Thymidylate synthase polymorphisms and mRNA expression are independent chemotherapy predictive markers in esophageal adenocarcinoma patients

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Abstract. Thymidylate synthase (TS) is known to have polymorphisms in the 5' and 3' untranslated region (UTR). These polymorphisms have been reported to be associated with high TS expression and chemoresistance to 5-FU. The aim of this study was to examine the prognostic roles of the 5'-UTR and 3'-UTR TS polymorphisms in esophageal adenocarcinoma patients, as well as their relation with TS mRNA expression. Eighty-three patients with esophageal adenocarcinoma were assessed. Thirty-four had received 5-FU containing chemotherapy and 49 were treated with surgery alone. Surgically resected tumor tissues were analyzed for TS genotype and TS mRNA expression using a quantitative real-time RT-PCR method. No survival difference was seen between the patients with 3RG allele (3RG group) and non-3RG group among surgery-alone patients. However, among patients with a history of 5-FU-based chemotherapy, the non-3RG group showed significantly better overall survival compared to the 3RG group (p=0.02). Moreover, whereas chemotherapy produced a significant increase in survival for the non-3RG group patients, those in the 3RG group obtained no survival benefit from chemotherapy. When patients were classified by low or high TS mRNA expression levels, low TS expressers obtained survival benefit from chemotherapy while high TS expressers did not, although there was no difference of median TS mRNA levels between 3RG and non-3RG group. The 3'-UTR polymorphism was not associated with overall survival. These results suggest that the status of the TS 5'-UTR polymorphism and TS mRNA expression are independent predictive markers for survival benefit from 5-FU-based therapy.

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Introduction

Esophageal adenocarcinoma is one of the fastest rising cancers in the Western countries, the incidence of which has increased dramatically in USA (1). Even for those who underwent a curative surgical resection, 5-year survival rates remain very low, ranging from 20 to 40% (2,3). Therefore combined modality treatment approaches, such as systemic chemotherapy, are necessary for those patients. Fluorouracil has been used in the treatment of gastrointestinal cancer and still remains the cornerstone for esophageal adenocarcinoma (4). The primary mechanism of action of fluorouracil is the inhibition of the DNA-synthetic enzyme thymidylate synthase (TS). In several studies, TS expression has been reported to be associated with response to 5-fluorouracil (5-FU) therapy and prognosis; with high TS levels in most cases associated with poorer response and survival (5-8).

TS gene is known to have several polymorphisms in its 5' untranslated region (UTR) and 3'-UTR. The first studied polymorphism is a tandem repeat sequence in 5'-UTR, which consists of either two or three 28-bp repeated sequences (9). The triple repeat (3R) allele has been associated with high TS mRNA and/or protein expression, and patients with a homozygous 3R genotype have generally shown less response to 5-FU related chemotherapy and poorer survival when compared to the patients with the homozygous double repeat (2R) genotype (10-13). In addition, a G/C single nucleotide polymorphism (SNP) within the 3R allele segregates the 3R allele into 3RG and 3RC (14,15). The 3RC allele apparently can abolish the increased transcriptional activity of the 3R variant *in vitro*, by altering a transcription factor-binding site (15). Another polymorphism, a 6-bp insertion/deletion (ins/del) in the 3'-UTR (16) has been associated with TS mRNA stability *in vitro*, and intratumoral TS mRNA

Table I. Patient characteristics.

	5'-UTR genotype			3'UTR genotype			TS mRNA levels		
	3RG group (n=30)	Non-3RG group (n=52)	P-value	ins/ins (n=28)	ins/del, del/del (n=52)	P-value	High (>2.79) (n=44)	Low (≤2.79) (n=33)	P-value
Age (years)									
≤65	12	27	0.36	15	23	0.49	22	15	0.69
>65	18	25		13	29		22	18	
Gender									
Male	24	41	1.00	24	39	0.39	33	27	0.48
Female	6	11		4	13		11	6	
Histotype									
Moderate	10	26	0.17	12	22	1.00	23	13	0.32
Poor	19	25		15	29		21	19	
Stage									
I	2	2		2	1		1	2	
II	6	15	0.65	9	11	0.37	14	6	0.53
III	19	32		15	36		26	22	
IV	3	3		2	4		3	3	
Chemo status									
Never	17	31		15	31		26	22	
Adjuvant	7	9		6	10		9	6	
Reccurence	3	9	0.54	4	8	0.77	7	4	0.88
Adjuvant and rec	1	2		2	1		2	1	
Neoadjuvant	2	1		1	2		Excluded		
TS mRNA levels									
High (>2.79)	15	29	0.63	15	28	0.74			
Low (≤2.79)	13	20		10	22				

