Original paper

Impact of TGF-β1 expression and -509C>T polymorphism in the TGF-β1 gene on the progression and survival of gastric cancer

Julian Ananiev[1], Irena Manolova[2], Elina Aleksandrova[1], Maya Gulubova[1]

[1]Department of General and Clinical Pathology, Medical Faculty, Trakia University Stara Zagora, Bulgaria
[2]Department of Biology, Immunology and Genetics, Medical Faculty, Trakia University, Stara Zagora, Bulgaria

The aim of this study was to examine the expression of TGF-β1 and TGF-β receptor type II (RII) and the impact of the -509C>T single nucleotide polymorphism (SNP) in the gene in relation to clinicopathological factors in gastric cancer (GC).
Using immunohistochemistry we investigated 43 patients with GC for expression of TGF-β1 and TGF-β-RII. Consequently, RFLP-PCR was performed to analyze the presence of -509C>T polymorphism in the TGF-β1 gene.
We found that 72.1% of GCs had cytoplasmic TGF-β1 expression and 27.9% were negative. The TGF-β1 receptor type II was expressed on tumor cell membranes in 58.1%. TGF-β1 positivity in tumor cytoplasm correlated positively with TGF-β1-RII expression in tumor cytoplasm in 67.4% of cases ($\chi^2 = 8.02$; $p = 0.005$). Also, the results showed that patients with low and moderate tumor differentiation had TGF-β1-RII positivity in 53.3% and 81.8% resp. ($\chi^2 = 6.58$; $p = 0{,}037$). The analysis of genotype distribution of the -509C>T SNP in the promoter region of TGF-β1 gene and clinical stage distribution revealed that among the 32 patients in III-IV clinical stage 53.1% were heterozygous (TC), 34.4% were homozygous for the C-allele and 12.5% were homozygous for the variant T-allele ($\chi^2 = 3.31$; $p = 0.069$).
In conclusion the expression of TGF-β1 was related to shorter survival time and rapid progression for the GC patients. Additionally, the variant T-allele of the studied polymorphism was associated with worse prognosis for GC patients.

Key words: gastric cancer, TGF-β1, -509C>T SNP.

Introduction

Gastric cancer (GC) is one of the most common malignant tumors worldwide and the third cause of neoplasm related death [1]. About one million new cases of GC were estimated to have occurred in 2012 (952,000 cases, 6.8% of the total) making it the fifth most common malignancy after cancers of the lung, breast, colon/rectum and prostate [2]. Different factors such as obesity, gastroesophageal reflux disease and chronic gastritis are thought to contribute to its deadliness, and unfortunately between 23% and 34% of the patients were diagnosed in advanced stages [3, 4].

As the survival rate of patients with GC is rather low, even in the developed countries, apart from the variety of prognostic markers, new independent parameters are being investigated. To date many tumor-associated antigens either intracellular or on the cell surface have been identified.

Transforming growth factor β1 (TGF-β1) is a multifunctional cytokine that can induce growth inhibi-

tion, apoptosis, and differentiation of gastrointestinal epithelial cells [4]. TGF-β1 is encoded on chromosome 19q13.1 and it is a 44.3kDa protein that is usually secreted as an inactive compound consisting of a homodimer non-covalently linked to a latency-associated peptide homodimer. The active protein binds to type II TGF-β1 receptors which form heterodimers with TGF-β1 type I receptors. This results in receptor-mediated serine-threonine kinase activity involving phosphorylation of members of the SMAD family of transcription factors and activation/inhibition of various genes, depending on the state of cell transformation, including in GC [5, 6, 7, 8].

In addition, there is no significant data in public sources about the role of the -509C>T single nucleotide polymorphism, protein expression and correlations with clinical parameters and progression of GC patients. One previous publication has described a case-control study investigating whether the *TGF-β1* -509 C>T SNP can modify the risk of gastric cancer [9].

The aim of the present study was to investigate a group of 43 patients with GC immunohistochemically for TGF-β1 and TGF-β1-RII expression and to analyze the genotype distribution regarding the -509C>T *TGF-β1* SNP using RFLP-PCR technique. We also evaluated the relation between the collected data and some clinical and pathological parameters of the investigated group of patients.

Material and methods

Patients and samples

We investigated biopsy specimens collected from 43 patients who underwent gastrectomy in The University Hospital in Stara Zagora, Bulgaria between 2007 and 2014. The tissue samples were collected in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. Twenty seven of the patients are male and sixteen female, with age range from 22 to 80 years (63.4 years mean). Some clinicopathological findings of patients were performed (Table I).

