RESEARCH ARTICLE

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Evaluation of TS and ENOSF1 Variants as a Biomarker in Response to Neoadjuvant Chemotherapy based on 5FU in Gastric Cancer Patients

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Abstract

Objective: Neoadjuvant chemotherapy with 5-fluorouracil (5FU) is one of the most effective treatment options for gastric cancer patients. However, treatment response varies significantly between patients based on their genetic profile. The purpose of this study was to determine the association between thymidylate synthase (TS) and enolase superfamily member 1 (ENOSF1) polymorphisms, treatment response, and overall survival in patients with gastric cancer. **Methods:** The TS and ENOSF1 variants were analyzed in formalin-fixed paraffin-embedded (FFPE) tissue from 100 gastric cancer patients receiving neoadjuvant 5FU-based chemotherapy. Polymerase chain reaction (PCR) amplification and restriction fragment length polymorphism (RFLP) were used to determine TS polymorphisms' genotypes, and the Tetra Arms PCR method was used to identify ENOSF1 polymorphisms. Patients were followed for up to five years, and the association between variants, treatment response, and overall survival (OS) was examined. **Results:** There was a significant association between the TS 5' UTR polymorphism and response to treatment in patients with gastric cancer who received neoadjuvant 5FU therapy (P=0.032). Patients with the 2R3R genotype responded better to treatment, whereas those with the 3R3R genotype did not respond to treatment. Patients with the 2R2R and 3R3R genotypes had the longest and shortest median survival times, respectively, and the observed differences were significant (p=0.003). There was a statistically significant relationship between rs2612091 and chemotherapy response (P=0.017). Patients with genotype AG did not respond to treatment. **Conclusion:** This study established that the TS 5' UTR and ENOSF1 rs2612091 polymorphisms could be used to predict treatment response and overall survival in patients with gastric cancer who received neoadjuvant chemotherapy based on 5FU.

Keywords: Gastric cancer- chemotherapy- personalized medicine- TS- ENOSF1

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Introduction

According to GLOBOCAN 2020, gastric cancer (GC) is the fifth most common type of cancer and the fourth leading cause of cancer death worldwide (Sung et al., 2021). Most patients with gastric cancer are diagnosed in the advanced stages of the disease, with a poor prognosis and a 5-year survival rate of between 5% and 15% (Jianwei et al., 2013). Chemotherapy is the standard treatment for advanced gastric cancer (Jianwei et al., 2013). Chemotherapy resistance can diminish the effect of chemotherapy. Whether intrinsic or acquired, chemotherapeutic resistance is a complex and multifactorial phenomenon (Shi and Gao, 2016). 5FU is the most frequently used chemotherapy agent in advanced gastric cancer, either alone or in combination with other drugs (Shitara et al., 2010; Matsusaka and Lenz, 2015).

The thymidylate synthase gene is located on chromosome 18p, contains seven exons, and encodes the TS enzyme (Meulendijks et al., 2016; Meulendijks et al., 2017; Gallegos-Arreola et al., 2018). The TS gene is required for DNA replication and repair, and fluoropyrimidines inhibit the TS enzyme (Shitara et al., 2010; Meulendijks et al., 2016; Meulendijks et al., 2017). Overexpression of TS is associated with poorer treatment outcomes in patients treated with fluoropyrimidines for GC and colorectal cancer (Meulendijks et al., 2017). Genetic polymorphisms in the genes encoding metabolizing enzymes and drug targets have been shown to play a significant role in the variability of response to treatment in GC patients (Matsusaka and Lenz, 2015; Meulendijks et al., 2016; Shi and Gao, 2016; Meulendijks et al., 2017).

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The TS promoter contains a 28-bp sequence in the 5'-untranslated enhanced region (5' UTR) and is presented as a double (2R) or triple tandem repeat (3R) allele. The 3R and 2R alleles are polymorphic and are associated with TS expression, 5FU response, 5FU toxicity. The 2R/2R genotype has significantly lower levels of TS mRNA than either the 3R/3R or the 2R/3R genotypes. The homozygous 2R/2R genotype is associated with increased susceptibility to 5FU toxicity and drug sensitivity (Lima et al., 2013; Meulendijks et al., 2016; Ab Mutalib et al., 2017; De Mattia et al., 2020).

