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Involvement of gene polymorphisms of thymidylate synthase in gene expression, protein activity and anticancer drug cytotoxicity using the NCI-60 panel

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ABSTRACT

A significant association has been established, in clinical studies, between the expression or activity of thymidylate synthase (TYMS) and the efficiency of fluorouracil. TYMS expression is partly under the dependence of gene polymorphisms in the 5′ and 3′ untranslated regions (UTR), but conflicting results have been obtained about their roles on fluorouracil efficiency. In this study, we wanted to use the National Cancer Institute (NCI) panel of 60 human tumour cell lines to clarify this problem. Three relevant polymorphisms of the *TYMS* gene were studied: (i) the 5′UTR tandem repeat of 28-bp (2R/3R polymorphism); (ii) the single nucleotide polymorphism (SNP) within the second repeat (3C/3G polymorphism); (iii) the 3′UTR 6-bp deletion (+6/−6 polymorphism). Allele frequencies were close to those expected in a Caucasian population (2R/3C/3G: 53/29/18%; +6/−6: 68/32%), but the proportion of heterozygous genotypes was lower than expected from allele frequencies. The 2R and 3G alleles were significantly associated with the +6 and the −6 alleles, respectively. There was a significant association between the presence of the 3G allele and TYMS mRNA expression and catalytic activity, particularly in p53-mutated cell lines. However, no significant correlation existed between fluorouracil cytotoxicity, as extracted from the NCI databases, and TYMS expression, activity or polymorphisms.

1. Introduction

Thymidylate synthase (TYMS) is the target enzyme of fluorouracil, an antimetabolite widely used in the treatment of colorectal, head-and-neck and breast cancers. It is a key enzyme for the synthesis of the DNA-specific nucleotide thymidylate, by catalysing the reductive methylation of deoxyuridylic acid into thymidylic acid in the presence of N^5,N^{10}-methylene tetrahydrofolate as a cofactor.[1] Fluorouracil and other fluoropyrimidines, as well as other folate analogues such as raltitrexed, exert their cytotoxic action by depleting the thymidylate pool, leading to the activation of apoptotic pathways and to cell death.[2]

The intracellular level of TYMS has been long ago recognised as a determinant of fluorouracil cytotoxicity *in vitro*[3,4] and *in vivo*.[5–8] Response of colorectal cancers to infusional fluorouracil has been associated to low expression levels of thymidylate synthase by several groups, using different methods addressing mRNA, protein or enzyme activity, and in palliative as well as in adjuvant settings.[9] The correlation between low thymidylate synthase expression and fluorouracil response is also displayed in other malignancies which benefit

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from fluorouracil therapy, such as pancreatic cancer and hepatocellular carcinoma.[10,11]

The transcription of the *TYMS* gene does not involve TATA or CAAT boxes, but an 80% proportion of CG bases is present in the 5′ untranslated region (UTR) of the gene. Kaneda et al.[12] have identified in this region a tandem repeat of a 28-bp sequence harbouring a CGCCGCG motif able to form secondary stem-loop structures which regulates the transcription level of the *TYMS* gene. In addition, Chu et al.[13] have identified a specific site for the fixation of TYMS at the level of the 5′ UTR of its own mRNA, involving the repeated sequence and the translation initiation codon, which would play a major role in TYMS translational regulation.

Several polymorphisms are present in the *TYMS* gene promoter and interfere with the mechanism of regulation of TYMS expression. A first variation lies in the number of repeats of the 28 bp sequence, which is either present in duplicate (2R) or in triplicate (3R).[14] In addition, a C > G transversion can occur in the second repeat when three repeats are present, giving rise in that case to two possibilities for the 3R allele: 3C or 3G.[15] A polymorphism is also present at the level of the 3′UTR of *TYMS*, consisting of the presence or absence of a 6 bp sequence close to the polyadenylation signal.[16]

These polymorphisms have been shown to be associated with the expression of TYMS and, consequently, with the sensitivity to fluorouracil. The presence of three repeats, at the heterozygous or homozygous states, was first shown to be associated with higher protein expression and lower fluorouracil efficiency.[17,18] However, opposite results were found later in terms of patients' survival after adjuvant fluorouracil treatment,[19,20] but in that case the DNA used to determine the gene variation originated from normal tissue or peripheric blood cells instead of tumour tissue. It was later suggested that only the 3G allele was associated with these features. The 2R/2R, 2R/3C and 3C/3C diplotypes were thus characterised by lower gene expression while the 2R/3G, 3C/3G and 3G/3G diplotypes by higher gene expression.[1,15] Concerning the 3′ UTR polymorphism, it was shown that the deletion of the 6 bp sequence was associated with low gene expression,[22] which would lead to a better efficiency of fluorouracil,[21] but, there again, opposite results were found by another group.[20]