DNA extraction

Genomic DNA was extracted from fresh tumor biopsy tissues using a genomic DNA purification kit (NucleoSpin Tissue, Macherey-Nagel, Duren, Germany) and DNA from the controls was isolated from blood samples (NucleoSpin Blood L, Macherey-Nagel, Duren, Germany) according to manifacturer's protocol. After that extracted DNA was stored at –80°C until further use. The concentration of resulting DNA was measured spectrophotometrically at 260 nm by NanoVue TM Spectrophotometer (Healthcare, Buckinghamshire, UK). The ratio of absorptions at 260 vs. 280 nm was used to assess the purity of the DNA samples.

Genotyping of TGFβ1 -509 C>T polymorphism (rs1800469)

Genotyping was performed by PCR restriction length polymorphism (RFLP) assay. The PCR primers for amplification of the studied polymorphism were as follows: 5'-CAGTAATGTATGGGGTCGCAG-3' (forward) and 5'-GGTGTCAGTGGGAGGAGGG-3' (reverse). Sample DNA was amplified in 20 ml of a reaction mixture, containing 1×PCR buffer, 0.4 mmol/l of each primer, 0.2 mmol/l dNTPs, 1.5 mmol/l $MgCl_2$, and 1 U of Taq polymerase (Fermentas Ltd). The PCR profile included initial denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 63°C for 1 minute, and extension at 72°C for 1 minute. Final elongation was performed for 7 minutes at

Table I. Clinical and morphological characteristics of the patients

PARAMETER	NUMBER (%)
Clinical data	(n = 43)
Gender	
Male	27 (62.8)
Female	16 (37.2)
Age (years)	
median	63.4
(range)	(22-80)
T stage	
T1-2	8 (18.6)
T3-4	35 (81.4)
N stage	
N0	10 (23.3)
N1-3	33 (76.7)
Metastases	
No	35 (81.4)
Yes	8 (18.6)
Clinical stage	
I	3 (7.0)
II	9 (20.9)
III	18 (41.9)
IV	13 (30.2)
Histological data	
Differentiation grade of tumor	
low	30 (68.8)
moderate	11 (25.6)
high	2 (4.7)
Histological type	
intestinal	34 (79.1)
diffuse	9 (20.9)

72°C. PCR amplification was performed on QCycler (QuantaBiotech Ltd, UK).

Amplification was checked electroforetically on a 2% agarose gel. The PCR product was digested with 10U Eco81I in a total volume of 20 ml of 1xTango buffer (Fermentas Ltd) at 37°C overnight. The restriction yields two fragments of 117 bp and 36 bp in the presence of the ancestral C-allele. The fragments were separated on a 3% agarose gel and stained with ethidium bromide (0.5 mg/ml). For all genotype analysis laboratory personal were blinded to subjects' status; agarose restriction fragment gels were interpreted by at least two independent readers.

In each PCR run, heterozygous control template and negative template controls were used to ensure accuracy.

Immunohistochemistry

Gastric cancer tissues from all 43 patients were analyzed immunohistochemically. Specimens were fixed in 10% buffered formalin, embedded in paraffin and then cut to 4 μm thickness. Next step were dewaxed and endogenous peroxidase was blocked for 5 minutes with blocking reagent according to the protocol. Then the slides were washed 3 times with PBS and incubated with primary antibody for 1 hour. Reactions were carried out with rabbit anti-human TGF-β1 antibody (sc-146, Santa Cruz Biotechnology, USA) in a dilution 1 : 50 and rabbit anti-human TGFβRII antibody (sc-400, Santa Cruz Biotechnology, USA) in a dilution 1 : 50. After that slides washing 3 times the slides were incubated with detection system EnVision™ FLEX+, Mouse, High pH, (Link) (K8002, DAKO) and then washed again. In the last step probes were incubated with DAB substrate-chromogen and contra stained with Mayer's hematoxylin.

Table II. Distribution of the expression of immunohistochemically markers and -509C/T polymorphism of TGF-β1

Parameter	Number (%)
TGF-β 1 expression	
Yes	31 (72.1)
No	12 (27.9)
TGF-β RII expression	
Yes	25 (58.1)
No	18 (49.1)
-509C/T polymorphism of TGF-β1	
TC	24 (55.8)
CC	15 (34.9)
TT	4 (9.3)

The analysis was performed according to the manufacturer's protocols and final score for TGF-β1 and TGFβRII expression were obtained according to immunostaining intensity in tumor epithelial cells and were designated as negative – score 0, or positive 1+.

