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Involvement of gene polymorphisms of the folate pathway enzymes in gene expression and anticancer drug sensitivity using the NCI-60 panel as a model

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ABSTRACT

Folate, a vitamin of the B group involved in one-carbon group metabolism, plays an important role in DNA synthesis and methylation. Several polymorphisms in the genes involved in folate uptake and biotransformations have been shown to be associated to the risk of cancer and to anticancer drug response. We studied common polymorphisms in *MTHFR* ($N^{5,10}$-methylene-tetrahydrofolate reductase), *MTHFD1* ($N^{5,10}$-methylene-tetrahydrofolate dehydrogenase), *MTR* (methionine synthetase) and *SLC19A1* (reduced folate carrier) in the panel of 60 human tumour cell lines established by the NCI for anticancer drug screening and we tentatively associated these polymorphisms with gene expression and drug cytotoxicity as extracted from the public database of the Developmental Therapeutic Programme. We observed a consistent and highly significant association between the presence of the variant C allele of the A>C1298 polymorphism of *MTHFR* and the sensitivity to many anticancer drugs belonging to the classes of antifolates, antimetabolites, alkylating agents and, to a lesser extent, topoisomerase inhibitors. In contrast, the T variant allele of the C>T677 variation of *MTHFR* was rather associated to lower sensitivity of the cell lines towards anticancer drugs (alkylating agents, antifolates and antimetabolites) but with much lower effects than the A>C1298 variation. The polymorphisms of the other genes studied were not associated with differences in anticancer drug sensitivity, but the expression of the *SLC19A1* gene was significantly correlated with the sensitivity to several drugs (antifolates, thiopurines, nitrosoureas, and DACH-platinum drugs). We concluded that the NCI-60 panel may constitute a good starting point for implementing clinical studies aimed at discovering and validating predictive genetic markers of drug efficacy and/or toxicity.

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1. Introduction

Folate, a vitamin of the B group involved in one-carbon group metabolism, plays an important role in DNA synthesis and methylation (see Ref. [1] for review). Under the form of $N^{5,10}$-methylene-tetrahydrofolate, it is used as a cofactor for thymidylate synthesis; under the form of N^{10}-formyl-tetrahydrofolate, it is used in several reactions for purine biosynthesis; under the form of N^{5}-methyltetrahydrofolate, it is used to transform homocysteine into methionine, which, after conjugation with an adenine nucleotide, is the methyl donor for DNA methylation. Several enzymes play a major role in folate metabolism. Among them, methylene-tetrahydrofolate reductase (MTHFR) converts $N^{5,10}$-methylene-tetrahydrofolate to N^{5}-methyl-tetrahydrofolate and, therefore, may limit the production of dTMP via thymidylate synthase (TYMS) and enhance methyl availability for DNA methylation. The methylation of homocysteine is catalysed by methionine synthase (MTR), which requires vitamin B12 as a cofactor. Methylene-tetrahydrofolate dehydrogenase (MTHFD1) is a trifunctional NADP-dependent cytoplasmic enzyme that possesses three distinct enzymatic activities: $N^{5,10}$-methylene-tetrahydrofolate dehydrogenase, $N^{5,10}$-methenyltetrahydrofolate cyclohydrolase and N^{10}-formyltetrahydrofolate synthetase, which successively catalyse the different steps generating the one-carbon donor used for purine synthesis. Fig. 1 presents a simplified scheme of the folate pathways in cells. Alterations in expression and/or activity of any of these enzymes may have important consequences at different levels of cell metabolism, especially cell proliferation, in view of the role of folates in DNA synthesis and methylation.

Tetrahydrofolate is carried into cells via a transporter called the reduced folate carrier (RFC), which belongs to the SLC19A family of transporters. Several anticancer agents called "antifolates", such as methotrexate, exert their cytotoxic effect via interference with the folate pathway. Resistance to these drugs may occur, among other factors, by an altered cell uptake. They may have various enzyme targets in the cell, some of them limited to a single enzyme and other having multiple distinct targets. Methotrexate acts preferentially as an inhibitor of dihydrofolate reductase (DHFR), while raltitrexed interacts mainly with TYMS. Pemetrexed, in contrast, can interfere with several enzymes of the folate pathways. Folate coenzymes generally act as poly-γ-glutamyl conjugates and some of the therapeutic antifolates may require activation through folyl-polyglutamyl synthase (FPGS) activity (see Ref. [2] for review).

The genes which encode the enzymes and transporter involved in these pathways display several single nucleotide polymorphisms (SNPs). Two common polymorphisms in the *MTHFR* gene have been described, both of which reduce enzyme activity. The C to T substitution at nucleotide 677 converts an alanine to a valine at position 222 of the polypeptide chain. It is associated with reduced enzyme activity and causes increased plasma homocysteine levels associated with lower folate status. Human cells harbouring the homozygous T/T genotype have 30% of the in vitro enzyme activity seen in those with the C/C wild-type. Heterozygous C/T cells show 65% of normal enzyme activity.[3] The variant T/T genotype is present in about 10–12% of Caucasian and Asian populations while heterozygous genotype constitutes approximately 40% of these populations. A second common polymorphism has been reported that results in an A to C substitution at position 1298, leading to an amino-acid substitution glutamate to alanine at position 429 that also leads to decreased enzyme activity but to a lesser extent,[4,5] without