Functional G>C single-nucleotide polymorphism (rs2853542) is located in the second repeat of the 3R allele. The combination of this variant's 3R and G alleles has been associated with increased transcription efficiency, whereas the 3R and C allele combination have the same transcription efficiency as the 2R allele (Meulendijks et al., 2016; Gallegos-Arreola et al., 2018; Ntavatzikos et al., 2019; Hamzic et al., 2020). Another polymorphism that has been studied for its effect on chemotherapy response is the presence of a 6-bp insertion/deletion (ins/del) sequence (TTAAAG) in the 3' UTR (1494del6) of TS (rs151264360) (Jianwei et al., 2013; Lima et al., 2013; Hernando-Cubero et al., 2017; Ntavatzikos et al., 2019). This variant has been shown to increase the TS expression, resulting in decreased chemosensitivity to 5FU (Jianwei et al., 2013; Lima et al., 2013; Hernando-Cubero et al., 2017; Ntavatzikos et al., 2019; De Mattia et al., 2020).

The ENOSF1 gene is located adjacent to TS and is hypothesized to code both a protein and an antisense transcript, thereby regulating TS's mRNA expression either (Ab Mutalib et al., 2017; Meulendijks et al., 2017; Hamzic et al., 2020). The rs2612091 variant in the ENOSF1 gene's intronic regions is associated with ENOSF1 mRNA expression and capecitabine toxicity (Rosmarin et al., 2015; Meulendijks et al., 2017). Several studies indicate that ENOSF1 may be more sensitive to the cytotoxic effects of fluoropyrimidines than TS (Matsusaka and Lenz, 2015; Meulendijks et al., 2017). Recently, there has been a heightened interest in the association between rs2612091 and treatment response or survival following fluoropyrimidine treatment, but a dearth of knowledge in this area remains (Lecomte et al., 2004; Matsusaka and Lenz, 2015; Rosmarin et al., 2015; Meulendijks et al., 2017).

The purpose of this study was to determine the association between TS and ENOSF1 gene variants and drug response in patients with gastric cancer.

Materials and Methods

Patients

Between 2012 and 2018, 100 formalin-fixed paraffin-embedded (FFPE) tissues with gastric cancer were recruited from the Cancer Institute of Imam Khomeini Hospital in Tehran, Iran. Prior to gastrectomy, all patients received neoadjuvant chemotherapy with 5FU. The response to 5FU treatment was evaluated using the most recent protocol for examining specimens from patients with stomach carcinoma (Shi et al., 2017). Tehran Azad University of Science and Research's Medical Ethics Committee approved this study (IR.IAU.SRB.REC.1397.110).

DNA extraction

DNA was extracted from normal tissue from each patient's FFPE samples. All samples were stained with H&E and examined by a pathologist before being mechanically microdissected. The dissected specimens were deparaffinized and genomic DNA was extracted according to the manufacturer's instructions using the QIAmp DNA FFPE Tissue Kit (Qiagen, Germany).

TS Genotyping

TPCR was used to amplify the variable number of tandem repeats (VNTR) variants of the 5′ UTR flank of TS (rs45445694) using previously described primers. The PCR reactions were performed in a total volume of 22 μL containing 50 ng of genomic DNA, 10 pmol of each primer, 5% DMSO, and 2X Hot start PCR Master Mix (Amplicon, Danmark). The PCR was initiated with 15 minutes of denaturation at 95°C, followed by 37 cycles of 95°C for one minute, 64°C for 45 seconds, and 72°C for 45 seconds, followed by a 10-minute final extension at 72°C. PCR products were visualized on 3% agarose gel. The 210 bp and 238 bp bands correspond to the 2R and 3R alleles, respectively.

TRFLP was used to determine the single nucleotide polymorphism (SNP) G>C in the second repeat of the 3R allele (rs2853542, abbreviated 3RG or 3RC). To detect the SNP of a G to a C substitution, the VNTR PCR products were digested with HaeIII for 20 hours at 37 ºC (Thermo Fisher, USA), the digested fragments were separated on an 8% acrylamide gel, and the SNP genotype was determined as previously described in(Lecomte et al., 2004; Shitara et al., 2010; Arévalo et al., 2014).

APCR RFLP with DraI restriction enzyme was used to amplify a 6-bp ins/del variant at the 3′ UTR region of TS (rs151264360), as previously reported in (Gosens et al., 2008; Shitara et al., 2010; Arévalo et al., 2014).

ENOSF1 Genotyping

The candidate variants ENOSF1, rs2612091, and rs2741171 were genotyped using Tetra Arms PCR. SNP sequences were obtained from the db SNP database at ncbi.nlm.nih.gov, followed by primer design at primer1.soton.ac.uk/primer1.html and validation with Beacon Designer and BLAST. Table 1 shows the primer sequences.

The PCR mixture reaction contained 2X Hot start PCR Master Mix Blue (Amplicon, Denmark), 5 pmol of each primer, and between 50 and 100 ng of genomic DNA. Table 2 details the PCR cycle. Amplification products were analyzed on 3% agarose gel, and genotypes were determined.

