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ASSOCIATION OF THYMIDYLATE SYNTHASE POLYMORPHISMS WITH GASTRIC CANCER SUSCEPTIBILITY

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We investigated in a case-control study a possible role of thymidylate synthase gene (*TS*) polymorphisms for gastric cancer susceptibility. Lymphocyte genomic DNA from 134 Italian gastric cancer patients and 139 controls was used for genotyping two polymorphisms in the *TS* 5′-untranslated region (5′-UTR); a double (2R) or triple (3R) 28-bp repeat and a G/C polymorphism within the triple repeats allele (3G allele). Samples were also genotyped at a 6-bp deletion/insertion (del6 or ins6) polymorphism at position 1494 in the *TS* 3′-untranslated region (3′-UTR). Unconditional regression with odd ratios (OR) and 95% confidence intervals (CI), haplotype and linkage disequilibrium analyses were used to investigate the association of the polymorphisms with the disease. The global allelic distribution was in Hardy-Weinberg equilibrium. Genotypes with the 3G allele (2R/3G, 3C/3G, 3G/3G) were significantly more frequent in patients than controls and were associated with gastric cancer risk (OR = 2.06; 95% CI = 1.26–3.35). A significant risk was also observed for carriers of the del6 allele in the 3′-UTR. Odds ratios for combined 3G-del6/ins6 and 3G-del6/del6 genotypes were 2.59 (95% CI = 1.36–4.94) and 2.81 (95% CI = 1.22–6.64), respectively. The 3G-del6 haplotype showed a significant association with the disease ($p = 0.01$). Polymorphisms in the *TS* gene may contribute to gastric cancer susceptibility and this finding deserve further investigation in the context of novel strategies for gastric cancer prevention. *In vitro*, 3G genotypes have been related to high *TS* mRNA expression, which may underlie one of the possible etiologic mechanisms.

Key words: *gastric neoplasms; thymidylate synthase; polymorphism; susceptibility*

Over the past decades, a steady decline has occurred in gastric cancer incidence and mortality in many countries, but stomach cancer remains the second most frequent cancer worldwide, with about 12% of cancer deaths each year.[1] A major strategy for facing this health care problem is the identification of at risk individuals, for prevention and early detection of the disease. The knowledge on molecular alterations, which are involved in the carcinogenesis process of gastric carcinomas, may lead to new, and hopefully more effective means for controlling this lethal disease.[2] In this perspective, the role of genetic polymorphisms in gastric cancer risk has provoked increasing interest in recent years.[3]

TS is the key enzyme that catalyzes the conversion of dUMP to dTMP and the sole *de novo* source of thymidylate in the cell. Specific polymorphisms in the *TS* gene are detectable in the general population and they have been correlated with different levels of *TS* mRNA and protein expression, and the likelihood of response to chemotherapeutic agents that inhibit the TS enzyme.[4,5]

Recent studies have determined a potential role for *TS* gene polymorphisms in colorectal cancer susceptibility.[6–8] Experimental investigations have proposed some mechanisms for explaining the potential etiologic contribution of *TS* polymorphisms in cancer. Polymorphisms leading to high TS enzyme levels may cause perturbances in the folate availability[9] and imbalances in the deoxynucleotide pool in the cell.[10] These conditions have been experimentally associated with DNA damage, altered DNA replication and impaired mechanisms of DNA repair.[11–13] The TS enzyme functions as an RNA binding protein for translational repression of its own, and other cellular mRNAs.[14–17] These molecular interactions may induce dysregulation in the cell proliferation and the mechanisms affecting cell senescence and apoptosis.