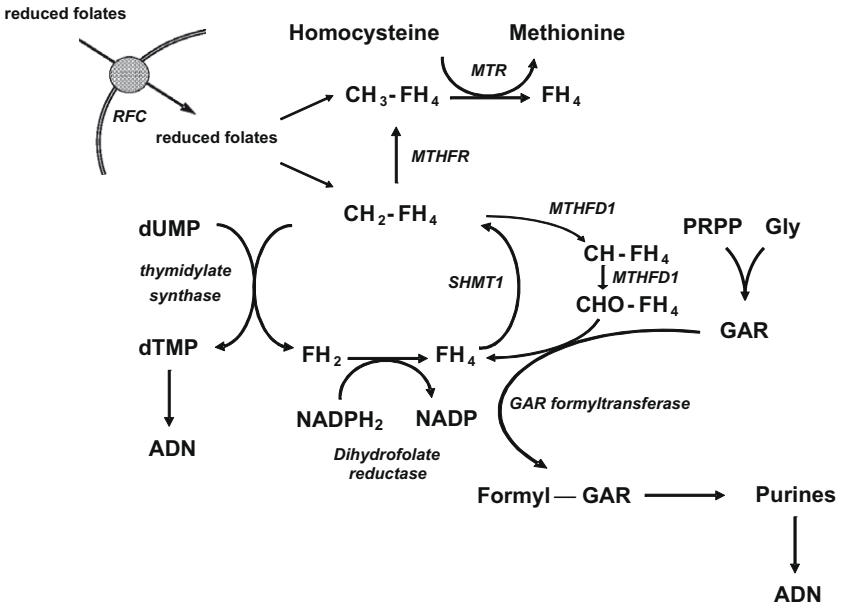

Fig. 1 – Schematic representation of the folate pathways. Abbreviations as follows: CH-FH_4: $N^{5,10}$-methenyltetrahydrofolate; CH_2-FH_4: $N^{5,10}$-methylenetetrahydrofolate; CH_3-FH_4: N^{5}-methyltetrahydrofolate; CHO-FH_4: N^{10}-formyltetrahydrofolate; GAR: phosphoribosylglycinamide; MTHFD1: methylenetetrahydrofolate dehydrogenase; MTHFR: methylene tetrahydrofolate reductase; MTR: methionine synthase; RFC: reduced folate carrier; and SHMT1: serine hydroxymethyltransferase.

significant differences in homocysteine plasma levels. These two polymorphisms (C>T677 and A>C1298) are in strong linkage disequilibrium.[6] They have been extensively studied in a wide variety of pathologies such as thrombosis, because homocysteine is both thrombo- and atherogenic, tube neural defects, rheumatoid arthritis, because methotrexate is used for the treatment of rheumatoid arthritis, and cancer. In cancer, MTHFR polymorphisms have been studied first as potential risk factors of cancer occurrence (see Refs. [7–10] for meta-analyses) and also as possible factors of response to antifolates and to inhibitors of TYMS (fluoropyrimidines) (see Refs. [11–13] for reviews).

In the *MTR* gene, an A>G polymorphism at position 2756 replaces aspartic acid by glycine in the protein-binding region of the enzyme (codon 919).[14] This functional polymorphism also causes an increase in homocysteine levels through decreased methionine synthesis and may be associated with DNA hypomethylation, which may alter cancer risk. This polymorphism has been studied especially in relation with the susceptibility to develop various cancers without consistent findings.[8,15,16] A polymorphism in *MTHFD1* gene (1958 G>A) results in the substitution of a conserved arginine amino-acid by a glutamine at position 653. Despite the role of this enzyme in folate pathway, this polymorphism has been little explored in cancer.[17,18] Finally, a G>A polymorphism at position 199 of the *SLC19A1* gene, which encodes the RFC transporter, converts a histidine to an arginine at codon 27. This polymorphism also affects folate and homocysteine levels.[19] Moreover, as this transporter is involved in methotrexate uptake, it can be hypothesised that an altered transport may influence drug toxicity and/or clinical outcome.[20]

It can be hypothesised that genetic polymorphisms in *MTHFR*, *MTR*, *MTHFD1* and *SLC19A1* genes may not only modify susceptibility to different pathologies and especially cancers, but may also account for part of the large interpatient variability in the therapeutic response to anticancer agents. Such clinical studies, although necessary, are complex and have often provided conflicting results. It remains difficult to demonstrate the role of a given polymorphism in efficacy and/or toxicity of a treatment in relatively small cohorts of patients. In order to clarify these discrepancies and to establish relationships between these polymorphisms and cellular sensitivity to anticancer drugs, we have studied these SNPs in the NCI-60 human tumour cell lines panel. The NCI-60 model consists of a panel of 60 human tumour cell lines of various origins which has served for the primary screening of thousands of potential anticancer drugs.[21] We and others have used the NCI-60 model to show the relationships between the presence of a given polymorphic variation and the *in vitro* sensitivity to many anticancer drugs,[22–25] as it had been originally done for establishing relationships between gene expression profiles and chemosensitivity.[26] The free availability of the databases elaborated by the Developmental Therapeutic Programme (DTP) of the National Cancer Institute (NCI) allows to establish genotype/phenotype associations on a large number of drugs without performing drug cytotoxicity assays. The fact that the cell lines are of tumour origin adds a special advantage when the polymorphisms studied concern genes encoding drug targets or proteins involved in DNA repair, since the activity of such proteins in the tumour is expected to play a major role in drug cytotoxicity. On the contrary, it would be more interesting to study polymorphisms in genes responsible for drug metabolism or transport in germline cells rather than in tumour cells, since they are expected to play a role in drug toxicity in the clinical setting. Using the NCI-60 model, in this paper we show that the A>C1298 SNP of the *MTHFR* gene plays a prominent role in *in vitro* chemosensitivity, which gives some clues for the understanding of clinical reports and for the implementation of prospective clinical studies aimed at developing predictive pharmacogenetic tests.

