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Relationships between promoter polymorphisms in the thymidylate synthase gene and mRNA levels in colorectal cancers

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Abstract

Thymidylate synthase (TS) intratumoural expression may be a prognostic marker and predict outcome of 5-fluorouracil (5-FU)-based chemotherapy in colorectal cancer patients. The *TS* gene promoter enhancer region contains two different polymorphisms which can influence *TS* mRNA transcriptional and translational efficiency: a polymorphic tandem repeat sequence (2 or 3 repeats; 2R and 3R) and a single nucleotide polymorphism (SNP), G > C, within the second repeat of the 3R alleles. We studied the relationship between tumoural *TS* mRNA expression levels and *TS* gene polymorphisms in the colonic mucosa of 48 colorectal cancer patients. The 3R/3R genotype was characterised by higher *TS* mRNA levels in the tumour than the 2R/2R–2R/3R genotypes ($P = 0.071$). Regarding the relationship with the SNP polymorphism, a statistically significant difference in *TS* gene expression between the 3RG/3RG genotype and 2R/2R–2R/3RC–2R/3RG genotype subset was observed ($P = 0.017$). No statistically significant correlation was observed between experimental data and baseline clinical-pathological characteristics as well as clinical outcome in the relatively small patient series investigated. This is the first study reporting an association between the *TS* intra-repeat SNP and gene expression levels in colorectal cancer patients. These results suggest that in 3R/3R patients, the G > C polymorphism may be an important factor in determining *TS* mRNA expression levels, and warrant further investigation of the role of *TS* promoter polymorphisms as predictors of sensitivity to 5-FU-based chemotherapy in larger case series.

Keywords: Thymidylate synthase; Polymorphisms; mRNA levels; Colorectal cancer; Fluoropyrimidine chemotherapy

1. Introduction

Thymidylate synthase (TS) is a key enzyme in the *de novo* synthesis of deoxythymidine monophosphate (dTMP), an essential precursor of DNA, which catalyses

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the methylation of deoxyuridine monophosphate to dTMP [1]. The critical role of TS in nucleotide metabolism has made it an important target of a variety of chemotherapeutic agents including 5-fluorouracil (5-FU), 5-FU prodrugs such as capecitabine, and novel folate-based *TS* inhibitors such as raltitrexed and pemetrexed, which are used in the treatment of colorectal and other solid tumours [2].

Resistance to fluoropyrimidines and other TS inhibitors may occur through a variety of mechanisms including elevated intracellular TS levels resulting from increases in TS transcription [3] and translation [4].

In colorectal cancer patients TS intratumoural expression may predict the sensitivity to 5-FU and other TS inhibitor-based chemotherapy [5,6], and may also be an important prognostic marker [7]. High TS expression in early stage colorectal cancer patients seems to predict poorer overall survival in both chemotherapy-treated and untreated patients following surgery [7]. Also, metastatic colorectal cancer patients with high TS levels are unlikely to respond to infusional treatment with 5-FU, whereas patients with low levels of this enzyme have higher than average response rates [8,9].

Two different polymorphisms have been described in the *TS* gene promoter region. The first one is represented by a variation in the number of a 28-base pair tandemly repeated sequence [10]. Although there have been reports of more than three repeats within certain populations, the vast majority of *TS* alleles harbour either a double- or a triple repeat, creating genotypes defined as 2R/2R, 2R/3R, and 3R/3R, respectively. This polymorphism modulates *TS* gene expression [4,10] and translational efficiency [11], as shown by the observation that *TS* genes with the 3R allele have greater expression activity than those with the 2R sequence in a transient expression assay in cancer cells [10].

Recently, a single nucleotide polymorphism (SNP), G > C, involving the twelfth nucleotide of the second repeat of the 3R alleles, has been described. The two alleles are defined as 3RG and 3RC, respectively. The 3RC allele can abolish the increased transcriptional activity of the 3R variant *in vitro*, by altering a transcription factor-binding site [12].

Increased expression of the *TS* gene [13] and/or of the TS protein in gastrointestinal cancer [14] is associated with the *TS* 3R allele. Patients with gastrointestinal cancer (mainly colorectal), who were homozygous for the 3R variant were found to have higher average tumour levels of *TS* mRNA [13] or TS protein [14] and were less likely to respond to 5-FU treatment compared with 2R/2R homozygotes and 2R/3R heterozygotes in the metastatic [13,15], neoadjuvant [16] and adjuvant settings [17].

