FU IC₅₀ with FA. Optimal FU IC₅₀ corresponded to the IC₅₀ obtained in the presence of optimal FA, that is, allowing 90% of the maximal F value to be reached. Correlations between TS activity and FU IC₅₀s were tested by means of Pearson correlation, after logarithm transformation that allows the Gaussian distribution to be fitted. All other statistics were performed by means of nonparametric tests: Spearman rank correlation, Kruskal–Wallis test, Mann–Whitney test. For this latter test, mut vs wt comparison was performed by merging homozygous and heterozygous mutated variants vs homozygous wt cell lines. Statistics were performed on SPSS software (Chicago, USA).

RESULTS

Cell line sensitivity to FU ± FA, intracellular reduced folates and TS activity

Table 1 summarises cell line sensitivity to FU alone or in combination with optimal FA concentration. In two cell lines (CAL51 and HUTU 80), FU cytotoxicity was not enhanced by FA. The basal intracellular CH₂FH₄ concentration was detectable in nine cell lines out of the 19 investigated. Thymidylate synthase activity (Table 2) ranged between 6.7 and 82.8 pmol min⁻¹ mg⁻¹ protein (mean 27). The greater the FU sensitivity, the lower the TS activity (P = 0.078 and 0.032, in the absence and presence of optimal FA concentration, respectively). Basal CH₂FH₄ was not linked to FU sensitivity.

5’ and 3’ TS polymorphisms

Distribution of 5’ TS genotype (Table 2) was 36.8% 2R/2R (n = 7), 31.6% 2R/3R (n = 6) and 31.6% 3R/3R (n = 6). Distribution of 3’ TS genotype (Table 2) was 57.9% 6 bp/6 bp (n = 11), 21.1% 6 bp/0 bp (n = 4) and 21.1% 0 bp/0 bp (n = 4). Cell doubling time was not linked to 5’ or 3’ TS genotypes.

Thymidylate synthase activity was significantly different according to 5’ TS genotype, heterozygous cell lines exhibiting significantly higher TS activities than homozygous ones (median 20, 40 and 17 pmol min⁻¹ mg⁻¹ in 2R/2R, 2R/3R and 3R/3R, respectively; Kruskal–Wallis P = 0.050, Figure 3A). No significant relationship was observed between TS activity and 3’ TS genotype (Kruskal–Wallis P = 0.23, Figure 3B). Whether in the absence or presence of FA, FU sensitivity was not statistically associated with either 5’ or 3’ TS polymorphism (Figure 4).

C677T and A1298C MTHFR polymorphisms

Distribution of C677T genotype (Table 2) was 31.6% C/C (n = 6), 52.6% C/T (n = 10), 15.8% T/T (n = 3) and that of A1298C was 52.6% A/A (n = 10), 36.8% A/C (n = 7) and 10.5% C/C (n = 2) (Table 2).

From Tables 1 and 2, it appears that basal CH₂FH₄ concentrations were not detectable in 5/6 homozygous wt (C/C) 677 genotype, whereas detectable concentrations were observed in 7/13 mut (T/T and C/T) 677 genotype. Also, basal CH₂FH₄ concentrations were below detection limit in 7/10 homozygous wt (A/A) 1298 genotype, in contrast with detectable concentrations observed in 5/9 mut (C/C and A/C) 1298 genotype. The above observations, which did not reach statistical significance, are depicted in Figure 5. Of note, in the three cell lines with homozygous wt genotype for both 677 and 1298 (CAL85-2, CAL120, HS766T), CH₂FH₄ was always below the detection limit.

