[Frontiers in Bioscience 9, 2484-2494, September 1, 2004]

PHARMACOGENOMICS OF THYMIDYLATE SYNTHASE IN CANCER TREATMENT

Peter V. Danenberg

Department of Biochemistry and Molecular Biology, Norris Cancer Center, Keck School of Medicine, University of Southern California, Los Angeles, CA 90033

TABLE OF CONTENTS

1. ABSTRACT

Cancer drugs such as 5-fluorouracil (5-FU) that target the enzyme thymidylate synthase (TS) have been and are still being widely used in cancer treatment, but as with other anti-cancer drugs, the majority of tumors do not respond to the treatment, whereas the patients still suffer drug-related toxicity. The most recent attempts at improving cancer treatment have taken the pharmacogenetic approach of identifying biochemical response determinants for response, so that patients with suboptimal determinants who unlikely to respond can be identified prior to treatment. Studies to date indicate that high intratumoral levels of TS gene expression or TS protein generally predict for non-response, whereas low levels are associated with a high response rate. Measuring these determinants requires tumor tissue and, in the case of gene expression, a technically demanding quantitative PCR procedure. Thus, considerable interest was generated by data suggesting that the variable number of a 28 base-pair (bp) segment in the promoter region of the TS gene was associated with TS gene expression and/or protein expression, as well as with tumor response to 5-FU therapy, toxicity and patient survival. However, not all studies have obtained the same results, so that the role of this TS polymorphism as a predictor of treatment outcome is still not clear and is currently under evaluation. This review will summarize pharmacogenomic studies of TS that were aimed at elucidating the function of this genetic polymorphism.

2. INTRODUCTION

2.1. Most current cancer treatment is empirical, "one-size-fits all" chemotherapy

The most frustrating aspect of cancer chemotherapy as it is currently practiced is the unpredictable variability of response among patients, ranging from complete pathological response at one extreme to progression in the face of the drug at the other extreme. Generally, the number of favorable responses is in the minority. A typical example is that of the commonly used drug 5-fluorouracil (5-FU). When used as a single agent against colorectal cancer, 5-FU elicits a response in only about 20% of patients in most large, randomized clinical trials (1,2), despite attempts at improved efficacy through biomodulation and/or variations in delivery schedules. Thus, a large majority of treated patients derive no tangible benefit from having received their chemotherapy, but still are subjected to drug toxicity, significant risk, and delay in treatment that might have been effective.

To improve cancer chemotherapy, much effort and expense has gone into developing or discovering new drugs that might elicit a higher response rate. However, this has proved to be very difficult. Five decades after its appearance, 5-FU still remains one of the most active drugs available and is often the standard against which new drugs are compared. Only recently have some new drug combinations such as 5-FU and CPT-11 (3) or 5-FU plus oxaliplatin (4) have pushed response rates of colorectal cancers to about 40%. Currently, drugs targeted at specific oncogenes such as the EGFR-directed agent C225, and the VEGF-directed agent avastin are being developed and tested (5). Some of the initial clinical results appear promising, especially in combination with conventional chemotherapy but the ultimate benefits of such drugs will not be apparent for some time yet.

2.2. A rational molecular approach: "Tumor tailored" chemotherapy through pharmacogenetics

Because experience over the years gives little reason to hope that any one drug or combination of drugs

will come close to producing a response in all cancer patients, the realization has dawned that the best chance for a significant improvement in cancer treatment is to make use of existing drugs in a more rational manner by treating only patients who are most likely to be responsive to particular drugs. This approach is based on the principle that inter-individual variations in tumor response or drug-related toxicity are due to genetic differences in biochemical factors, such as drug metabolizing enzymes, repair enzymes, drug targets and others. Provided that a) a number of different agents are available to treat a particular tumor type and b) sensitivity determinants can be identified for each agent and analyzed in patients, it should be possible to elicit a response in most patients the first time by tailoring therapy to fit the chemosensitivity profile of the tumor. This idea itself is not new, but in the past it has simply been technologically impossible to perform the necessary analyses of tumor response determinants quantitatively in pre-treatment tumor or normal tissue biopsies. However, the development of ultrasensitive analytical technologies such as real-time PCR, immunohistochemistry and DNA microarray chips has made possible a renewed flourishing of pharmacogenetics, defined as the study of the genetic basis for inter-individual differences in drug disposition and effects. Pharmacogenetics can embrace studies at the three genetic levels of DNA (mutations, polymorphisms, methylation), mRNA (quantitative gene expression) and protein (function, quantity, enzyme activity, postranslational processing). The new field of pharmacogenomics is a sub-category of pharmacogenetics, and concentrates on examining how heritable variations in DNA influence inter-individual differences in response to drugs and toxicities.

The pharmacogenetics of the enzyme thymidylate synthase (TS), due to its role as a target for a number of widely used anti-cancer drugs, has been much studied over the last 20 years or so. This review will discuss recent pharmacogenomic studies of thymidylate synthase (TS), concentrating on TS gene polymorphisms as response determinants.

3. THE TS PROMOTER CONTAINS A 28-BASE PAIR (BP) TANDEM REPEAT SEQUENCE THAT MAY REGULATE TS EXPRESSION

3.1 TS expression predicts tumor response to TS-directed drugs

TS catalyzes the reductive methylation of 2'-deoxyuridylate by 5,10-methylenetetrahydrofolate to form thymidylate and dihydrofolate. Inhibition of TS has been an intensively explored approach to cancer chemotherapy. Because TS is the only *de novo* source of the thymine base and the reaction catalyzed by TS is one of the rate-limiting steps in DNA synthesis (6), inhibition of TS rapidly shuts off DNA synthesis and triggers apoptosis and other cell-death processes. However, this mechanism does not provide much selectivity for cancer cells and thus TS inhibitors also cause considerable toxicity. TS is the major target for 5-fluorouracil (5-FU), which for almost 50 years has been one of the mainstay drugs for treatment of many cancers. 5-FU inhibits TS by forming a covalent ternary complex among 5,10-methylenetetrahydrofolate, TS and 5-fluoro-2'-deoxyuridylate (FdUMP), the active metabolite of 5-FU (6). Since the appearance of 5-FU, other fluoropyrimidine-based therapies such as 5-fluoro-2'-deoxyuridine, UFT, S-1 and capecitabine as well as folate-based TS inhibitors such as ratitrexed, pemetrexed and nolatrexed have been developed. Cells exposed to TS inhibitors *in vitro* acquire resistance by up-regulating TS expression and raising intracellular TS levels, usually *via* gene amplification (7). These observations led to a number of studies attempting to relate TS gene or protein levels in tumors with outcome to 5-FU-based therapy (8-12). Most studies have consistently agreed that both TS mRNA and TS protein expressions do vary considerably among tumors and that sensitivity of various tumors to 5-FU-based chemotherapy is related to the intratumoral level of TS, with higher TS expression generally associated with lower response rate and shorter survival. Moreover, high TS levels in tumors have also been shown to be associated with worse prognosis (13,14).