By analysing the G > C SNP within the 28-bp tandem repeat sequence of the *TS* gene, Kawakami and colleagues [18], demonstrated that colorectal cancer patients with *TS* genotypes, classified as high expression type (2R/3RG, 3RG/3RC and 3RG/3RG), did not benefit from postoperative adjuvant therapy with oral fluoropyrimidines, while patients with *TS* genotypes classified as low expression type (2R/2R, 2R/3RC and 3RC/3RC) survived longer if treated postoperatively with oral fluoropyrimidines as compared to untreated controls. The SNP located within the promoter region of the *TS* gene was also found to be associated with response to 5-FU-based chemotherapy and survival in patients treated for metastatic colorectal cancer [19]. However, no studies on the association between this *TS* intra-repeat SNP and gene expression levels in colorectal cancer patients have been published so far.

The purpose of the present study was to analyse the relationship between tumour *TS* gene expression levels and the two above described *TS* polymorphisms in normal colonic mucosa from colorectal cancer patients at various stages of disease who were undergoing surgery and post-operative 5-FU-based chemotherapy.

2. Materials and methods

2.1. Tissue sampling

Primary tumour and corresponding colonic mucosa explants obtained from colorectal patients at surgery were frozen in liquid nitrogen until molecular analysis. Immediately after resection, the tumour sample was divided into two equal portions after washing and removal of necrotic tissues. One portion was fresh frozen in liquid nitrogen until the time of RNA extraction, and the other portion was embedded in paraffin to confirm histologically that it did not contain significant contamination by normal tissues, necrotic tissues, and lymphocytes.

2.2. Relative quantitative analysis of TS gene expression by reverse transcription and real time PCR (RT-PCR) assay

Total RNA was extracted by a standard protocol based on the guanidine thiocyanate-phenol/chloroform extraction method [20]. Semiquantitative analysis of *TS* mRNA was performed using the LightCycler *TS* mRNA Quantification Kit^{Plus} (Roche Diagnostics S.p.A. Roche Applied Science, Monza, Italy) following a two-step procedure. In the first step, cDNA was reverse transcribed from 100 ng of total RNA using AMV reverse transcriptase and *TS* and glucose-6-phosphate dehydrogenase (*G6PDH*, reference gene) specific primers. In the second step, a 111 bp fragment of *TS* cDNA was amplified by PCR in a LightCycler apparatus (Roche Diagnostics S.p.A. Roche Applied Science, Monza, Italy) using specific primers. The *TS* amplicon

was detected by fluorescence with a specific pair of hybridisation probes (fluorescence resonance energy transfer, FRET). The reference gene *G6PDH* was amplified using the same cDNA preparation but in a separate PCR reaction tube. A calibrator RNA was included in the kit for relative quantification. *TS* PCR amplification products were quantitatively analysed as follows: the amount of target *TS* was calculated as a ratio of the amount of target (T) gene to the amount of reference R gene (*G6PDH*). Then the ratio of target to reference (T:R) of the sample was divided by T:R of the calibrator to compensate for run-to-run differences. Analysis was performed using a dedicated quantification software (The LightCycler Relative Quantification Software, Roche Diagnostics S.p.A. Roche Applied Science, Monza, Italy).

2.3. Analysis of the TS promoter enhancer region polymorphisms

Genomic DNA extraction was performed by a standard phenol/chloroform method. DNA was amplified by PCR using the following primers: forward 5′-GTGGCTCCTGCGTTTCCCCC, reverse 5′-CTCCGAGCCGGCCACAGG. PCR analysis was performed in a total volume of 30 μl containing 200 ng DNA, 1.25 mM $MgCl_2$, 2 mM deoxynucleotide triphosphates, 3 μl of dimethyl sulfoxide (DMSO), 40 pmol of each primer and 1.25 U of Taq polymerase. After 35 cycles of amplification (denaturation at 95 °C for 30 s, annealing at 63 °C for 30 s and elongation at 72 °C for 30 s), PCR products were electrophoresed onto a 2.5% agarose gel. Products of 213 base pairs (2R/2R), 241 base pairs (3R/3R), or both of these products (2R/3R), depending on the *TS* genotype, were observed. Fifteen microliters of the amplification products from samples showing the presence of the 3R allele were subsequently digested with the restriction enzyme *Hae*III, which allows recognition of the intra-repeat G/C SNP. Digested PCR products were electrophoresed onto 15% non-denaturing polyacrylamide gels. *Hae*III digestion yielded several lower molecular weight bands, with a 94-base pairs band specific for the C allele.

2.4. Statistical analysis

Relationships between *TS* mRNA expression and genotypes were analysed on the following subsets of patients: two subgroups selected on the basis of *TS* mRNA high or low expression (cut off = median value); two subgroups selected on the basis of repeat length allele combinations: 3R/3R genotypes ($n = 16$), and 2R/2R–2R/3R genotypes ($n = 32$); and three subgroups obtained from additional investigation of the intra-repeat SNP: 2R/2R–2R/3RC–2R/3RG ($n = 31$), 3RG/3RG ($n = 6$) and 3RG/3RC genotypes ($n = 7$).