Looking for a cellular model able to resolve these discrepancies and provide clear indications on the relationships between *TYMS* gene polymorphisms and fluorouracil activity, we wanted to see whether the NCI-60 panel could serve as a surrogate tool able to provide answers to this problem. The NCI-60 panel consists of 60 human tumour cell lines in culture and was initially established for high-throughput screening of natural products and synthetic molecules, on the basis of their antiproliferative properties.[23] In addition, a number of molecular markers and gene expression profiles have been determined in the panel, allowing to establish relationships between chemo-sensitivity or -resistance and the molecular features of the cells.[24]

We have recently studied several gene polymorphisms in the NCI-60 panel and have established relationships with the cytotoxicity of anticancer drug families.[25] Since the cell lines are tumoural, they can present many somatic genetic alterations, distinct from an actual constitutive polymorphism present in the patient who hosted the tumour. We have shown, however, that the NCI panel could represent a valuable model to study the role of gene variations on anticancer drug activity.[26] In the present study, we have determined the three known polymorphisms of *TYMS* and tentatively related these polymorphisms to the TYMS mRNA expression and catalytic activity as well as to fluorouracil *in vitro* cytotoxicity.

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 (PCR) 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. Electrophoresis was performed before and after digestion on 10% polyacrylamide gels. The presence of a variation was translated into the occurrence of a restriction site on the PCR product, leading to two shorter products. This technique allowed the unambiguous discrimination between variant homozygous, common homozygous and heterozygous cell lines, for the polymorphism of the 5′UTR (2R/3C/3G) and that of the 3′UTR (+6/−6). Sequencing was performed on 18 randomly chosen PCR products from the various genotypes of the variations studied. Concordance with RFLP was obtained in 100% of the cases.

We used the following primers for the 5′ variation: sense: 5′ AGGCGCGCGGAAGGGGTCCT 3′; antisense: 5′ TCCGAGCCGGCCACAGGCAT 3′. The amplification was performed using the Invitrogen PCR enhancer system with 1.5 mM of $MgSO_4$. The 2R/3R variation was first identified by direct electrophoresis of the PCR products on 12% polyacrylamide gels: bands of 114 or 141 bp can be easily identified. The PCR products were then digested by *HaeIII*, which specifically cleaves the 3G allele, and subjected to polyacrylamide gel electrophoresis.

For the 3′UTR variation, we used the following primers: sense: 5′ CAAATCTGAGGGAGCTGAGT 3′; antisense: 5′ CAGATAAGTGGCAGTACAGA 3′. The PCR was performed with 1 mM $MgCl_2$. The PCR products were digested by *DraI*, which specifically cleaves the +6 allele, and subjected to polyacrylamide gel electrophoresis.

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. [24] for details): alkylating or platinating agents acting on N^7 of guanine; other alkylating agents, acting on N^2 and 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 out 136 remained unclassified because of

disagreement about their precise mechanism of action. It was first possible to directly compare the mean IC_{50} values of each drug in the various genotypes, and it was also possible to use a paired Student's *t* test to analyse the data related to drug families. Due to the number of tests performed (136), we considered as significant only the *P* values lower than 4×10^{-4}.

The expression of TYMS was also extracted from the NCI database. Six different datasets are available, one obtained by Western blots, one by quantitative RT-PCR and four by microarray analysis using three different types of microarrays. We chose to focus our analyses on the RT-PCR data which are considered as the reference method to quantify mRNA products. Since no data on TYMS catalytic activity were present in the database, we evaluated this activity using the cytosols prepared by sonication of the cell pellets and ultracentrifugation at 100,000 g. The method used had been described by Etienne et al.[27] it uses radiolabelled [^{3}H]dUMP (Moravek Biochemicals Brea, CA, USA, specific activity: 14.3 Ci/mmol) which is converted by the enzyme, in the presence of N^5,N^{10}-methylene tetrahydrofolate, into tritiated water which is estimated in a beta scintillation counter. The cofactor was synthesised from (6R,S)-5,6,7,8-tetrahydrofolic acid hydrochloride (Schircks Laboratories, Jona, Switzerland) according to Peters et al.[28]

The Chi-square test was used for comparing the distribution of the cell lines among genotypes. Pearson coefficients of correlation were calculated for comparing continuous variables. To study the pharmacological parameters as a function of the genotype, we analysed the variances of fluorouracil IC_{50} and TYMS expression and activity values for each genotype and calculated the significance of the differences in mean values, taking into account the unbalanced size of the groups.

