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Potency of Thymidylate Synthase in Predicting Prognosis and Therapeutic Response in Colorectal Cancer Patients

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Abstract:- Thymidylate synthase (TS), a critical enzyme in 5-Flourouracil (5-FU) metabolism, plays a significant role in predicting survival and therapeutic response in colorectal cancer (CRC) patients. Present study evaluated prognostic as well as predictive utility of TS VNTR polymorphism, TS mRNA expression and its protein level in CRC patients. Total 143 CRC patients were enrolled. TS VNTR polymorphism and TS mRNA expression were studied by PCR and semiquantitative RT-PCR, respectively. Immunohistochemistry was carried out to examine TS protein expression. TS polymorphism showed that majority of the patients were carriers of 3R allele (2R/3R+3R/3R; 80%) compared to those with 2R/2R genotype (20%). TS mRNA expression was noted in 80% of patients and 77% showed cytoplasmic TS immunopositivity. With regard to prognosis, a trend of reduced relapse-free survival (RFS) was noted with carriers of 3R allele (2R/3R+3R/3R) in rectal cancer subgroup (P=0.065). Further, high TS mRNA expression was associated with unfavorable RFS in total CRC patients (P=0.038), as well as in the subgroups of early stage (P=0.073), rectal cancer (P=0.059) and single drug 5-FU treated patients (P=0.029). On the other side, low TS protein expression showed poorer RFS (P=0.050) and overall survival (OS) (P=0.071) in colon cancer subgroup. It can be concluded that TS could be beneficial prognostic and predictive marker to identify patients with poor clinical outcome and thus might be useful to choose effective treatment modalities for CRC patients.

Keywords:- *TS, Colorectal Cancer, VNTR polymorphism, mRNA, Protein, 5-FU, Immunohistochemistry, Prognosis*

I. INTRODUCTION

Colorectal cancer (CRC) is the third most lethal and life-threatening cancer with 10% incidence and 9.4% mortality rate worldwide according to Globocan 2020 [1]. Over past half a century, the antineoplastic drug 5-Fluorouracil (5-FU) has constituted the backbone of chemotherapeutic treatment in CRC. 5-FU has capacity to enhance DNA damage, resulting in cell growth arrest and apoptosis. However, drug resistance of cancer cells plays a major role in diminishing the clinical efficacy of 5-FU [2]. Despite extensive research, overcoming drug resistance remains the most challenging task for 5-FU and related chemotherapeutic drugs in CRC. Therefore, identifying molecular markers that can anticipate 5-FU efficacy would be most demanding to facilitate individualized treatment in CRC patients.

The core enzyme in 5-FU metabolism is thymidylate synthase (TS), which plays a critical role in one of the most crucial of the 3 pathways of 5-FU anabolism [3]. It is a rate-limiting enzyme which catalyses the reductive methylation of dUMP to dTMP, for which 5,10-MTHF (CH_2THF) is the methyl donor [4]. Once dTMP is synthesized intracellularly, it gets metabolized to dTTP (the triphosphate form), a necessary precursor for biosynthesis of DNA. This process is necessary to produce thymine nucleotide, which is required for synthesis and repair of DNA. Human TS is regarded as an

anticancer drug target because of its key role in DNA replication [5]. However, TS levels are often found to be altered, which promotes 5-FU resistance [3]. Notably, high TS levels are associated with poor prognosis and resistance to 5-FU in CRC patients [6]. The reason for differences in TS levels might be polymorphisms in the TS gene which regulate TS gene expression. A 28 bp variable number tandem repeat (VNTR) polymorphism consisting of either double repeats (2R) or triple repeats (3R) within the 5' enhancer region of TS gene promoter has been identified [7]. This polymorphic region seems to be functional and may alter expression of TS gene [8]. Moreover, it has been described that the tandem repeat sequences of TS promoter region enhance transcription and translation [9]. Several findings reported that TS gene having triple repeats showed greater expression and activity than those with double repeats both in vitro and in vivo [7, 10-11]. Studies in CRC patients have reported that homozygous 3R/3R genotype may be associated with high TS expression and lower response to 5-FU-based therapy [8, 12]. Thus, it may be postulated that TS VNTR polymorphism alters the expression levels of TS and also its enzymatic activity, which might have an effect on the clinical outcome in CRC patients. Hence, present study intended to evaluate the role of TS VNTR polymorphism, its gene expression and protein levels and their correlation with clinical and pathological variables in CRC patients. Also prognostic alongwith predictive role of TS was examined.