3RG group, 2R/3RG, 3RC/3RG, 3RG/3RG; non-3RG group, 2R/2R, 2R/3RC, 3RC/3RC; P-value, Fisher's exact test.

expression *in vivo* (17). Some previous studies reported finding an association between the 3'-UTR polymorphism and prognosis of cancer patients (18,19) while others reported no associations (20-22).

The aim of this study was to examine the prognostic and predictive roles of the 5'-UTR and 3'-UTR polymorphisms in esophageal adenocarcinoma patients, as well as their relation with TS mRNA expression.

Materials and methods

Patient characteristics. Eighty-two patients with esophageal adenocarcinoma who underwent esophagectomy in University of Southern California hospital between 1992 and 2004 were assessed. Written informed consent was obtained from every patient according to the institutional regulations. Patient characteristics are listed in Table I. Thirty-four patients had received 5-FU-based chemotherapy during their treatment period (16 patients with adjuvant chemotherapy, 12 patients with chemotherapy upon recurrence, 3 patients with both adjuvant and upon recurrence, and 3 patients with neoadjuvant therapy), and 48 patients had never received 5-FU.

Microdissection. Formalin-fixed paraffin embedded (FFPE) tumor specimens were obtained from surgically-resected blocks. FFPE specimens were cut into serial sections with a thickness of 10 μm. For the pathological diagnosis, one slide was stained with H&E and evaluated by pathologist. Other sections were stained with nuclear fast red (NFR,

American MasterTech Scientific Inc., Lodi, CA) to enable visualization of histology. Laser capture microdissection (P.A.L.M. Microlaser Technologies AG, Munich, Germany) was performed in all the tumor samples to ensure that only tumor cells were dissected. Adjacent normal esophageal mucosa was dissected from the slide using a scalpel.

RNA extraction from FFPE samples. Extraction of RNA from FFPE tumor specimens and adjacent non-cancerous tissues was performed as described previously (23). After RNA isolation, cDNA was prepared from each sample as described previously (24).

Genomic DNA extraction. After the aqueous phase was removed for RNA extraction in the procedure above, 300 μl of GITC/Sarc solution and 150 μl of 1 M Tris-HCl (pH 8.0) (Invitrogen, Carlsbad, CA) were added into the remained organic phase, followed by 600 μl of pre-mixed phenol/chloroform/isoamyl alcohol (pH 8.05-8.35) (Invitrogen). The tubes were voltexed for 15 sec, placed on ice for 15 min, and then centrifuged at 13,000 rpm for 8 min in a chilled (8˚C) centrifuge. The upper aqueous phase (800-1000 μl) was carefully removed and placed in a 2-μl tube. Glycogen (10 μl) and 1000 μl of isopropanol were added and the samples were vortexed for 10-15 sec. The tubes were placed at -20˚C for 30-45 min to precipitate the DNA. The samples were then centrifuged at 13,000 rpm for 7 min in a chilled (8˚C) centrifuge. The supernatant was poured off and 500 μl of 75% ethanol was added. The tubes were centrifuged at 13,000 rpm for 6 min in a chilled (8˚C) centrifuge. The supernatant was carefully poured off so as not to disturb the DNA pellet and the samples were quick-spun for 15 sec at 13,000 rpm. The remaining ethanol was removed with a 20 μl pipette and the samples air-dried for 15 min. The pellet was re-suspended in 50 μl of 5 mM Tris.

Real-time PCR quantification of mRNA expression. Quantitation of TS mRNA and an internal reference gene (ß-actin) was done using a fluorescence based real-time detection method [ABI PRISM 7900 Sequence detection System (TaqMan®) Perkin-Elmer (PE) Applied Biosystem, Foster City, CA, USA], as described previously (25). The sequence of primers and probes of TS and ß-actin were previously described (25).

Gene expression values (relative mRNA levels) are expressed as ratios (differences between the Ct values) between the genes of interest (TS in this case) and an internal reference gene (ß-actin) that provides a normalization factor for the amount of RNA isolated from a specimen.