The Wilcoxon RankSum test was used to assess the correlation between *TS* gene expression and the *TS* tandem repeat polymorphism, and the Kruskal–Wallis test to assess the correlation between *TS* gene expression and *TS* SNP genotypes.

The correlation of *TS* gene expression with clinical and pathologic characteristics was analysed using the Spearman test (age), the Wilcoxon RankSum test (sex and histotype) and the Kruskal–Wallis test (tumour site, stage and grading).

The correlation between the different *TS* polymorphisms and the clinical and pathological characteristics was analysed using the Fisher exact test for sex, grading, tumour site, histotype and stage parameters and the Kruskal–Wallis test for age.

Overall and disease-free survival in every subset of patients were estimated by the Kaplan–Meier method and compared using the log-rank test.

Analyses were carried out using the Stata Corp. 2001 Stata Statistical Software, Release 7.0 College Station, TX, Stata Corporation. P values <0.05 were considered significant.

3. Results

The case series of 48 colorectal cancer patients was comprised of 20 females and 28 males; their median age was 60 years, ranging between 23 and 76. The site of the primary tumour was right colon in 8 cases, left colon in 20 cases, transverse in 4, and rectum in 16 cases; primary tumour stage was Dukes' B in 22 patients, C in 20, D in 6. The histotype was adenocarcinoma in 45 cases and mucinous in 3; the grading was G2 in 41 patients, G3 in 4 and not determined in 3 patients. All patients received fluoropyrimidine-based chemotherapy postoperatively: in 42 cases as adjuvant and in 6 as palliation (Table 1).

The median time interval between surgery and postoperative treatment was 35 days, ranging between 22 and 87 days in the adjuvant setting ($n = 42$). The same parameter for the 6 patients with synchronous metastases was 36.5 days (range 25–48 days).

Disease recurrence occurred in 10 out of 42 completely resected patients; the median time between surgery and this event was 14 months (range 4–32). The median follow-up for the 32 disease-free patients was 88.5 months (range 17+;114+).

A marked interindividual variation in *TS* mRNA expression in the 48 colorectal cancer samples was observed: *TS*/*G6PDH* ratios varied between 0 and 10.72 (>100 fold) with a median value of 1.52. This value was also used to discriminate between patients with high and low *TS* mRNA expression in the two subpopulations of patients receiving adjuvant or palliative chemotherapy after surgery.

Table 1
Main clinical and pathological characteristics of colorectal cancer patients

No. of patients	48
Age (years)	
<60	22
⩾60	26
Sex	
M	28
F	20
Site of primary tumours	
Right colon	8
Transverse colon	4
Left colon	20
Rectum	16
Dukes' stage	
B	22
C	20
D	6
Histotype	
Adenocarcinoma	45
Mucinous	3
Grading	
G2	41
G3	4
ND	3
Type of 5-FU-based chemotherapy	
Adjuvant	42
Palliative	6

ND, not determined.

The genotype frequencies of the *TS* tandem repeat polymorphism in colonic mucosa were 18.7% (2R/2R), 47.9% (2R/3R) and 33.3% (3R/3R).

A trend for increased *TS* gene expression in patients with the 3R/3R genotype *versus* those with the 2R/2R–2R/3R genotypes was noted (the median *TS*/*G6PDH* ratio was 2.22 for 3R/3R and 1.36 for 2R/2R–2R/3R patients; $P = 0.071$) (Fig. 1, panel a).

SNP analysis was performed in 44 colonic mucosa samples, due to unavailability of DNA from 4 samples for further analysis. The frequency of each *TS* SNP genotype was 20.5% (2R/2R), 27.3% (2R/3RG), 22.7 (2R/3RC), 13.6% (3RG/3RG), and 15.9% (3RG/3RC). We compared the subgroup including the 2R/2R, 2R/3RC, and 2R/3RG genotypes ($n = 31$) with the subgroups comprised of the 3RG/3RG ($n = 6$) or of the 3RG/3RC ($n = 7$) genotypes. We observed a statistically significant difference in *TS* gene expression levels only between the first (2R/2R, 2R/3RC, and 2R/3RG genotypes) and the second subgroup (3RG/3RG genotype) (median *TS*/*G6PDH* ratios 1.42 and 5.27, respectively) ($P = 0.017$) (Fig. 1, panel b). Median *TS* mRNA expression value in the third subgroup (3RG/3RC patients) was not substantially different from that observed in the first subgroup (median *TS*/*G6PDH* ratios 2.01 and 1.42, respectively, $P = 0.55$) (Fig. 1, panel b).