II. MATERIALS & METHODS

➢ *Patients*

Total 143 untreated histologically confirmed patients with CRC registered at 'The Gujarat Cancer & Research Institute' from 2007 to 2014 were included in present study. Institutional Review and Ethics Committee have approved this study. Written consents were obtained for all the patients undergoing surgery at the Department of Surgical Oncology prior to sample collection. The chemotherapeutic treatment was given to 113 out of 143 patients. The chemotherapeutic regimen includes either single agent therapy (5-FU+leucovorin or oral Capecitabine), or combination therapy [5-FU+Oxaliplatin (OX)]. The follow-up period was 36 months and complete details of follow-up were obtained for 114 CRC patients. Hence, overall survival (OS) analysis was performed for 114 patients. Relapse-free survival (RFS) analysis could be performed for 101 out of 114 patients as 13 patients died because of persistent disease and were not comprised for RFS. Further, survival analysis was also studied in the subsets of early stage, advanced stage; as well as colon cancer and rectal cancer. Moreover, the predictive potency of TS on survival was evaluated in accord with adjuvant treatment. Hence, patients were also divided into single drug group (treated with 5-FU alone) and combined drug group (treated with combined 5-FU+OX), irrespective of RT. With regard to adjuvant treatment, RFS was performed in 83 out of 101 patients, while OS was evaluated in 94 out of 114 patients.

➢ *Sample Collection*

For all 143 CRC patients, primary tumor tissue specimen collection was carried out on ice straight from the operation theatre. Then, tumor portion selection from collected specimen was performed by a pathologist and it was divided into 2 portions, snap frozen in liquid nitrogen and conserved at -80°C. One tumor portion was used for DNA extraction and subsequently for TS polymorphism study. The other part was used for total RNA extraction to study TS mRNA expression. To examine TS protein expression by immunohistochemistry, paraffin embedded blocks of tumor tissues of all the patients were retrieved from Department of Histopathology of the institute.

➢ *TS VNTR Polymorphism by PCR*

DNA extraction was carried out from frozen tumor tissues using phenol-chloroform extraction method. The extracted DNA samples were quantified using Lambda Hind III digest by agarose gel electrophoresis. DNA samples were also analysed spectrophotometrically at 260 and 280 nm to check the purity of samples. Polymerase chain reaction (PCR) was carried out to study TS VNTR polymorphism. PCR was performed in a total reaction volume of 50 µl using PCR core kit (Qiagen, USA) and adding 0.1 µg of genomic DNA. Primers used for amplification of TS were as follows: Forward: 5' GTG GCT CCT GCG TTT CCC CC 3' and Reverse: 5' GCT CCG AGC CGG CCA CAG GCA TGG CGC GG 3'. The following conditions were applied in a PCR system: initial denaturation at 94°C for 3 minutes; amplification for 35 cycles (denaturation temperature 95°C for 1 minute, annealing temperature 60°C for 30 seconds, extension temperature 72°C for 1 minute); final extension temperature 72°C for 7 minutes. Then the separation of PCR products was performed on 2.5% ethidium bromide-stained agarose gel.

➢ *TS mRNA Expression by Semi-Quantitative RT-PCR*

Total RNA was isolated from frozen tumor tissues using RNA iso-plus solution (Takara). The quantification of extracted RNA was carried out spectrophotometrically at 260 nm and 280 nm. Amplification of mRNA was carried out using two-step RT-PCR method. First, cDNA synthesis using iScript cDNA synthesis kit (Bio-Rad) and then PCR amplification of cDNA was carried out using Taq PCR Core kit (Qiagen). The primers used were: Forward: 5' TTG GAC AGC CTG GGA TTC TC 3' and Reverse: 5' AGC TGG CGA TGT TGA AAG G 3'. The following conditions were used to perform PCR: initial denaturation at 94°C for 3 minutes; amplification for 35 cycles (denaturation temperature 94°C for 1 minute, annealing temperature 58°C for 45 seconds, extension temperature 72°C for 1 minute); final extension temperature 72°C for 10 minutes. Then PCR product separation was performed on 2% ethidium bromide-staine agarose gel.

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➢ *TS Protein Expression by Immunohistochemistry*

Formalin fixed paraffin embedded blocks of tumor tissues of CRC patients were utilized to study immunohistochemical localization of TS. The primary antibody used was TS mouse monoclonal (Clone TS 106: sc-33679, Santa Cruz) and dilution applied was 1:50. The immunohistochemical staining was performed using Mouse and Rabbit specific HRP/DAB (ABC) Detection IHC kit (Abcam) and the procedure carried out in accord to manufacturer's protocol. To retrieve the antigenicity prior to applying primary antibody, tissue sections were heated in 10mM tri-sodium citrate buffer solution (pH-6.0) in a pressure cooker for 20 minutes. Mounting was performed on stained sections and scoring was conducted under light microscope. The percentage of positive cells and staining intensities were estimated independently for all the sections. Scoring of TS immunostaining was performed using modified histoscore (H-score) method [13].