Evaluation of TS polymorphisms. The promoter region of the TS gene in 5'-UTR was amplified by PCR using the primers and PCR conditions described previously (13). The number of tandem repeats was analyzed by Agilent 2100 bioanalyzer and DNA 500 Assay kit (Agilent Technologies Inc., Palo Alto, CA). A G/C polymorphism in triple repeat sequence was analyzed as described previously (14). A 6-bp insertion/deletion polymorphism in 3'-UTR region was analyzed as previously described (16). Since the genotype from cancer tissue and that from non-cancerous tissue are not always the same in case of loss of heterozygosity (LOH) (13,14) adjacent non-cancerous esophageal mucosa was also assessed, and the genotype from non-cancerous tissue was compared to the genotype from cancer tissue in each patient.

Statistical analysis. Overall survival was measured from the date of surgery to the date of death (any cause) or the date last seen. Survival curves were generated using the Kaplan-Meier method. The log-rank statistic was used to compare survival distributions. Fisher's exact test was used for comparison of patient characteristics, and for assessing linkage disequilibrium between 5'-UTR and 3'-UTR polymorphism. The Mann-Whitney U test was used to compare the median TS mRNA values between groups. The maximal χ^2 method of Miller and Siegmund and Halpern was adapted to determine which TS gene expression value (optimal cut point) best segregated patients into poor- and good-prognosis subgroups (in terms of likelihood of survival). The Cox proportional hazards model was used to assess the prognostic importance of parameters. All reported P-values are two-sided and statistical significance was set at the 0.05 level for the P-value.

Results

The distribution of 5'-UTR genotype and 3'-UTR genotype. We followed the convention established by previous studies and classified the 5'-UTR genotypes into the 3RG group (2R/3RG, 3RC/3RG, 3RG/3RG) and the non-3RG group (2R/2R, 2R/3RC, 3RC,3RC), while the 3'-UTR genotypes were classified into the Ins group (ins/ins) and Del group (ins/del, del/del) (17,26,27). Significant linkage disequilibrium was observed between two polymorphism groups (p=0.002). Whereas the Del genotype was distributed evenly between the 3RG and non-3RG genotypes, the Ins genotype was predominantly associated with the non-3RG group.

Frequency of LOH in 5'-UTR locus. The 5'-UTR genotype was compared between cancer and non-cancerous (normal) tissue in order to evaluate LOH in this locus. Among 39 patients with informative heterozygous 5'-UTR genotype in their normal tissue, 17 patients showed LOH in their cancer tissue (43.6%). In 3 of these patients, the LOH led to an absence of the 3G allele in their cancer tissue, and thus a genotype discrepancy between cancer and normal tissue in the 5' UTR region (3/39=7.7%).

TS 5'-UTR genotype and prognosis. In the entire set of patients, those in the non-3RG group showed significantly better overall survival compared to patients in the 3RG group (p=0.038). Among the patients who had received 5-FU related chemotherapy (5-FU(+) patients), a survival difference was observed between the 3RG and the non-3RG groups (p=0.018) (Fig. 1B). However, among the patients who had never received 5-FU related chemotherapy (5-FU(-) patients), no significant survival difference was seen between these 2 groups (p=0.34) (Fig. 1A). Overall survival in 5-FU(+) patients was significantly longer than 5-FU(-) patients in the non-3RG group (p=0.0180), while no significant difference

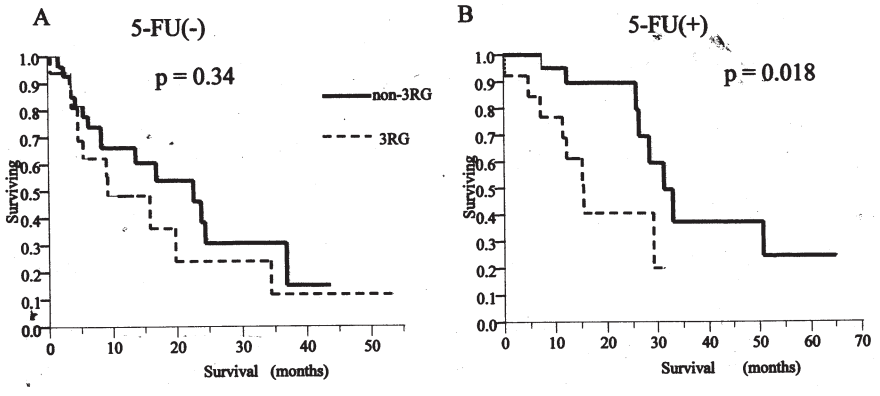

Figure 1. Kaplan-Meier analysis of overall survival in patients with esophageal adenocarcinoma according to TS 5'-UTR polymorphism. (A) In the patients who had received 5-FU related chemotherapy. (B) In the patients who had never received 5-FU.