2. Materials and methods

Frozen cell pellets from 59 of the 60 NCI cell lines of the panel were kindly provided by Dr. S. Holbeck, Cancer Therapeutic Branch, NCI, Bethesda. One cell line, MDA-N, is no longer available in the NCI panel.

Genomic DNA was extracted from cell pellets using QIAamp® DNA minikit from Qiagen. It was quantified by spectrophotometry. Polymerase chain reactions (PCRs) were performed on genomic DNA using appropriate primers (see below). Polymorphisms were detected using restriction fragment length polymorphism (RFLP) techniques on PCR products, using appropriate restriction enzymes (see below). Electrophoresis was performed before and after digestion of PCR products on 2.5% agarose gels. This technique allowed the unambiguous discrimination between variant homozygous, common homozygous and heterozygous cell lines for the polymorphisms studied. Two independent determinations were performed and provided exactly the same results. Sequencing by the Sanger method was performed on 10 randomly chosen PCR products from the various genotypes of the variations studied. Concordance with RFLP was obtained in 100% of the cases.

The polymorphisms that were determined and the conditions of determination are presented in Table 1. The PCRs conditions for *MTHFR*, *SLC19A1* and *MTHFD1* gene polymorphisms were identical; 35 cycles were performed (30 s at 95 °C for denaturation, 30 s at 55 °C for hybridisation and 1 min at 72 °C for elongation). For *MTR*, annealing temperature was 60 °C for 1 min. AmpliTaq Gold polymerase (Applied Biosystems, Courtaboeuf, France) was used for the PCRs, which were performed by use of a thermocycler (GeneAmp PCR System 9600; Applied Biosystems).

After identification of the genotypes of each cell line, the IC_{50} values of 136 core drugs vis-à-vis the 59 cell lines, expressed as $-\log_{10}(IC_{50})$, were extracted from the NCI database (http://dtp.nci.nih.gov); mean values were calculated for common homozygous, variant homozygous and heterozygous cell lines and were compared by analysis of variance using a general linear model. Drugs were grouped as a function of their known mechanism of action into eight categories (see Ref. [25] for details): alkylating or platinating agents acting on N^7 of guanine; other alkylating agents, acting on N^2 or O^6 of guanine; antimetabolites; antifolates; topoisomerase I inhibitors; topoisomerase II inhibitors; spindle poisons, subdivided into vinca-alkaloid-type and taxane-type mechanisms of action. Only 18 drugs of 136 remained unclassified

Table 1 – PCR primers used for the determination of the polymorphisms.

Gene	Alteration	Primers	Restriction enzyme	PCR products (bp)
MTHFR				
rs1801133	C>T 677, ala222val	S: 5′ TGAAGGAGAAGGTGTCTGCGGGA 3′ AS: 5′ AGGACGGTGCGGTGAGAGTG 3′	*HinfI*	WT: 198 HT: 198 + 175 + 23 VAR: 175 + 23
rs1801131	A>C1298, glu429ala	S: 5′ CTTTGGGGAGCTGAAGGACTACTA 3′ AS: 5′ CACTTTGTGACCATTCCGGTTTG 3′	*MboII*	WT: 56 + 31 + 30 + 28 + 18 HT: 84 + 56 + 31 + 30 + 28 + 18 VAR: 84 + 31 + 30 + 18
SLC19A1				
rs1051266	G>A80, his27arg	S: 5′AGTGTCACCTTCGTCCCCTC 3′ AS: 5′CTCCCGCGTGAAGTTCTT 3′	*CfoI*	WT: 125 + 68 + 37 HT: 162 + 125 + 68 + 37 VAR: 162 + 68
MTHFD1				
rs2236225	G>A1958, arg653glu	S: 5′ CACTCCAGTGTTTGTCCATG 3′ AS: 5′ GCATCTTGAGAGCCCTGAC 3′	*MspI*	WT: 196 + 70 + 56 + 8 HT: 267 + 196 + 70 + 56 + 8 VAR: 267 + 56 + 8
MTR				
rs1805087	A>G2756, gly919asp	S: 5′ TGTTCCCAGCTGTTAGATGAAAATC 3′ AS: 5′ GATCCAAAGCCTTTTACACTCCTC 3′	*HaeIII*	WT: 211 HT: 211 + 131 + 80 VAR: 131 + 80

because of disagreement about their precise mechanism of action.

The χ^2 test was used for comparing the distribution of the cell lines among genotypes. Pearson coefficients of correlation were computed for comparing continuous variables (IC_{50} and gene expression data extracted from the NCI database). To study the pharmacological parameters as a function of the genotype, we analysed the variances of drug GIC_{50}s values or gene expression for each genotype and calculated the significance of the differences in mean values, using a general linear model taking into account the unbalanced size of the groups. Chemosensitivity could be studied independently for each drug or, after normalisation, for each drug group as already defined.