3. Results

Fig. 1 shows some representative electrophoretic profiles of PCR products before and after digestion with the appropriate restriction enzymes. Table 1 lists the 59 cell lines studied and their genotypic status for the polymorphisms considered. No special trend appeared concerning the presence or absence of a given variant as a function of the tissue of origin of the cells. There were 23 cell lines with the 2R/2R genotype, 17 with the 2R/3R genotype and 19 with the 3R/3R genotype. Among the 36 cell lines with a 3R allele, there were ten lines 2R/3C, seven lines 2R/3G, nine lines 3C/3C, six lines 3C/3G and four lines 3G/3G. In terms of allele frequency, and considering all cell lines are diploid, the 2R allele occurs with a frequency of 53%, and the 3C and the 3G alleles with frequencies of 29 and 18%, respectively. One cell line (the renal cell carcinoma 786-0) presented an unexpected RFLP profile, with two 28-bp repeats (2R/2R) and a cleavage site characteristic for the C > G transversion. The sequencing of the PCR product (Fig. 2) confirmed that this cell line was a 2C/2G heterozygote, with a new variant allele in the 28-bp repeat polymorphism. At the level of the 3′UTR, there were 32 lines with the +6/+6 genotype, 16 heterozygous cell lines +6/−6 and 11 −6/−6, giving a −6 allele frequency of 32%.

From these data, it was possible to estimate the frequency of the various haplotypes, based on the constitution of the different genotypes. Among the six possible haplotypes, the two major ones were those associating the 2R and +6 variants and the 3C and +6 variations (Table 2). There is a linkage disequilibrium between the polymorphisms of the 5′ and the 3′ UTRs: the 3G allele is significantly associated with the −6 allele and the 2R allele with the +6 allele ($P = 0.0011$ and 0.0001, respectively, with Chi-square test). In addition, there is, in the NCI-60 panel, a deviation from the Hardy–Weinberg rule, with a lower than expected proportion of heterozygous genotypes for both polymorphisms ($P = 0.005$ and 0.014, respectively, for the 5′ and the 3′ UTR polymorphisms). This can be interpreted as a loss of heterozygosity at the level of chromosome 18 which bears the *TYMS* gene.

We first looked for significant associations between the presence of the variations identified and TYMS expression and catalytic activity. TYMS expression was extracted from the NCI database and we evaluated TYMS catalytic activity in the cytosols of cell pellets (Fig. 3). It appeared that the 3R genotype was not significantly associated with differences in the average level of expression and catalytic activity, but the presence of at least one 3G allele was significantly associated with both TYMS expression and catalytic activity of (Table 3): the level of expression, as estimated by RT-PCR, was on average two times higher in cells lines harbouring at least one 3G allele than in those harbouring none (11.5 versus 19.2 units, $P = 0.034$); and the catalytic activity was in average 3.5 higher in the 3G cell lines than in the other ones (13.5 versus 46.2 pmol/min/mg prot., $P = 0.014$). As a general feature, the

