Influence of Thymidylate Synthase Expression on Survival in Patients with Colorectal Cancer

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Abstract

**Background:** Thymidylate synthase (TS) plays a critical role in nucleotide metabolism and is an important target for 5-fluorouracil (5-FU), the standard chemotherapeutic drug for treatment of colorectal cancer (CRC). **Aims and Methods:** The present study aimed to evaluate TS variable number tandem repeat sequences (VNTR) polymorphism by polymerase chain reaction and TS protein expression by immunohistochemistry and its association with clinicopathological parameters in untreated CRC patients (n = 100). Further, the prognostic and predictive role of TS has been evaluated. **Results:** For TS VNTR polymorphism, the observed frequencies of 2R/2R, 2R/3R, and 3R/3R genotypes were 22%, 51%, and 27%, respectively. When immunohistochemical localization was studied, cytoplasmic staining for TS was observed in 70% of patients. A significant inverse correlation was noted between TS protein expression and tumor, node, metastasis staging (P = 0.027), Dukes’ staging (P = 0.039), and lymph node status (P = 0.012) of CRC patients. However, there was no significant correlation between TS VNTR polymorphism and TS protein expression. On survival analysis, a significantly shorter overall survival (OS) was seen in CRC patients with negative protein expression (P = 0.031). Moreover, the subgroup of CRC patients treated only with surgery also showed a trend of poor OS in patients with negative TS protein expression (P = 0.058). However, neither TS polymorphism nor its protein expression was able to predict relapse-free survival. **Conclusion:** Negative TS protein expression may be related to unfavorable clinical outcome in CRC patients. However, further studies in a larger set of patients are necessary to better assess TS as a prognostic and predictive marker for 5-FU response in CRC patients.

**Keywords:** 5-fluorouracil, colorectal cancer, protein expression, thymidylate synthase, variable number tandem repeat polymorphism

**INTRODUCTION**

Although continued attempts have been made to improve the clinical outcome of colorectal cancer (CRC) patients, it remains a major health burden with nearly 1.36 million new cases and approximately 694,000 cases of disease-specific mortality worldwide according to GLOBOCAN 2012 estimates. Surgery with adjuvant chemotherapy is considered the standard treatment for CRC patients with high-risk stage II and advanced disease. 5-fluorouracil (5-FU), the main chemotherapy drug, has been used for single or combination therapy to treat CRC patients in both adjuvant and advanced settings. During the course of disease, approximately 50% of CRC patients develop local recurrence or distant metastasis, and the efficacy of 5-FU differs greatly among these individuals. Therefore, the identification of potential molecular markers involved in the 5-FU activity mechanism is necessary to predict 5-FU efficacy and prognosis in CRC patients.

Since the introduction of 5-FU by Heidelberger et al. in 1957, it has remained the basis of therapeutic regimens used in the treatment of many human malignancies including CRC. Action of 5-FU is primarily mediated through the inhibition of thymidylate synthetase (TS). TS is a key enzyme in folate metabolism catalyzing the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) and provides the sole de novo source of thymidine, which is necessary for DNA synthesis and repair. When 5-FU enters the cell, it gets converted to the

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active metabolite 5-fluoro-2-dUMP (5-FdUMP) and forms a stable ternary complex with TS. Due to TS inhibition, DNA synthesis shuts off rapidly triggering apoptosis and other cell death processes. Therefore, TS represents a key target for 5-FU and other fluoropyrimidine-based therapies and it plays a key role in cancer therapy and probably in the prevention of cancer.