3.2. Variability of the number of TS repeats among individuals and populations

Although the intracellular TS level appears to be an important determining factor for response to TS-directed therapy, surprisingly little is definitively known about the mechanism(s) by which TS gene expression is regulated. In the late 1980's, Kaneda *et al* (15) discovered that the 5' untranslated region of the TS gene contained a 28 bp tandemly triple-repeated sequence, which appeared to be involved in regulating the translation efficacy of the gene presumably due to stem-loop formation between inverse complementary sequences. It was subsequently found that in humans, this TS enhancer region (TSER) is polymorphic in the numbers of the 28 bp repeat (16). The DNA of most people contains either a double tandem repeat (2R/2R), a triple repeat (3R/3R) or a heterozygous (2R/3R) genotype and interestingly, the distribution of the alleles was found to differ among ethnic populations (16,17). The frequency of the homozygous 3R genotype among Chinese was found to be about 2-fold higher than among Caucasians (67% *vs.* 38%, respectively), while only 2% of the Chinese population had a 2R/2R genotype compared to about 20% for Caucasians (17). In rare cases, higher order repeats including the 4R, 5R and 9R alleles have been found. The 9R allele so far has been found at a low frequency exclusively in one African group (Ghanaians) whereas 4R is more widely distributed among various other African populations (18). The origin of such differences was hypothetically ascribed to environmental pressures that would favor better survival of individuals with higher TS expression *(e.g.*, low thymidine intake in the diet) (16).

3.3. The relationship of TS polymorphism repeat number and TS expression

The discovery by Horie *et al* (16) that the human TS gene contained double as well as triple repeats of the 28 bp segment prompted them to compare the transcription activities of the TS promoter containing either the 2R or the 3R repeats. They linked a reporter gene to the TS promoters and found that the transcription activity of the 3R promoter was 2.6-fold greater than that of the 2R

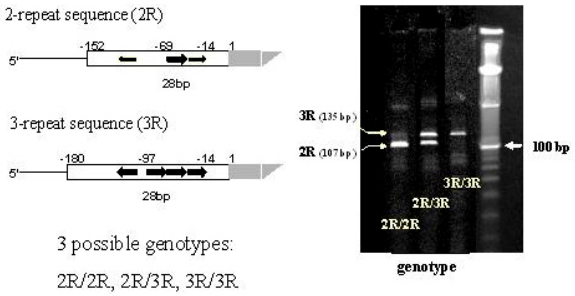

Figure 1. The structure of the thymidylate synthase promoter with variable numbers of a 28 base-pair segment. Upper diagram shows a double repeat (2R) and the lower diagram shows a triple repeat (3R) in the TSER. The segments are separated by gel electrophoresis (right) after PCR using primers that flank the polymorphic region.

promoter. This first-ever identification of a genomic polymorphism as a distinct molecular regulatory factor for TS expression, coming at a time of increasing awareness of the possible role of genetic polymorphisms as determinants of drug effectiveness and of the role of TS expression as a determinant of tumor chemosensitivity to 5-FU-based therapy, stimulated a flurry of further investigations into the biochemical role and the medical consequences of this TS polymorphism. The possibility of a genomic polymorphism in TS as an indicator of chemosensitivity to 5-FU caused considerable excitement because germline DNA alterations can be detected in readily available normal tissue such as peripheral blood, without the necessity for delving into tumor tissues. As researchers in the field are well aware, accessing tumor tissue can be technically difficult, depending on the type of cancer, and can also be fraught with regulatory hurdles and administrative difficulties in obtaining review board approvals and patient consent. Moreover, because genomic polymorphisms are stable sequence alterations of DNA, the assay methods used to determine the presence or absence of the particular genomic lesion of interest utilize relatively easy sequence-analysis technologies. For example, the 2R and 3R TS genotypes can be easily identified by performing a PCR reaction using primers flanking the polymorphic region. The 2R and 3R segments give rise to PCR products of different lengths, which are readily separated by gel electrophoresis (figure 1).

Following the initial *in vitro* study of Horie *et al* (15), Kawakami *et al* (19) carried out the first *in vivo* test of the regulatory effects of TSER polymorphisms. These investigators measured the TS protein content by a radioligand-binding assay making use of the covalent complex between [^{3}H]FdUMP and TS (20) in 70 gastrointestinal adenocarcinomas, 21 of which had been treated with UFT, a fluoropyrimidine based therapy. Among the 21 UFT-treated patients, the 3R/3R genotypes had significantly higher TS content than 2R/3R genotypes, while in the UFT-untreated group the same (but statistically non-significant) trend was observed. The frequency of the 2R/2R genotype in this group was only about 5%, typical for East Asian populations but too low a number for evaluating the correlation with TS content. TS gene expression was not determined in this study.

To study the *in vivo* regulation of gene expression by TSER polymorphisms, Pullarkat *et al* (21) analyzed tumor tissue samples from a group of 52 patients with disseminated colorectal cancer who had received 5-FU-based therapy. TS gene expression (relative mRNA levels) was measured by quantitative reverse transcription (RT)-PCR (22). The genotype distribution in this study was 29, 50, 21% as 3R/3R, 2R/3R and 3R/3R, respectively. The reported results showed a remarkably linear correlation of TS gene expression with TS genotype: the TS expression values were 9.4, 5.5 and 2.6 in 3R/3R, 2R/3R and 2R/2R tumors, respectively, corresponding to a 3.6-fold difference between the homozygous 2R and 3R genotypes. Moreover, a similar association was reported in 26 normal liver tissue samples: TS expressions were 8.2, 4.4 and 3.2 in 3R/3R, 2R/3R and 2R/2R genotypes, respectively.

Although the above study seemed to confirm that the 2R and 3R differentially regulate TS gene expression *in vivo*, studies published subsequently have given discrepant results. Kawakami *et al* (23) independently investigated the relationship of TS gene and protein expression with TSER genotype in colorectal cancer specimens from Japanese patients. TS mRNA isolated from 130 surgically obtained tumor specimens was quantitated by RT-PCR and TS protein was quantitated in 92 samples by the FdUMP ligand-binding assay. These values were matched to the TS genotypes of the samples, which again showed a genotype distribution typical of East Asians populations with 4% (5/130) 2R/2R, 30% (45/130) 2R/3R and 40% (79/130) 3R/3R. In this group of patients, there was no difference in gene expression based on TSER genotype. On the other hand, cancer tissues with 3R/3R genotype had significantly higher TS protein expression level than those with 2R/3R or 2R/2R genotype. These results suggested that the efficiency of TS mRNA translation rather than transcription is responsible for genotype-dependent difference in TS protein expression. *In vitro* analysis using *TS* 5'-UTR-luciferase reporter constructs showed that the RNA with 3-repeat sequence was translated 3 to 4 times more efficiently than that with 2-repeat sequence. Thus, the results from both the *in vitro* and *in vivo* studies were consistent in showing that TS mRNA with 3-repeat sequence has greater efficiency of translation than that with 2-repeat sequence.

Similar results were obtained by Ishida *et al* (24). These investigators quantitated TS mRNA in 115 gastric cancer specimens by RT-PCR and TS protein by FdUMP binding in 72 of the samples. In this study, no correlation was observed between TS genotype and mRNA expression, but tumors with the 3R/3R genotype had higher TS protein content than the 2R/2R genotypes, again consistent with an effect of TSER polymorphism on translation but not transcription.