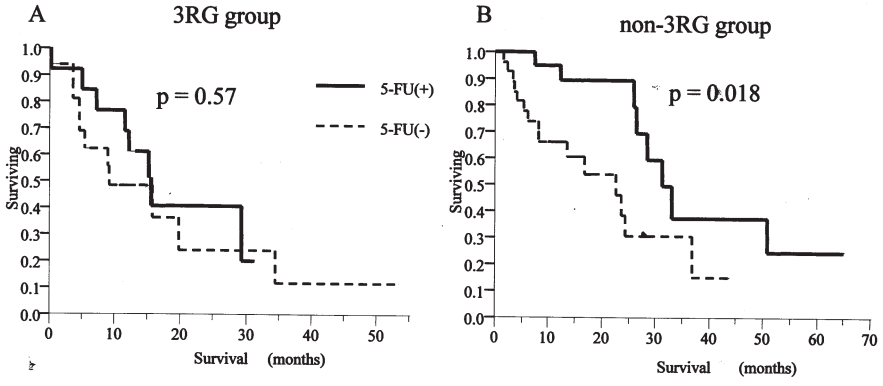

Figure 2. Kaplan-Meier analysis of overall survival in patients with esophageal adenocarcinoma according to a history of 5-FU treatment. (A) In patients who had at least one 3RG allele. (B) In the patients who did not have a 3RG allele.

Table II. TS mRNA levels in each genotypes.

	5'-UTR genotype			3'-UTR genotype		
	3RG group	Non-3RG group	P-value	ins/ins	ins/del, del/del	P-value
Cancer	3.62 (1.01-32.16)	3.34 (0.80-19.54)	0.73	3.23 (0.8-19.54)	3.48 (1.14-32.16)	0.94
Normal	1.79 (0.77-3.51)	1.59 (0.34-4.61)	0.40	1.85 (0.34-3.28)	1.62 (0.77-4.61)	0.33

TS mRNA, median (range); 3RG group, 2R/3RG, 3RC/3RG, 3RG/3RG; non-3RG group, 2R/2R, 2R/3RC, 3RC/3RC; p-value, Mann-Whitney U test.

was observed between survival of 5-FU(+) patients and 5-FU(-) patients in the 3RG group (p=0.57) (Fig. 2).

TS mRNA expression level and genotypes. The patients who had received neoadjuvant chemotherapy were excluded from assessing TS mRNA expression because mRNA levels might be changed after chemotherapy. TS mRNA expression levels were assessable in 77 patients. No significant difference was found between median TS mRNA expression levels of the 3RG and the non-3RG groups in either cancer tissue (p=0.73) or in normal tissue (p=0.40). Likewise, no difference was observed between median TS mRNA levels of the two 3'-UTR genotype groups either in cancer tissue (p=0.9418) or in normal tissue (p=0.33) (Table II).

TS mRNA expression level and prognosis. In the entire set of patients, no significant survival difference was observed between those who had higher TS mRNA level than the cut-off value (2.79) and those with low TS mRNA level (p=0.5103). For patients not receiving post-operative chemotherapy (5-FU-), the survival curves of high TS and low TS expressers were practically superimposable (p=0.69), while