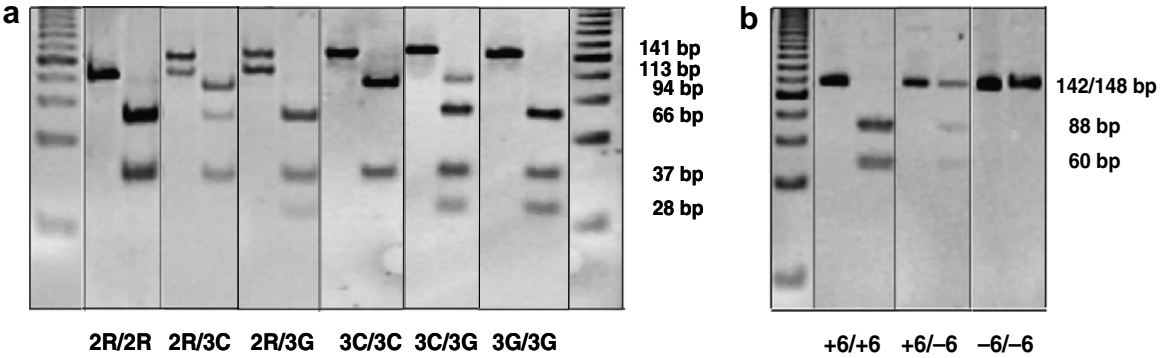

Fig. 1 – Representative electrophoretic patterns of PCR products before (left lanes) and after (right lanes) digestion for the identification of TYMS polymorphisms. (a) 5′UTR polymorphisms. (b) 3′UTR polymorphism. The deduced diplotypes are indicated below each couple of lanes.

Table 1 – Polymorphisms of the TYMS genes found in the NCI-60 panel

Tumour type	Cell line	5′UTR	3′UTR	TYMS expression	TYMS activity	p53 status
Leukaemia	CCRF-CEM	3C/3G	+6/−6	31.9	203	Mut
	HL-60	2R/3C	+6/+6		18.0	Mut
	K-562	2R/2R	+6/+6	3.24	46.7	Mut
	MOLT-4	2R/2R	+6/+6	67.8	114	WT
	RPMI-8226	3C/3G	+6/+6	11.9	6.63	Mut
	SR	3C/3G	−6/−6	4.68	69.1	WT
Lung cancer	A549/ATCC	2R/3C	+6/+6		0.621	WT
	EKVX	3C/3G	−6/−6	9.51	17.3	Mut
	HOP-62	2R/3C	+6/+6	11.4	31.8	Mut
	HOP-92	2R/3C	+6/+6	1.61	1.73	Mut
	NCI-H226	2R/3C	+6/+6		1.93	Mut
	NCI-H23	2R/3G	+6/−6	28.9	278	Mut
	NCI-H322M	2R/2R	+6/+6	11.2	0.273	Mut
	NCI-H460	3C/3C	+6/+6	9.30	1.10	WT
	NCI-H522	3C/3C	+6/−6	12.7	1.20	Mut
Colon cancer	COLO-205	2R/2R	+6/+6	10.6	1.94	Mut
	HCC-2998	3C/3C	+6/−6	11.0	11.3	Mut
	HCT-116	2R/2R	−6/−6	23.4	39.6	WT
	HCT-15	2R/2R	+6/+6	15.4	2.73	Mut
	HT29	2R/2R	+6/+6	15.1	2.30	Mut
	KM12	3C/3C	+6/+6	5.64	0.756	Mut
	SW-620	2R/2R	+6/+6	9.01	5.16	Mut
Central nervous system	SF-268	2R/2R	+6/+6	7.38	6.62	Mut
	SF-295	3C/3C	+6/−6	8.08	0.798	Mut
	SF-539	3C/3C	−6/−6	4.59	1.75	WT
	SNB-19	2R/2R	−6/−6	1.31	7.67	Mut
	SNB-75	2R/2R	+6/+6	4.02	3.30	Mut
	U251	2R/3C	+6/−6	11.0	3.01	Mut
Melanoma	LOXIMVI	2R/2R	+6/+6	11.5	6.31	WT
	MALME-3M	2R/2R	+6/+6	19.7	9.98	WT
	M14	2R/3G	+6/−6	18.4	52.0	Mut
	SK-MEL-2	2R/3G	+6/+6	30.7	40.0	Mut
	SK-MEL-28	2R/3G	+6/−6	14.5	28.6	Mut
	SK-MEL-5	3C/3G	−6/−6	19.0	31.1	WT
	UACC-257	3C/3C	+6/−6	4.93	7.86	WT
	UACC-62	3G/3G	−6/−6	52.3	0.752	WT
Ovarian cancer	IGROV1	3G/3G	+6/−6	12.4	11.8	WT
	OVCAR-3	2R/3C	−6/−6	8.34	13.4	Mut
	OVCAR-4	2R/2R	+6/+6	8.57	0.43	WT
	OVCAR-5	2R/2R	+6/+6	14.5	25.6	Mut
	OVCAR-8	2R/2R	+6/+6	10.7	9.25	Mut
	SK-OV-3	2R/2R	−6/−6	21.3	1.62	Mut
Renal cancer	786-0	2R/2R	+6/−6	8.12	4.34	Mut
	A498	2R/2R	+6/+6	2.41	1.91	WT
	ACHN	3G/3G	−6/−6	12.1	7.05	WT
	CAKI-1	2R/3C	+6/+6	11.9		WT
	RXF-393	2R/3C	+6/−6	15.8	26.1	Mut
	SN-12C	2R/2R	+6/+6	6.29	0.914	Mut
	TK-10	3C/3C	+6/+6	6.83	15.0	Mut
	UO-31	2R/3G	+6/−6	6.38	0.533	WT
Prostate cancer	PC-3	3C/3G	+6/−6	18.9	1.09	Mut
	DU-145	2R/2R	+6/+6		11.1	Mut
Breast cancer	MCF-7	3C/3C	+6/+6		9.57	WT
	NCI/ADR-RES	2R/2R	+6/+6	8.86	7.58	Mut
	MDA-MB-231	2R/3C	+6/+6		62.1	Mut
	HS578T	2R/3G	+6/−6		5.62	Mut
	MDA-MB-435	2R/3G	+6/−6	17.3	7.67	Mut
	BT-549	3G/3G	−6/−6		25.0	Mut
	T-47D	2R/2R	+6/+6		28.6	Mut