Statistical analysis

SPSS version 26.0 software package (SPSS, Chicago, IL, USA) was used for statistical analysis. The association between the TS and ENOSF1 genotypes, clinicopathological characteristics, and neoadjuvant chemotherapy response was determined using the χ^2 test and Monte-Carlo method with a 2-sided significance level

of 0.05. The Monte-Carlo method was utilized for TS SNP and TS 3' UTR analysis. OS (overall survival) was defined as the time between the start of first-line chemotherapy and the date of death from any cause. Kaplan-Meier estimates and log-rank tests were employed in univariate analysis of overall survival. The Cox-regression method was used for OS multivariate analysis. The results were considered to be statistically significant when bilateral p-values were < 0.05.

Results

Patients' characteristics and treatment

This study enrolled 100 patients with advanced gastric cancer treated with neoadjuvant 5FU chemotherapy. Out of the 100 patients, 70 (70%) were male, while 30 (30%) were female. Patients ranged in age from 27 to 81 years, with a mean of 58.10 ± 11.43. All patients were followed for a maximum of five years; 24 developed progressive disease, and 47 died. Histopathological responses of the patients were classified as follows: 57 patients (13 with a complete response, 15 with a partial response, and 29 with a moderate response) were classified as responders, while 43 patients were classified as nonresponders (poor response).

TS and ENOSF1 genotyping results

Table 3 summarizes the genotype distributions of variants. Of the 100 samples analyzed, 25 patients lacked the TS SNP variant.

Correlations of TS, ENOSF1 genotypes with histopathological features and responses

This study examined the correlation between the TS and ENOSF1 genotypes in 100 patients with gastric adenocarcinoma who received neoadjuvant chemotherapy and studied their response to treatment. Table 3 summarizes the results of correlations between polymorphisms and pathologic responses. The test showed a significant association between the TS 5' UTR polymorphism and response to neoadjuvant chemotherapy (p=0.032). The 2R3R genotype had a significantly better

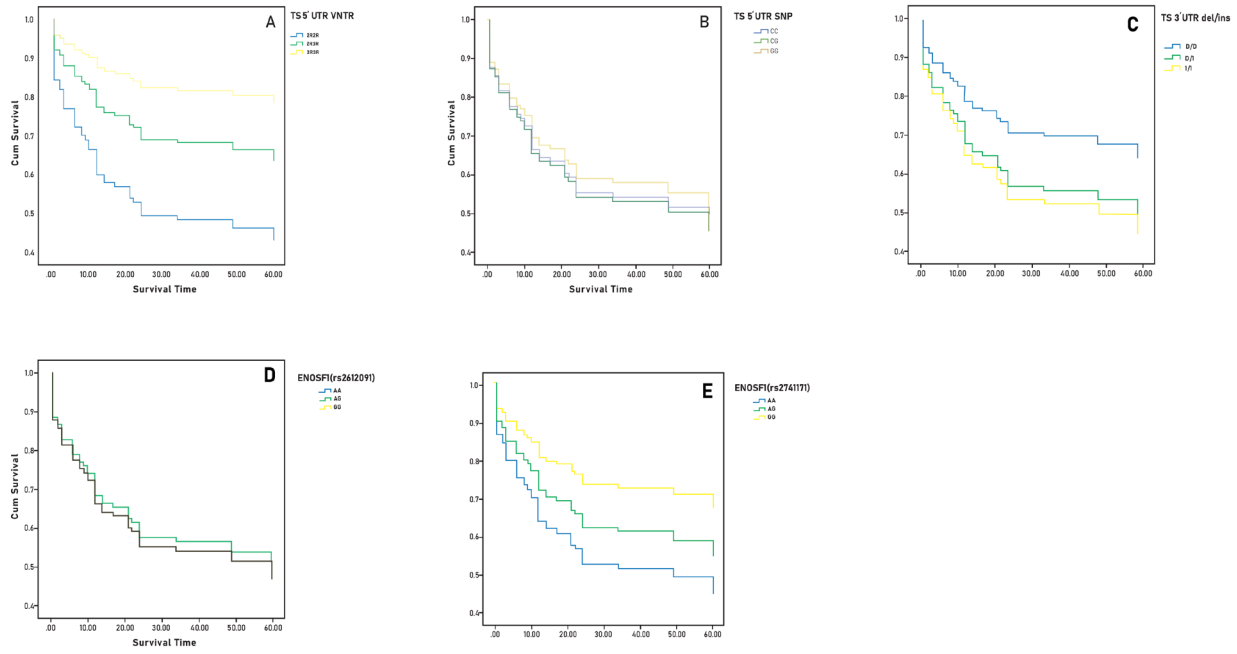

Figure 1. Kaplan-Meier Curve for OS According to A) TS 5' UTR, B) TS 5' UTR SNP, C) TS 3' UTR, D) ENOSF1 (rs2612091) and E) ENOSF1 (rs2741171) Groups. Comparisons were made using log-rank tests