Analysis of the correlations between experimental data (*TS* mRNA expression and promoter polymorphisms) and baseline clinical and pathological characteristics showed no statistically significant differences (data not shown).

Relationships between clinical outcome (disease recurrence and death) and *TS* gene expression or genotype for the 42 patients undergoing radical surgery followed by adjuvant chemotherapy are reported in Table 2.

The other 6 patients with synchronous metastases (5 at hepatic level, one at peritoneal level) that persisted after surgery belonged to the same group of genotypes (3 patients 2R/2R; 1 patient 2R/3RG; 2 patients 2R/3RC) and 5 out of 6 presented low levels of *TS* gene expression. Due to the limited number of patients and the non-homogeneous distribution, it was not possible to evaluate the predictive role of experimental parameters in this disease setting.

Three disease stabilisation and 3 disease progression were observed. Two patients, undergoing curative surgery after chemotherapy became disease-free; one of these relapsed at 25 months, the other was disease-free at the time of analysis (49 months). The median time to progression was 3 months (range 2–25).

The median overall survival time was not achieved in patients undergoing radical surgery ($n = 42$) nor in the entire case series ($n = 48$) with a median follow-up of 94 months. This parameter was instead 14 months in the 6 patients with metastatic disease at surgery.

The overall survival in our entire case series ($n = 48$) and in that of cases with completely resected tumours ($n = 42$) was similar in patients with *TS* mRNA levels lower than the median value and in those with higher levels. Disease-free survival (DFS) was also similar in patients with completely resected tumours and high *TS* expression compared with patients having low *TS* expression ($P =$ ns). No consistent differences in OS and DFS were observed among patients with 2R/2R and 2R/3R or 3R/3R genotypes and among 2R/2R–2R/3RC–2R/3RG and 3RG/3RG or 3RG/3RC genotypes (Fig. 2).

4. Discussion

The tandem repeat length polymorphism of the *TS* 5′ regulatory region has been shown to influence *TS* expression levels [4,10,14], and its predictive value for treatment outcome has been demonstrated following the finding that colorectal cancer patients with one or two 2R alleles benefit from 5-FU-based chemotherapy to a greater extent than those with two copies of the 3R allele [13,15–17].

Our results show that in normal colonic mucosa the 3R/3R genotype patient subset displays increased *TS* mRNA levels compared to the 2R/2R–2R/3R subset with a trend to statistical significance ($P = 0.071$). However, 6