Still a different result was obtained by Etienne *et al* (25), who measured TS enzyme activity by a radiological assay in 88 liver metastases and 54 primary colorectal tumors. Interestingly, the median TS activity was found to be the highest in the heterozygotic 2R/3R primary tumors and lowest in the 3R/3R genotype tumors, with the 2R/2R tumors having an intermediate value. The TS activity

Table 1. TSER polymorphisms and TS expression: Summary of studies to date

The study measured	Result	Reference
Reporter gene activity linked to 2R or 3R promoter	3R promoter had 2.6-fold higher transcriptional activity.	Horie *et al* (16)
TS protein level in gastrointestinal cancers	3R had higher TS protein content than 2R genotypes.	Kawakami *et al* (19)
TS gene expression, TS protein content in 130 CRC patients; reporter gene activity linked to 2R or 3R promoter	No differenceseen among genotypes in gene expression; protein levels higher in 3R/3R; reporter transcription activity was the same for 2R and 3R promoters.	Kawakami *et al* (23)
TS gene expression in 50 5-FU-treated CRC patients	TS gene expression ratios 3.7:2.1:1 in 3R/3R, 2R/3R, 2R/2R, respectively.	Pullarkat *et al* (21)
TS mRNA in 115 gastric cancer specimens and TS protein in 72 patients	TS gene expression did not differ among TSER genotypes; TS protein was higher in 3R/3R.	Ishida *et al* (24)
TS enzyme activity in 88 liver metastases and 54 primary colorectal tumors	TS activity was highest in 2R/3R tumors both in primary tumor and metastases.	Etienne *et al* (25)
TS gene expression, TS protein by IHC in stage I and II NSCLC specimens	3R/3R had higher median TS gene expression and TS protein.	Shintani *et al* (26)
TS gene expression in 30 colorectal cancers	TS gene expression was 1.5-fold higher in 3R-only tumors than in 2R-only tumors.	Uchida *et al* (42)

ratios were about 5:2:1 for the 2R/3R, 2R/2R and 3R/3R genotypes, respectively. Mean TS activity was also highest in the 2R/3R metastatic tissues than in the other genotype metastases, although the differences did not reach significance.

Shintani *et al* (26) determined TS gene expression by RT-PCR, TS protein by immunohistochemistry (IHC) and TS genotype in 48 stage I and II non-small-cell lung cancer (NSCLC) specimens. They found significantly higher TS mRNA levels in 3R/3R genotype tumors than in the combined 2R/2R and 2R/3R genotype tissues. In addition, the 3R/3R genotypes expressed higher levels of TS protein: 74% were TS-positive by IHC compared to 14% for the combined 2R/2R and 2R/3R genotypes. However, different results for NSCLC were obtained in an unpublished study from this author's laboratory. We analyzed specimens from 82 NSCLC patients who were treated with surgery only and found no difference in the median TS gene expressions and ranges of expression among the 3 genotypes. In this group of NSCLC patients, high TS gene expression was a significant prognostic factor for worse survival. Thus, if the 3R/3R genotype were tightly linked to high TS expression, the presence of this genotype should predict for worse survival than the 2R/2R or 2R/3R genotypes. However, the median survival among 3R/3R genotype patients did not differ from that of the other genotypes.

The results of these studies on the association between TSER polymorphic repeat number and TS expression are summarized in table 1.

3.4 The relationship of TSER polymorphisms and clinical outcome from 5-FU-based therapy

A major reason for interest in the TSER polymorphisms was the possible medical implications of the apparent connection of the TSER genotype to the response of cancer patients to fluoropyrimidine-based therapy, mediated presumably by the influence of the number of repeats on TS gene or protein expression. As will be discussed below, the clinical studies are also marked by a number of discrepant results.

Pullarkat et al (21) not only related TSER genotype status to TS gene expression but also to clinical outcome in colorectal cancer patients treated with 5-FU-based therapy. Tumor response rates in their study were high for 2R/2R patients (4/8; 50%) but the presence of a 3R segment in the TSER caused dramatic declines in response rates to 15% (3/20) for the 2R/3R group and 9% (2/22) for the 3R/3R tumors. Median survival was longer for the 2R/2R patients (16.2 months) compared to ones with 3R genotypes (8.5 and 8.3 months for 2R/3R and 3R/3R, respectively), although these differences did not reach statistical significance. Of added interest, the TSER polymorphism status also was associated with normal tissue toxicity in an inverse manner: grade 3 (severe) toxicity was seen in 63% (5/8) 2R/2R patients but in only 27% (6/22) in the 3R/3R group and in 32% (6/19) of the 2R/3R group. Thus, the TSER genotype seemed to be simultaneously a predictor for tumor response as well as for toxicity to normal tissue. The same group (Park et al, ref. 27) also investigated tumor response to the 5-FU prodrug Xeloda (capecitabine) in metastatic colorectal cancer patients as a function of TSER polymorphism status. In this study, only 24 patients were analyzed, but the results were similar to those of their initial study of 5-FU-treated patients (21). Individuals with the 2R/2R genotype had a response rate of 75% (3/4), compared to 8% (1/8) for 2R/3R patients and 25% (2/8) for 3R/3R patients. Toxicity with respect to TSER polymorphism status followed the same trend as in the 5-FU study (21), that is, higher in the 2R/2R group, but the differences did not reach statistical significance.

Table 2. TSER polymorphisms and clinical outcome: Summary of studies to date

The study involved:	Result	Reference
65 rectal cancer patients treated with 5-FU	Higher probability of downstaging seen for 2R/2R patients than 3R/3R patients.	Villafranca *et al* (31)
50 metastatic CRC patients treated with 5-FU	2R/2R had better response than 3R/3R; toxicity higher for 2R/2R than 3R/3R	Pullarkat *et al* (21)
221 CRC patients tretaed with 5-FU and surgery or surgery alone	2R/2R received more benefit from 5-FU than 3R/3R, although statistics were disputed by Ulrich & Potter (29)	Iacopetta *et al* (28)
24 metastatic CRC patients treated with capecitabine	2R/2R had better response than 3R/3R or 2R/3R.	Park *et al* (27)
70 stage I and II NSCLC patients	Disease-free survival correlated with TS gene expression but not polymorphism status.	Shintani *et al* (26)
30 esophageal cacner patients	2R-only tumor genotypes (2R/2R+2R/loss) had higher response rate and longer survival than 3R-only tumor genotypes	Uchida *et al* (42)

Iacopetta *et al* (28) investigated the relationship between TSER status and chemotherapy benefit in a group of 221 Dukes C stage colorectal cancer patients. These investigators compared patients treated with surgery alone and those given 5-FU-based adjuvant chemotherapy. They reported that although the 3R/3R group seemed to have a short-term benefit from the chemotherapy, no long-term benefit was apparent. In contrast, the 2R/2R and 2R/3R combined group did show a significant long-term survival benefit from chemotherapy. The statistical methodology in this study, however, came under criticism in a comment article by Ulrich & Potter (29), who held that the observed 38% survival benefit among the 3R/3R patients was not actually significantly different from the 48% increase in survival of the 2R/2R+2R/3R group and that the determination of survival benefit should not have used surgery-alone patients as the comparison group but should only have compared patients who received chemotherapy.

Etienne *et al* (25) performed a prospective study to examine, among other factors, the association between TS activity and TSER polymorphism status in primary tumor and metastases with clinical outcomes in 103 metastatic colorectal cancer patients receiving 5-FU-folinic acid. Low TS activity in the metastatic tissue was the only factor associated with tumor response (49% for low TS vs. 21% for high TS), whereas the 2R/2R, 2R/3R and 3R/3R genotype tumors all had almost identical response rates (all about 37%). The authors stated that these results "clearly demonstrate that TS genotype cannot serve as substitute for TS activity in order to predict responsiveness." High TS activity and 2R/3R TSER polymorphism status in the primary tumor, but not in the metastases, both had similar prognostic values for worse survival. Although supporting a link between TS genotype and TS expression, their observation that it was the 2R/3R genotype with the highest activity and worst prognosis contrasts with all other studies.