Table 1. Primer Sequences for ENOSF1 Variants

Gene	Primer sequences	Product size (bp)	Tm (ºC)
ENOSF1			
rs2612091	Forward outer primer 322 TGTGCATGATTCAGAATGTGACAAAATGG 360	390	70
	Reverse outer primer 721 AAAAGAGACTCTTCACAGGGAGGTCAGCC 693		70
	Forward inner primer (A allele) 476 CTGGACATCCAGTGGCTCCTCAATCA 501	247	71
	Reverse inner primer (G allele) 528 GGTACAGTCTTTAGGAGGAGCCGTGCAC 501	197	70
ENOSF1			
rs2741171	Forward outer primer 243 CAATTTCCTGCCACAGCCAAAATTTCTC 270	454	70
	Reverse outer primer 696 TGACTCTCAGAGTGCACAAGCAGCACTT 669		70
	Forward inner primer (A allele) 476 GGGTTTCACCATGTTGATCAGGTGGA 501	222	70
	Reverse inner primer (G allele) 530 GCGGATCACCTGAGGTCAGGAGTATGATAC 501	288	70

Table 2. The PCR Cycle for ENOSF1 Variants

	Temperature	Time	Number of cycle
Tetra Arms PCR (rs2612091)			
Initial denaturation	95ºC	10 min	1
Denaturation	95ºC	1 min	40
Annealing	62ºC	1min	
Extension	72ºC	1 min	
Final extension	72ºC	10 min	1
Tetra Arms PCR (rs2741171)			
Initial denaturation	95ºC	10 min	1
Denaturation	95ºC	1 min	40
Annealing	60ºC	1 min	
Extension	72ºC	1 min	
Final extension	72ºC	10 min	1

†, Kruskal-Wallis ANOVA; two sided P.value<0.05

response to chemotherapy than other genotypes, while the 3R3R genotype had a significantly worse response. Similarly, there was a significant association between the ENOSF1 (rs2612091) polymorphism and response to treatment (p=0.017), with patients carrying the AG genotype having a poorer response to treatment. There was no significant association between the TS 5' UTR SNP, the TS 3' UTR SNP, or the ENOSF1 (rs2741171) polymorphism and pathologic responses (all p>0.05).

Correlations between the TS and ENOSF1 genotypes and survival

Table 4 and Figure 1 show the median survival times of patients based on their polymorphism genotypes and their hazard ratios (HR). As depicted in the table and figure, there was a significant difference in the median survival time following neoadjuvant chemotherapy with 5FU in patients with the 2R2R and 3R3R genotypes (p=0.003). Additionally, in the Cox regression model, the risk of death was lower in patients with 3R3R and 2R3R genotypes than patients with 2R2R genotypes (reference group), with p=0.24 and p=0.47, respectively. There was no statistically significant relationship between the TS 5' UTR SNP and the TS 3' UTR del/ins genotypes and median survival time (p=0.92, 0.84). The risk of death ratio was not statistically significant between the genotypes of both polymorphisms (p>0.05).

There was no association between genotypes and median survival time or HR in different genotypes for ENOSF1 variants in the Cox regression model (p>0.05).

Discussion

In recent years, pharmacogenetic analysis has been used to investigate the association between drug pathway-associated germline polymorphisms and chemotherapy

Table 3. The PCR Cycle for ENOSF1 Variants

Polymorphism	Genotypes	No. of patients	Treatment		p value
		(n=100)	Responders	non-responders	
TS 5' UTR	2R2R	25 (25%)	14 (56%)	11 (44%)	
	2R3R	54 (54%)	36 (66.7%)	18 (33.3%)	
	3R3R	21 (21%)	7 (33.3%)	14 (66.7%)	
TS 5' UTR SNP	3RC3RC, 2R3RC	50 (50%)	29 (58%)	21 (42%)	0.29
	3RG3RG, 2R3RG	23 (23%)	9 (39.1%)	14 (60.9%)	
	3RC3RG	2 (2%)	0 (0%)	2 (100%)	
TS 3' UTR	del6/del6	3 (3%)	3 (100%)	0 (0%)	0.26
	del6/ins6	81 (81%)	47 (58%)	34 (42%)	
	ins6/ins6	16 (16%)	7 (43.8%)	9 (56.3%)	
ENOSF1 (rs2612091)	AA	29 (29%)	15 (51.7%)	14 (48.3%)	
	AG	48 (48%)	23 (47.9%)	25 (52.1%)	
	GG	23 (23%)	19 (82.6%)	4 (17.4%)	
ENOSF1 (rs2741171)	AA	16 (16%)	11 (68.8%)	5 (31.3%)	0.065
	AG	36 (36%)	15 (41.7%)	21 (58.3%)	
	GG	48 (48%)	31 (64.6%)	17 (35.4%)	