TS gene is polymorphic. In the 5′-terminal regulatory region of TS gene promoter (5′UTR), a functional polymorphism of the tandemly repeated sequences has been reported by Horie et al. This promoter region contains either triple (TS 3R) or double (TS 2R) repeats of a 28-bp sequence. Three major genotypes exist for TS: (1) homozygous for two tandem repeats (2R/2R); (2) homozygous for three tandem repeats (3R/3R); and (3) heterozygous for both alleles (2R/3R). This polymorphic region of TS gene may alter its gene expression level. TS gene polymorphisms may result in modulation of activity level of enzyme and affect the DNA methylation and synthesis and in turn affect cancer susceptibility. Several studies have reported that TS genes with 3R allele have higher expression activity than those with 2R allele in vitro and in vivo. Moreover, the tandem repeat sequences of TS promoter region appear to function as an enhancer of transcription and translation. Thus, TS protein expression has been associated with the length of tandem repeats. Several studies showed that high TS protein expression was associated with decreased 5-FU response and unfavorable outcomes in CRC. Several reports have suggested that patients having low TS levels showed improved clinical outcome compared to those having high TS levels in prostate cancer, nonsmall cell lung cancer (NSCLC), and CRC. Hence, the evaluation of the TS genotype and protein expression may be useful to select 5-FU therapy for only those patients who have a higher possibility of response. The present study was conducted to explore the potential significance of TS 28 bp variable number tandem repeat sequence (VNTR) polymorphism and TS protein expression in primary CRC patients and to correlate results with established clinicopathological parameters. Further, the prognostic and predictive value of TS VNTR polymorphism and its protein expression have also been evaluated.

**Materials and Methods**

A total of 100 treatment-naive patients with histologically confirmed CRC seen at The Gujarat Cancer and Research Institute, Ahmedabad, India, between 2007 and 2013 were enrolled in this study. Before primary tumor tissue collection, written consent was obtained from the patients who underwent surgery at the department of surgical oncology. The detailed clinical history (age, gender, anatomic site, family history of cancer, habit, histopathological findings, treatment given, appearance of recurrence/metastases, and disease outcome) was noted from the case files maintained at the medical record department of the institute. Pathologic staging was performed according to Modified Dukes classification and TNM classification with the World Health Organization grading System. Primary treatment offered to all patients was surgery or surgery followed by adjuvant chemotherapy and/or radiotherapy. The main chemotherapeutic treatment included 5-FU and leucovorin, oral capecitabine, or in combination with oxaliplatin. The study was approved by the Institutional Review and Ethical committees.

**Sample collection**

To detect TS VNTR polymorphism, surgically removed colorectal specimens were collected on ice directly from the operation theater and transported to the research laboratory. The viable tumor tissue was selected by a pathologist and divided into two portions. One tumor portion was submitted for routine histopathological evaluation and another tumor portion was snap frozen in liquid nitrogen and preserved at −80°C until DNA extraction. Isolation of DNA from the snap-frozen tumor tissue was performed only after receiving the confirmed positive histopathology report for malignancy. For immunohistochemical localization, paraffin-embedded tumor tissue blocks were collected from the pathology department of the institute.

**Thymidylate synthase variable number tandem repeat polymorphism by polymerase chain reaction**

DNA was extracted from the frozen tumor tissues obtained immediately after the surgery. Approximately, 30–50 mg of tumor tissue was homogenized properly and washed with phosphate-buffered saline, centrifuged, and pellet was dissolved in Tris-NaCl-EDTA buffer (pH 8.0), subsequently followed by proteinase K (100 ng/ml) digestion and overnight incubation at 37°C. DNA was then extracted by phenol–chloroform extraction method. The quantification of extracted DNA samples was performed by agarose gel electrophoresis using Lambda Hind III digest. Furthermore, the purity of the DNA samples was checked spectrophotometrically at 260 and 280 nm. For TS VNTR polymorphism study, polymerase chain reaction (PCR) analysis was performed in a total volume of 50 µl using PCR core kit (Qiagen, USA). Primers used for TS amplification were 5′-GTG GCT CCT GCG TTT CCC CC-3′ (forward) and 5′-GCT CCG AGC CGC CCA CAG GCA TGG CGC GG-3′ (reverse). 0.1 µg of genomic DNA was added per reaction. PCR was performed in a Mastercycler gradient (Eppendorf, Germany) using the following conditions: initial denaturation at 94°C for 3 min followed by 35 cycles of amplification (denaturation at 95°C for 1 min, annealing at 60°C for 30 s, and extension at 72°C for 1 min) and final extension at 72°C for 7 min. The amplified products were electrophoresed on 4% agarose gel. Products at 220 bp (homozygous of double repeat variants 2R/2R), 250 bp (homozygous of triple repeat variants 3R/3R), or 220 and 250 bp (heterozygous of double and triple repeat variants 2R/3R) depending on the TS genotype were obtained.