TYMS gene polymorphisms were determined as described in Materials and methods. TYMS expression is extracted from the NCI database and expressed in arbitrary units. TYMS activity was determined as described in Material and methods and is expressed as pmol/min/mg proteins. The p53 status of the cell lines (mutated or wild-type) was extracted from the NCI database.

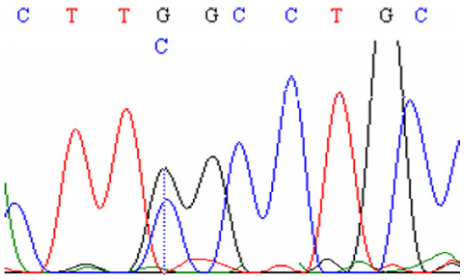

Fig. 2 – DNA sequence analysis of the 5′UTR PCR product in the 786-0 cell line, showing the unique 2C/2G genotype.

Table 2 – Frequency of the TYMS gene haplotypes found in the NCI-60 panel

Haplotype	Frequency (%)
2R/+6	46.6
2R/−6	6.8
3C/+6	18.6
3C/−6	10.2
3G/+6	2.5
3G/−6	15.3

overall correlation between the levels of TYMS expression and activity was highly significant ($r = 0.47$, $P = 4 \times 10^{-4}$). No association between the +6/−6 polymorphism and TYMS expression or activity appeared as significant (Table 3).

We then looked for correlations between the presence of a given polymorphism and the cytotoxicity of individual drugs of the NCI database. No significant association with $P < 4 \times 10^{-4}$ (due to Bonferroni correction) could be found with any of the 136 drugs, especially fluorouracil and the other fluoropyrimidines, and any polymorphism or combination of polymorphisms. Similarly, no association appeared when the drugs were pooled as a function of their mechanism of action. It must be emphasised at this point that there was no significant correlation between the levels of expression or catalytic activity of TYMS and the IC_{50} of fluorouracil as extracted from the databases ($r = 0.02$ and -0.02, respectively).

Because the cytotoxicity of fluorouracil appeared as extremely dependent upon the p53 status of the cell lines, with the wild-type cell lines being 5-fold more sensitive than the p53-mutated ones, we repeated the quest of significant associations between *TYMS* gene polymorphisms and TYMS expression and catalytic activity, as well as fluorouracil cytotoxicity, in the subset of the 40 p53-mutated cell lines of the NCI panel. It appears that the association between the presence of a 3G allele in the *TYMS* genotype and the expression and activity of TYMS was much more significant than in the whole NCI-60 panel: TYMS expression was 2-fold higher and TYMS activity 6-fold higher in the 3R allele-containing cells than in the other cell lines ($P = 5 \times 10^{-5}$ and 5×10^{-3}, respectively). However, there again, no relationship appeared between the cytotoxicity of fluorouracil and TYMS expression, activity or genotype.