* The $\chi 2$ test was used for the correlation study; ** The Monte-Carlo test used for TS 5' UTR SNP and TS 3' UTR polymorphisms; * A p-value < 0.05 was considered as significant

Table 4. Median Survival Time and HR for Patients with Various Genotypes.

Polymorphism	Genotypes	Survival time (months)		Hazard ratio (HR)	
		Mean (95% CI)	p value	HR (95% CI)	p value
TS 5' UTR VNTR	2R2R	23.44 (13.84-33.04)	0.003	1 (reference group)	
	2R3R	39.36 (32.62-46.1)		0.47 (0.25-0.87)	0.02
	3R3R	47.17 (37.3-57.04)		0.24 (0.09-0.64)	0.005
TS 5' UTR SNP	3RC3RG	32 (4.28-59.72)	0.92	1 (reference group)	
	2R3RG, 3RG3RG	47.84 (38.52-57.15)		0.51 (0.07-4.18)	0.53
	2R3RC, 3RC3RC	38.88 (31.82-45.94)		0.82 (0.11-6.1)	0.85
TS 3' UTR	del6/del6	31.67 (23.13-40.2)	0.84	1 (reference group)	
	del6/ins6	37.22 (31.57-42.86)		1.64 (0.22-11.9)	0.63
	Ins6/ins6	33.22 (19.93-45.51)		1.82 (0.23-14.62)	0.57
ENOSF1 (rs2612091)	AA	30.71 (21.37-40.4)	0.17	1 (reference group)	
	AG	36.39 (28.97-43.8)		0.73 (0.39-1.37)	0.32
	GG	45.8 (36.17-55.44)		0.47 (0.2-1.08)	0.08
ENOSF1 (rs2741171)	AA	36.84 (24.62-49.07)	0.97	1 (reference group)	
	AG	37.41 (28.93-45.88)		0.92 (0.39-2.16)	0.85
	GG	36.79 (29.31-44.28)		1 (0.45-2.23)	0.99

(Lecomte et al., 2004; Meulendijks et al., 2016; Shi and Gao, 2016; Hernando-Cubero et al., 2017). This study examined genetic polymorphisms in the TS and ENOSF1 genes in patients with advanced gastric cancer to determine a possible association with response to neoadjuvant chemotherapy with 5FU and overall survival.

The role of various polymorphisms in the TS gene in chemotherapy response remains unknown. Our study found that patients with the 2R3R genotype responded better to neoadjuvant chemotherapy than those with the 3R3R genotype (p=0.032). Overall survival was also significantly better in patients with the 3R3R and 2R3R genotypes than those with the 2R2R genotype (p=0.003). Consistent with the current study, a better response to a 5FU regimen has been reported in patients with gastrointestinal cancer who have the 2R/3R genotype than those who have the 3R/3R genotype. Additionally, another study demonstrated that patients with rectal cancer who have the 2R/2R or 2R3R genotypes might benefit from preoperative chemoradiotherapy (Yang et al., 2017).

Studies have demonstrated a significant inverse relationship between the number of 28-bp tandem repeats in the TS promoter region and the severity of toxicity (Pullarkat et al., 2001; Lecomte et al., 2004; Schwab et al., 2008). Dotor et al. reported that patients with colorectal cancer (CRC) who had the 3R/3R genotype had a better survival rate, consistent with our findings (Dotor et al., 2006). However, multiple studies have demonstrated an association between the 3R/3R genotype and a poor response to adjuvant chemotherapy for colon and rectal cancers (Iacopetta et al., 2001; Marsh et al., 2001; Pullarkat et al., 2001; Villafranca et al., 2001). There has been a significant increase in the incidence of toxicity to fluoropyrimidines in patients with colorectal cancer who are homozygous for the 2R variant of the TS gene [7, 16, 19, 28-29]. Patients with gastroesophageal cancer who had a 2R/2R genotype and received perioperative chemotherapy had statistically superior overall survival than those with a 2R/3R or 3R/3R genotype (Smyth et al., 2017).