**Thymidylate synthase protein expression by immunohistochemistry**

Immunohistochemistry (IHC) was performed to detect the protein expression in primary tumors of CRC patients. Briefly, formalin-fixed paraffin-embedded tumor tissue blocks were cut into 4 µm thick sections using Leica microtome and
were mounted on 3-aminopropyltriethoxysilane-coated glass slides. The immunohistochemical staining was carried out using primary mouse monoclonal TS antibody (1:50 dilution, clone 106, Santa Cruz, USA) and mouse and rabbit specific HRP/DAB (Avidin-Biotin Complex) detection IHC kit from Abcam, as per manufacturer’s protocol recommendations. Antigenicity was retrieved by heating the tissue sections in 10 mM trisodium citrate buffer (pH-6.0) solution for 20 min in a pressure cooker before application of the primary antibody. All sections were scored independently by two independent pathologists in a blinded manner. The percentage of positive cells and the staining intensities was separately assessed in primary tumor tissues \( N = 100 \). Modified histoscore (H-score) method was used to combine the percentage of TS expressing cell staining and staining intensity. More specifically, the staining intensity was assessed on a four-point scale from negative (0), weak (1), moderate (2), and strong intensity (3). The extent of the staining was expressed as percentage of positive cells (0%–100%) by 10% intervals. The TS histoscore was calculated by multiplying the intensity level by percentage of positive cells, resulting in a value between 0 and 300. Based on histoscore levels, patients were divided into two groups: patients having negative TS expression (H-score <30) and those having positive TS expression (H-score ≥30). This was done on the basis that the CRC patients with ≤10% TS-positive cells with staining intensity: 0, 1, or 2 or those with weak staining intensity (1) with 0%–20% of positive cells making final histoscore maximum 20 out of 300 were considered to have negative expression for TS, while the rest were considered as having TS-positive expression.

**Statistical analysis**

The data were analyzed using the Statistical Package for the Social Sciences (SPSS) software version 17 (SPSS Inc., USA). Two-tailed Chi-square \( (\chi^2) \) test was used to assess the associations of TS polymorphism and protein expression with clinicopathological parameters; and intercorrelation between TS polymorphism and protein expression. Relapse-free survival (RFS) and overall survival (OS) were calculated using Kaplan–Meier estimates, and the survival curves were compared using Log-rank test. \( P \leq 0.05 \) was considered statistically significant.

**RESULTS**

The patient and tumor characteristics are shown in Table 1. The patients were followed up for a minimum period of 3 years or until death within that period. Complete follow-up details were obtained in 75% \( (75/100) \) CRC patients and were included for OS analysis. Among these, 9% \( (7/75) \) of patients were not included for the RFS analysis as they died due to persistent disease. Therefore, 68/75 CRC patients were included for RFS analysis.

**Distribution of thymidylate synthase variable number tandem repeat polymorphism**

Three types of genotypes were detected for TS VNTR polymorphism: 2R/2R homozygous at 220 bp, 3R/3R homozygous at 250 bp, and 2R/3R heterozygous at