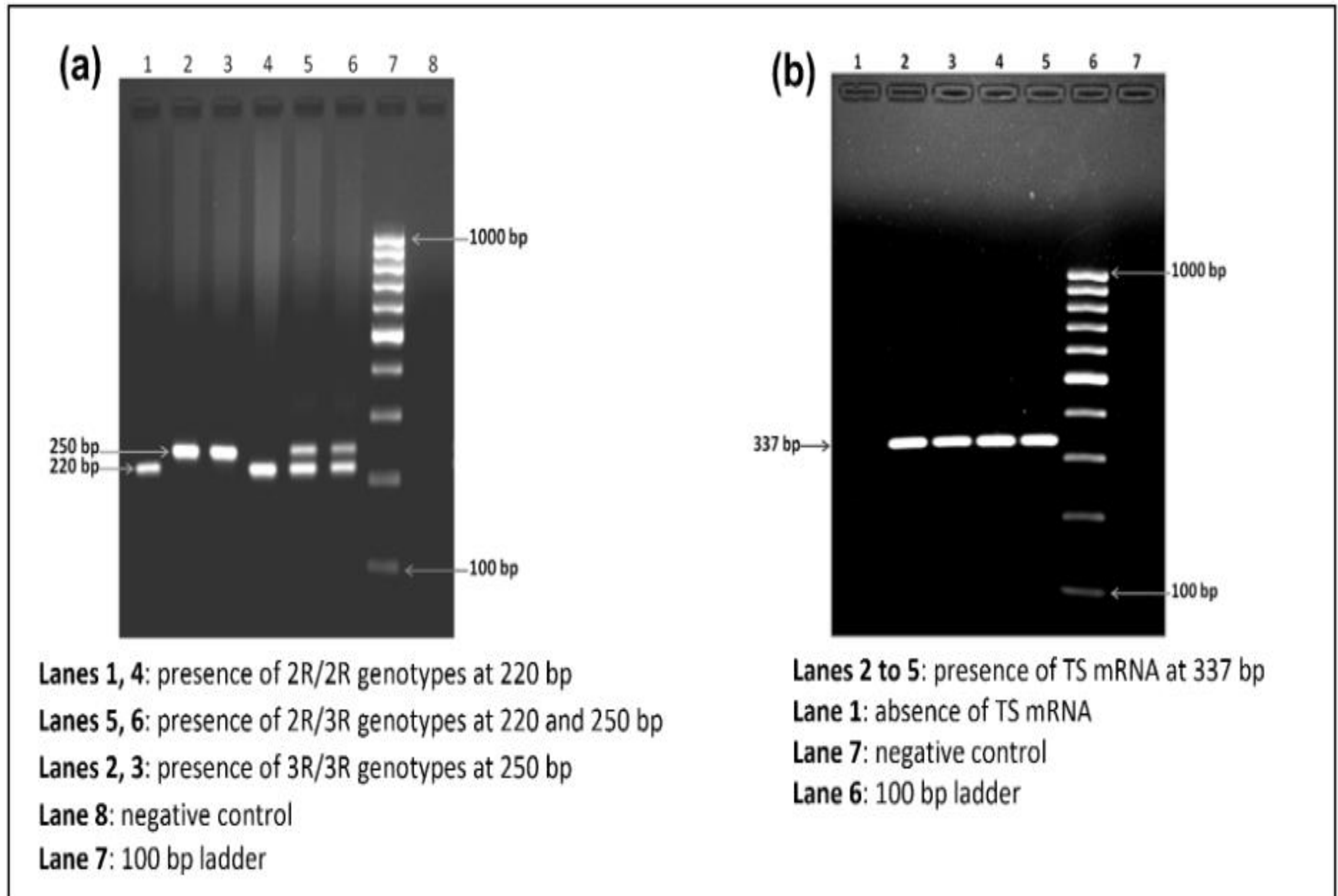

Fig 1 Representative Image of (a) TS VNTR Polymorphism (b) TS mRNA Expression on Agarose Gel in CRC Patients

➢ *Statistical Analysis*

Statistical Package for Social Sciences (SPSS) software was utilized for statistical analysis. Hardy-Weinberg equilibrium (HWE) by a goodness-of-fit Chi-square (χ^2) test was used to study the distribution of genotypes in CRC patients by comparing the observed genotype frequencies to the expected ones. To estimate the correlation of TS VNTR polymorphism, its mRNA and protein expression with clinical and pathological parameters, two-tailed χ^2 test was performed. Spearman's correlation coefficient (r) method was performed to calculate the correlation between two parameters. Kaplan-Meier estimates was used to study RFS and OS analysis and the difference in survival curve was determined by Log rank test. P value ≤0.05 was considered to be significant.

III. RESULTS

➢ *Incidence of TS VNTR Polymorphism, TS mRNA and TS Protein Expression*

TS VNTR polymorphism exhibited three types of genotypes (Fig. 1a). The genotypic frequencies showed 20% (29/143) of patients with 2R/2R homozygous genotype, 50% (72/143) with 2R/3R heterozygous genotype and 30% (42/143) with 3R/3R homozygous genotype. The genotype distribution of this polymorphism in CRC patients followed HWE (χ^2=0.033, P=0.854). The TS mRNA expression was detected in 80% (114/143) of patients. The product size of the amplified TS mRNA molecule was 337 bp (Fig. 1b). The median IDV for TS was 21505.0 counts/mm^2, with a range of 0 to 1114985.0 counts/mm^2 and 21505.0 counts/mm^2 was considered as cut-off point. Based on it, the patients were categorised into low (<21505.0 counts/mm^2) and high (≥21505.0 counts/mm^2) mRNA expression groups. TS immunoreactivity was detected in 77% (110/143) of patients.