The first and extensively studied functional polymorphism in the *TS* promoter is a VNTR, which consists of either 2 or 3 28-bp repeated sequence in the 5′-UTR.[18] The 3R allele has been related to enhanced *TS* translational efficiency[19] and TS expression[20] when compared to the 2R allele and the greatest effect is observed in the presence of the homozygous 3R/3R genotype. A G/C polymorphism within the 3R VNTR produces 2 additional alleles (3G or 3C) at this locus.[21,22] The 3G allele has been associated with increased reporter gene activity at both the transcriptional[21] and translational[22] levels *in vitro*. *In vivo*, 3G-containing genotypes (2R/3G, 3C/3G, 3G/3G) have been found to correlate with high *TS* mRNA expression.[23] More recently, a 6-bp deletion in the *TS* gene

Abbreviations: CI, confidence intervals; del6, allele with 6-bp deletion in the 3′-untranslated region; dTMP, deoxythymidine-5′-monophosphate; dTTP, deoxythymidne-5′triphosphate; dUMP, deoxyuridine-5′-monophosphate; ins6, allele with 6-bp insertion in the 3′-untranslated region, OR, odds ratio; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; 3R, three repeats; 2R, two repeats; TS, thymidylate synthase; UTR, untranslated region; VNTR, variable number of tandem repeats polymorphism.

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has been found in the 3′-UTR.[24] The results of 3 recent investigations have shown conflicting findings on the functional consequences of this novel polymorphism.[23,25,26]

According to these data, we have speculated that polymorphisms in the *TS* gene may have a role in gastric cancer susceptibility and a tested this hypothesis in a case-control study.

MATERIAL AND METHODS

Human samples and clinicopathologic data

Peripheral blood samples from consecutive patients with sporadic gastric cancer and healthy controls were collected in 5 medical oncology units in Central Italy (Urbino, Pesaro, Senigallia, Ancona, Rome). The inclusion criteria for eligible patients were: Caucasian ethnicity, residency in one of the 5 geographical areas and lack of family history of cancer. The same criteria plus lack of personal history of cancer were adopted for controls. Current and former blood donors were used as healthy controls and they were identified through the pools of blood donors available at each participating Institution. Controls were randomly selected with frequency matching to cases by age (±2 years) and gender.

Before study inclusion, eligible patients and healthy controls were interviewed about personal/family medical history, education, tobacco smoking and alcohol intake. Pedigrees were traced back for at least 3 generations and laterally to second- and third-degree relatives. The threshold of 20 g/day of alcohol intake was estimated as approximately 2 cans of beer, 2 glasses of wine or 2 shots of spirit. The diagnosis of gastric cancer was confirmed by 2 independent pathologists after reviewing tumour blocks. The ethical requirements were verified by the internal review boards and all participants gave their written informed consent.

Analysis of TS *genotypes*

Lymphocyte genomic DNA was extracted from peripheral blood samples. For the analysis of the VNTR and G/C polymorphisms in the 5′-UTR, PCR and PCR-RFLP method were used as described previously.[22] Briefly, a fragment containing the repeats was amplified using primers *TS* 25: 5′-AGGCGCGCGGAAGGGGTCCT-3′ and *TS* 18: 5′-TCCGAGCCGGCCACAGGCAT-3′. Aliquots of amplified fragments were separated on a 3% agarose gel. The fragments containing 3 (3R) or 2 (2R) repeats sized 141 or 113 bp, respectively. Samples showing the 2R/3R or 3R/3R genotypes were further analyzed for the G/C polymorphism by RFLP. HaeIII digestion of the 3R fragment produced 66-, 37-, 28- and 10-bp bands for the 3G allele and 94-, 37- and 10-bp bands for the 3C allele after separation on a 3% agarose gel. Accordingly, *TS* 5′-UTR genotypes were classified into either 2R/2R, 2R/3G, 2R/3C, 3G/3G, 3G/3C or 3C/3C as described previously.[22]

The 3′-UTR polymorphism was analyzed by PCR followed by electrophoresis of amplified fragments using Spreadex gel (Elchrom Scientific, Switzerland). Fragments containing the 6-bp deletion were amplified using primers *TS* 63 5′-CAAATCTGAGGGAGCTGAGT-3′ and *TS* 64 5′-CAGATAAGTGGCAGTACAGA-3′ in a reaction containing 1 × TaKaRa Taq buffer, 200 μM deoxyribonucleotide triphosphates, 500 nM each primer, 1 U of TaKaRa Taq DNA polymerase (hot start version), and 100 ng of genomic DNA. The cycling conditions were 1 cycle of 94°C for 3 min, 30 cycles of 94°C for 40 sec, 58°C for 60 sec, and 72°C for 40 sec and a final extension at 72°C for 5 min. PCR reactions were analyzed on EL300-S100 Spreadex gel to allow separation into expected fragment sizes of 148 and 142 bp.