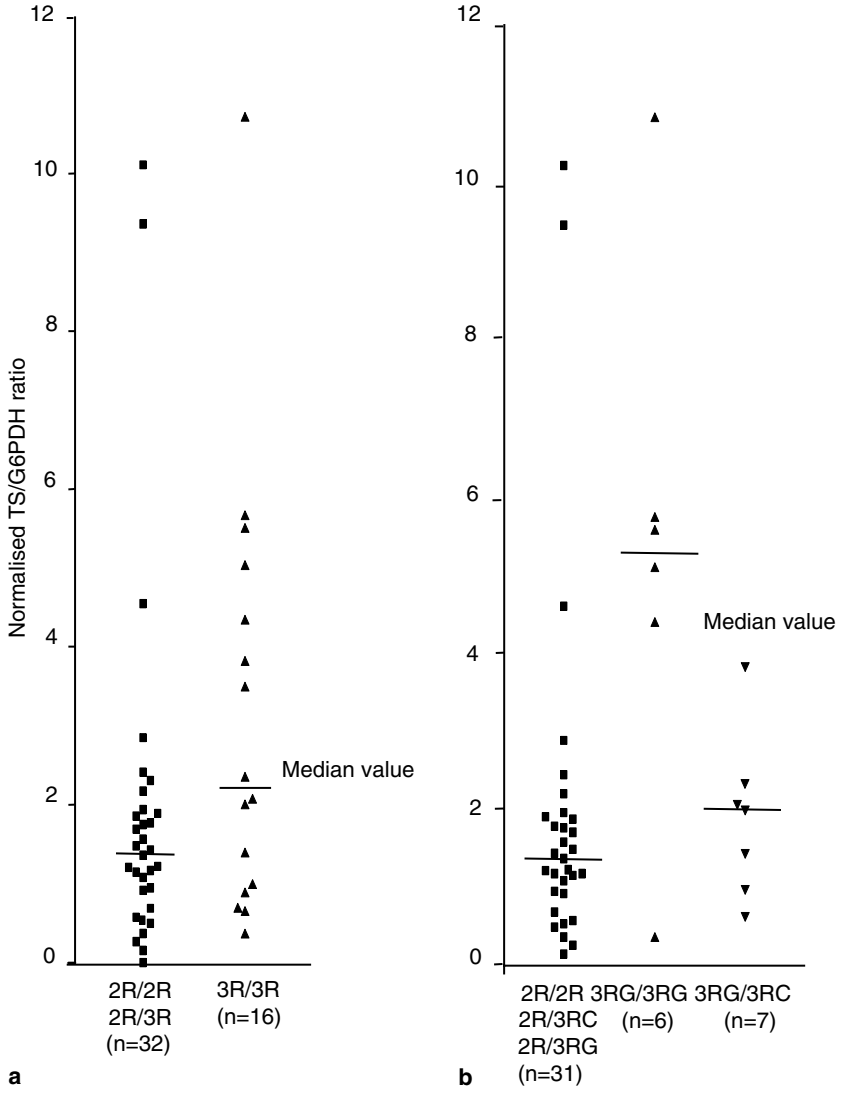

Fig. 1. Relationship between *TS* mRNA expression and *TS* polymorphisms: (a) tandem repeat length polymorphism in 48 patients: 3R/3R genotype *vs.* 2R/2R and 2R/3R genotypes ($P = 0.071$); (b) single nucleotide polymorphism (SNP) analysis in 44 patients: subgroup 1 (TS genotypes 2R/2R–2R/3RC–2R/3RG) *vs.* subgroup 2 (3RG/3RG genotype) *vs.* subgroup 3 (3RG/3RC genotype). A statistically significant difference ($P = 0.017$) was observed when comparing subgroup 1 with subgroup 2.

out of 16 patients (37.5%) with 3R/3R genotypes had a *TS* expression level lower than or just above the median value of the 2R/2R–2R/3R subset. Our results are in agreement with those of other authors who observed low *TS* levels in a subset of patients with the 3R/3R genotype [13].

This phenomenon may be due to the presence of a single G > C base change within the second repeat in the 3R genotype. This SNP has been shown to alter a critical residue in the upstream stimulatory factors (USF) E-box consensus element that abolishes the binding of USF regulatory proteins [12].

Indeed, analysis of the G > C SNP in our case series demonstrates that 3 out of 6 low *TS* expressing patients with a 3R/3R genotype had the G > C base change in the second tandem repeat unit of the *TS* gene. In two of the remaining patients SNP analysis was not performed, and in one patient a 3RG/3RG genotype was detected. These findings provide evidence for a relationship between the G > C SNP and *TS* expression levels, confirming its importance for the regulation of *TS* transcription.

Despite the limited number of patients, our results suggest that in 3R/3R patients the G > C polymorphism plays an important role in determining *TS* mRNA expression levels and that combined evaluation of this SNP and of the *TS* gene tandem repeat sequence polymorphism may provide more effective prediction of sensitivity to 5-FU-based chemotherapy.

Avoiding contamination of tumour samples by normal tissues may be critical when quantitatively assessing *TS* gene expression that is significantly higher in

Table 2
Thymidylate synthase (*TS*) gene expression, *TS* genotypes and clinical outcome in completely resected patients

	No. of patients	No. of recurrence	Time to progression		No. of deaths	Time to death	
			Median (mo.)	Range (mo.)		Median (mo.)	Range (mo.)
Low expression	19	5 (26.3%)	16	6–32	4 (21.0%)	52	28–86
High expression	23	5 (21.7%)	12	4-26	5 (21.7%)	27	13–86
Total	42	10 (23.8%)			9 (21.4%)		
2R/2R, 2R/3R	26	7 (26.9%)	17	4–32	6 (23.1%)	42.5	13–86
3R/3R	16	3 (18.7%)	12	11–16	3 (18.7%)	39	28–46
Total	42	10 (23.8%)			9 (21.4%)		
2R/2R, 2R/3RG, 2R/3RC	25	7 (28.0%)	17	4–32	6 (24.0%)	42.5	13–86
3RG/3RG	6	1 (16.6%)	12	NA	1 (16.6%)	39	NA
3RG/3RC	7	1 (14.3%)	11	NA	1 (14.3%)	12	NA
Total	38[a]	9 (23.7%)			8 (21.0%)		

NA, not applicable.
[a] Single nucleotide polymorphism (SNP) analysis was performed in 38 patients, due to unavailability of DNA from 4 samples for further analysis.

tumours than in normal adjacent tissues [21,22]. For this purpose, previous studies on quantitative analysis of *TS* gene expression in surgical explants and biopsies have relied on histological confirmation of specimen nature and composition [23,24]. Our study is in keeping with this approach. The meta-analysis by Popat and colleagues [7] has provided accepted evidence of the predictive/prognostic role of *TS* gene expression obtained by this methodology.