| Characteristics | n (%) |
|-----------------|-------|
| **Age (range: 23–86 years) median: 51 years** |       |
| <51             | 49 (49) |
| ≥51             | 51 (51) |
| **Gender**      |       |
| Female          | 40 (40) |
| Male            | 60 (60) |
| **Habit**       |       |
| No              | 53 (53) |
| Yes             | 47 (47) |
| **Diet**        |       |
| Vegetarian      | 65 (65) |
| Mixed           | 35 (35) |
| **Anatomic site** |     |
| Colon           | 43 (43) |
| Rectum          | 57 (57) |
| **Tumor size**  |       |
| T1 + T2         | 32 (32) |
| T3 + T4         | 68 (68) |
| **TNM stage**   |       |
| I               | 23 (23) |
| II              | 40 (40) |
| III             | 33 (33) |
| IV              | 4 (4)  |
| **Dukes stage** |       |
| B               | 63 (63) |
| C               | 33 (33) |
| D               | 4 (4)  |
| **Histology**   |       |
| Adenocarcinoma  | 70 (70) |
| Mucinous + signet ring cell adenocarcinoma | 30 (30) |
| **Histologic grade** | |
| I               | 19 (19) |
| II              | 70 (70) |
| III             | 11 (11) |
| **Lymph node status** | |
| Absent          | 65 (65) |
| Present         | 35 (35) |
| **Vascular permeation** | |
| Absent          | 82 (82) |
| Present         | 18 (18) |
| **Treatment**   |       |
| Surgery alone   | 16 (16) |
| Surgery + chemotherapy | 47 (47) |
| Surgery + chemotherapy + radiotherapy | 33 (33) |
| Surgery + radiotherapy | 4 (4)  |
| **Preoperative circulating CEA (ng/ml) (n=82)** | |
| <5.0            | 46 (56) |
| ≥5.0            | 36 (44) |
| **Recurrence/metastasis (n=68)** | |
| Absent          | 62 (91) |

Contd...
220/250 bp [Figure 1]. The TS 2R/2R homozygous genotype was observed in 22% (22/100), 3R/3R homozygous genotype in 27% (27/100), and 2R/3R heterozygous genotype in 51% (51/100) of CRC patients.

**Incidence of thymidylate synthase protein expression**

The localization of TS protein expression was observed within the cytoplasm of the epithelial cells of invasive tumors of colon and rectum. TS-positive protein expression was observed in 70% (70/100) of patients whereas 30% (30/100) of patients showed TS-negative protein expression. Representative pattern of TS protein expression in CRC patients is shown in Figure 2.

**Correlation of thymidylate synthase with clinicopathological parameters**

No significant correlation was observed between TS VNTR polymorphism and any of the clinicopathological parameters. On the other hand, when TS protein expression was correlated with clinicopathological parameters, a significantly higher TS expression was observed in early-stage patients as compared to advanced stage patients ($\chi^2 = 4.905, r = -0.221, P = 0.027$) and in Dukes B patients as compared to Dukes C and D patients ($\chi^2 = 5.616, r = -0.207, P = 0.039$) [Figure 3]. Moreover, TS protein expression showed a significant inverse correlation with lymph node status of CRC patients ($\chi^2 = 6.332, r = -0.252, P = 0.012$) [Figure 3].

**Intercorrelation between thymidylate synthase variable number tandem repeat polymorphism and TS protein expression**

Two-tailed $\chi^2$-test revealed that no significant correlation between TS VNTR polymorphism and TS protein expression ($\chi^2 = 2.987, r = +0.026, P = 0.797$).

**Prognostic evaluation of clinicopathological parameters**

Univariate survival analysis showed that none of the clinicopathological parameters emerged as useful prognostic predictors for RFS in CRC patients ($n = 68$). However, for predicting OS, vascular permeation (Log rank = 8.664, df = 1, $P = 0.003$) and Dukes stage (Log rank = 19.087, df = 2, $P < 0.001$) were significant prognostic factors in studied patients ($n = 75$) [Table 2].

**Prognostic evaluation of thymidylate synthase variable number tandem repeat polymorphism and TS protein expression**

Univariate survival analysis indicated that TS VNTR polymorphism did not predict RFS (Log rank = 2.864, df = 2, $P = 0.239; n = 68$) and OS (Log rank = 1.251, df = 2, $P = 0.535$; $n = 75$). Likewise, TS protein expression did not significantly predict RFS (Log rank = 0.580, df = 1, $P = 0.446$), whereas it emerged as the significant prognosticator for OS (Log rank = 4.670, df = 1, $P = 0.031$) [Figure 4].