In a small study of 24 colorectal cancer patients receiving a bolus 5-FU regimen, Marsh *et al* (30) found that 40% of the responding patients but only 22% of the non-responders had a 2R/2R genotype. Median survival was longer for the 2R/2R genotype (16 months) than for the 3R/3R genotype patients (12 months). No data on heterozygous patients were reported.

Shintani *et al* (26) studied the association of TS gene expression, TSER polymorphism status and TS protein content (measured by IHC) with clinical outcome in stage I and II NSCLC patients treated only with surgery. They found that even though there was an association between TS mRNA levels and intensity of IHC staining, and between the 3R/3R genotype and higher TS mRNA levels, the only factor that significantly correlated with disease-free survival was TS mRNA expression. The hazard ratio of high TS expression compared to low TS was 5.4. Disease free survivals did not differ among the TSER genotype groups nor between IHC-positive and IHC-negative tumors.

Villafranca *et al* (31) showed that among 65 patients with rectal cancer who received 5-FU-based chemoradiation as neo-adjuvant therapy, patients who had the 3R/3R genotype had a lower probability (22%) of experiencing downstaging than those with the 2R/2R or 2R/3R genotypes (60%) (27). In addition, the 3R/3R genotype was an indicator of nodal-positive pathological stage. A trend toward improved survival was seen in the 2R/2R and 2R/3R groups compared with the 3R/3R group.

A summary of these studies is presented in table 2.

4. OTHER POLYMORPHISMS IN THE TS GENE

In a recent study, Mandola *et al* (32) showed that the 28 bp TSER tandem repeats contain consensus elements that bind to upstream stimulatory factor (USF) proteins and that USF-1 and USF-2 binding enhances transcription of TS. The transcriptional activity of 3R-reporter gene construct was somewhat greater than that of the 2R construct, suggesting that the additional USF binding element in 3R segments could account for the supposedly higher TS mRNA levels in 3R/3R genotypes. These authors found a G→C single-nucleotide polymorphism (SNP) within the USF protein binding element at the 12th nucleotide of the second repeat of the 3R segment. They showed that, whereas phosphorylated USF-1 bound the normal consensus sequence, the G→C substitution abolished binding. *In vitro* transcription of reporter genes showed that a 3RC-containing promoter caused a lower transcription rate than 3RG, similar to that of the 2R segment. The frequency of the 3RC allele among all 3R alleles showed a variation of 56%, 47%, 28% and 37% for whites, Hispanics, African Americans and Singapore

Chinese, respectively. The same G→C SNP was simultaneously discovered by Kawakami & Watanabe (33), but these investigators found that translation efficacy rather than transcription was the process affected by the G→C substitution. Whereas there were no functional differences between 2RG and 2RC, the 3RC allele had a 4-fold lower translational activity *in vitro* than did 3RG, again similar to that of 2R. On the basis of the *in vitro* data, the 2R/3RG, 3RC/3RG and 3G/3G alleles were designated as high-expression genotypes and 2R/2R, 2R/3C and 3C/3C as the low-expression alleles. Survival analysis showed that colorectal cancer patients who received adjuvant oral fluoropyrimidines benefited only if they possessed one of the low-expressing genotypes. Although the overall frequency of the 3RC allele was similar to that reported by Mandola *et al* (32), Japanese females were noted to have lower frequency of the 3RG allele than males, which the authors speculated might be associated with a known gender difference in benefits from 5-FU-based chemotherapy.

By searching expressed sequence tag (EST) databases, Ulrich *et al* (34) identified a 6-bp variation starting at bp 1494 in the 3'-untranslated region of the TS mRNA. The allele frequency of the 6-bp deletion was 0.29 (+6bp/+6bp, 48%; +6bp/-6bp, 44%; -6bp/-6bp, 7%). The authors proposed that this polymorphism may be of relevance to chemotherapy response because changes in the 3'-untranslated region could affect mRNA stability and thereby alter mRNA levels and protein levels. In an abstract from the 2002 AACR meeting, Lenz *et al* (35) reported that this 6 bp deletion predicts TS mRNA expression in colorectal tumors. At the date of this writing, the only information about the effect of the 1494del6 polymorphism on tumor response is an abstract from the 2003 ASCO meeting (36) which reported that patients homozygous for the 6 bp segment (+6bp/+6bp) had an odds ratio of 2.0 for response to 5-FU-based therapy.

The possibility of 3 different polymorphisms in the same gene obviously complicates effort aimed at understanding the effects of each individual polymorphism. As pointed out by Evans and McLeod (37), the presence of two independent polymorphisms leads to 9 possible combinations of alleles and 9 possible phenotypes. In the case of TS, if the G→C SNP in one 3R segment is added to the mix, there are 18 possible alleles (assuming that all 3 polymorphisms occur independently of each other), all of which theoretically may give rise to a different phenotype in terms of promoting TS expression and altering chemosensitivity. Every study to date has only examined one TS polymorphism at a time without taking into account the presence or absence of other polymorphisms. Future studies should address questions about the possible interplay between polymorphisms with potentially different effects on TS expression or chemosensitivity. For example, assuming that a 3R/3R genotype decreases the sensitivity of tumors to 5-FU and a +6bp/+6bp genotype increases it, what would be the effect of the simultaneous presence both genotypes? Would the opposing effects counteract each other or would one be dominant? Would the effects of 2R/2R and +6bp/+6bp in the same gene be additive for sensitizing cells to 5FU? In addition, the G→C SNP in 3R, which makes the 3R behave more like a 2R in terms of promoting transcirption or translation, also must be taken into consideration. The consequences of failing to take into account the effects of multiple polymorphisms may be especially pronounced in studies with small numbers of patients because of the greater possibility of skewed distributions of alleles. It seems reasonable to believe that a true assessment of the effects of each individual polymorphism is not possible if the presence of the other polymorphisms constitutes an unknown variable.

5. THE EFFECTS OF LOSS OF HETEROZYGOSITY (LOH) AT THE TS LOCUS

The expectation that genetic polymorphisms determined in DNA from normal tissues can be used as tumor markers is based on the assumption that the germline genotype in the normal tissue is identical to that in cancer tissue. Although this assumption may be true in many cases, it is known not to be always true in the case of *TS* genotype. The TS gene has been localized to the telomeric region of the short arm of chromosome 18 at chromosome band 18p11.32 (38). Chromosome 18 is known to be a site of frequent deletions in a high percentage of colorectal cancers (39). The TS gene itself is not likely to be the target of deletions because it is an essential gene, but if TS is in proximity to the real target gene(s) of chromosome 18 deletions, it may in many cases be contained within the deleted DNA segment. When LOH occurs at the *TS* locus, the tumor genotype of homozygous patients will be the same as that in normal tissue, but in the case of heterozygous 2R/3R individuals, the tumor will have either a 2R/loss or 3R/loss genotype. Zinzindohoue *et al* (40) first suggested the idea that LOH at the *TS* locus could give rise to different TS expression levels in the tumor and thus alter the predicted clinical outcome. These investigators reported an LOH frequency of 63% (19/30) in colorectal hepatic metastases. Kawakami *et al* (41) obtained an almost identical result when analysis of the TS genotype in colorectal tumors from 2R/3R individuals showed a 62% (31/50) frequency of allelic imbalance. Because the presence of 3R (either 3R/3R or 3R/2R genotype) has usually been associated with less favorable clinical outcomes than if 3R is not present, we investigated the question of whether patients with 3R/loss tumors fare worse those with 2R/loss tumor genotypes or whether the occurrence of LOH makes no difference and the germline genotype determines outcome (42). We studied 30 colorectal cancer patients treated with the fluoropyrimidine-based combination S-1, all of whom had Stage IV disease. The response rate to S-1 in this group of patients was 13/30 (43%). The heterozygous 2R/3R genotype was found in 22/30 normal tissues, whereas 10 (45%) of the matched cancer tissues showed only the 2R-sequence band (2R/loss) and 7 cancer tissues (32%) showed only the 3R-sequence band (loss/3R). This corresponds to an LOH frequency of 77%, somewhat higher than previously observed. The data from this study are summarized in table 3 and illustrated in figure 2. 2R/3R patients with a 2R/loss genotype in their tumors had strikingly better response rate from the treatment than