In our series, one patient with a 3RG/3RG genotype had a low *TS* expression level whereas 4 patients with 2R/2R ($n = 2$), 2R/3RC ($n = 1$) and 3RG/3RC ($n = 1$) genotypes had high *TS* expression. This finding could be due to the influence of additional *cis* genetic variants involved in the control of *TS* transcriptional activity. For instance, a novel polymorphism that may play an important modulatory role in *TS* regulation has been recently discovered by searching the public Expressed Sequence Tag database [25]. This is represented by a deletion of 6 base pairs starting at position 1494 of the 3′UTR, which has been shown to be associated with mRNA instability and decreased intratumoural *TS* mRNA levels [26]. A recently published paper [27] challenges, however, the observation by Mandola and colleagues [26] showing that while the association between the tandem repeat polymorphism in the 5′ UTR of the *TS* gene and mRNA expression is confirmed, no relationship is seen between the polymorphism in the 3′ UTR and expression of the same gene. The latter evidences support the complexity of events that regulate TS activity and the necessity of a multifactorial approach to predict TS expression.

In our study, correlations between experimental data and baseline clinical and pathological characteristics of patients were not statistically significant. In addition, *TS* mRNA expression or *TS* gene polymorphisms were not correlated with survival. Our findings are in keeping with those obtained in larger case series showing no correlation between survival and *TS* levels [7,28,29] or tandem repeat polymorphism [30] in the adjuvant [7,28,30] or advanced disease setting [7,29]. However, other studies have shown a significant relationship between *TS* mRNA levels [7,9,24] or tandem repeat polymorphism [13,17,31] or G > C SNP [19] in the 5′ UTR of the *TS* gene and survival.

The prevalence of patients in early disease stage (i.e., Dukes' stage B 45.8%) in our study may be responsible for the low number of disease recurrences and deaths observed. Also, adjuvant 5-FU-based therapy performed in the vast majority (87.5%) of patients may have contributed to prolong survival of patients whose tumours express high *TS* levels while failing to do so in patients whose tumours express low *TS* levels as reported in some studies [32,33]. These factors, along with the relatively low number of patients, may have contributed to the observed lack of outcome differences in our study, even upon long-term follow-up (94 months).

In conclusion, the polymorphisms investigated in this study are factors that may help in the prediction of TS activity at the individual level and, if confirmed through analysis of larger case series, may be useful predictors of the clinical response to 5-FU.

Conflict of interest statement

There are no conflicts of interest (including financial and personal relationships with other people or organisation) for any author that could inappropriately have influenced this work.