**Prognostic evaluation of thymidylate synthase in the subgroups of colorectal cancer patients treated with surgery alone and with surgery followed by 5-fluorouracil-based adjuvant therapy**

In the subgroup of CRC patients treated with surgery alone ($n = 12$), the Kaplan-Meier univariate survival analysis demonstrated a borderline significant shorter OS in patients having negative TS protein expression as compared to those with positive TS expression (Log rank = 3.582, df = 1, $P = 0.058$) [Figure 5]. However, TS VNTR polymorphism had no prognostic value in this subgroup of patients. On the other hand, in the subgroup of patients treated with surgery followed by 5-FU-based therapy ($n = 61$), both TS VNTR polymorphism and protein expression had no prognostic significance.

**Discussion**

TS is a major enzyme for pyrimidine nucleotide synthesis and DNA damage repair. It might play a vital role in the regulation of the malignant potential of cancer.$^{[10]}$ 5-FU, the standard treatment for CRC, mainly acts by inhibiting the activity of TS enzyme. Data regarding TS expression as prognostic and predictive markers have been controversial. In the current study, the role of TS VNTR polymorphism and protein expression in CRC patients was investigated.

The present study demonstrated the predominance of 2R/3R heterozygous genotype (51%). Consistent with these results, Kristensen *et al.* found preponderance of 2R/3R genotype in 61% of CRC patients.$^{[10]}$ Contradictorily, the 3R/3R genotype was found in 50% of ALL patients.$^{[25]}$ Hu *et al.* also observed the prevalence of 3R/3R genotype to be 60% in patients with...
NSCLC. The variations in genotype frequencies might be due to ethnic and environmental differences.

In this study, cytoplasmic TS protein expression was observed by IHC in 70% of CRC patients. Similarly, Westra et al. demonstrated high TS expression in 86% of primary tumor samples in stage III colon cancer patients. Edler et al. also reported high TS expression in 76% of CRC patients compared to 24% of patients with low TS expression. Moreover, another study in NSCLC showed that TS-positive protein expression was found in 57.4% of patients.

No significant association of TS VNTR polymorphism and clinicopathological parameters was observed in this study. Similar findings were reported by Hu et al. in NSCLC and Takehara et al. in lung cancer. Sulzyc-Bielicka et al. also showed no significant differences between the frequencies of 5'-TSER genotypes and age, sex, Astler-Coller stage, grade, or introduced chemotherapy in CRC patients while a significant association was noted between 5'-TSER polymorphism and tumor location (P = 0.0042). Moreover, Fariña-Sarasqueta et al. demonstrated that TS VNTR polymorphism was significantly associated with T stage (P = 0.05) and age (P = 0.001) in stage III colon cancer patients. This association might identify a role of the TS gene polymorphisms in colon cancer risk.

On the other hand, positive protein expression of TS showed a significant inverse correlation with Dukes stage, TNM stage, and lymph node status in the present study. Such an inverse correlation suggests a probability of getting a better 5-FU response in CRC patients with positive TS expression if treated right at the earlier stage. One study by Johnston et al. reported

| Parameters                  | n  | Patients died n (%) | Log rank=8.664, df=1, P<0.003 |
|-----------------------------|----|---------------------|-------------------------------|
| Vascular permeation         |    |                     |                               |
| Absent                      | 71 | 7 (10)              |                               |
| Present                     | 4  | 2 (50)              |                               |

| Dukes stage                 |    |                     |                               |
| B                           | 49 | 4 (8)               |                               |
| C                           | 24 | 3 (12)              |                               |
| D                           | 2  | 2 (100)             |                               |

Log rank=19.087, df=2, P<0.001

**Table 2: Univariate survival analysis of clinicopathological parameters for overall survival in colorectal cancer patients (n=75)**

**Figure 2:** Representative photomicrographs of thymidylate synthase immunostaining in tumor tissue by IHC (×40)

**Figure 3:** Association of thymidylate synthase protein expression with (a) tumor, node, and metastasis stage (b) Dukes stage and (c) lymph node status
a significant association of high TS protein levels with more advanced Dukes stage in rectal cancer ($P < 0.01$). However, Zhao et al. demonstrated that TS protein expression was not related to gender, tumor status, nodal status, pathological stage, histology types, or treatment model in NSCLC patients, but the frequency of TS-positive tumors in patients <60 years was significantly higher than that in patients >60 years. Another study by Westra et al. indicated that low TS protein levels were correlated with only mucinous histology ($P = 0.04$) in adjuvantly treated stage III colon cancer patients.