3. Results

We present in Table 2 the distribution of the various genotypes of the five SNPs studied. With the exception of the C>T677 SNP in the *MTHFR* gene, all of them were in Hardy–Weinberg equilibrium, with no distortion of genotype distribution as compared to the distribution expected from allele frequencies (Table 3). For the C>T677 variation of the *MTHFR* gene, the proportion of heterozygous genotypes was lower than that expected and this could be interpreted as a loss of heterozygosity. Table 4 presents the combined *MTHFR* genotypes in the NCI-60 panel. Two combined genotypes were not encountered: the double homozygous variant T^{677}/T^{677}–C^{1298}/C^{1298} variant and the 677-heterozygous – 1298-variant homozygous genotypes (C^{677}/T^{677}–C^{1298}/C^{1298}). As a consequence, it appeared that the two SNPs of the *MTHFR* gene were in linkage disequilibrium, the C^{1298} variant being more frequently associated to the T^{677} variant than to the C^{677} common genotype. We have established the haplotype distribution for the *MTHFR* variations: by considering the haplotypes that were required to explain the genotypes observed, the C^{677}–A^{1298} haplotype was ascertained in 20 cell lines, the C^{677}–C^{1298} haplotype in 25 cell lines, the T^{677}–A^{1298} haplotype in 19 cell lines and the T^{677}–C^{1298} haplotype in only one cell line. The haplotype of 9 double heterozygous cell lines could not be ascertained.

We first looked for associations between the expression of the genes under study and the activity of the 136 core drugs of the DTP database. The only gene whose expression was significantly associated with drug sensitivity was the RFC transporter, *SLC19A1*. For this gene, there was a significant positive correlation between cell sensitivity and gene expression for most antifolates, including methotrexate and raltitrexed, but also for several drugs of different classes: thiopurine derivatives, DACH-platinum drugs, nitrosoureas and some taxane analogues. For these drugs, the coefficient of correlation between $-\log_{10}(IC_{50})$ and gene expression ranged between 0.4 and 0.55 ($10^{-5} < P < 10^{-3}$). An example of the correlation between the expression of the *SLC19A1* gene and the cytotoxicity of methotrexate is presented in Fig. 2.

We then looked for associations between the polymorphisms identified and the expression of the corresponding genes. In the NCI-60 panel, we observed such an association for the *MTHFD1* gene, whose expression was significantly increased in variant homozygous cell lines as compared to common homozygous cell lines (ratio = 1.44, $P = 0.002$), the heterozygous cell lines having an intermediate level of expression. In addition, when considering the haplotypes of the *MTHFR* gene, it appears that the C^{677}–A^{1298} haplotype was associated with significantly higher levels of *MTHFR* expression. In cell lines homozygous for this haplotype compared to cell lines homozygous for the C^{677}–C^{1298} or the T^{677}–A^{1298} haplotypes, the expression ratio was 1.46 ($P = 0.0010$). In cell lines containing at least one allele of the C^{677}–A^{1298} compared to those not containing this allele, the expression ratio was 1.29 ($P = 0.014$).

Table 2 – Polymorphisms of the genes found in the NCI-60 panel.

Tumour type	Cell line	MTHFR C>T 677	MTHFR A>C1298	SLC19A1 G>A80	MTHFD1 G>A1958	MTR A>G2756
Leukaemia	CCRF-CEM	HT	HT	HT	HT	WT
	HL-60	VAR	HT	HT	HT	WT
	K-562	WT	HT	WT	WT	HT
	MOLT-4	WT	VAR	VAR	WT	HT
	RPMI-8226	WT	HT	HT	WT	HT
	SR	WT	HT	WT	HT	WT
Lung cancer	A549/ATCC	WT	VAR	WT	HT	HT
	EKVX	WT	VAR	VAR	HT	WT
	HOP-62	VAR	WT	WT	WT	HT
	HOP-92	VAR	WT	WT	WT	WT
	NCI-H226	HT	HT	HT	HT	WT
	NCI-H23	WT	WT	WT	WT	VAR
	NCI-H322M	VAR	WT	WT	HT	HT
	NCI-H460	WT	VAR	WT	WT	WT
	NCI-H522	WT	WT	HT	WT	HT
Colon cancer	COLO-205	WT	WT	HT	HT	VAR
	HCC-2998	HT	HT	WT	HT	WT
	HCT-116	HT	WT	HT	VAR	WT
	HCT-15	WT	HT	WT	WT	WT
	HT29	HT	HT	HT	WT	WT
	KM12	VAR	WT	WT	WT	HT
	SW-620	HT	WT	HT	VAR	WT
Central nervous system	SF-268	WT	VAR	WT	WT	VAR
	SF-295	HT	HT	VAR	WT	WT
	SF-539	WT	HT	VAR	WT	WT
	SNB-19	WT	VAR	HT	WT	WT
	SNB-75	WT	HT	VAR	HT	HT
	U251	WT	VAR	HT	WT	WT
Melanoma	LOXIMVI	WT	VAR	HT	HT	WT
	MALME-3M	WT	HT	VAR	HT	WT
	M14	HT	HT	HT	VAR	WT
	SK-MEL-2	VAR	WT	HT	VAR	HT
	SK-MEL-28	WT	WT	HT	VAR	WT
	SK-MEL-5	VAR	WT	HT	HT	WT
	UACC-257	HT	WT	WT	HT	HT
	UACC-62	WT	VAR	HT	VAR	WT
Ovarian cancer	IGROV1	VAR	WT	HT	VAR	WT
	OVCAR-3	WT	WT	VAR	HT	WT
	OVCAR-4	WT	WT	HT	HT	WT
	OVCAR-5	WT	VAR	VAR	WT	WT
	OVCAR-8	WT	VAR	VAR	VAR	WT
	SK-OV-3	HT	WT	HT	WT	WT
Renal cancer	786-0	WT	HT	WT	WT	WT
	A498	VAR	WT	VAR	WT	HT
	ACHN	VAR	WT	WT	VAR	WT
	CAKI-1	WT	VAR	HT	VAR	WT
	RXF-393	WT	HT	WT	VAR	WT
	SN-12C	WT	VAR	VAR	HT	VAR
	TK-10	VAR	WT	WT	VAR	WT
	UO-31	WT	HT	WT	WT	WT
Prostate cancer	PC-3	HT	HT	VAR	HT	WT
	DU-145	VAR	WT	HT	HT	HT
Breast cancer	MCF-7	VAR	WT	VAR	VAR	WT
	NCI/ADR-RES	WT	VAR	VAR	VAR	WT
	MDA-MB-231	WT	VAR	VAR	WT	WT
	HS578T	HT	HT	WT	WT	WT
	MDA-MB-435	HT	HT	HT	VAR	WT
	BT-549	VAR	WT	WT	VAR	WT
	T-47D	VAR	WT	WT	VAR	WT