Statistical methods

The significance of the difference in the distribution of the polymorphisms among different groups was calculated using the χ^2 test. All allelic distributions were examined for deviations from their corresponding Hardy-Weinberg equilibrium. Unconditional logistic regression models were used to obtain OR and 95% CI, adjusting for age (age at diagnosis for case patients and age at selection for control subjects), sex, educational level (≤8 years and >8 years, indicating high school and beyond), alcohol intake (current drinker of ≥20 g of ethanol per day and non-drinker or drinker <20 g ethanol per day) and smoking status (current smoker and non-smoker). All values were two-sided and statistical significance was defined as $p < 0.05$. To better define possible roles of *TS* polymorphisms in gastric cancer risk, single allele or haplotype association with the disease was carried out and linkage disequilibrium between loci was assessed *via* the GLUE interface (available at: www.hgmp.mrc.ac.uk) using the Unphased software package. Linkage disequilibrium was estimated by r^2, which can range from 0 (random co-inheritance of alleles) to 1 (complete linkage disequilibrium).Values <0.33 suggest absence of strong linkage disequilibrium.[27]

RESULTS

The study population consisted of 134 gastric cancer patients and 139 healthy controls. There were 68 male and 66 females among gastric cancer patients and 71 males and 68 females among controls. According to Lauren's classification, 67 gastric cancer cases were of the intestinal histotype and 67 gastric cancer cases were of the diffuse histotype. The study population and the distribution of genotypes is shown in Table I. All allelic distributions were in Hardy-Weinberg equilibrium ($p > 0.127$).

Analysis of polymorphism in the 5′-UTR region

Neither the 2R/3R genotype nor the 3R/3R genotype showed significant OR for gastric cancer risk (Table II). The combined analysis of 2R/3R and 3R/3R genotypes (114 cases and 105 controls) in comparison with 2R/2R as reference genotype (18 cases and 31 controls) resulted in a non-significant OR of 1.86 (95% CI = 0.98–3.54).

The inclusion of the G/C polymorphism into analysis showed that carriers of at least one 3G allele (3G/3G, 2R/3G, 3G/3C) were significantly over represented in cases compared to controls ($p = 0.005$) (Table II). An overall 2.06 OR (95%CI = 1.26–3.35) estimated the gastric cancer risk associated with 3G genotypes (Table II). Single allele analysis supported an association of the 3G allele with the disease (frequency cases 31.8%, controls 22.4%, $p = 0.048$).

The frequency of the 3G-allele carriers was significantly higher in both intestinal ($p = 0.001$) and diffuse ($p = 0.04$) gastric cancer patients than controls. The OR for 3G-allele carriers was 3.0 (95%CI = 1.7–5.5) in the intestinal gastric cancer group and it was 1.4 (95%CI = 0.8–2.9) in the diffuse gastric cancer group.

Analysis of polymorphisms in the 3′-UTR region

The OR for gastric cancer was significantly elevated in del6/ins6 heterozygotes, but below the threshold of significance in del6/del6 homozygotes (Table III). The combined analysis of del6/ins6 and del6/del6 genotypes (95 cases and 77 controls) in comparison with ins6/ins6 as referent genotype (39 cases and 62 controls), showed a significant association with gastric cancer risk and an OR of 1.95 (95% CI = 1.17–3.24). Single allele analysis corroborated an association of the del6 allele with disease (frequency cases 43.9%, controls 34.2%, $p = 0.021$).