Whether in the absence or presence of FA, FU sensitivity (FU IC₅₀ or optimal (Opt) FU IC₅₀) was not linked to C677T genotype (C/C vs C/T vs T/T: nonsignificant, Figure 6; wt (C/C) vs mut (C/T + T/T): nonsignificant). In contrast, FU efficacy tended to be higher in mutated A1298C variants (C/C + A/C), both in ‘physiological-folate’ conditions (Figure 7A) and in the presence of optimal FA concentration (Figure 7B). In ‘physiological-folate’ conditions, FU IC₅₀ ranged from 0.6 to 25.4 μM (median 8.4) in the 10 cell lines exhibiting wt A/A 1298 variant, whereas IC₅₀ were comprised between 0.3 and 7.3 (median 2.9) in the nine cell lines exhibiting mutated A/C or C/C 1298 variants; this difference was very close to statistical significance (Mann–Whitney, P = 0.055). A similar pattern of distribution, although less significant (Mann–Whitney, P = 0.085), was observed for Opt FU IC₅₀ that is in the presence of optimal FA concentrations.

DISCUSSION

In the context of cancer treatment, pharmacogenetic exploration may result, in the future, in the replacement of tedious and heavy phenotypic explorations either at blood level (pharmacokinetics) or at tumour level (prognostic/predictive markers) by genetic analyses performed on easily obtainable DNA samples from
normal cells (blood or oral cavity brushing for instance). The observation that pharmacogenetic status faithfully reflects phenotypic changes at the target level is a prerequisite of clinical pharmacogenetic applications. In fact, from a theoretical point of view, genetic polymorphisms are identical in all tissues. However, the possibility of a clonal selection emerging from a heterozygous subject during the process of carcinogenesis cannot be ruled out. This strengthens the need to examine the impact of cancer-treatment-related gene polymorphisms at the tumoral target itself.

The aim of the present study was thus to analyse the impact at tumoral target level of polymorphisms of two major genes related to DNA synthesis, namely TS and MTHFR polymorphisms, which may influence FU cytotoxicity. To our knowledge, the present experimental study is the first one designed for this purpose. Moreover, the present model was specially controlled for reduced folate status (folate-free medium supplemented with physiological-compatible CH2FH4 concentrations) and experiments were conducted both in the absence and presence of optimal FA concentrations, in order to mimic the two opposite situations regarding FA supplementation in FU-based treated patients. To this end, we closely explored a panel of 19 cancer cell lines expressing spontaneous FU sensitivity and covering the major fluoropyrimidine-treated localisations (digestive tract, breast, head and neck). The 5′ TS and C677T MTHFR genotype frequencies were in the range of those reported in Caucasian populations (Marsh et al, 1999; Ueland et al, 2001), strengthening the relevance of the present model. The 3′TS and A1298C MTHFR polymorphisms have been less extensively studied in Caucasian populations, thus frequency comparisons with published data were difficult to perform. The chosen experimental approach did not allow allelotype analyses to be performed, since such analyses require large population studies.

Up to now, clinical studies investigating the influence of 5′ TS gene polymorphism on TS mRNA or protein level have given rather contrasting results. A retrospective study conducted on 52 colorectal tumour specimens reported that triple repeat homozygous (3R/3R) exhibit 3.6-fold higher TS mRNA levels as compared to double repeat homozygous (2R/2R) (Pullarkat et al, 2001). Another study conducted on 133 cancer biopsies (mostly colorectal cancer) showed no difference in TS mRNA level according to 5′ TS genotype, but demonstrated higher TS protein concentration in 3R/3R as compared to 2R/2R (Kawakami et al, 2001b). One of the major findings of the present study is that TS enzymatic activity is significantly influenced by the 5′ TS genotype. This result is somewhat surprising since TS activity was significantly higher in 2R/3R heterozygous cell lines (Table 2, Figure 3A). However, superimposable conclusions were drawn from the only available data on the link between TS activity and TS polymorphism, which we recently published on colorectal cancer patients (Etienne et al, 2002). Mandola et al (2003) recently described an additional G→C SNP within the second...
repeat of the triple tandem that may influence the transcriptional activity of the gene. Such an additional polymorphism in the 5’ regulatory region with functional consequences on transcriptional activity may complicate the links between TS activity and tandem repeat polymorphisms. This could explain the present unexpected data with a high TS activity in 2R/3R patients by Pullarkat et al (2001) in 50 patients receiving protracted FU infusion and by Park et al (2002) in 24 capecitabine-treated patients. Also, lower downstaging was demonstrated in 3R/3R tumours by Villafranca et al (2001) on 66 rectal cancer patients receiving FU-based chemoradiotherapy protocols. A significant shorter survival rate in 3R/3R patients receiving FU-based adjuvant chemotherapy was demonstrated by Iacopetta et al (2001). The value of 5’ TS genotype for predicting fluoropyrimidine responsiveness and its use as a surrogate of TS measurement at the target level is still far from being clearly established, and requires additional large-scale prospective clinical studies including the recently reported G→C SNP within the triple tandem repeat (Kawakami and Watanabe, 2003; Mandola et al, 2003).