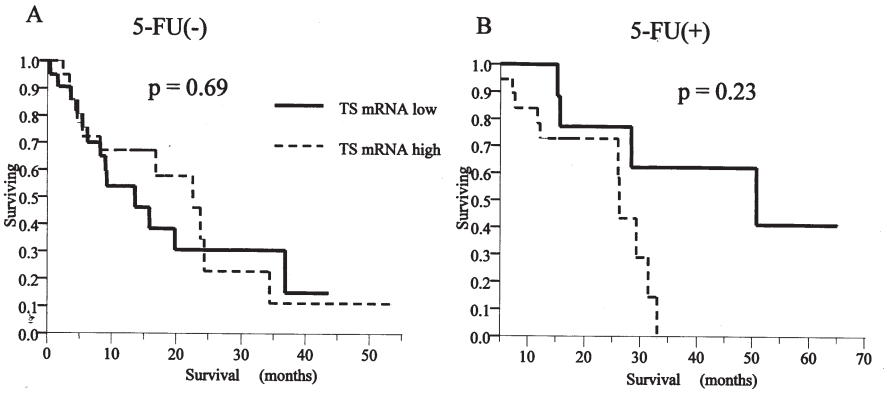

Figure 3. Kaplan-Meier analysis of overall survival in patients with esophageal adenocarcinoma according to TS mRNA expression levels. (A) In the patients who had received 5-FU related chemotherapy. (B) In the patients who had never received 5-FU.

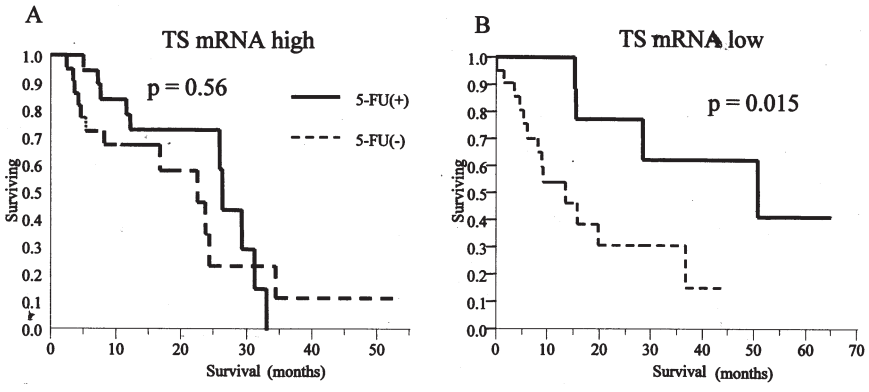

Figure 4. Kaplan-Meier analysis of overall survival in patients with esophageal adenocarcinoma according to a history of 5-FU treatment. (A) In patients with high TS mRNA levels. (B) In patients with low TS mRNA levels.

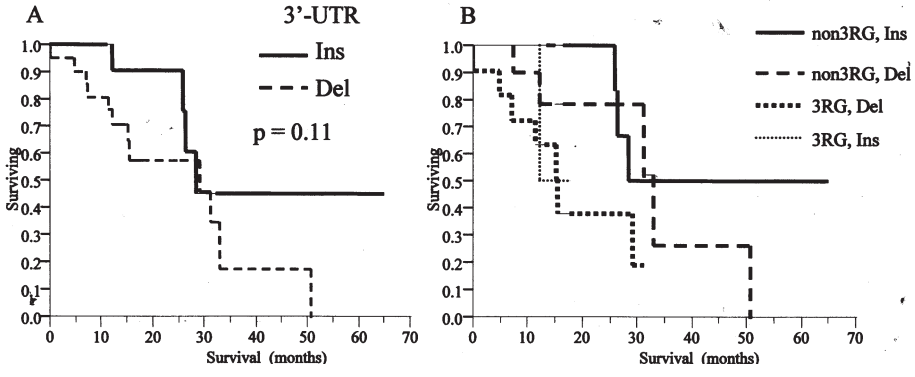

Figure 5. (A) Kaplan-Meier analysis of overall survival in patients with esophageal adenocarcinoma according to 3'-UTR polymorphism. 5'-UTR polymorphism was not taken into consideration. (B) Kaplan-Meier analysis of overall survival in patients with esophageal adenocarcinoma according to a combination of 5'-UTR and 3'-UTR polymorphism.

among 5-FU(+) patients, there was a trend toward better overall survival for low TS expressers compared to high expressers, although the difference did not reach significance (p=0.23) (Fig. 3). However, when the patients were separated according to high and low TS expression, we found a significantly longer overall survival in the low TS-5-FU(+) group compared to the low TS-5-FU(-) group (p=0.015), while among patients with high TS mRNA, no survival difference was observed between 5-FU(+) patients and 5-FU(-) patients (p=0.56) (Fig. 4).