The 1+, 2+ and 3+ staining intensities were observed in 22% (32/143), 29% (41/143) and 26% (37/143) of patients, respectively. The median H-score for TS was 60 with a range of 0 to 270, and 60 was considered as cut-off point to divide the patients into the groups of low (<60) and high (≥60) TS protein expression. Fig. 2 shows the representative negative and cytoplasmic staining pattern for TS.

➢ *Association of TS with Clinicopathological Parameters*

TS VNTR polymorphism could not show significant association with any of the clinicopathological parameters. Whereas, TS mRNA expression exhibited significant association with gender. A significantly higher TS mRNA expression was found in female patients versus male patients (P=0.030). On the other side, TS protein expression was found to be significantly associated with tumor location (P=0.012) and nodal status (P=0.032). Moreover, a trend of high TS immunoreactivity was noted in patients with rectal cancer (P=0.058), early stage (P=0.072), and absence of necrosis (P=0.064) in comparison with their respective counterparts. (Table 1)

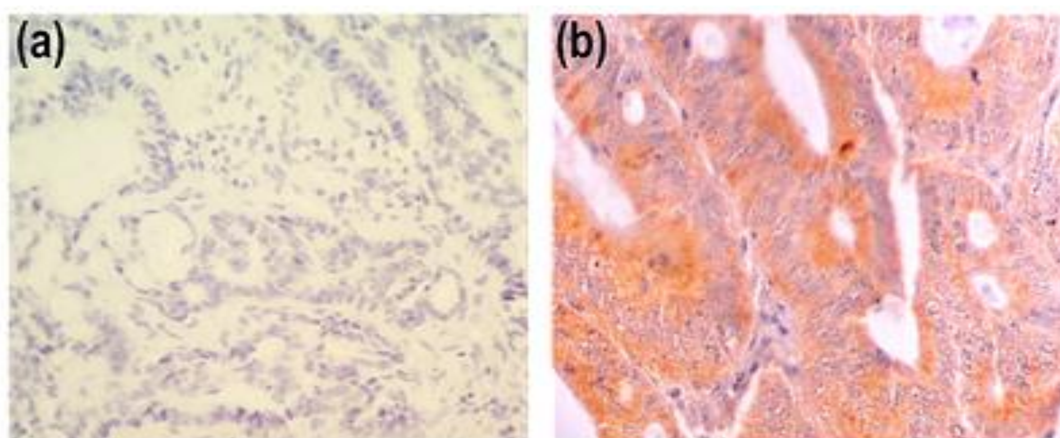

Fig 2 Representative Photomicrographs Showing Staining of TS (40x) (a) Negative Staining (b) Cytoplasmic Staining in Colon Adenocarcinoma

Table 1 Association of TS with Clinicopathological Parameters in CRC Patients (N=143)

Characteristics	N	TS					
		VNTR polymorphism		mRNA expression		Protein expression	
		2R/2R N (%)	2R/3R+3R/3R N (%)	Low N (%)	High N (%)	Low N (%)	High N (%)
Age (years)							
<52	68	14 (21)	54 (79)	29 (43)	39 (57)	32 (47)	36 (53)
≥52	75	15 (20)	60 (80)	41 (55)	34 (45)	35 (47)	40 (53)
		χ^2=0.008, r=+0.007, P=0.931		χ^2=2.062, r=-0.120, P=0.153		χ^2=0.002, r=+0.004, P=0.963	
Gender							
Female	58	11 (19)	47 (81)	22 (38)	36 (62)	25 (43)	33 (57)
Male	85	18 (21)	67 (79)	48 (57)	37 (43)	42 (49)	43 (51)
		χ^2=0.104, r=-0.027, P=0.749		**χ^2=4.742, r=-0.182, P=0.030**		χ^2=0.551, r=-0.062, P=0.461	
Tumor site							
Colon	69	12 (17)	57 (83)	38 (55)	31 (45)	38 (55)	31 (45)
Rectum	74	17 (23)	57 (77)	32 (43)	42 (57)	29 (39)	45 (61)
		χ^2=0.688, r=-0.069, P=0.410		χ^2=1.999, r=+0.118, P=0.160		**χ^2=3.618, r=+0.159, P=0.058**	
Tumor location							
Right side	45	07 (16)	38 (84)	23 (51)	22 (49)	28 (62)	17 (38)
Left side	98	22 (22)	76 (78)	47 (48)	51 (52)	39 (40)	59 (60)
		χ^2=0.906, r=-0.080, P=0.345		χ^2=0.123, r=+0.029, P=0.728		**χ^2=6.229, r=+0.209, P=0.012**	
Tumor size							
T2	36	07 (19)	29 (81)	18 (50)	18 (50)	17 (47)	19 (53)
T3	95	20 (21)	75 (79)	47 (49)	48 (51)	42 (44)	53 (56)
T4	12	02 (17)	10 (83)	05 (42)	07 (58)	08 (67)	04 (33)
		χ^2=0.148, r=+0.002, P=0.983		χ^2=0.281, r=+0.029, P=0.730		χ^2=2.160, r=-0.048, P=0.566	
Nodal status							
Negative	90	18 (20)	72 (80)	46 (51)	44 (49)	36 (40)	54 (60)