Further, the present study demonstrated no significant correlation between TS VNTR polymorphism and protein expression. In concordance with this study, several studies described no association between TS polymorphism and IHC staining of TS in patients with CRC and colon cancer. It might be because the final protein expression is the product of many regulatory changes such as transcription, posttranscriptional regulation, translation, and posttranslational regulation. Hence, the polymorphisms at the gene level resulting in the expression of specific mRNA and the expression of associated proteins are not always linearly proportional because modifications or aberrant changes may occur during these regulatory processes. Contradictory to present results, Boyle et al. showed that TS VNTR genotype was associated with TS protein expression ($P = 0.03$) in lung cancer. Several studies in CRC patients also demonstrated that as compared to 2R allele, the 3R sequence has greater transcriptional and translational efficiency. Moreover, TS protein expression was significantly related to TS 5'-UTR genotype ($P < 0.05$) in esophageal squamous cell carcinoma.

The present study revealed that at the end of 36 months follow-up, TS VNTR polymorphism failed to emerge as one of the prognostic factors for RFS and OS in CRC patients. Concordantly, Stoehlmacher et al. could not find any significant difference in the clinical outcome according to the TS 5'-UTR genotypes in CRC. Similar results were noted by Yim et al. in gastric cancer. In contrast, TS 5’UTR 3R genotype was correlated with better survival in CRC and higher response to 5-FU-based treatment in metastatic CRC. However, Salgado et al. demonstrated lower response rates in patients having TS 3R genotypes than those with TS 2R/2R homozygous genotypes in CRC. Moreover, the present study failed to show any prognostic significance of TS VNTR polymorphism in both subgroups of patients treated with surgery alone or treated with surgery followed by 5-FU-based therapy.

On the other hand, negative TS protein expression was found to be a significant prognosticator for poor OS but not for RFS. In concordance with this study, Zhao et al. found that TS-negative protein expression significantly correlated to lower OS in NSCLC patients. Another study by Zheng et al. also showed that high TS protein levels significantly correlated to better survival in resected NSCLC patients. Moreover, Cho et al. reported that a high TS expression may be related to a better outcome in gastric cancer patients. Contradictorily, Popat et al. indicated that CRC patients having high levels of TS had adverse OS compared to those with low TS levels. However, Findlay et al. failed to show a relationship of TS immunostaining with survival and response to chemotherapy in CRC patients.

Moreover, in the present study, although not significant, positive TS protein expression was associated with longer OS in the subgroup of CRC patients treated with surgery alone. Whereas, TS expression was having no prognostic value in the group of patients treated with surgery followed by adjuvant chemotherapy. Similarly, Karlberg et al. in CRC demonstrated that the subgroup of patients treated with surgery alone showed a prognostic significance while TS expression was having no prognostic value in the entire study group or in the group treated with surgery and adjuvant chemotherapy.
In addition, TS expression in the primary tumor only had a significant prognostic value among patients who were treated with surgery alone and not among the entire patient population including who received adjuvant FU-based chemotherapy in CRC.[43] Several other adjuvant studies in CRC reported that TS protein expression had no prognostic significance in the group of patients treated with adjuvant chemotherapy which is in accordance with the present results.[27,46] Allegra et al. showed similar finding in locally advanced colon cancer patients.[47] The primary mechanism of 5-FU resistance has been described as an increase in TS expression.[31] However, positive TS expression showed better outcome in the present study following another theory that, as compared to negative TS expression, positive TS expression might be related to more availability of TS for binding with 5-FU leading to TS inhibition and subsequently better response and survival.

**CONCLUSION**

TS VNTR polymorphism failed to predict clinical significance while negative TS protein expression might be useful to predict poor clinical outcome in CRC patients. However, further studies on a large series of patients are warranted to better understand the role of TS as a prognosticator of survival and predictive marker for 5-FU response in CRC patients.

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**Conflicts of interest**

There are no conflicts of interest.

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