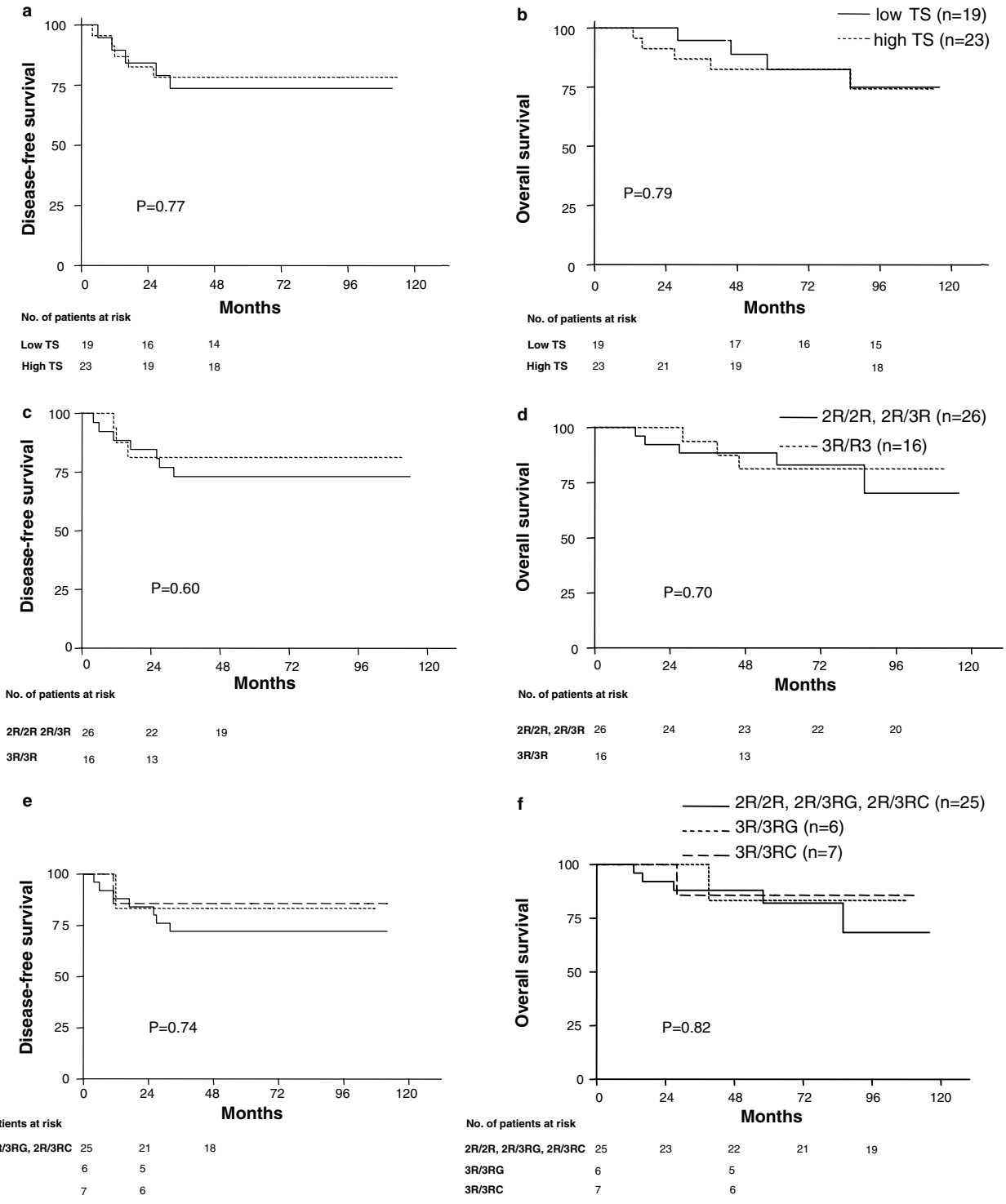

Fig. 2. (a) Disease-free and (b) overall survival of patients who received radical surgery and adjuvant post-operative chemotherapy according to *TS* gene expression. (c, d) tandem repeat length polymorphism and (e, f) SNP. *P* was calculated by log-rank test.