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Characteristics	N	TS					
		VNTR polymorphism		mRNA expression		Protein expression	
		2R/2R N (%)	2R/3R+3R/3R N (%)	Low N (%)	High N (%)	Low N (%)	High N (%)
Positive	53	11 (21)	42 (79)	24 (45)	29 (55)	31 (58)	22 (42)
		χ^2=0.012, r=-0.009, P=0.914		χ^2=0.453, r=+0.056, P=0.504		**χ^2=4.580, r=-0.179, P=0.032**	
TNM stage							
Early (I+II)	88	18 (20)	70 (80)	45 (51)	43 (49)	36 (41)	52 (59)
Advanced (III+IV)	55	11 (20)	44 (80)	25 (45)	30 (55)	31 (56)	24 (44)
		χ^2=0.004, r=+0.005, P=0.948		χ^2=0.437, r=+0.055, P=0.512		**χ^2=3.246, r=-0.151, P=0.072**	
Tumor differentiation							
Well	29	05 (17)	24 (83)	14 (48)	15 (52)	13 (45)	16 (55)
Moderate/Poor	114	24 (21)	90 (79)	56 (49)	58 (51)	54 (47)	60 (53)
		χ^2=0.208, r=-0.038, P=0.651		χ^2=0.007, r=-0.007, P=0.936		χ^2=0.060, r=-0.020, P=0.808	
Lymphatic permeation							
Absent	112	21 (19)	91 (81)	56 (50)	56 (50)	51 (45)	61 (55)
Present	31	08 (26)	23 (74)	14 (45)	17 (55)	16 (52)	15 (48)
		χ^2=0.748, r=-0.072, P=0.391		χ^2=0.227, r=+0.040, P=0.636		χ^2=0.360, r=-0.050, P=0.552	
Vascular permeation							
Absent	136	29 (21)	107 (79)	66 (48)	70 (52)	63 (46)	73 (54)
Present	07	00 (00)	07 (100)	04 (57)	03 (43)	04 (57)	03 (43)
		χ^2=0.786, r=+0.114, P=0.375		χ^2=0.003, r=-0.037, P=0.955		χ^2=0.029, r=-0.047, P=0.864	
Lymphocytic stromal response							
Absent	120	24 (20)	96 (80)	61 (51)	59 (49)	54 (45)	66 (55)
Present	23	05 (22)	18 (78)	09 (39)	14 (61)	13 (56)	10 (44)
		χ^2=0.000, r=-0.016, P=1.000		χ^2=1.058, r=+0.086, P=0.307		χ^2=1.029, r=-0.085, P=0.314	

- *Association of TS with Survival*

In relation to TS polymorphism, Kaplan-Meier survival analysis revealed that carriers of 3R allele (2R/3R+3R/3R) exhibited a trend of reduced RFS (27%, 12/45) as compared to patients having 2R/2R genotype (0%, 0/11; P=0.065) in rectal cancer subgroup (Fig. 3a).

With regard to TS mRNA expression, a significant decreased RFS was noted in total CRC patients having high TS mRNA expression (27%, 14/52) as compared to patients having low TS mRNA expression (10%, 5/49; P=0.038) (Fig. 3b). Further, in subgroup of early stage patients, a trend of unfavorable RFS was found in patients with high TS mRNA expression (20%, 6/30) versus patients having low TS expression (5%, 2/37; P=0.073; Fig. 3c). Moreover, in rectal cancer subgroup, patients having high TS mRNA expression (30%, 10/33) exhibited a trend of poor RFS in comparison with those with low TS mRNA expression (9%, 2/23; P=0.059; Fig. 3d).

When TS protein expression was analysed for survival, in colon cancer subgroup, a significant poor RFS was found in patients with low TS protein expression versus those having high TS expression (P=0.050). Moreover, colon cancer patients having low TS protein expression had a trend of adverse OS in comparison with those having high TS expression (P=0.071). (Table 2)

- *Association of TS with 5-FU/Oxaliplatin Based Treatment*

TS VNTR polymorphism and TS protein expression failed to emerge as predictive markers according to treatment given. However, in case of TS mRNA expression, Kaplan-Meier univariate survival analysis revealed that a significant high prevalence of disease relapse was noted in patients treated with single drug having high TS mRNA expression versus patients treated with combined drug having high TS mRNA expression (P=0.036). Importantly, in single drug group, patients with high TS mRNA expression were significantly related with reduced RFS (P=0.029). (Table 3)