In the multivariate analysis, TS 5'-UTR genotype was significantly associated with survival when adjusting stage (p=0.033).

3'-UTR genotype and prognosis. We found an unequal distribution (linkage disequilibrium) of the 2 different 3'-UTR genotypes between the 3RG and non-3RG 5'-UTR genotypes. Fig. 5A shows Kaplan-Meier survival curves of 3'-UTR Ins group and Del group among 5-FU received patients without sorting according to 5'UTR genotypes.

Although there appears to be a trend of survival difference between the two groups, it may due to the fact that the Ins group is strongly associated with the non-3RG group. When we compared the association of 3'-UTR genotypes with overall survival within the same 5'-UTR groups (Fig. 5B), we observed no survival difference between 3'-UTR Ins genotypes and Del genotypes in either the combined group of patients (p=0.43 within the 3RG group; p=0.28 within non-3RG group) or in the 5-FU(+) patients (p=0.78 within the 3RG group; p=0.25 within the non-3RG group).

Discussion

The goal of this study was to determine the interrelationships of 5'-UTR and 3'-UTR polymorphisms of the TS gene, TS gene expression and clinical outcome in esophageal cancer. In designing this study, we tried to avoid certain shortcomings apparent in some previous studies on this topic. First, in order to distinguish predictive from prognostic effects, we determined the association of these genetic factors with survival both in patients that had received 5-FU-based chemotherapy as well as patients that had not received chemotherapy. Secondly, we used microdissected tumor tissue to determine TS gene expression and polymorphism status because, as will be discussed below, tumor genotype in the case of heterozygous individuals may not reflect that in germ line tissue. Third, we analyzed TS polymorphisms at both the 5'UTR and 3'-UTR and thus we were able to assess the association of the 3'-UTR polymorphisms with outcome in the context of the same 5'-UTR groups.

Fig. 1 shows the non-3RG genotype to be significantly associated with better survival only in patients treated with 5-FU-based adjuvant chemotherapy (Fig. 1B) but not in the group of chemotherapy-untreated patients (Fig. 1A). The difference between these two profiles allows us to conclude that the non-3RG genotype is a predictive factor for 5-FU based therapy but not a prognostic factor, i.e., that the separation of the survival curves in Fig. 1B is due to the beneficial effects of the chemotherapy on non-3RG patients rather than intrinsically longer survival of such patients. The 3RG genotypes have been previously found to be significantly associated with worse response of gastric cancer treated with 5-FU-cisplatin (28) and worse prognosis for colorectal cancer patients treated adjuvantly with 5-FU-based therapy (14,27). However, none of these studies specifically separated predictive and prognostic effects of the TS polymorphisms.

The data further show that, whereas chemotherapy produces a substantially and significantly better survival benefit for the non-3RG patients, 3RG patients receive no significant benefit from chemotherapy. Fig. 2, which is a re-plot of the survival data by genotype, shows a striking difference between the genotype groups in the divergence of the survival curves of chemotherapy-treated and untreated patients, especially at earlier time points. A similar result was previously observed in colorectal cancer patients (14).

The 5'-UTR genotypes are thought to influence 5-FU-based chemotherapy by regulating intratumoral TS levels. Several studies agree on the point that non-3RG genotype tumors have better response due to lower TS levels, but disagree as to the mechanism by which the 5'-UTR polymorphisms control TS expression. Mandola *et al* (15) and Morganti *et al* (29) reported that the 3RC genotype results in lower TS gene expression compared to 3RG, whereas Kawakami and Watanabe (14) found no effect of the G-C SNP on TS gene expression but rather on TS protein expression. Our data show no relationship between TS mRNA levels and 5'-UTR genotype status and thus are more consistent with the hypothesis that the TS polymorphisms influence TS expression at the protein translation level. Another possibility is that differences in tumor response might arise from the effects of TS polymorphisms on folate levels. It has been known for some time that manipulations of intracellular folate pools can alter tumor sensitivity to 5-FU (30,31) and there are a number of studies indicating a relationship between TS polymorphisms, circulating folate levels and cancer risk (32-34). However, to date we are aware of no published studies specifically addressing differences in folate levels between 3RG and non-3RG genotypes.