The G>C (rs2853542) variant was associated with a decreased ability to respond to chemotherapeutic agents with each copy of the G allele (Castro-Rojas et al., 2017). Eugenio et al. demonstrate that the double polymorphism in the TS tandem repeat sequence is a more accurate predictor of 5FU-based chemotherapy outcome than the VNTR alone (Marcuello et al., 2004). Mandola et al. hypothesize that the effect of a 3R genotype on TS transcriptional activation may be related to the presence or absence of USF (upstream stimulatory factor) binding sites and the G>C single nucleotide polymorphism in the 3R allele's second repeat (Mandola et al., 2003). In a study conducted by Lima et al., 3RG homozygotes fared better in terms of survival (Lima et al., 2014). Our analysis of rs2853542 revealed no association with neoadjuvant chemotherapy response or overall survival in patients with advanced gastric cancer. Gusella et al. (2009) demonstrated that the G>C substitution did not predict clinical outcome, and Meulendijks et al. 2016) concluded that there was insufficient evidence to support the use of TS variants as biomarkers in the palliative setting.

The present study discovered a non-significant association between TS 3' UTR genotypes and response to neoadjuvant chemotherapy with 5FU and overall survival in patients with advanced gastric cancer. The presence of TS 1494 del 6bp in the 3' UTR region of the gene results in decreased mRNA stability and increased decomposition, thereby decreasing TS expression (Lima et al., 2014). Gao et al. (2013) demonstrated a significant association between the TS 3' UTR ins/ins genotype and poor survival in patients with advanced gastric cancer treated with capecitabine plus paclitaxel. Patients who were homozygous for the insertion had a significantly greater chance of survival than those who were homozygous for the deletion or heterozygous (Shitara et al., 2010).

Studies have demonstrated that the 6bp allele confers

a favorable prognosis in patients with advanced non-small-cell lung cancer (NSCLC) and colorectal cancer (Dotor et al., 2006; Lima et al., 2014). The rs151264360 6bp+/6bp+ genotype has been associated with a positive response to fluoropyrimidines (Castro-Rojas et al., 2017). MTHFR 1298A>C and TS 3' UTR ins/del polymorphisms in combination may predict 5FU treatment-related toxicity in colorectal cancer (Afzal et al., 2011). In comparison, some studies found that the TS 6bp del/ins variant did not reliably predict clinical outcomes (Gusella et al., 2009; Kristensen et al., 2010). Given the debate surrounding the TS 3' UTR insertion/deletion polymorphism, additional research is necessary to determine the role of TS in clinical outcomes.

Our study discovered a significant association between ENOSF1 rs2612091 genotypes and response to neoadjuvant chemotherapy with 5FU in gastric cancer, as the AG genotype was significantly associated with treatment non-response (p=0.017). There was no correlation between ENOSF1 rs2612091 genotypes and overall survival, however. There was no statistically significant association between the ENOSF1 rs2741171 variant and response to treatment or overall survival. According to Rosmarin et al., the ENOSF1 gene, which is located adjacent to TS, plays a critical role in regulating cell sensitivity to fluoropyrimidines (Meulendijks et al., 2016). In univariate analysis, the ENOSF1 rs2612091 G/G genotype was associated with a shorter OS in the overall population. After adjusting for TS VNTR, which interacts functionally with ENOSF1 rs2612091, the G/G genotype was nominally associated with inferior OS, while the 3R/3R genotype showed a trend toward inferior OS.

These findings suggest that TS and ENOSF1 variants may predict OS in patients receiving palliative care for GC. There was a significant effect of ENOSF1 rs2612091 G>A on OS in patients with locally advanced disease. The rs2612091 G allele has been shown to be causally and independently associated with inferior OS. Moreover, with each additional rs2612091 G allele, an increasingly poor outcome was observed. The association between 5FU/capecitabine toxicity and TS polymorphisms previously reported appears to be explained by the rs2612091 polymorphism (Rosmarin et al., 2015). Analysis of mRNA expression data demonstrated rs2612091 is associated with ENOSF1 expression and not with TS expression (Rosmarin et al., 2015).

In summary, in this study, we investigated the association between TS and ENOSF1 gene variants and response to neoadjuvant chemotherapy with 5FU and overall survival in gastric cancer. The results indicated that the 2R3R genotype was associated with a better response to treatment and the 3R3R genotype with a worse response. Furthermore, the 3R3R and 2R3R genotypes had a higher overall survival rate than the 2R2R genotype. The AG genotype of the rs2612091 variant was more prevalent among nonresponder patients. No statistically significant association was observed between other TS and ENOSF1 variants in this study. Additional research is required to investigate and establish the role of these variants in patient response to treatment and survival in other populations.