4. Discussion

The distribution of the *TYMS* genotypes of the NCI-60 panel was in agreement with the distribution found in Caucasian populations: the 2R/3C/3G allele proportions were 48, 29 and 23%, respectively, in the study of Krajinovic et al.[29] working on leukaemic Canadian children of European descent, and 50, 28 and 22% in the study of Graziano et al.[30] in an Italian population, while we found 53, 29 and 18% in the NCI-60 panel. This distribution is different from the one observed in Japanese subjects by Kawakami and Watanabe[15]: 17, 40 and 43%. On the 3′ UTR, the frequency of the −6 allele is 27% in the study of Krajinovic et al.[28] 34% in the study of Graziano et al.[30] and 32% in our study. Similarly, the distribution of haplotypes obtained by combining the 5′ and the 3′ UTR variations does not differ significantly between Caucasian populations and the NCI panel.

These results indicate first that the linkage disequilibrium we observed is a general feature and that the two extremities of the *TYMS* gene belong to the same haplotype block. This also indicates that, although the NCI-60 panel is constituted of tumour cell lines, with a large number of gene rearrangements, the overall structure of the *TYMS* gene still persists in the genetic background. One of the features which may characterise tumour cell lines in this respect is the loss of heterozygosity which is likely to have occurred in a significant number of cell lines, without any possibility of identifying precisely which ones, among the homozygous lines, are in

Table 3 – Fluorouracil cytotoxicity, TYMS mRNA expression and TYMS catalytic activity in the 59 cell lines of the panel, according to the 5′ and 3′ UTR polymorphisms of the TYMS gene

	Fluorouracil ($-\log_{10}IC_{50}$)	TYMS expression (arbitrary units)	TYMS activity (pmol/min/mg prot.)
2R/2R	4.61 ± 0.11	13.3 ± 2.9	14.9 ± 5.2
2R/3R	4.42 ± 0.17	14.7 ± 2.1	35.7 ± 16.3
3R/3R	4.82 ± 0.17	13.9 ± 2.8	22.2 ± 10.7
+6/+6	4.64 ± 0.12	12.6 ± 2.3	15.4 ± 4.2
+6/− 6	4.57 ± 0.13	14.7 ± 1.9	40.2 ± 20.1
− 6/− 6	4.65 ± 0.23	15.6 ± 4.5	19.5 ± 6.3
2R/2R + 2R/3C + 3C/3C	4.65 ± 0.10	11.5 ± 1.7	13.4 ± 3.3
2R/3G + 3C/3G + 3G/3G	4.55 ± 0.15	19.2 ± 3.0*	46.2 ± 18.6*
No 3G allele, p53 mutated	4.45 ± 0.10	9.57 ± 0.9	11.8 ± 2.7
3G allele, p53 mutated	4.32 ± 0.20	20.2 ± 2.6***	64.0 ± 20.4**

Results are indicated as means ± s.e.m.
Significant differences: *$p < 0.05$; **$p < 0.01$; ***$p < 0.0001$.