As many previous studies have, we also found an association between TS gene expression and clinical outcome. In general, low TS expression has predicted for better response to chemotherapy as well as better survival (35,36), although there are several studies that seem to show the opposite relationship, i.e., that patients with higher intratumoral TS levels received more benefit from treatment (37,38). This apparent contradiction in the effects of TS on tumor response has never really been explained satisfactorily (39). In theory as well as intuitively, it makes more sense that drug therapy directed at inhibition of TS should be more effective against tumors with lower TS expression. The results of the present study are consistent with this expectation in that 5-FU-based chemotherapy significantly increased survival of patients with low TS expression, whereas it had no effect on survival among patients with high TS mRNA levels. Moreover, our results also show that TS gene expression as a predictive factor for 5-FU-based therapy is independent of TS polymorphism status. Thus, patients who have both low TS mRNA and the non-3RG genotype represent a group that may derive the most benefit from chemotherapy, while those with high TS mRNA and 3RG genotype would be predicted to have the worst prognosis.

Previous studies have suggested that in addition to its role as the target for fluoropyrimidines, TS is also a prognostic factor in patients not treated with chemotherapy (38,40,41) and it has even been suggested that TS is an oncogene (42). However, we saw no significant survival difference between the high and low TS expressers in the group of patients who had not received 5-FU chemotherapy. Thus, our data do not support the idea that in esophageal cancer the TS expression level in tumors is a prognostic marker independent of chemotherapy. Whether the discrepancy between our results and the earlier studies is due to patient selection, the tumor type or the precise mode of treatment is not clear at this time.

To better evaluate the prognostic and predictive roles of TS polymorphisms, we isolated genomic DNA from tumor tissues. The use of peripheral blood samples is a less invasive, more convenient and lower-cost method for obtaining DNA, but the problem is that the genotype of the germ-line tissue is not always the same as that of the cancer

due to loss of heterozygosity (LOH) observed at the TS locus in chromosome 18q (43). LOH at the TS locus occurs at a frequency of 62-76% in colorectal cancer (44,13) and 53% in esophageal adenocarcinoma (Kuramochi *et al,* Proc ASCO 22: abs. 842, 2000). An LOH event in the tumor cells of heterozygous 3RG/2R individuals that resulted in loss of the 3RG allele would confer a 2R genotype on the tumor, thereby changing the unfavorable prognosis indicated by the normal tissue genotype to a favorable one (13). In the present set of patients, LOH at the 5'-UTR locus of heterozygous patients led loss of the 3RG allele in the tumor in only 3 cases. However, if these 3 patients are re-classified into the 3RG group based on their normal tissue genotype (i.e., ignoring the LOH events in the tumors), the difference between the survival curves of the 3RG and non-3RG groups is no longer statistically significant (p=0.2) (data not shown). This result again demonstrates the necessity for analyzing tumor genotype when studying relationships between TS polymorphisms and clinical end-points involving tumor response. Not doing so can at the least alter the true significance value of any findings from such studies and in some cases, may result in the reporting of significant relationships as non-significant.

Several previous studies have reported an association of the TS 3'-UTR polymorphism with prognosis (18,19), whereas others observed no significant relationship (20-22). However, assigning a predictive or prognostic role to the 3'-UTR is complicated by linkage disequilibrium between the 5'-UTR and 3'-UTR polymorphisms (17,22,45,46). In this study, we have shown a significantly frequent association of the non-3RG 5'-UTR genotype with the 3'-UTR Ins polymorphism. Thus, by identifying 3'-UTR Ins genotypes for study, we are also selecting for a predominantly non-3RG genotype in the 5'-UTR and cannot therefore with any certainty ascribe regulatory function to the 3'-UTR polymorphism. Our strategy for avoiding such ambiguities was to compare the effects of the 3'-UTR polymorphism on survival within the same 5'-UTR group. When we did this, there was no significant association between 3'-UTR polymorphism status and patients' prognosis.

In conclusion, 5'-UTR polymorphism status and TS mRNA levels are autonomous predictive factors for the effect of 5-FU based chemotherapy in esophageal cancer but are not prognostic factors independent of chemotherapy. Determining whether there is a 3RG or non-3RG genotype in the 5'-UTR of TS together with TS mRNA expression may provide a clinically useful test for distinguishing patients who may benefit from 5-FU-based adjuvant therapy from those who would not benefit.

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