Author Contribution Statement

K.A. conducted the study, performed the statistical analysis, and prepared the manuscript, F.A. supervised data collection and evaluated the manuscript, I.S. supervised the project and evaluated the manuscript, M.E. conducted data collection and evaluated the manuscript, Sh.I. consulted on data collection and analysis, and evaluated the manuscript

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Approval

This study was approved by the Tehran Azad University of Science and Research's Medical Ethics Committee (IR.IAU.SRB.REC.1397.110).

Ethical Declaration

Tehran Azad University of Science and Research's Medical Ethics Committee (IR.IAU.SRB.REC.1397.110).

Data Availability

The ownership of the data is reserved by the Tehran Azad University of Sciences.

Study Registration: The study was not registered in any database.

Conflict of Interest

None.

References

Ab Mutalib N-S, Md Yusof NF, Abdul S-N, et al (2017). Pharmacogenomics DNA biomarkers in colorectal cancer: current update. *Front Pharmacol*, 8, 736.

Afzal S, Gusella M, Vainer B, et al (2011). Combinations of polymorphisms in genes involved in the 5-Fluorouracil metabolism pathway are associated with gastrointestinal toxicity in chemotherapy-treated colorectal cancer patients. *Clin Cancer Res*, **17**, 3822-9.

Arévalo E, Castañón E, López I, et al (2014). Thymidylate synthase polymorphisms in genomic DNA as clinical outcome predictors in a European population of advanced non-small cell lung cancer patients receiving pemetrexed. *J Transl Med*, **12**, 1-9.

Castro-Rojas CA, Esparza-Mota AR, Hernandez-Cabrera F, et al (2017). Thymidylate synthase gene variants as predictors of clinical response and toxicity to fluoropyrimidine-based chemotherapy for colorectal cancer. *Drug Metab Pers Ther*, **32**, 209-18.

De Mattia E, Roncato R, Palazzari E, et al (2020). Germline and somatic pharmacogenomics to refine rectal cancer patients selection for neo-adjuvant chemoradiotherapy. *Front*