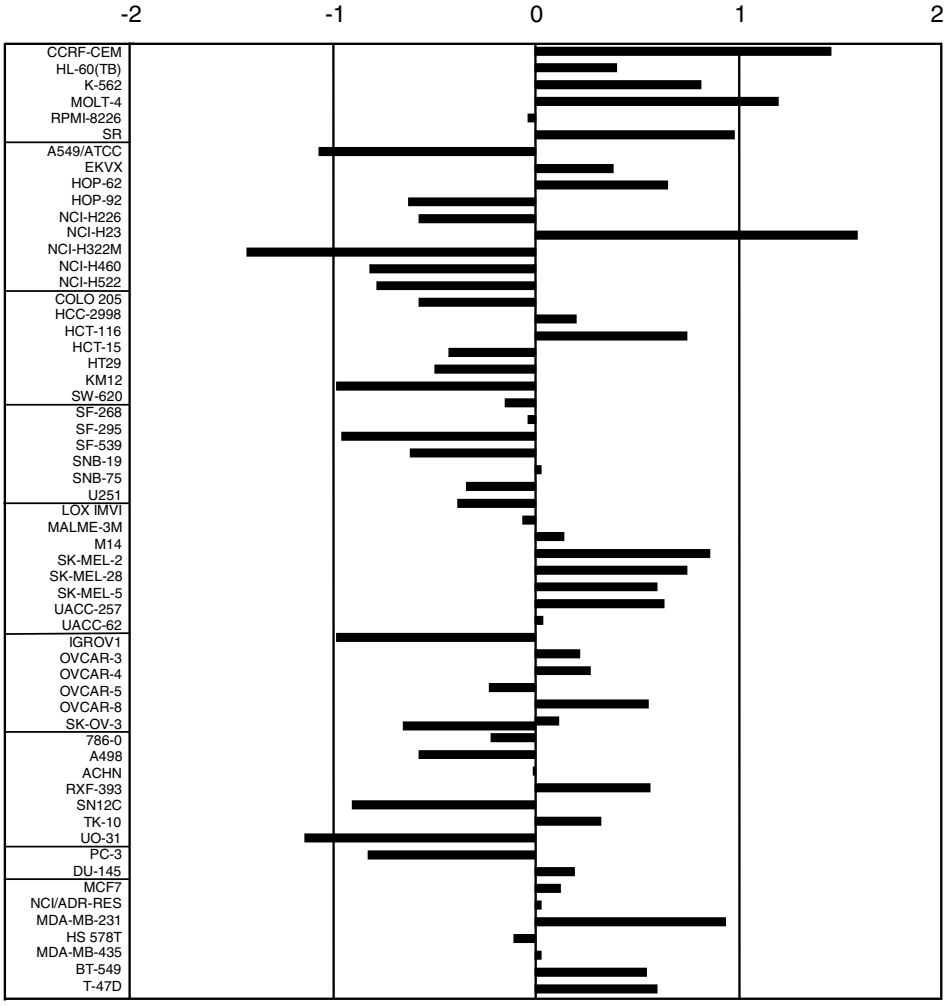

Fig. 3 – Representation of TYMS catalytic activity in the NCI-60 panel. The activities, after $\log_{10}$ transformation, were normalised; activities higher than the average value in the 60 cell lines are represented as bars oriented to the right, and those lower than the average to the left.

fact hemizygous. Loss of heterozygosity on chromosome 18p has been shown to occur in colorectal cancers and may modify the tumour response to fluorouracil of heterozygous patients when the 3R allele is lost.[31] Since our study precisely concerns the tumour response to anticancer drugs, and not the epidemiology of *TYMS* polymorphisms, this loss of heterozygosity does not hamper the validity of our approach.

We observed a significant association between the presence of the 3G allele and both the mRNA expression and catalytic activity of thymidylate synthase. This is in agreement with the observations of Kawakami et al.[21] in gastric cancer and of Morganti et al.[32] in colorectal cancer. It had been first shown that the 3R allele was associated with high *TYMS* expression,[17,18] but this observation was not made in all studies.[19,20] The fact that high TYMS expression appears to be associated with a better prognosis of colorectal cancer, independently of chemotherapy,[33] is a confounding factor that may explain these discrepancies. However, the discovery of the fact that the 3R allele could be either 3C or 3G, the second one only being associated with high *TYMS* expression, may also explain why the role of the 3R allele was not found in all studies.[15,21,33]

Among the NCI-60 cell lines, the presence of a 3R allele is not enough to determine high TYMS expression, only the 3G alleles display this property. It is now well accepted that genotypes 2R/3G, 3C/3G and 3G/3G are associated *in vivo* to high TYMS expression, while the genotypes 2R/2R, 2R/3C and 3C/3C are not.[34] However, the role of the −6 allele in destabilising mRNA, which had been shown by Mandola et al.[22] has no apparent consequence on TYMS expression in the NCI-60 panel. In fact, the possible role of the −6 allele on mRNA stability may be hidden by the linkage disequilibrium which associates the 3G allele (which confers high transcription/translation rate) and the −6 allele (which confers poor mRNA stability). Only two cell lines of the panel associated the presence of the 3G allele with a +6/+6 3′ UTR genotype, which is not enough for statistical comparisons.