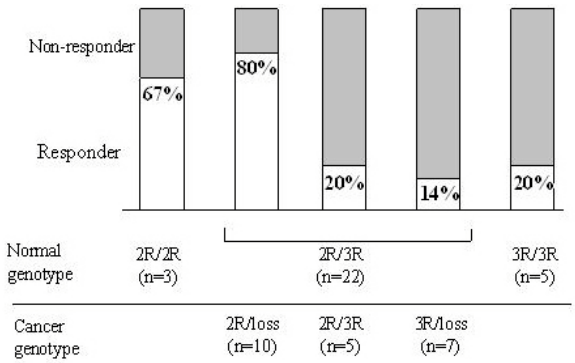

Figure 2. The effects of loss of heterozygosity at the TS locus on tumor response to S-1, a fluoropyrimidine-based therapy. Data taken from ref. 42.

patients with the 3R/loss genotype, as well as survival (333 days vs. 203 days), supporting the idea that tumor genotype, not germline genotype, is the determinant of response to chemotherapy. The observation that heterozygous patients with a 2R/loss genotype had a long survival, whereas patients with a 2R/3R tumor genotype had a short survival similar to homozygous 3R/3R genotype suggests that 3R is a negative effector of outcome (rather than 2R being a positive effector). The message from this study is that if TS polymorphisms are to be used as pharmacogenomic predictors of clinical outcome, genotyping of only normal tissue alone is insufficient and TS polymorphism status must be determined in the tumor tissue.

With regard to the effects of the TSER polymorphism on gene expression, it appears from comparing the TS gene expression values of 2R/loss and 3R/loss tumors that the 3R causes a 1.5-fold elevation of TS gene expression (table 3). The question we raised in our paper (42) was whether such a modest difference in TS levels between 2R and 3R genotype tumors is sufficient to account for the rather dramatic difference in response rate and survival. We concluded that it was not sufficient, and that other or additional mechanisms contribute to the difference in clinical outcome between 2R and 3R patients. Our suspicion at present is that effects of TSER polymorphisms on intracellular folate levels may play a major role.

6. TSER POLYMORPHISMS AND INTRACELLULAR FOLATE LEVELS

This section will summarize the threads of evidence for the making the case that TSER polymorphisms can influence response to TS-directed drugs and toxicity to patients by means of folate pool perturbations. It has been known for some time that low folate levels are associated with greater toxicity to TS and DHFR inhibitors and that the administration of folic acid can significantly alleviate this toxicity while preserving the anti-tumor activity of the drug (43,44). This was the strategy used by clinical investigators associated with Eli Lilly Co. to improve the safety profile of their TS-directed antifolate pemetrexed (Alimta) (45,46). Providing further support for the role of the folate level as a drug toxicity determinant are studies on the effect of mutations in methylenetetrahydrofolate reductase (MTHFR), a central enzyme in folate metabolism. The C677T polymorphism of MTHFR produces an enzyme with only 30% wild-type activity and is associated with overall decreased total folate levels, as indicated by elevated cysteine levels (47,48). Toffoli *et al* (49) showed that the MTHFR 677T/T polymorphism genotype was associated with severe toxicity during adjuvant treatment of breast cancer with CMF (cyclophosphamide/methotrexate/5-FU). Significant associations have also been observed between toxicity and the MTHFR 677TT genotype in treatment of ovarian cancer and marrow transplant patients with methotrexate (MTX) (50,51).

Folate pools can also impact tumor response to TS-directed and other folate analog drugs. Leucovorin is often given to patients along with 5-FU in order to raise intracellular reduced folate levels and thus presumably enhance the inhibition of TS by FdUMP (52). An increase in reduced folate levels associated with the MTHFR C677T genotype (53,54) would be expected to improve response to 5-FU. Indeed, Cohen *et al* (55) showed that among responders to 5-FU, the frequency of the 677T/T MTHFR genotype was 2.86-fold greater than the 677C/T or 677C/C genotypes. In a series of cell lines, Sohn *et al* (56) observed that the MTHFR C677T mutation altered folate pools and led to greater chemosensitivity to 5-FU, whereas Etienne *et al* (57) observed a marked trend for greater 5-FU efficacy in cells containing A1298C variants of MHTFR, although not in cells with the C677T polymorphism. These effects of MTHFR alterations on sensitivity to 5-FU must be an indirect result of folate pool alterations because MTHFR does not interact with 5-FU.

There is a fairly extensive literature on the association between MTHFR polymorphisms that cause low folate status and the incidence of various diseases such as colorectal polyps, colon cancer, neural-tube defects and cardiovascular disease (47). Similar links between folate pools, TSER polymorphisms and disease have been provided by several recent studies, albeit with somewhat discrepant results. Skibola *et al* (58) found TSER 2R/3R individuals to be at a 2.8-fold lower risk of acute lymphocytic leukemia (ALL) compared to 2R/2R, whereas the 3R/3R genotype conferred a 4-fold level of protection, and when the 3R/3R genotype was combined with a 1420CT/TT genotype of serine hydroxymethyltransferase, an enzyme that provides one-carbon units for folate metabolism, a dramatically lower (14-fold) risk resulted. The presence of at least one 2R allele was also associated with a 1.6-fold increased risk of malignant lymphoma (59). In contrast, Chen *et al* (60) found that the risk of colorectal cancer was highest among 3R/3R individuals, intermediate for 2R/3R individuals (risk ratio 0.86) and lowest among 2R/2R genotypes (risk ratio 0.59). In this study, the 1494del6 polymorphism in TS did not influence either cancer risk, survival, or modification of plasma folate levels. Interestingly, individuals with the 2R/2R genotype had the lowest plasma folate levels, and a non-significantly better survival, even though for all patients together, better

Table 3. Effects of LOH at the TS locus on response to fluoropyrimidine-based therapy and survival (data from ref. 42)

Germline genotype	Tumor genotype	Response rate	Median survival days (range)	TS gene expression (range)
All patients		43%	215 (98-627)	
2R/2R (n=3)	2R	67% (2/3)	224	2.9 (1.3-4.1)
3R/3R (n=5)	3R	20% (1/4)	150 (74-268)	3.3 (1.6-12.0)
2R/3R (n=22)	2R/loss (n=10)	80% (8/10)	333 (241-468)	2.4 (0.6-3.4)
	3R/loss (n=7)	14% (1/6)	203 (152-279)	3.7 (2.1-10.7)
	2R/3R (n=5)	20% (1/4)	143 (127-184)	3.0 (1.6-19.2)
	Total 3R (3R/loss + 3R/3R) (n=10)	20% (2/10)	181 (151-243)	3.7 (1.6-12.0)
	Total 2R (2R/loss + 2R/2R (n=13)	10/13 (77%)	308 (233-418)	2.5 (0.6-4.1)

survival outcome was associated with high plasma folates. A different relationship between TSER polymorphism and folate level was reported by Trinh *et al* (61), who found that 3R/3R individuals in a Chinese population had the lowest levels of plasma folates and, for those with low folate consumption, an elevated plasma homocysteine. Ulrich *et al* (62) found that 3R/3R genotype individuals with low folate consumption had increased the risk of colon adenomas relative to 2R/2R, whereas high folate levels caused the ratios to reverse and rendered 2R/2R individuals at greater risk. These studies provide evidence for a connection among TSER polymorphism status, folate pools and risk of cancer.