Pharmacol, **11**, 897.
Dotor E, Cuatrecases M, Martínez-Iniesta M, et al (2006). Tumor thymidylate synthase 1494del6 genotype as a prognostic factor in colorectal cancer patients receiving fluorouracil-based adjuvant treatment. *J Clin Oncol*, **24**, 1603-11.
Gallegos-Arreola MP, Zúñiga-González GM, Sánchez-López JY, et al (2018). TYMS 2R3R polymorphism and DPYD [IVS] 14+ 1G> A mutation genes in Mexican colorectal cancer patients. *Acta Biochim Pol*, **65**, 227-34.
Gosens MJ, Moerland E, Lemmens VP, et al (2008). Thymidylate synthase genotyping is more predictive for therapy response than immunohistochemistry in patients with colon cancer. *Int J Cancer*, **123**, 1941-9.
Gusella M, Frigo A, Bolzonella C, et al (2009). Predictors of survival and toxicity in patients on adjuvant therapy with 5-fluorouracil for colorectal cancer. *Br J Cancer*, **100**, 1549-57.
Hamzic S, Kummer D, Froehlich TK, et al (2020). Evaluating the role of ENOSF1 and TYMS variants as predictors in fluoropyrimidine-related toxicities: An IPD meta-analysis. *Pharmacol Res*, **152**, 104594.
Hernando-Cubero J, Matos-García I, Alonso-Orduña V, et al (2017). The role of fluoropirimidines in gastrointestinal tumours: From the bench to the bed. *J Gastroint Cancer*, **48**, 135-47.
Iacopetta B, Grieu F, Joseph D, et al (2001). A polymorphism in the enhancer region of the thymidylate synthase promoter influences the survival of colorectal cancer patients treated with 5-fluorouracil. *Br J Cancer*, **85**, 827-30.
Jianwei L, Changming G, Jianzhong W, et al (2013). Polymorphism in the methylenetetrahydrofolate reductase and thymidylate synthase gene predicts for response to fluorouracil-based chemotherapy in advanced gastric cancer patients. *J Int Transl Med*, **1**, 4-12.
Kristensen M, Pedersen P, Melsen G, et al (2010). Variants in the dihydropyrimidine dehydrogenase, methylenetetrahydrofolate reductase and thymidylate synthase genes predict early toxicity of 5-fluorouracil in colorectal cancer patients. *J Int Med Res*, **38**, 870-83.
Lecomte T, Ferraz J-M, Zinzindohoué F, et al (2004). Thymidylate synthase gene polymorphism predicts toxicity in colorectal cancer patients receiving 5-fluorouracil-based chemotherapy. *Clin Cancer Res*, **10**, 5880-8.
Lima A, Azevedo R, Sousa H, et al (2013). Current approaches for TYMS polymorphisms and their importance in molecular epidemiology and pharmacogenetics. *Pharmacogenomics*, **14**, 1337-51.
Lima A, Seabra V, Martins S, et al (2014). Thymidylate synthase polymorphisms are associated to therapeutic outcome of advanced non-small cell lung cancer patients treated with platinum-based chemotherapy. *Mol Biol Rep*, **41**, 3349-57.
Mandola MV, Stoehlmacher J, Muller-Weeks S, et al (2003). A novel single nucleotide polymorphism within the 5′ tandem repeat polymorphism of the thymidylate synthase gene abolishes USF-1 binding and alters transcriptional activity. *Cancer Res*, **63**, 2898-904.
Marcuello E, Altés A, del Rio E, et al (2004). 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*, **112**, 733-7.
Marsh S, McKay JA, Cassidy J, et al (2001). Polymorphism in the thymidylate synthase promoter enhancer region in colorectal cancer. *Int J Oncol*, **19**, 383-6.
Matsusaka S, Lenz H-J (2015). Pharmacogenomics of fluorouracil-based chemotherapy toxicity. *Exp Opin Drug Metab Toxicol*, **11**, 811-21.
Meulendijks D, Jacobs BA, Aliev A, et al (2016). Increased risk of severe fluoropyrimidine-associated toxicity in patients carrying a G to C substitution in the first 28-bp tandem repeat of the thymidylate synthase 2 R allele. *Int J Cancer*, **138**, 245-53.
Meulendijks D, Rozeman E, Cats A, et al (2017). Pharmacogenetic variants associated with outcome in patients with advanced gastric cancer treated with fluoropyrimidine and platinum-based triplet combinations: a pooled analysis of three prospective studies. *Pharmacogenomics J*, **17**, 441-51.
Ntavatzikos A, Spathis A, Patapis P, et al (2019). TYMS/KRAS/BRAF molecular profiling predicts survival following adjuvant chemotherapy in colorectal cancer. *World J Gastrointest Oncol*, **11**, 551.
Pullarkat S, Stoehlmacher J, Ghaderi V, et al (2001). Thymidylate synthase gene polymorphism determines response and toxicity of 5-FU chemotherapy. *Pharmacogenomics J*, **1**, 65-70.
Rosmarin D, Palles C, Pagnamenta A, et al (2015). A candidate gene study of capecitabine-related toxicity in colorectal cancer identifies new toxicity variants at DPYD and a putative role for ENOSF1 rather than TYMS. *Gut*, **64**, 111-20.
Schwab M, Zanger UM, Marx C, et al (2008). Role of genetic and nongenetic factors for fluorouracil treatment-related severe toxicity: a prospective clinical trial by the German 5-FU Toxicity Study Group. *J Clin Oncol*, **26**, 2131-8.
Shi C, Berlin J, Branton P, et al (2017). Protocol for the examination of specimens from patients with carcinoma of the esophagus, version 4.0. 0.0. Northfield, IL: College of American Pathologists.
Shi W-J, Gao J-B (2016). Molecular mechanisms of chemoresistance in gastric cancer. *World J Gastroint Oncol*, **8**, 673.
Shitara K, Muro K, Ito S, et al (2010). Folate intake along with genetic polymorphisms in methylenetetrahydrofolate reductase and thymidylate synthase in patients with advanced gastric cancer. *Cancer Epidemiol Prev Biomarkers*, *19*, 1311-9.
Smyth E, Zhang S, Cunningham D, et al (2017). Pharmacogenetic analysis of the UK MRC (medical Research Council) magic trial: association of polymorphisms with toxicity and survival in patients treated with perioperative epirubicin, cisplatin, and 5-fluorouracil (ECF) chemotherapy. *Clin Cancer Res*, **23**, 7543-9.
Sung H, Ferlay J, Siegel RL, et al (2021). Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*, **71**, 209-49.
Villafranca E, Okruzhnov Y, Dominguez MA, et al (2001). Polymorphisms of the repeated sequences in the enhancer region of the thymidylate synthase gene promoter may predict downstaging after preoperative chemoradiation in rectal cancer. *J Clin Oncol*, **19**, 1779-86.
Yang Y, Wu G, Jin L, et al (2017). Association of thymidylate synthase polymorphisms with the tumor response to preoperative chemoradiotherapy in rectal cancer: a systematic review and meta-analysis. *Pharmacogenomics J*, **17**, 265-73.

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