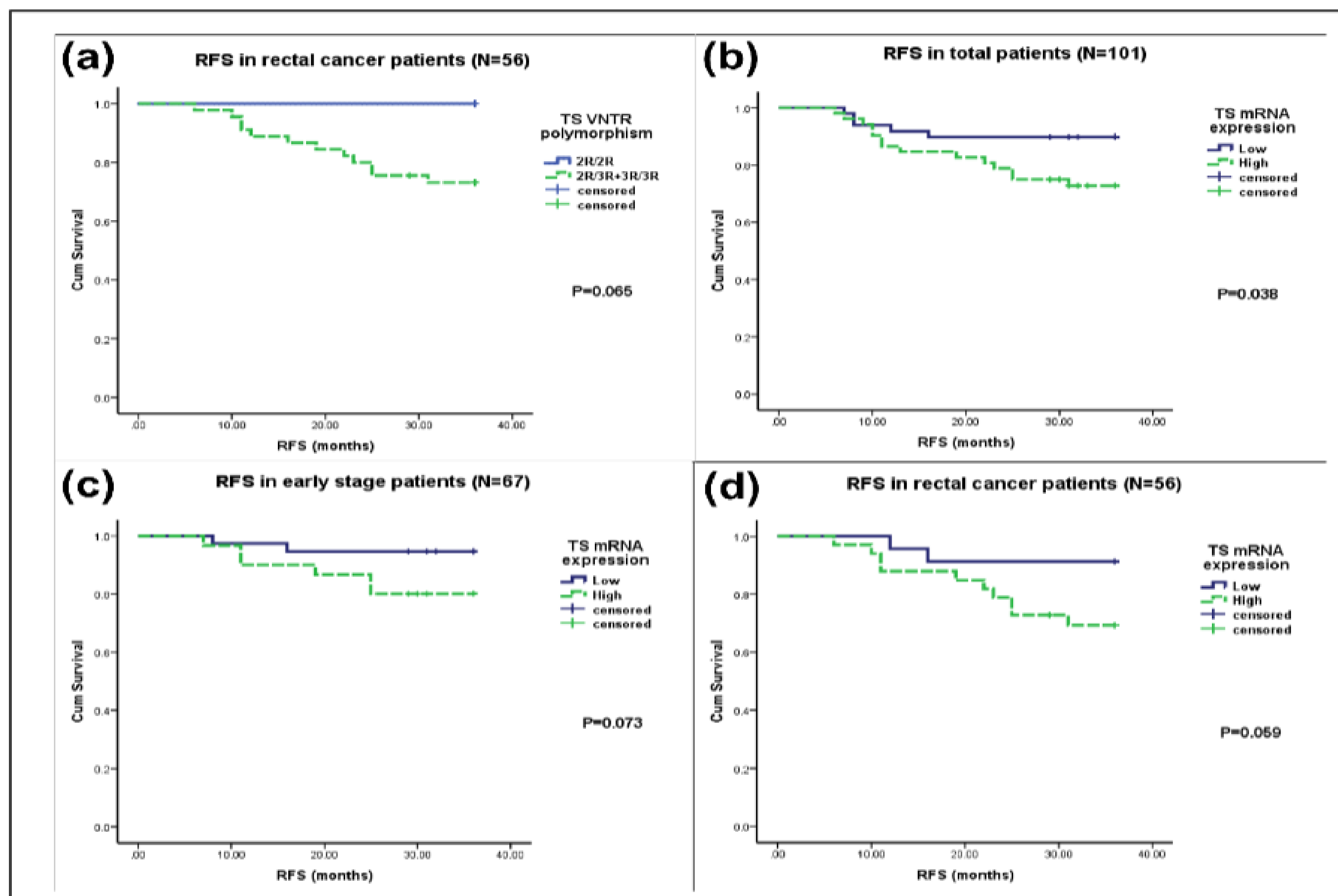

Fig 3 Kaplan-Meier Survival Curves for RFS (a) TS VNTR Polymorphism in Rectal Cancer Patients (b) TS mRNA Expression in Total Patients (c) TS mRNA Expression in Early Stage Patients (d) TS mRNA Expression in Rectal Cancer Patients

Table 2 Univariate Survival Analysis for RFS and OS in Relation to TS Protein Expression in Colon Cancer Patients

TS protein expression	RFS			OS		
	N	No recurrence N (%)	Recurrence N (%)	N	Alive N (%)	Dead N (%)
Colon cancer patients	**45**			**51**		
Low	23	17 (74)	06 (26)	27	18 (67)	09 (33)
High	22	21 (96)	01 (04)	24	21 (88)	03 (12)
		Log rank=3.851, df=1, **P=0.050**			Log rank=3.260, df=1, **P=0.071**	

IV. DISCUSSION

5-FU, the standard therapy for CRC, preliminary works by blocking the functioning of TS enzyme. It is well known that TS, a prime target enzyme for the synthesis of pyrimidine nucleotide and for repairing of DNA damage, plays a crucial role to regulate the carcinogenic potential in cancer [14-15]. Although prognostic together with predictive role of TS VNTR polymorphism, TS mRNA and its protein expressions in CRC have been well documented, its clinical significance in Indian population remains yet to be elucidated. Therefore, present study investigated the clinical utility of TS VNTR polymorphism, TS mRNA expression and its protein expression in patients with CRC.