Table 3 – Distribution of the genotypes of MTHFR, MTR, MTHFD1 and SLC19A1 in the NCI-60 panel.

	Common homozygous	Heterozygous	Variant homozygous	Variant allele frequency (%)	Distortion from Hardy–Weinberg equilibrium
MTHFR C>T677	31 (53%)	13 (22%)	15 (25%)	36.4	Yes ($P = 0.02$)
MTHFR A>C1298	24 (41%)	20 (34%)	15 (25%)	42.4	No
SLC19A1 G>A80	21 (36%)	23 (39%)	15 (25%)	44.9	No
MTHFD1 G>A1958	23 (39%)	20 (34%)	16 (27%)	44.1	No
MTR A>G2756	42 (71%)	13 (22%)	4 (7%)	17.8	No

Table 4 – Distribution of the genotypes of MTHFR combined genotypes in the NCI-60 panel.

C>T677 variation	A>C1298 variation	No. of cell lines
C^{677}/C^{677}	A^{1298}/A^{1298}	6
	A^{1298}/C^{1298}	10
	C^{1298}/C^{1298}	15
C^{677}/T^{677}	A^{1298}/A^{1298}	4
	A^{1298}/C^{1298}	9
	C^{1298}/C^{1298}	0
T^{677}/T^{677}	A^{1298}/A^{1298}	14
	A^{1298}/C^{1298}	1
	C^{1298}/C^{1298}	0

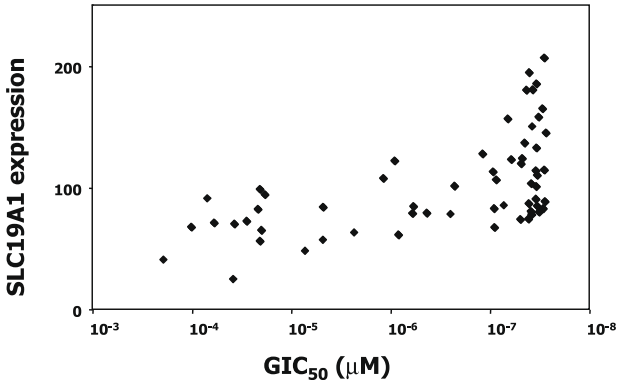

Fig. 2 – Correlation between the cytotoxicity of methotrexate (GIC_{50} in μM, abscissa) and the expression of the reduced folate carrier as obtained from Affymetrix U133 microarrays (arbitrary units, ordinates) ($r = 0.428$, $P < 0.001$).

We finally analysed the associations between the polymorphisms identified and the cytotoxicity of the core 136 drugs of the DTP database. No consistent relationship was found between the presence or the absence of the variations of the *SLC19A1*, *MTR* and *MTHFD1* genes and the sensitivity of the cell lines to any anticancer agent. In contrast, the *MTHFR* A>C1298 gene polymorphism was significantly associated to the sensitivity to numerous anticancer drugs, belonging to several classes: antifolates, antimetabolites, alkylating agents and, to a lesser extent, topoisomerase I and topoisomerase II inhibitors. The cell lines homozygous for the variant allele (C^{1298}) were significantly more sensitive to most anticancer drugs than the cell lines homozygous for the common allele, with the heterozygous cell lines behaving intermediately. For instance, the ratio of IC_{50} values for homozygous common and variant cell lines reached 6 for methotrexate ($P = 0.05$), 13 for cytarabine ($P = 0.008$) and 1.7 for melphalan ($P = 0.03$) (Fig. 3). When drug classes were considered as a whole, the mean IC_{50} ratios between variant and common homozygous cell lines were 4.9 for antifolates ($P = 5.3 \times 10^{-6}$), 2.8 for antimetabolites ($P = 8 \times 10^{-4}$) and 1.7 for alkylating agents ($P = 1.8 \times 10^{-9}$) (Fig. 4). Concerning the *MTHFR* C>T677 gene variation, only minor effects of the polymorphism were detected. The cell lines harbouring the T^{677} variation at the homozygous state were significantly less sensitive to anticancer drugs than the cell lines harbouring no variant allele. This approached significance for some scattered drugs only. When drug classes were considered as a whole, the mean IC_{50} ratios were 2.0 for antifolates ($P = 2.3 \times 10^{-4}$), 1.4 for antimetabolites ($P = 0.011$) and 1.3 for alkylating agents ($P = 5.9 \times 10^{-5}$). When considering *MTHFR* haplotypes, the highest cell chemosensitivity was exhibited by C^{677}–C^{1298} cell lines and the lowest by T^{677}–A^{1298} cell lines.