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References

1. Santi DV, McHenry CS, Sommer H. Mechanism of interaction of thymidylate synthase with 5-fluorodeoxyuridylate. *Biochemistry* 1974, **13**, 471–481.
2. Rose MG, Farrell MP, Schmitz JC. Thymidylate synthase: a critical target for cancer chemotherapy. *Clin Colorectal Cancer* 2002, **1**, 220–229.
3. Shibata J, Aiba K, Shibata H, *et al*. Detection and quantitation of thymidylate synthase mRNA in human colon adenocarcinoma cell line resistant to 5-fluorouracil by competitive PCR. *Anticancer Res* 1998, **18**, 1457–1463.
4. Kaneda S, Takeishi K, Ayusawa D, *et al*. Role in translation of a triple tandemly repeated sequence in the 5′-untranslated region of human thymidylate synthase mRNA. *Nucleic Acid Res* 1987, **15**, 1259–1270.
5. Bertino JR, Banerjee D. Is the measurement of thymidylate synthase to determine suitability for treatment with 5-fluoropyrimidines ready for prime time? *Clin Cancer Res* 2003, **9**, 1235–1239.
6. Di Paolo A, Chu E. The role of thymidylate synthase as a molecular biomarker. *Clin Cancer Res* 2004, **10**, 411–412.
7. Popat S, Matakidou A, Houlston RS. Thymidylate synthase expression and prognosis in colorectal cancer: a systematic review and meta-analysis. *J Clin Oncol* 2004, **22**, 529–536.
8. Johnston PG, Lenz HJ, Leichman CG, *et al*. Thymidylate synthase gene and protein expression correlate and are associated with response to 5-fluorouracil in human colorectal and gastric tumors. *Cancer Res* 1995, **55**, 1407–1412.
9. Leichman CG, Lenz HJ, Leichman L, *et al*. Quantitation of intratumoral thymidylate synthase expression predicts for disseminated colorectal cancer response and resistance to protracted-infusion fluorouracil and weekly leucovorin. *J Clin Oncol* 1997, **15**, 3223–3229.
10. Horie N, Aiba H, Oguro K, *et al*. 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* 1995, **20**, 191–197.
11. Kawakami K, Salonga D, Park JM, *et al*. Different lengths of a polymorphic repeat sequence in the thymidylate synthase gene affect translational efficiency but not its gene expression. *Clin Cancer Res* 2001, **7**, 4096–4101.
12. Mandola MV, Stoehlmacher J, Muller-Weeks S, *et al*. 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* 2003, **63**, 2898–2904.
13. Pullarkat ST, Stoehlmacher J, Ghaderi V, *et al*. Thymidylate synthase gene polymorphism determines response and toxicity of 5-FU chemotherapy. *Pharmacogenomics J* 2001, **1**, 65–70.
14. Kawakami K, Omura K, Kanehira E, *et al*. Polymorphic tandem repeats in the thymidylate synthase gene is associated with its protein expression in human gastrointestinal cancers. *Anticancer Res* 1999, **19**, 3249–3252.
15. Marsh S, McKay JA, Cassidy J, *et al*. Polymorphism in the thymidylate synthase promoter enhancer region in colorectal cancer. *Int J Oncol* 2001, **19**, 383–386.
16. Villafranca E, Okruzhnov Y, Dominguez MA, *et al*. 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* 2001, **19**, 1779–1786.
17. Iacopetta B, Grieu F, Joseph D, *et al*. 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* 2001, **85**, 827–830.
18. Kawakami K, Watanabe G. Identification and functional analysis of single nucleotide polymorphism in the tandem repeat sequence of thymidylate synthase gene. *Cancer Res* 2003, **63**, 6004–6007.
19. Marcuello E, Altés A, Del Rio E, *et al*. Single nucleotide polymorphism in the 5′ tandem repeat sequences of thymidylate synthase gene predicts for response to fluorouracil-based chemotherapy in advanced colorectal cancer patients. *Int J Cancer* 2004, **112**, 733–737.
20. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* 1987, **162**, 156–159.
21. Mini E, Biondi C, Morganti M, *et al*. Marked variation of thymidylate synthase and folylpolyglutamate synthetase gene expression in human colorectal tumors. *Oncol Res* 1999, **11**, 437–445.
22. Odin E, Wettergren Y, Nilsson S, *et al*. Altered gene expression of folate enzymes in adjacent mucosa is associated with outcome of colorectal cancer patients. *Clin Cancer Res* 2003, **9**, 6012–6019.
23. Ichikawa W, Uetake H, Shirota Y, *et al*. Combination of dihydropyrimidine dehydrogenase and thymidylate synthase gene expressions in primary tumors as predictive parameters for the efficacy of fluoropyrimidine-based chemotherapy for metastatic colorectal cancer. *Clin Cancer Res* 2003, **9**, 786–791.
24. Lenz HJ, Hayashi K, Salonga D, *et al*. p53 point mutations and thymidylate synthase messenger RNA levels in disseminated colorectal cancer: an analysis of response and survival. *Clin Cancer Res* 1998, **4**, 1243–1250.
25. Ulrich CM, Bigler J, Velicer CM, *et al*. Searching expressed sequence tag databases: discovery and confirmation of a common polymorphism in the thymidylate synthase gene. *Cancer Epidemiol Biomarkers Prev* 2000, **9**, 1381–1385.
26. Mandola MV, Stoehlmacher J, Zhang W, *et al*. A 6 bp polymorphism in the thymidylate synthase gene causes message instability and is associated with decreased intratumoral TS mRNA levels. *Pharmacogenetics* 2004, **14**, 319–327.
27. Merkelbach-Bruse S, Hans V, Mathiak M, *et al*. Associations between polymorphisms in the thymidylate synthase gene, the expression of thymidylate synthase mRNA and the microsatellite instability phenotype of colorectal cancer. *Oncol Rep* 2004, **11**, 839–843.
28. Allegra CJ, Parr AL, Wold LE, *et al*. Investigation of the prognostic and predictive value of thymidylate synthase, p53, and Ki-67 in patients with locally advanced colon cancer. *J Clin Oncol* 2002, **20**, 1735–1743.
29. Johnston PG, Benson 3rd AB, Catalano P, *et al*. Thymidylate synthase protein expression in primary colorectal cancer: lack of correlation with outcome and response to fluorouracil in metastatic disease sites. *J Clin Oncol* 2003, **21**, 815–819.
30. Tsuji T, Hidaka S, Sawai T, *et al*. Polymorphism in the thymidylate synthase promoter enhancer region is not an efficacious marker for tumor sensitivity to 5-fluorouracil-based oral adjuvant chemotherapy in colorectal cancer. *Clin Cancer Res* 2003, **9**, 3700–3704.
31. Etienne MC, Chazal M, Laurent-Puig P, *et al*. Prognostic value of tumoral thymidylate synthase and p53 in metastatic colorectal cancer patients receiving fluorouracil-based chemotherapy: phenotypic and genotypic analyses. *J Clin Oncol* 2002, **20**, 2832–2843.
32. Johnston PG, Fisher ER, Rockette HE, *et al*. The role of thymidylate synthase expression in prognosis and outcome of adjuvant chemotherapy in patients with rectal cancer. *J Clin Oncol* 1994, **12**, 2640–2647.
33. Edler D, Glimelius B, Hallstrom M, *et al*. Thymidylate synthase expression in colorectal cancer: a prognostic and predictive marker of benefit from adjuvant fluorouracil-based chemotherapy. *J Clin Oncol* 2002, **20**, 1721–1728.