We were unable to show any significant relationship between fluorouracil cytotoxicity and the presence of *TYMS* gene polymorphisms. It was already possible, from the *in silico* analysis of the NCI database, to discover that TYMS expression was in no way related to fluorouracil cytotoxicity. This is puzzling because of the general observation that fluorouracil efficiency in patients is associated with TYMS expression

and/or activity, which are in turn under the dependence of *TYMS* gene polymorphisms.[9,35] In an *in vitro* study on 19 cell lines in culture, Etienne et al.[27] have shown that there was no correlation between TYMS catalytic activity and fluorouracil IC_{50}; however, when this parameter was evaluated in the presence of a high concentration of reduced folates, then a significant correlation appeared ($r = 0.610$, $P = 0.0027$). This is in relation to the fact that the tumour cell lines have highly variable intracellular concentrations of reduced folates; the presence of the cofactor considerably enhances the activity of fluorouracil, which is well known in the clinics, where fluorouracil is now always combined to leucovorin.[2] The determination of fluorouracil cytotoxicity in the NCI-60 panel has been done without folate addition, and fluorouracil IC_{50}s must be dependent on reduced folate intracellular levels rather than on TYMS expression. As a consequence, no direct effect of TYMS polymorphisms on fluorouracil cytotoxicity can be found in the NCI-60 panel with the current database.

Yawata et al.[36] also studied the TYMS polymorphisms at the 5′ UTR of the gene in a collection of 30 human tumour cell lines. They found no relationship between the genotype and TYMS expression at the mRNA level and no relationship either with fluorouracil cytotoxicity. However, they observed a significant relationship between the genotype and the cytotoxicity of fluorodeoxyuridine, the deoxyribonucleoside derived from fluorouracil, with significantly higher IC_{50} values in cell lines harbouring the 3G allele at the homozygous state. No indications on the reduced folate concentrations of the cell lines were given in this study.

We have observed that, in the cell lines having a mutated p53, the relationship between *TYMS* gene polymorphism and TYMS expression or activity was much more significant than in the cell lines having a wild-type p53. This would suggest that a different regulation of TYMS expression at the transcription and/or translation levels occurs upon the loss of genomic stability induced by p53 mutation. It would be of interest to study the regulation of *TYMS* transcription and translation in isogenic models differing only by the p53 status. It should be mentioned that there is a significant correlation between the expression of several enzymes involved in the folate pathway and TYMS expression or activity: this is the case for methionine synthase and for serine hydroxymethyl transferase 1. Coordinate regulations of these enzymes can be inferred from these observations.

There is a distortion in the relative levels of TYMS mRNA expression and its catalytic activity as a function of the genotype. TYMS mRNA levels are twice higher in the 3G allele-containing cell lines than in the other ones while TYMS catalytic activity is three times higher, and even six times higher when considering only the p53-mutated cell lines. This may be related to the fact that the 3G allele is involved not only in increased transcription rate through USF binding,[37] but also in increased translation level, as shown by Kawakami and Watanabe.[15] According to Chu et al.[13] TYMS translation rate is autoregulated by fixation of the protein on a stem-loop motif of 36 bp at the 5′UTR of its own mRNA. This is precisely the zone where the two polymorphisms occur and the genotype-dependency of translation rates may be related to different binding affinity or stability. Using an *in silico* approach, we have compared the secondary structures of the 5′ UTRs of the three mRNA species, 2R, 3C and 3G, over 100 bp length,[38] [http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi]. The same hairpin stem-loop structure containing the translation initiation site was found for all three mRNAs. However, structural differences may influence TYMS mRNA binding and explain differences in translation rate according to the genotype. Furthermore, this modelling allowed us to calculate the minimal free energy of each type of mRNA, using the algorithm of Zuker and Stiegler.[39] It appeared that the 3G mRNA ($\Delta G_0 = -57.2$ kcal/mol) was more stable than the 3C mRNA ($\Delta G_0 = -53.7$ kcal/mol), which was more stable than the 2R mRNA ($\Delta G_0 = -48.3$ kcal/mol). The increased mRNA stability of the 3G allele may explain the increased protein activity associated with this genotype.

In conclusion, the NCI-60 model is able to provide interesting information about the role of *TYMS* gene polymorphisms on TYMS mRNA expression and catalytic activity. The importance of reduced folates in the cytotoxicity of fluorouracil has to be emphasised, as well as the importance of the p53 status on the transcriptional and translational regulation of TYMS. Selecting patients for fluorouracil therapy as a function of their polymorphic thymidylate synthase status has been envisaged[40]: such studies will have to take into account not only *TYMS* gene polymorphisms, both in normal and tumour tissue,[31] but probably also the p53 status of the tumour.

Conflict of interest statement

None declared.

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