4. Discussion

The allele frequency and genotype distribution of the polymorphic variations studied here are in agreement with those already published in Caucasian populations.[6–10,15–20] The linkage disequilibrium between the two SNPs of *MTHFR* is a classical feature; more specifically, the absence of the double variant homozygous genotype and the low frequency of the T^{677}–C^{1298} haplotype have been observed in many epidemiologic studies.[6–10] There is, in the NCI-60 panel, a smaller proportion of heterozygous cell lines for the *MTHFR* gene, which was significant only for the C>T677 variation ($P = 0.02$). According to T^{677} allele frequency, one would have expected 8 T/T^{677} homozygous genotypes, whereas 15 of these were identified among the NCI-60 cell lines. This can be interpreted as a loss of heterozygosity and would argue in favour of a contribution of the *MTHFR* gene to oncogenesis, as can be inferred from its association with cancer risk. Alternatively, the presence of a tumour suppressor gene in the vicinity of this gene could explain this observation.

As already shown for other polymorphisms,[22–25] the NCI-60 panel represents a useful tool for the understanding of the complex relationships between gene variations and cell sensitivity to anticancer drugs. In our study, only one gene among the four genes selected appears to harbour common variations which may be associated to anticancer drug cytotoxicity: the *MTHFR* gene. This gene has been especially studied for several years in relation to sensitivity to antifolates and to fluoropyrimidines, as it is involved in the availability

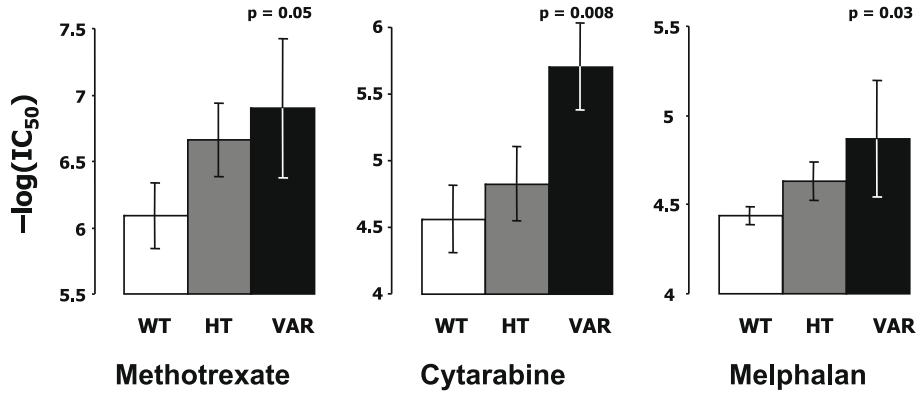

Fig. 3 – Association between the *MTHFR* A>C1298 gene polymorphism of the cell lines of the NCI-60 panel and the cytotoxicity of three representative drugs. The IC_{50}s of the each drug towards the 60 cell lines IS expressed as $-\log_{10}(IC_{50})$. Data are means ± s.e.m. White columns: common homozygous cell lines; grey columns: heterozygous cell lines; and black columns: variant homozygous cell lines.

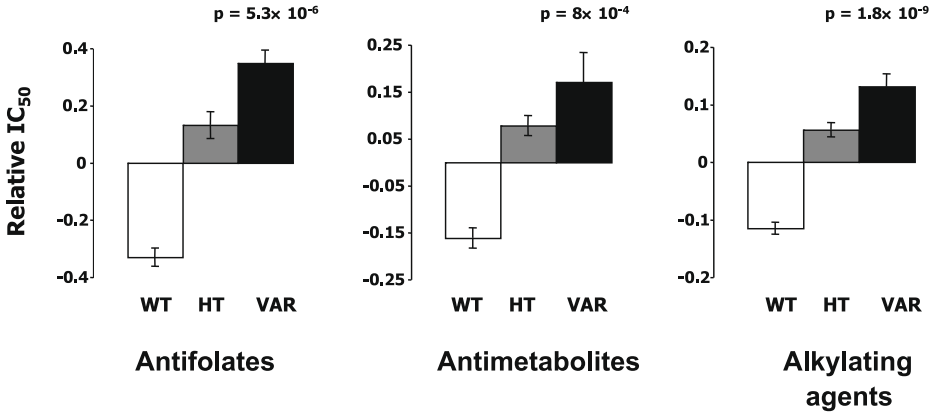

Fig. 4 – Association between the *MTHFR* A>C1298 gene polymorphism of the cell lines of the NCI-60 panel and the cytotoxicity of three drug classes agents. The IC_{50}s of the drugs of each class towards the 60 cell lines, expressed as $-\log_{10}(IC_{50})$, were normalised to zero, the values higher than the mean with a positive sign and the values lower than the mean a negative sign. Same legend as Fig. 3.

of $N^{5,10}$-methylene-tetrahydrofolate, the methyl donor to TYMS for the synthesis of thymidylate. Both polymorphisms have been shown to induce a reduction in enzyme activity, which does not exceed 30% of that of the wild-type enzyme for the C>T677 variation but appears less important for the A>C1298 variation.[3,5] As a consequence, an effect of the polymorphisms of this enzyme on the cytotoxicity of antifolates and fluoropyrimidines was expected.