Current study reported 20% 2R/2R, 50% 2R/3R and 30% 3R/3R genotypes for TS VNTR polymorphism in CRC patients; demonstrating a preponderance of 2R/3R genotype. Concordant with present results, Hitre et al. [16] reported 18% 2R/2R, 48% 2R/3R and 34% 3R/3R and Salgado et al. [17] showed 15% 2R/2R, 52% 2R/3R and 33% 3R/3R frequencies in CRC. Additionally, predominance of 2R/3R genotype with 61% was reported in CRC patients [9]. On the contrary, the high frequency of 3R/3R genotype was noted in patients with CRC in Japanese population [18] and with non-small cell lung cancer (NSCLC) in Chinese population [19]. Similar result was observed in patients with acute lymphoid leukemia (ALL) in Kashmiri (Indian) population [20]. These variations in the frequency of genotypes might be attributed to ethnic differences as well as variability in disease type and environmental factors.

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Table 3 Univariate Survival Analysis for RFS in Relation to TS mRNA Expression in CRC Patients Treated with Adjuvant 5-FU/5-FU+OX Based Therapy (N=83)

TS mRNA expression	N	No recurrence N (%)	Recurrence N (%)	Log rank	df	P	Log rank statistics
Single drug: 5-FU (N=55)							
Low	28	25 (89)	03 (11)	4.768	1	**0.029**	Log rank=4.388 df=1 **P=0.036**
High	27	17 (63)	10 (37)				
Combined drug: 5-FU+OX (N=28)							
Low	12	10 (83)	02 (17)	0.258	1	0.612	
High	16	12 (75)	04 (25)				

Present study could not identify TS VNTR polymorphism as a significant prognostic factor for both RFS and OS as well as predictive factor for 5-FU/ 5-FU+OX based therapy in CRC patients. Similarly, several studies failed to find any significant association of TS genotypes with clinical outcome and response to 5-FU drug in CRC [21] and colon cancer patients [22]. However, lower response rates to 5-FU based therapy was noted in patients having TS 3R/3R genotypes in CRC [17]. Moreover, higher response rates with 2R/2R genotypes as compared to those with 3R allele observed in CRC patients treated with 5-FU based therapy [8]. It has also been described that 3R/3R homozygous patients are least likely to respond to 5-FU therapy than 2R/2R homozygous or 2R/3R heterozygous patients [23]. Hence, one of the reasons for poor clinical outcome in CRC patients with 3R allele might be resistance to 5-FU based therapy. Since, the prime mechanism of resistance to 5-FU has been demonstrated as an increase in the expression of TS [22], it might be expected that 3R allele carriers may be associated with high TS expression which leads to resistance to 5-FU and poor survival. Result of present study support this theory as it was observed that although not significant, carriers of 3R allele were associated with a poor RFS as compared to 2R/2R genotype in the subset of rectal cancer patients. One study in CRC also indicated a decline in median survival with rising numbers of TS repeats [24].

Several studies reported that the tandem repeat sequences of TS 5’UTR polymorphism may be linked with the enhancement of transcription and translation [8,11]. Hence, present study evaluated TS expression at transcript level and examined its prognostic and predictive role in CRC. It was observed that high TS mRNA expression was related with poor RFS in total patients as well as in the subgroup of patients with early stage. In accordance with present study, high TS mRNA was significantly related with reduced survival as compared to low TS mRNA levels in CRC patients [25]. Further, one study also showed poor clinical outcome in patients with greater TS mRNA expression in CRC [26]. Contradictorily, CRC patients with high TS mRNA expression were noticed to have a significantly lower risk for relapse (P=0.030) and death (P=0.015) [27]. On the other hand, numerous studies have reported that TS mRNA expression was not associated with survival in patients with CRC [28], esophaegeal adenocarcinoma [29], NSCLC [30] and hepatocellular carcinoma [31]. Additionally, the current study also observed association of high TS mRNA with poor RFS in patients with rectal cancer suggesting the likely role of TS mRNA as a prognostic marker to predict recurrence in a subset of rectal cancer patients, which is an aggressive tumor phenotype. Similarly, high TS mRNA predicts distant recurrence in patients with rectal cancer [32]. Further, when patients were stratified according to adjuvant treatment, present study showed that high TS mRNA level was also associated with poor RFS in the subgroup of patients treated with adjuvant 5-FU (single drug) based therapy. In concordance with this, association of high TS mRNA with decreased response rates was noted in CRC patients treated with 5-FU [33]. Contrary, patients with low tumoral TS mRNA levels developed tumor recurrence more frequently than those with high tumoral TS levels in CRC patients treated with adjuvant 5-FU [34]. Moreover, high TS mRNA was associated with greater response to 5-FU and irinotecan-based combination therapy in CRC [35]. Present study also showed better clinical outcome in patients having high TS mRNA expression treated with 5-FU+OX based combination therapy when compared to those having high TS mRNA treated with single drug 5-FU, suggesting the beneficial role of combination therapy in patients having high TS mRNA expression.