When the histotypes were analyzed separately, only gastric cancer patients of the diffuse histotype showed a significantly higher frequency of the del6-allele carriers ($p = 0.001$). The OR for del6-allele carriers was 1.4 (95% CI = 0.7–2.8) in the intestinal gastric cancer group and 2.6 (95% CI = 1.5–4.8) in the diffuse gastric cancer group.

Combined analysis of polymorphisms and haplotypes

The combined analysis of the 5′-UTR VNTR-G/C polymorphism with the 3′-UTR del6/ins6 polymorphism is shown in Table IV. A significant association with gastric cancer risk was observed for combinations of the 3G-containing genotypes with del6/ins6 and del6/del6 genotypes.

TABLE I – CHARACTERISTICS OF THE STUDY POPULATION AND DISTRIBUTION OF GENOTYPES IN 139 CONTROLS, 134 GASTRIC CANCER CASES WITH 67 OF THE INTESTINAL HISTOTYPE AND 67 OF THE DIFFUSE HISTOTYPE[1]

	Controls	Gastric cancer cases	Intestinal	Diffuse
Age[a], mean years (range)	58 (33–77)	59 (30–83)	61 (32–80)	58 (30–83)
Gender				
Male	71 (51)	68 (51)	35 (52)	33 (49)
Female	68 (49)	66 (49)	32 (48)	34 (51)
Educational level				
≤ 8 years	84 (60)	79 (58)	36 (53)	43 (64)
> 8 years	55 (40)	55 (42)	31 (47)	24 (36)
Smoking status				
Current smokers	46 (34)	50 (37)	24 (36)	26 (38)
Non-smokers	93 (66)	84 (63)	43 (64)	41 (62)
Alcohol intake				
Current drinkers	69 (50)	73 (54)	37 (55)	36 (53)
Non-drinkers or <20g/day	70 (50)	61 (46)	30 (45)	31 (47)
5′-UTR				
>3R[2]	3 (2)	2 (1)	1 (1)	1 (1)
3R/3R	31 (22)	38 (28)	22 (33)	16 (24)
2R/3R	74 (54)	76 (57)	35 (52)	41 (60)
2R/2R	31 (22)	18 (14)	9 (14)	9 (14)
>3R[2]	3 (2)	2 (1)	1 (1)	1 (1)
3G/3G	10 (7)	12 (10)	7 (10)	5 (7)
2R/3G	28 (20)	42 (32)	21 (31)	21 (31)
3G/3C	13 (9)	19 (14)	10 (16)	9 (14)
2R/2R	31 (22)	18 (13)	9 (14)	9 (14)
2R/3C	46 (34)	34 (25)	14 (21)	20 (30)
3C/3C	8 (6)	7 (5)	5 (7)	2 (3)
3′-UTR				
del6/del6	18 (13)	22 (17)	14 (21)	8 (12)
del6/ins6	59 (42)	73 (54)	32 (48)	41 (60)
ins6/ins6	62 (45)	39 (29)	21 (31)	18 (28)

Values are n (%) except for age.– [2]Rare genotypes not been included in further analyses.

TABLE II – ANALYSIS OF *TS* POLYMORPHISMS IN THE 5′-UTR REGION[1]

Genotypes	Controls *n* (%)	Cases *n* (%)	χ^2	OR (95% CI)
2R/2R	31 (23)	18 (14)		Referent
2R/3R	74 (54)	76 (57)	$p = 0.13$	1.76 (0.91–3.43)
3R/3R	31 (23)	38 (29)		2.11 (0.99–4.46)
2R/2R + 2R/3C + 3C/3C	85 (62)	59 (44)	$p=0.005$	Referent
2R/3G + 3C/3G + 3G/3G	51 (38)	73 (56)		2.06 (1.26–3.35)

[1]Chi-square test for comparing distributions of genotypes in cases and controls. Odds ratios from the unconditional regression model with 95% confidence intervals.