Statistical analysis

The SPSS 19.0 software for Windows was used for statistical analysis. The descriptive statistical tests, including the mean, standard deviation, and median, were calculated according to the standard methods and program protocol. The frequency of distribution of immunohistochemical staining and the clinicopathological parameters in 2×2 contingency tables was analyzed by χ^2-test. Survival was calculated from the date of operation to the date of death or of the last follow-up. Cumulative survival curves were drawn by the Kaplan-Meier method ant the difference between curves was analyzed by the log-rank test. For all statistical analysis, $p < 0.05$ was considered to be statistically significant.

Results

Expression of TGF-β1 and TGF-β1-RII in tumor tissue and corellations with clinical and pathological factors

After immunohistochemical examination we found that 31 cases (72.1%) of GCs had cytoplasmic TGF-β1 expression and 12 (27.9%) were negative (Fig. 1A). Some of the normal epithelial and inflammatory cells also were marked by TGF-β1. The TGF-β1 receptor type II was expressed on tumor cell membranes in 25 (58.1%) of the cancers (Fig. 1B).

Furthermore, we compared the cases on the base of expression of two markers, and found that TGF-β1 positivity in tumor cytoplasm correlated with TGF-β1-RII expression in tumor cytoplasm in 67.4% of cases ($\chi^2 = 8.02$; $p = 0.005$, data not shown in table). Also, our results showed that patients with low and moderate tumor differentiation had TGF-β1-RII positivity in 53.3% and 81.8% resp. ($\chi^2 = 6.58$; $p = 0{,}037$). In addition, we also observed a positive correlation between TGF-β1- RII expression and stage – 56.3% of the patients in III and IV clinical stage were positive for TGF-β1-RII ($\chi^2 = 6.81$; $p = 0{,}078$, tendency).

No correlation was observed between TGF-β1 and TGF-β1-RII expression and other clinicopathological factors (Table III).

-509C>T TGF-β1 polymorphism correlations

After RFLP-PCR analysis of 43 cases of GCs, 24 (55.8%) of the cases had genotype TC, 15 (34.9%) – CC, and the rest 4 cases (9.3%) had TT genotype (Fig. 2).

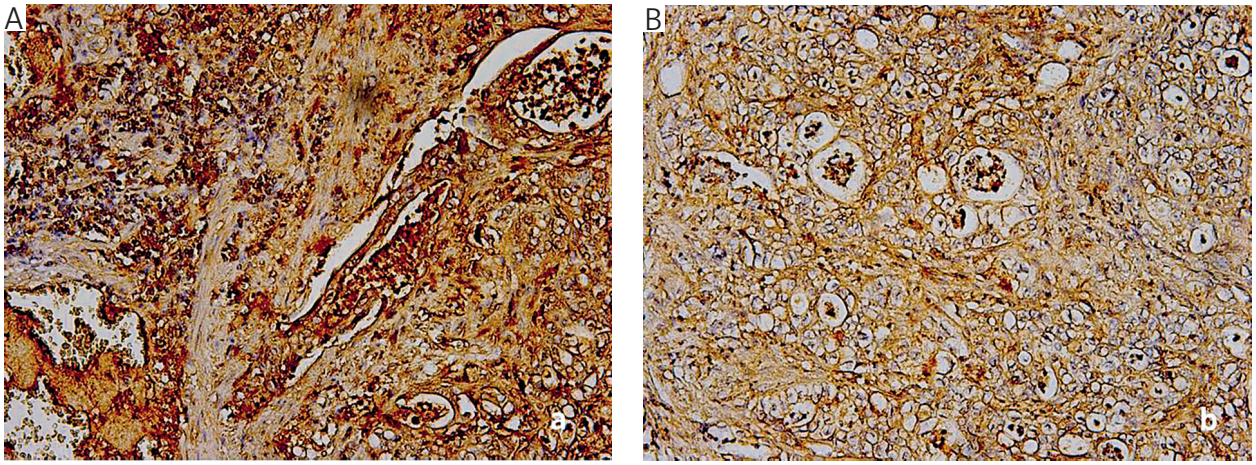

Fig. 1. A) Tumor cells cytoplasm TGF-β1 expression in gastric cancer intestinal type (original magnification 200×); B) tumor cells membrane TGF-β1-RII expression in gastric cancer intestinal type (original magnification 200×)