Measurement of TS protein in CRC has been found to be beneficial due to the key role of this enzyme in the clinical response to 5-FU-based therapy [36]. Immunohistochemistry detected cytoplasmic TS immunostaining in 77% of CRC patients, which is consistent with our previous study [37]. Similarly, cytoplasmic TS staining observed in 90% of stage III colon cancer patients [38]. Moreover, 76% of CRC patients exhibited higher TS protein expression versus 24% of patients having low TS expression [36]. On the other side, in NSCLC, cytoplasmic TS immunostaining was detected in 57.4% of patients [15]. TS is usually examined to be a cytoplasmic enzyme. However, previous observations also revealed nuclear expression of TS in human cells [39-40]. Cytoplasmic alongwith nuclear staining of TS was found in 61% of patients with CRC [41].

In present study, TS protein expression failed to show association with survival in CRC patients. Concordantly, several studies could not find an association between TS protein expression and survival in CRC patients [42-44]. Moreover, TS expression was not associated with prognosis in patients with gastric cancer [45-46]. On the other side, Johnston et al. [39] were the first to suggest that TS protein expression might have a prognostic importance in primary

CRC as they found that patients having high TS expression had a significant poor 5-year DFS and OS as compared to those having low TS expression in rectal cancer. Further, CRC patients with high TS levels had unfavorable survival when compared to those with low levels of TS [47-48]. Similarly, tumors with TS negative expression had better clinical outcome than tumors with TS positive expression in gastric and gastro-oesophageal adenocarcinoma [49]. Moreover, association of high TS expression with poor survival and decreased response to 5-FU based therapy was noted in gastric cancer [50]. Contradictorily, several studies revealed that positive/high TS protein expression associated with favourable clinical outcome in patients with NSCLC [15, 30] and gastric cancer [51]. The present study shows concordance with these findings, but only in the subset of colon cancer patients, probably suggesting that colon is presumed to be biologically less aggressive tumor site compared to rectum. Therefore, colon cancer patients with high TS protein levels might likely to be more sensitive to adjuvant therapies and hence shows better survival; contradicting the theory of association between high TS levels and 5-FU resistance.

Further, when TS polymorphism, TS mRNA and its protein expressions were intercorrelated, present study demonstrated no significant association between them. In accordance with this, several reports failed to find significant correlation between TS polymorphism and TS immunostaining in patients with CRC [52] and colon cancer [53]. Moreover, TS VNTR polymorphism was not associated with TS mRNA expression in CRC, whereas patients with 3R/3R genotype showed a significantly higher TS protein expression versus those with the 2R/3R genotype [11]. Further, it has been reported that 3R allele has greater transcriptional capacity than 2R allele in CRC patients [8]. Yu et al. [54] described that 3R/3R genotype detected significantly higher IRS (immunoreactive score) of TS compared to those with 2R/3R and 2R/2R genotypes in human colon cancer. While, TS activity was significantly higher in 2R/3R tumors as compared with 2R/2R or 3R/3R in metastatic CRC [55]. Additionally, in NSCLC, no significant association of TS mRNA expression observed with its protein expression, while patients with the 3R/3R genotype had a significantly higher TS protein expression level compared to those with the 2R/3R genotype ($P<0.05$) [30] suggesting that TS genotype affects TS protein expression through a post-transcriptional mechanism. In present study, no intercorrelation between TS polymorphism and expression of mRNA and protein levels might be due to variable doses and schedules of 5-FU-based chemotherapy and different tumor stages of the patients [56]. Moreover, the association of high TS mRNA and low TS protein expression with poor prognosis in present study might be described by one hypothesis that there are many regulatory levels of gene expression, such as post-transcriptional regulation, translation and post-translational regulation. Final protein expression is not entirely controlled by these regulatory levels and hence the expression of specific mRNA and the expression of associated proteins is not always linearly proportional [26].

V. CONCLUSION

TS VNTR polymorphism demonstrated association of 3R allele with poor RFS in patients with rectal cancer. Hence, it could be useful factor to predict unfavorable prognosis in rectal cancer patients. Additionally, high TS mRNA expression correlated with reduced RFS in CRC patients. Moreover, patients with high TS mRNA had additional benefit to combined drug therapy than single drug therapy in predicting RFS. Therefore, TS mRNA expression emerged as a significant prognostic and predictive marker for RFS in CRC patients. Further, Low TS protein expression may be poor prognosticator for RFS and OS in colon cancer patients. Thus, TS could be useful prognostic as well as predictive marker to identify patients with poor clinical outcome and thus might be beneficial to choose effective treatment modalities for CRC patients.

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