7. SUMMARY AND PERSPECTIVE

When all of the available data are considered, it is still unclear whether and to what extent variable numbers of the 28 bp TSER polymorphism influences TS expression, although the consensus of the available clinical data seems to be that possession of the 3R genotype is associated with worse outcome from treatment. To try to account for the discrepant and sometimes contradictory data in the literature, one can bring in all the "usual suspects" such as small study sample number, lack of standardized methodologies for measuring protein and gene expression, suboptimal samples consisting of different mixtures of cells, tissue-specific differences and study populations with different allele distributions. In addition to these general problems, there are several other TS-specific factors that are likely to have affected the results and conclusions from the various studies in unpredictable ways. The first is that besides the 28 bp TSER repeat, the TS gene has been shown to contain two other polymorphisms, which preliminary evidence indicates have biological function of their own. Thus, it is likely that the observed TS expression levels, tumor response and toxicity may be complicated functions of multiple TS gene alterations. A second important factor that has not been taken into account in most studies is LOH at the TS locus in tumors, which in heterozygous 2R/3R individuals will confer a different genotype to the tumor than found in normal tissue. Thus, in the case of 2R/3R genotypes, predictions based only on TS genotyping in normal tissue may not be valid. Thirdly, there is evidence that the TSER repeat status is associated with changes in folate pools, which provides another factor besides TS expression level that could influence tumor response and toxicity to TS-directed drugs. The message emerging from all the studies on TS pharmacogenomics is that it is probably naive to think that just the presence or absence of any one polymorphism by itself will be an adequate predictive factor for the design of individualized therapy due to the influence of other, often unsuspected factors that could counteract, reinforce or cancel the predicted effect of the polymorphism.

8. ACKNOWLEDGEMENT

The author has been supported by NIH grants CA71716, CA84424 and CA85790.

9. REFERENCES

1. J.L. Grem: 5-Fluorouracil plus leucovorin in cancer therapy. In: Principles and Practice of Oncology Update Series, 2(7). Eds: De Vita, Jr., VT, Hellman S & Rosenberg SA, J.B. Lippincott, Philadelphia (1988).

2. Moertel CG: Chemotherapy for colorectal cancer. *New Engl J Med* 330, 1136-1144 (1994)

3. Saltz LB, J.V. Cox, C. Blanke, L.S. Rosen, L. Fehrenbacher, M.J. Moore, J.A. Maroun, S.P. Ackland, P.K. Locker, N. Pirotta, G.L. Elfring & L.L. Miller: Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. Irinotecan Study Group. *New Engl J Med* 343, 905-14 (2000)

4. Bleiberg H & A. de Gramont: Oxaliplatin plus 5-fluorouracil: clinical experience in patients with advanced colorectal cancer. *Semin Oncol* 25:32-39(1998)

5. Gill S, R.R. Thomas & R.M Goldberg: New targeted therapies in gastrointestinal cancers. *Current Treatment Options in Oncology 4:393-403 (2003)*

6. Danenberg, PV: Thymidylate synthetase – a target enzyme in cancer chemotherapy. *Biochim Biophys Acta* 473: 73-92 (1977)

7. Wang W, S. Marsh, J. Cassidy & H.L. McLeod: Pharmacogenomic dissection of resistance to thymidylate synthase inhibitors. *Cancer Res* 61: 5505-5510 (2001)

8. Huang CL, H. Yokomise, S. Kobayashi, M. Fukushima, S. Hitomi & H. Wada: Intratumoral expression of thymidylate synthase and dihydropyrimidine dehydrogenase in non-small cell lung cancer patients treated with 5-FU-based chemotherapy. *Int J Oncol* 17: 47-54 (2000)

9. Nishimura R, K. Nagao, H. Miyayama, M. Matsuda, K. Baba, Y. Matsuoka, H. Yamamashita, M. Fukuda, A. Higuchi, A. Satoh, T. Mizumoto & R. Hamamoto: Thymidylate synthase levels as a therapeutic and prognostic predictor in breast cancer. *Anticancer Res* 19: 5621-5626 (1999)

10. Salonga D, K.D. Danenberg, M. Johnson, R. Metzger, S. Groshen, D.D. Tsao-Wei, H.-J. Lenz, C.G. Leichman, R.B. Diasio & P.V. Danenberg: Colorectal tumors responding to 5-fluorouracil have low gene expression levels of dihydropyrimidine dehydrogenase, thymidylate synthase, and thymidine phosphorylase. *Clin Cancer Res* 6: 1322-1327 (2000)

11. Shirota Y, J. Stoehlmacher, J. Brabender, Y.P. Xiong, H. Uetake, K.D. Danenberg, S. Groshen, D.D. Tsao-Wei, P.V. Danenberg & H.-J. Lenz: ERCC1 and thymidylate synthase mRNA levels predict survival for colorectal cancer patients receiving combination oxaliplatin and fluorouracil chemotherapy. *J Clin Oncol* 19: 4298-4304 (2001)

12. Yeh KH, C.T. Shun, C.L. Chen, J.T. Lin, W.J. Lee, P.H. Lee, Y.C. Chen & A.L. Cheng, A.L. High expression of thymidylate synthase is associated with the drug resistance of gastric carcinoma to high dose 5-fluorouracil-based systemic chemotherapy. *Cancer* 82: 1626-1631 (1998)

13. Kralovanszky J, I. Koves, Z. Orosz, C. Katona, K. Toth, P. Rahoty, F. Czegledi, T. Kovacs, B. Budai, L. Hullan & A, Jeney: Prognostic significance of the thymidylate biosynthetic enzyme in human colorectal tumors. *Oncology* 62: 167-174 (2002)

14. Nakagawa T, F. Tanaka, Y. Otake, K. Yanagihara, R. Miyahara, K. Matuoka, T. Takata, T. Yamada, M. Fukushima & H. Wada: Prognostic value of thymidylate synthase expression in patients with p-stage I adenocarcinoma of the lung. *Lung Cancer* 35: 165-70 (2002)

15. Kaneda S, K. Takeishi, D. Ayusawa, K. Shimizu, T. Seno & S. Altman: Role in translation of a triple tandemly repeated sequence in the 5'-untranslated region of human thymidylate synthase mRNA. *Nucleic Acids Res* 15: 1259-70 (1987)

16. Horie N, H. Aiba, K. Oguro, H. Hojo & K. Takeishi: Functional analysis and DNA polymorphism of the tandemly repeated sequences in the 5'-terminal regulatory region of the human gene for thymidylate synthase. *Cell Struct Funct* 20: 191-197 (1995)