TABLE III – ANALYSIS OF *TS* POLYMORPHISMS IN THE 3′-UTR REGION[1]

Genotypes	Controls *n* (%)	Cases *n* (%)	χ^2	OR (95% CI)
ins6/ins6	62 (45)	39 (29)		Referent
del6/ins6	59 (42)	73 (54)	$p = 0.03$	1.92 (1.13–3.28)
del6/del6	18 (13)	22 (17)		2.04 (0.96–4.33)

[1]Chi-square test for comparing distributions of genotypes in cases and controls. Odds ratios from the unconditional regression model with 95% confidence intervals.

Individual haplotype frequencies and linkage disequilibrium analyses are shown in Table V. Haplotype analysis indicated a significant association of the 3G-del6 haplotype with disease. Estimates of linkage disequilibrium using r^2 suggest that the 5′-UTR VNTR-G/C double polymorphism and the 3′-UTR del6/ins6 locus are not in strong linkage disequilibrium.

DISCUSSION

To the best of our knowledge, this is the first study investigating the relevance of *TS* polymorphisms for gastric cancer susceptibility. Although the VNTR in the 5′-UTR alone, did not provide evidence for a role in the disease, the 3G allele resulting from the VNTR-G/C double polymorphism correlated with gastric cancer risk. A potential etiologic role also emerged for the del6 polymorphism in the 3′-UTR. Haplotype analysis indicates the greatest association with disease occurs when the 3G- and the del6-alleles appear together. When divided into histological subtypes, the association between the 3G allele and diffuse gastric cancer risk as well as between the del6 allele and intestinal gastric cancer risk did not attain the threshold of statistical significance. These findings suggest that the observed associations may depend on the histologic cancer subtype. The distribution of the 3G allele and of the del6 allele in intestinal and diffuse gastric carcinomas was similar. The lack of a significant association between *TS* polymorphisms and subtypes of gastric cancer may be a Type 2 statistical error, which reflects the lack of statistical power to detect a mild risk. The lack of statistical power may also explain the non-significant increase in overall gastric cancer risk in the presence of the del6/del6 genotype.

To date, only 3 case-control studies have addressed the role of *TS* polymorphisms in cancer susceptibility and they have been carried out in colorectal cancer.[6–8] The only study investigating both the 5′-UTR VNTR-G/C double polymorphism and the 3′-UTR del6/ins6 variant was carried out by Stoehlmacher *et al.*[8] Akin to our results, they observed that the highest risk (OR = 1.5) for colorectal cancer was associated with the presence of both the

TABLE IV – ANALYSIS OF COMBINED *TS* GENOTYPES[1]

Combined genotypes		Controls *n* (%)	Cases *n* (%)	OR (95% CI)
5′UTR	3′UTR			
2R/2R + 2R/3C + 3C/3C	ins6/ins6	53 (39)	29 (22)	Referent
2R/2R + 2R/3C + 3C/3C	ins6/del6	28 (21)	28 (21)	1.82 (0.91–3.65)
2R/2R + 2R/3C + 3C/3C	del6/del6	4 (3)	2 (2)	0.98 (0.15–5.29)
2R/3G + 3C/3G + 3G/3G	ins6/ins6	7 (5)	9 (7)	2.34 (0.79–6.96)
2R/3G + 3C/3G + 3G/3G	ins6/del6	31 (22)	44 (33)	2.59 (1.36–4.94)
2R/3G + 3C/3G + 3G/3G	del6/del6	13 (10)	20 (15)	2.81 (1.22–6.46)

[1]Odds ratios from the unconditional regression model with 95% confidence intervals.

TABLE V – INDIVIDUAL HAPLOTYPE AND LINKAGE DISEQUILIBRIUM ANALYSES IN 134 GASTRIC CANCER CASES AND 139 CONTROLS

Haplotype	Cases %	Controls %	χ^2	r^2-cases[2]	r^2-controls[2]
2R-del6	9.4	9.3	0.8[1]	0.15	0.11
2R-ins6	33.7	40.6	0.07	0.15	0.11
3C-del6	8.7	7.5	0.6	0.01	0.01
3C-ins6	16.3	19.9	0.2	0.01	0.01
3G-del6	25.8	17.2	0.01	0.26	0.23
3G-ins6	6.0	5.1	0.6	0.26	0.23

[1]*p* values of the chi-square test for comparing distributions of haplotypes in cases and controls.– [2]Linkage disequilibrium estimates for haplotypes: r^2 values (see text) range from 1 (complete disequilibrium) to 0 (random co-inheritance of alleles).