Several studies attempting to relate *MTHFR* gene polymorphisms and methotrexate efficacy and/or toxicity in rheumatoid arthritis have been performed, but reached conflicting conclusions. An association between the presence of the T^{677} or the C^{1298} variant alleles and methotrexate efficiency was evidenced by Kurzawski and colleagues,[27] while the homozygous genotypes C/C^{677} and A/A^{1298} were associated to clinical improvement in the study of Wessels and colleagues.[28] The A^{1298} allele was associated to methotrexate-induced adverse events by Hughes and colleagues,[29] while the C^{1298} allele had a protective effect in the study of Bohanec Grabar et al.[30] Similar studies relating *MTHFR* polymorphisms and methotrexate activity have been performed in cancer patients. Several studies concluded that the variant T^{677} allele was associated with increased toxicity of methotrexate in leukaemia,[31] breast[32] and ovarian[33] cancer, which can be interpreted as higher methotrexate cytotoxicity. In contrast, an increased risk of relapse was noticed in acute lymphoblastic leukaemia children treated with methotrexate,[34] which would suppose less drug efficiency. In an *ex vivo* study on paediatric acute lymphoblastic leukaemia cells, patients with the C^{1298} variant allele showed in contrast decreased sensitivity to methotrexate, but no effect of the T^{677} allele was noticed.[35] Using the NCI-60 model, we observed an important and highly significant association between the *in vitro* cytotoxicity of methotrexate and other antifolates and the presence of the C^{1298} allele, but, on the contrary, antifolate cytotoxicity was rather associated with the C^{677} common allele than with the variant allele. This is not inconsistent, since there is a clear linkage between the two polymorphisms, the variant C^{1298} allele being much more frequently harboured by common homozygous C/C^{677} cell lines and, conversely, the variant T^{677} allele being more frequently harboured by the common homozygous A/A^{1298} cell lines (see Table 4).

Concerning 5-fluorouracil, the presence of the T^{677} or of the C^{1298} variant allele has been significantly associated with responsiveness to this drug in patients with colorectal[36–38] or oesophageal[39] cancer, but in contrast less toxicity and shorter survival were associated with the T^{677} genotype in another study,[40] while another study did not find any predictive value of *MTHFR* gene polymorphisms.[41] The T^{677} and the C^{1298} alleles have been shown to be associated with a high cytotoxic gfactivity of fluorouracil, respectively, in transfected colon and breast cancer cell lines[42] and in a panel of colon cancer lines spontaneously bearing the variation.[43] This was attributed to the well-known enhancing effect of $N^{5,10}$-methylene-tetrahydrofolate levels on 5-fluorouracil cytotoxicity by stabilisation of the ternary complex between the active folate, 5-fluoro-dUMP and TYMS. In our hands, no association between 5-fluorouracil cytotoxicity and the presence of any *MTHFR* polymorphism was detected in the NCI-60 panel. One study has concerned raltitrexed, a folate analogue targeting TYMS[44]; homozygous patients for the T^{677} allele developed less toxicity compared with other patients. In that case, it is hypothesised that high intracellular levels of $N^{5,10}$-methylene-tetrahydrofolate in T/T^{677} patients might have competed with raltitrexed for its binding to TYMS to decrease its effectiveness. However, in the NCI-60 panel, raltitrexed cytotoxicity was significantly higher in cell lines bearing the variant C^{1298} allele (expression ratio: 11.2, $P = 0.007$). The reason for these discrepancies is discussed later.

In addition to their effect on the modulation of antifolate activity, the alterations of folate availability associated with *MTHFR* gene polymorphisms have been related to many physiological or pathological situations, including thrombosis and risk of cardiovascular disease, neural tube defects and birth mortality, as well as cancer. It is one of the 11 genes for which "the variations have a noteworthy association with cancer" in a recent review of meta-analyses and pooled studies.[45] As the variant enzymes display lower activity than the common ones,[2,4] an effect can be expected on methylation patterns and aberrant DNA synthesis and repair, since folate deficiency has been associated with uracil misincorporation into DNA and DNA double strand breaks during uracil excision repair, thus increasing the risk of chromosomal aberrations after radiation- or chemically-induced DNA damage.[46] One would expect, therefore, an increased risk of developing a cancer in individuals bearing one of the variant *MTHFR* alleles. However, the molecular epidemiology studies appear conflicting and the role of the C>T677 variation in cancer risk appears different according to the organ site: the variant T^{677} appears to be associated with a protective role against colorectal cancer[6,9] and acute lymphoblastic leukaemia[7] and with an increased risk of gastric[10] and pancreatic[47] cancer. The consequences of the A>C1298 variation generally appear less important than those of the C>T677 variation, although a major protective effect against the risk of acute lymphoblastic leukaemia was associated to the C/C^{1298} homozygous variant.[48]

The role of the folate pool in the various enzymes associated to DNA synthesis and repair explains the potential role of *MTHFR* gene polymorphisms in cancer risk. It certainly also explains the association of these polymorphisms with the *in vitro* cytotoxicity of many anticancer drugs in the NCI-60 model, especially DNA-damaging agents. We observed a consistent and highly significant association between the presence of the C^{1298} variation and the chemosensitivity of the cell lines, whereas the T^{677} variation was rather associated to lower drug cytotoxicity. By analogy with the role of chemical carcinogens in cancer occurrence, we can infer that the T^{677} variation would be protective against xenobiotic aggressions and the C^{1298} variation, on the contrary, enhancing cell chemosensitivity. To the best of our knowledge, no clinical study has sought to establish a relationship between *MTHFR* polymorphisms and response to DNA-interacting anticancer agents, all the researches having been conducted in response to antifolates and fluoropyrimidines.