Methylenetetrahydrofolate reductase is a key enzyme of the folate metabolic pathway (Figure 1). Two SNPs (C677T and A1298C) associated with altered phenotypes have been described for this enzyme (Frosst et al, 1995; Weisberg et al, 1998). The mutated forms of these variants (i.e. 677TT and 1298CC) exhibit significantly lower enzymatic activity, and should theoretically lead to an accumulation of intracellular CH₂FH₄ concentrations as compared to wt forms. Consequently, C677T and A1298C
MTHFR polymorphisms may influence the pharmacodynamics of fluoropyrimidines since they control the intracellular concentration of the specific reduced folate required for optimal TS inhibition. This hypothesis is supported by preclinical and clinical data (Danenberg and Danenberg, 1978; Houghton et al., 1981; Rustum et al., 1987; Keyomarsi and Moran, 1988; Chéradame et al., 1997a,b) demonstrating the impact of CH2FH4 intratumoral concentrations on FU cytotoxicity. Recent clinical studies have suggested that MTHFR polymorphisms may be associated with methotrexate pharmacodynamics (Ulrich et al., 2001; Urano et al., 2002). However, no study has so far reported their impact on fluoropyrimidine sensitivity (Wisotzkey et al., 1999). First of all, the present data closely concur with the theoretical impact of C677T and A1298C polymorphisms on CH2FH4 intracellular pool: five out of six homozygous wt C677 variants and seven out of ten homozygous wt A1298 variants exhibited nondetectable CH2FH4 concentrations, in contrast to six out of 13 T/T variants and C/T 677 variants, and four out of nine C/C or A/C 1298 variants (Figure 5, Tables 1 and 2). These data agree with those of Kawakami et al. (2001a) who recently reported relationships between MTHFR genotypes and tumoral-reduced folates in gastrointestinal cancer.

The C677T genotype did not significantly influence FU cytotoxicity, whether in the absence or presence of optimal FA supplementation (Figure 6). However, FU sensitivity was related to A1298C MTHFR genotype: homozygous mutated cell lines (C/C) were the more sensitive, homozygous wt (A/A) were the more resistant, heterozygous cell lines (A/C) exhibiting intermediary sensitivity (Figure 7, Tables 1 and 2). The influence of A1298C MTHFR polymorphism on FU sensitivity was observed irrespective of the absence or presence of optimal FA supplementation. These stimulating results show a trend close to statistical significance (P = 0.055 and 0.085 in the absence and presence of FA, respectively) and are thus consistent with the initial hypothesis that MTHFR genotypes associated with altered enzymatic activity may be more FU sensitive.

Altogether, it is hoped that the present data will encourage future studies to consider not only TS polymorphisms but also MTHFR polymorphisms as potential predictors of fluoropyrimidine responsiveness and/or toxicity. Both tumoral and constitutional genotype analyses should be taken into account in clinical prospective studies, particularly regarding the design of future FU-based chemotherapy study.
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