3G and the del6 alleles. Chen *et al.*[7] found a protective role for the 5′-UTR 2R/2R genotype, whereas Adleff *et al.*[6] reported a higher frequency of the 3R/3R-ins6/del6 genotype in cancer cases than controls.

Experimental data indicate some possible mechanisms underlying the association of the 5′-UTR 3G variant with cancer susceptibility. The 3G allele showed higher transcriptional[21] and translational[22] reporter gene activity than other alleles, *in vitro*. *In vivo*, 3G-containing genotypes (2R/3G, 3C/3G, 3G/3G) have been found to correlate with high *TS* mRNA expression.[23] High TS activity in the cell causes perturbances in the folate pool[9] and the DNA precursor levels, with imbalances in the dUMP/dTMP ratio and dTTP excess.[9] These conditions may increase sensitivity to DNA-damaging agents and at the same time, they may cause DNA replication errors, leading to cancer risk.[11–13] *In vitro* studies support a TS binding and inhibiting activity to mRNAs of molecules critically involved in the cell cycle regulation, as p21, and apoptosis, as p53.[14–17] As a result, high TS expression may contribute to the neoplastic transformation because of inappropriate cell proliferation or escape from senescence and apoptosis.

Less clear is the possible etiological role for the del6 polymorphism in the 3′-UTR region. The hypothesized interference and decreased *TS* mRNA level associated with this variant was suggested from recent *in vitro* experimental data. Mandola *et al.*[25] found a higher stability of chimeric mRNA composed of a luciferase reporter and the 3′-UTR ins6 variant compared to a corresponding del6 construct. In another experimental investigation in human cancer cell lines, however, no significant influence of the 3′-UTR genotypes on the TS enzymatic levels was observed.[26] *In vivo*, colorectal carcinomas that were genotyped for both *TS* 5′-UTR and *TS* 3′-UTR polymorphisms showed a trend for higher *TS* mRNA levels in tumor with ins6/ins6 genotypes than in tumors with del6/del6 genotypes. This difference was not statistically significant, however, and it was limited to tumors with concomitant 2R/2R and 3C/3C genotypes in the *TS* 5′UTR.[23]

In the present study, carriers of the 3G allele showed an OR of 2.06 and carriers of the del6 allele showed an OR of 1.95. We cannot exclude that the OR of del6 is in part due to linkage disequilibrium with a functional 3G allele or vice versa. The observed linkage disequilibrium with 3G is not strong enough to fully explain the increased OR of del6. The gradual increase of the OR in the combined analysis (2.59 for del6/ins6-3G genotype, and 2.81 for del6/del6-3G genotype) suggests that both loci may contribute independently to gastric cancer risk. Additional studies are needed to elucidate functional effects of the del6 allele, functional interactions between del6 and 3G alleles and to examine the alternative hypothesis of linkage disequilibrium with an as yet unidentified susceptibility locus.

In conclusion, these findings indicate that the 3G allele in the 5′-UTR and the del6 polymorphism in the 3′-UTR of the *TS* gene are associated with gastric cancer risk in the studied population. The higher OR associated with the combination of the 3G and the del6 allele implies that both loci together could serve as a better marker that the single *TS* polymorphisms. However, given the relatively small sample of the present investigation and the variable frequency of the *TS* polymorphisms in different populations,[28] large epidemiologic studies with ethnicity comparisons are needed. These future investigations could also consider the possibility of concomitant analyses of plasma folate levels and combined analyses with polymorphisms in other genes that are linked to folate metabolism.[29,30] The confirmation of the etiological role for *TS* variants in gastric cancer will represent the basis for novel screening strategies in this lethal disease.

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