Table III. Correlations between TGF-β -509C/T polymorphism, TGF-β expression and its receptor – RII, and clinico-morphologicaly factors

	TGF-β1 POLYMORPHISM				TGF-β1 EXPRESSION			TGF-β–RII EXPRESSION		
	TC	CC	TT	P	YES	NO	P	YES	NO	P
Tumor				0.603			0.282			0.782
T1-2	5	3	–		7	1		5	3	
T3-4	19	12	4		24	11		20	15	
Nodules				0.509			0.330			0.184
N0	6	4	–		6	4		4	6	
N1-3	18	11	4		25	8		21	12	
Metastases				0.201			0.839			0.782
M0	21	12	2		10	25		20	15	
M1	3	3	2		6	2		5	3	
Stage				0.069			0.405			0.078
I-II	7	4	–		9	2		7	4	
III-IV	17	11	4		22	10		18	14	
Differentiation				0.155			0.783			0.037
Low	16	11	3		22	8		16	14	
Medium	11	3	1		8	3		9	2	
Well	3	0	1		1	1		-	2	
Histology type				0.976			0.214			0.560
Intestinal	19	12	3		26	8		19	15	
Diffuse	5	3	1		5	4		6	3	

The analysis of genotype distribution of the -509C>T SNP in the promoter region of *TGFβ1*-gene and clinical stage distribution revealed that among the 32 patients in III-IV clinical stage 53.1% were heterozygous (TC), 34.4% were homozygous for the C-allele and 12.5% were homozygous for the variant T-allele ($\chi^2 = 3.31$; $p = 0.069$) (Table III). No statistically significant correlation between genotype distribution and other parametrs was found.