The fact that the two *MTHFR* gene variations, which have the same ultimate consequences (reduction in enzyme activity) may lead to opposite consequences in terms of *in vitro* drug sensitivity, appears puzzling. However, it should be borne in mind that the two variations do not occur at the same site: the alanine-to-valine substitution at codon 222 (C>T677) lies at the binding site of the MTHFR cofactor, flavine–adenine dinucleotide, while the glutamate-to-alanine substitution at codon 429 (A>C1298) lies in the S-adenosylmethionine (SAM) regulatory domain of the enzyme. It has been shown that SAM inhibits MTHFR[49] and that this feedback loop is essential for methyl group biogenesis and prevention of $N^{5,10}$-methylene-tetrahydrofolate depletion. In the absence of this regulation, especially by sequence alteration of this domain, MTHFR would direct more one-carbon units to 5-methyl-tetrahydrofolate and to SAM synthesis.[49] Therefore, it appears possible that the C^{1298} variation might affect this regulation and, as suggested by Wiemels and colleagues,[50] act through a different way than the T^{677} variation, the variant Val^{429} enzyme tending to accumulate N^5-methyl-tetrahydrofolate and the variant Ala^{222} enzyme to accumulate $N^{5,10}$-methylene-tetrahydrofolate. The variant Ala^{429} enzyme would facilitate xenobiotic-induced DNA damage, while the variant Val^{222} enzyme would in contrast prevent against the risk of uracil incorporation into DNA. In addition, this would also explain the lack of hyperhomocysteinaemia in individuals with homozygous C/C^{1298} variant genotype. Absence of association between *MTHFR* gene polymorphisms and cancer or leukaemia risk may result from opposite functional consequences of the two polymorphisms and from the linkage disequilibrium observed between them. Few epidemiologic studies were designed with enough power to study the association of distinct haplotypes with cancer risk. Such studies should be designed on subgroups on the population studied, including only patients homozygous for the three frequent haplotypes (C^{677}/A^{1298}, C^{677}/C^{1298}, T^{677}/A^{1298}); or patients having at least one of these.

A slight but significant association between *MTHFR* gene polymorphisms and gene expression was detected, the cell lines harbouring the two common forms of the gene displaying a higher expression. However, no relationship between gene expression and cell chemosensitivity could be detected. One would have expected that a decrease in gene expression would have the same effect as the reduction of enzyme activity induced by the polymorphisms. The reason why this is not observed may come from the important variability of gene expression profiles in cells in culture. First, microarray

expression data may not be fully reliable for technical reasons: the choice of the probe, the quality of mRNAs, the conditions of hybridisation offer a large number of possible pitfalls in microarray experiments. In addition, gene expression profiles give only a snapshot of the mRNA populations present at a given moment, under precise conditions. It has been shown that many factors greatly influence the gene expression profiles of cells in culture: the degree of cell confluence, the source of the foetal calf serum used to grow them, etc. From one cell line to another one, these conditions might not be the same at the time the cells were harvested, and comparative profiling cannot give reliable results, independently of any technical reason. Large variations may occur within a short period of time for many genes, and the expression levels obtained may not be representative of the mean concentration of mRNAs within a 3-d period, i.e. the duration of cell growth in the presence of an anticancer agent for cytotoxicity evaluation. In contrast, the presence of a gene polymorphism is constant and not subject to sporadic, uncontrolled variations. We think, therefore, that our observations are not in contradiction with the fact that the *MTHFR* C>T677 and A>C1298 variations, which are responsible for alterations in enzyme activity or regulation, have major consequences on anticancer drug cytotoxicity while *MTHFR* gene expression levels do not.

Apart from the *MTHFR* gene polymorphisms, no significant association between the polymorphisms of the folate pathway and drug chemosensitivity was evidenced. The expression of the reduced folate carrier, which is involved in folate cell uptake, is positively correlated with the cytotoxicity of several drugs. This is the case for 5-fluorouracil, whose activity is dependent upon the presence of $N^{5,10}$-methylene-tetrahydrofolate; this is the case also for methotrexate and other antifolates, which enter cells through RFC; this is even the case for several alkylating agents, especially nitrosoureas and diaminocyclohexane platinum drugs, which can be related to the fact that folate availability may have effects on xenobiotic-induced DNA damage, as already discussed for *MTHFR* gene variants. The *MTHFD1* and the *MTR* gene polymorphisms and gene expression levels did not appear in relation with anticancer drug cytotoxicity in the NCI-60 panel despite they sometimes appear to have some association with the risk of cancer.[51] Their influence of the availability on folate pools may be insufficient for determining an effect on xenobiotic aggressiveness in this *in vitro* situation.

Conflict of interest statement

There is no actual or potential conflict of interest, including any financial, personal or other relationships with people or organisations, that could have inappropriately influenced this work.

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