17. Marsh S, E.S. Collie-Duguid, T. Li, X. Liu & H.L. McLeod: Ethnic variation in the thymidylate synthase enhancer region polymorphism among Caucasion and Asian populations. *Genomics* 58: 310-312 (1999)

18. Marsh S, M.M. Ameyaw, J. Githang'a, A. Indalo, D. Ofori-Adjei & H.L. McLeod: Novel thymidylate synthase enhancer region alleles in the African population. *Hum Mutat* 16: 528 (2000)

19. Kawakami K, K. Omura, E. Kanehira, & Y. Watanabe: Polymorphic tandem repeats in the thymidylate synthase gene is associated with its protein expression in human gastrointestinal cancers. *Anticancer Res* 19: 3249-3252 (1999)

20. Moran RG, C.P. Spears & C. Heidelberger: Biochemical determinants of tumor sensitivity to 5-fluorouracil: ultrasensitive methods for the determination of 5-fluoro-2'-deoxyuridylate, 2'-deoxyuridylate, and thymidylate synthetase. *Proc Natl Acad Sci USA* 76:1456-60 (1979)

21. Pullarkat ST, J. Stoehlmacher, V. Ghaderi, Y.P. Xiong, S.A. Ingles, A. Sherrod, R. Warren, D.D. Tsao-Wei, S. Groshen & H.J. Lenz: Thymidylate synthase gene polymorphism determines response and toxicity of 5-FU chemotherapy. *Pharmacogenomics J* 1: 65-70 (2001)

22. Leichman CG, H.-J. Lenz, L. Leichman, K. Danenberg, J. Baranda, S. Groshen, W. Boswell, R. Metzger, M. Tan & P.V. Danenberg: Quantitation of intratumoral thymidylate synthase expression predicts for disseminated colorectal cancer response and resistance to protracted infusion 5-fluorouracil and weekly leucovorin. *J Clin Oncol* 15: 3223-3229 (1997)

23. Kawakami K, D. Salonga, J.M. Park, K.D. Danenberg, H. Uetake, J. Brabender, K. Omura, G. Watanabe & P.V. Danenberg: Different lengths of polymorphic repeat sequence in the thymidylate synthase gene affect translational efficiency but not its gene expression. *Clin Cancer Res* 7: 4096-4101 (2001)

24, Ishida Y, K. Kawakami, Y. Tanaka, E. Kanehira, K. Omura & G. Watanabe: Association of thymidylate synthase gene polymorphism with its mRNA and protein expression and with prognosis in gastric cancer. *Anticancer Res* 22: 2805-2809 (2002)

25. Etienne MC, M. Chazal, P. Laurent-Puig, N. Magne, C. Rosty, J.L. Formento, M. Francoual, P. Formento, N. Renee, E. Chamorey, A. Bourgeon, J.F. Seitz, J.R. Delpero, C. Letoublon, D. Pezet & G. Milano: Prognostic value of tumoral thymidylate synthase and p53 in metastatic colorectal cancer patients receiving fluorouracil-based chemotherapy: phenotypic and genotypic analyses. *J Clin Oncol* 20: 2832-2843 (2002)

26. Shintani Y, M. Ohta, H. Hirabayashi, H. Tanaka, K. Iuchi, K. Nakagawa, H. Maeda, T. Kido, S. Miyoshi & H. Matsuda: New prognostic indicator for non-small-cell lung

cancer, quantitation of thymidylate synthase by real-time reverse transcription polymerase chain reaction. *Int J Cancer* 104: 790-795 (2003)

27. Park DJ, J. Stoehlmacher, W. Zhang, D.D. Tsao-Wei, S. Groshen & HJ Lenz: Thymidylate synthase gene polymorphism predicts response to capecitabine in advanced colorectal cancer. *Int J Colorectal Dis* 17: 46-49 (2002)

28. Iacopetta B, F. Grieu, D. Joseph & H. Elsaleh: A polymorphism in the enhancer region of the thymidylate synthase promoter influences the survival of colorectal cancer patients treated with 5-fluorouracil. *Br J Cancer* 85: 827-830 (2001)

29. Ulrich CM & J.D. Potter: Thymidylate synthase polymorphism and survival of colorectal cancer patients treated with 5-fluorouracil. *Br J Cancer* 86:1365 (2002)

30. Marsh S, J.A. McKay, J. Cassidy & H.L. McLeod: Polymorphism in the thymidylate synthase promoter enhancer region in colorectal cancer. *Int J Oncol* 19: 383-386 (2001)

31. Villafranca, E., Okruzhnov, Y., Dominguez, M. A., Garcia-Foncillas, J., Azinovic, I., Martinez, E., Illarramendi, J. J., Arias, F., Martinez Monge, R., Salgado, E., Angeletti, S. and Brugarolas, A. Polymorphisms of the repeated sequences in the enhancer region of the thymidylate synthase gene promoter may predict downstaging after preoperative chemoradiation in rectal cancer. *J Clin Oncol 19:* 1779-1786, 2001.

32. Mandola MV, J. Stoehlmacher, S. Muller-Weeks, G. Cesarone, M.C. Yu, H.J. Lenz & R.D. A novel single nucleotide polymorphism within the 5' tandem repeat polymorphism of the thymidylate synthase gene abolishes USF-1 binding and alters transcriptional activity. *Cancer Res* 63: 2898-904 (2003)

33. Kawakami K & G. Watanabe: Identification and functional analysis of single nucleotide polymorphism in the tandem repeat sequence of thymidylate synthase gene. *Cancer Res* 63: 6004-6007 (2003)

34. Ulrich CM, J. Bigler, C.M. Velicer, E.A. Greene, F.M. Farin & J.D. Potter: Searching expressed sequence tag databases: discovery and confirmation of a common polymorphism in the thymidylate synthase gene. *Cancer Epidemiology, Biomarkers & Prevention* 9: 1381-1385 (2000)

35. Lenz HJ, W. Zhang, S. Zahedy, J. Gil, M. Yu & J. Stoehlmacher: A 6 base-pair deletion in the 3' UTR of the thymidylate synthase (TS) gene predicts TS mRNA expression in colorectal tumors. A possible candidate gene for colorectal cacner risk. *Proc Am Assoc Cancer Res* 43: 660 (2002)

36. McLeod HL, D.J. Sargent, C.S. Fuchs, R.K. Ramanathan, S.K. Williamson, B.P. Findlay, S.N. Thibodeau, G.M. Petersen & R.M. Goldberg: Pharmacogenetic analysis of systemic toxicity and response after 5-fluorouracil (5FU)/CPT-11, 5FU/oxaliplatin (oxal) or CPT-11/oxal therapy for advanced colorectal cancer. *Proc Am Assoc Clin Oncol* 22: 252 (2003)

37. Evans WE & H.L. McLeod: Pharmacogenomics--drug disposition, drug targets, and side effects. *New Engl J Med* 348: 538-549 (2003)

38. Hori T, E. Takahashi, D. Ayusawa, K. Takeishi, S. Kaneda & T. Seno: Regional assignment of the human thymidylate synthase (TS) gene to chromosome band 18p11.32 by nonisotopic *in situ* hybridization. *Hum Genet* 85: 576-80 (1990)

39. Vogelstein B, E.R. Fearon, S.E. Kern, S.R. Hamilton, A.C. Preisinger, Y. Nakamura, & R. White: Allelotype of colorectal carcinomas. *Science* 244: 207-211 (1989)

40. Zinzindohoue F, J.-M. Ferraz & P. Laurent-Puig: Thymidylate synthase promoter polymorphism. *J Clin Oncol* 19: 3442 (2001)

41. Kawakami K, Y. Ishida, K.D. Danenberg, K. Omura, G. Watanabe & P.V. Danenberg: Functional polymorphism of the thymidylate synthase gene in colorectal cancer accompanied by frequent loss of heterozygosity. *Jpn J Cancer Res* 93: 1221-1228 (2002)

42. Uchida K, H. Hayashi, K. Kawakami, S. Schneider, J. M. Yochim, H. Kuramochi, K. Takasaki, K.D. Danenberg & P. V. Danenberg: Loss of heterozygosity at the thymidylate synthase (*TS*) locus on chromosome 18 affects tumor response and survival in individuals heterozygous for a 28-bp polymorphism in the TS gene. *Clin Cancer Res* 15: 433-439 (2004)

43. Morgan SL, J.E. Baggott, W.H. Vaughn, P.K. Young, J.V. Austin, C.L. Krumdieck & G.S. Alarcon: The effect of folic acid supplementation on the toxicity of low-dose methotrexate in patients with rheumatoid arthritis. *Arthritis Rheum* 33: 9-18 (1990)

44. Alati T, J.F. Worzalla, C. Shih, J.R. Bewley, S. Lewis, R.G. Moran & G.B. Grindey: Augmentation of the therapeutic activity of lometrexol -(6-R)5,10-dideazatetrahydrofolate- by oral folic acid. *Cancer Res* 56: 2331-2335 (1996)

45. Worzalla JF, C. Shih & R.M. Schultz: Role of folic acid in modulating the toxicity and efficacy of the multitargeted antifolate, LY231514. *Anticancer Res* 18: 3235-3239 (1998)

46. Hanauske AR, V. Chen, P. Paoletti & C. Niyikiza: Pemetrexed disodium: a novel antifolate clinically active against multiple solid tumors. *Oncologist.* 6: 363-373 (2001)

47. Ulrich CM, K. Robien & H.L. McLeod: Cancer pharmacogenetics: polymorphisms, pathways and beyond. *Nature Reviews Cancer* 3: 912-20 (2003)

48. Allen RH, S.P. Stabler, D.G. Savage & J. Lindenbaum: Metabolic abnormalities in cobalamin (vitamin B12) and folate deficiency. *FASEB J* 7: 1344-1353 (1993)

49. Toffoli G, A. Veronesi, M. Boiocchi & D. Crivellari: MTHFR gene polymorphism and severe toxicity during adjuvant treatment of early breast cancer with cyclophosphamide, methotrexate, and fluorouracil (CMF). *Annals of Oncology.* 11: 373-4 (2000)

50. Toffoli G, A. Russo, F. Innocenti, G. Corona, S. Tumolo, F. Sartor, E. Mini & M. Boiocchi: Effect of methylenetetrahydrofolate reductase 677C-->T polymorphism on toxicity and homocysteine plasma level after chronic methotrexate treatment of ovarian cancer patients. *Int J Cancer* 103: 294-299 (2003)

51. Ulrich CM, Y. Yasui, R. Storb, M.M. Schubert, J.L. Wagner, J. Bigler, K.S. Ariail, C.L. Keener, S. Li, H. Liu, F.M. Farin & J.D. Potter: Pharmacogenetics of methotrexate: toxicity among marrow transplantation patients varies with the methylenetetrahydrofolate reductase C677T polymorphism. *Blood.* 98: 231-234 (2001)

52. Park JG, J.M. Collins, A.F. Gazdar, C.J. Allegra, S.M. Steinberg, R.F. Greene & B.S. Kramer: Enhancement of fluorinated pyrimidine-induced cytotoxicity by leucovorin in human colorectal carcinoma cell lines. *JNCI* 80: 1560-1564 (1988)

53. Bagley PJ & J. Selhub: A common mutation in the methylenetetrahydrofolate reductase gene is associated with an accumulation of formylated tetrahydrofolates in red blood cells. *Proc Natl Acad Sci USA* 95: 13217-13220 (1998)

54. Kawakami K, K. Omura, E. Kanehira & G. Watanabe: Methylenetetrahydrofolate reductase polymorphism is associated with folate pool in gastrointestinal cancer tissue. *Anticancer Res.* 21: 285-289 (2001)

55. Cohen V, V. Panet-Raymond, N. Sabbaghian, I. Morin, G. Batist & R. Rozen: Methylenetetrahydrofolate reductase polymorphism in advanced colorectal cancer: a novel genomic predictor of clinical response to fluoropyrimidine-based chemotherapy. *Clin Cancer Res* 9: 1611-1615 (2003)

56. Sohn KJ, R. Croxford, Z. Yates, M. Lucock & Y.I. Kim: Effect of the methylenetetrahydrofolate reductase C677T polymorphism on chemosensitivity of colon and breast cancer cells to 5-fluorouracil and methotrexate. *JNCI* 96: 134-144 (2004)

57. Etienne MC, K. Ilc, J.L. Formento, P. Laurent-Puig, P. Formento, S. Cheradame, J.L. Fischel & G. Milano: Thymidylate synthase and methylenetetrahydrofolate reductase gene polymorphisms: relationships with 5-fluorouracil sensitivity. *Br J Cancer* 90: 526-34 (2004)

58. Skibola CF, M.T. Smith, A. Hubbard, B. Shane, A.C. Roberts, G.R. Law, S. Rollinson, E. Roman, R.A. Cartwright & G.J. Morgan: Polymorphisms in the thymidylate synthase and serine hydroxymethyltransferase genes and risk of adult acute lymphocytic leukemia. *Blood* 99: 3786-3791 (2002)

59. Hishida A, K. Matsuo, N. Hamajima, H. Ito, M. Ogura, Y. Kagami, H. Taji, Y. Morishima, N. Emi & K. Tajima: Associations between polymorphisms in the thymidylate synthase and serine hydroxymethyltransferase genes and susceptibility to malignant lymphoma. *Haematologica* 88: 159-166 (2003)

60. Chen J, D.J. Hunter, M.J. Stampfer, C. Kyte, W. Chan, J.G. Wetmur, R. Mosig, J. Selhub & J. Ma: Polymorphism in the thymidylate synthase promoter enhancer region modifies the risk and survival of colorectal cancer. *Cancer Epidemiology Biomarkers & Prevention* 12: 958-962 (2003)

61. Trinh BN, C.N. Ong, G.A. Coetzee, M.C. Yu & P.W. Laird: Thymidylate synthase: a novel genetic determinant of plasma homocysteine and folate levels. *Hum Genet* 111: 299-302 (2002)

62. Ulrich CM, J. Bigler, R. Bostick, L. Fosdick & J.D. Potter: Thymidylate synthase promoter polymorphism, interaction with folate intake, and risk of colorectal adenomas. *Cancer Res* 62: 3361-3364 (2002)

Key Words: pharmacogenomics, thymidylate synthase, polymorphism, gene expression, tumor response, 5-fluorouracil, Review

Send correspondence to: Dr. Peter V. Danenberg, USC/ Norris Cancer Center, 1441 Eastlake Ave., Rm 5318, Los Angeles, CA 90033, Tel: 323-865-0518, 818-681-2757, Fax: 323-865-3478, E-mail: pdanenbe@usc.edu