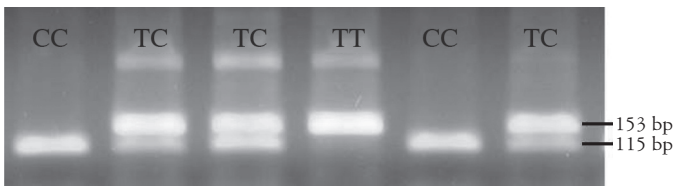

Fig. 2 Representative image of TGF-β1 -509 C/T genotypes

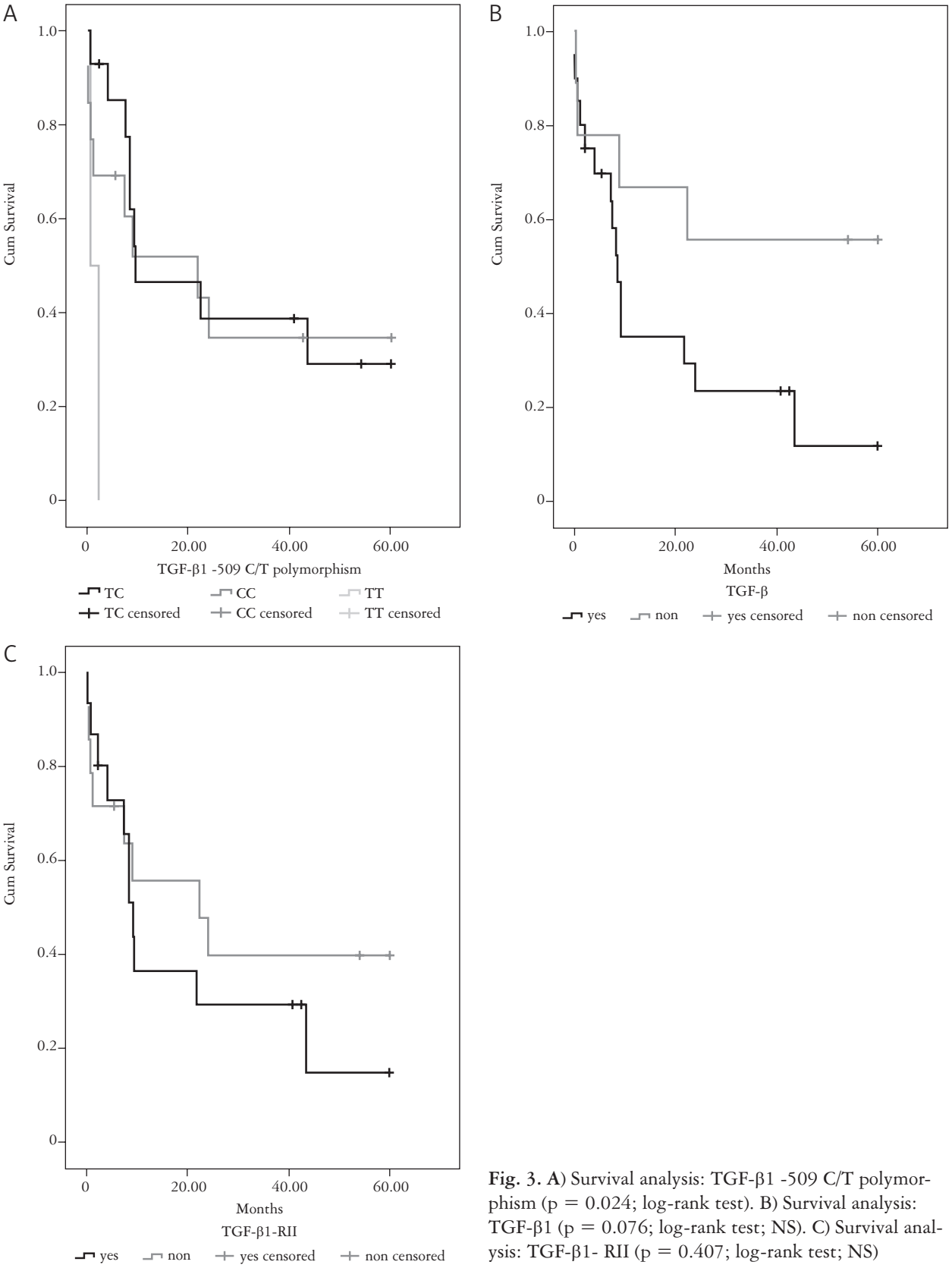

Fig. 3. A) Survival analysis: TGF-β1 -509 C/T polymorphism (p = 0.024; log-rank test). B) Survival analysis: TGF-β1 (p = 0.076; log-rank test; NS). C) Survival analysis: TGF-β1- RII (p = 0.407; log-rank test; NS)

Associations with patients'survival

Survival data were available for 29 patients.

For analyzing the impact of TGF-β1, TGF-β1 receptor type II expression and -509C>T *TGF-β1* SNP on the survival after surgery, the patient group was dichotomized according to expression in GCs tissues and polymorphism genotyping. At the end of the follow-up, 10 (34.5%) of the patients survived,

with median survival period of 25.05 months (range from 0.3 to 60.1 months).

With respect to the studied polymorphism it appeared that the carriers of TT-genotype had the shortest median survival compared to the carriers of the heterozygote genotype (CT) and the CC-genotype ($p = 0.024$, Log-rank test; Fig. 3A). After analysis of proteins expression we found that the survival time was shorter for the TGF-β1 and TGF-β-RII positive cases, compared with the survival time for negative GCs, although the differences did not reach statistical significance ($p = 0.076$ and $p = 0.407$ Log-rank test; Fig. 3B, C).

Discussion

As a socially significant disease affecting as much as 700 000 people worldwide every year [10], gastric cancer remains a huge challenge to researchers and intense efforts are put in identifying potential biomarkers for detection and early diagnosis in order to be prolonged patients' survival and chances for cure as prognosis is inversely related to the stage of the disease.

According to our obtained data a significant association between the -509C>T SNP and the stage of the disease and survival in the patients' group was observed, as well as between the expression of TGF-β1-RII and the differentiation stage of tumor. There was no significant association between the expression of TGF-β1 and other factors.

Also, our results indicate the involvement of TGF-β1 and other proteins of the TGF-β pathway at different gastric pathology. In metaplastic and dysplastic lesions the reactivity to TGF-β1 was more intense than in normal mucosa, and also there was a relation to the histological subtype of GC – a strong reactivity both as intensity and number of positive cells, especially in the diffuse variant of this type of neoplasms [11].

In our previous work we have reported that the positive rate of TGF-β1 in intestinal type gastric cancers were significantly higher than those in diffuse type gastric cancers and it was lower in moderate and high differentiated tumors. Also, we concluded that the TGF-β1 positive patients had a shorter median survival compared to TGF-β1-negative patients who underwent gastrectomry or curative resection. This is in agreement with our statement that upregulation of TGF-β1 is common in various types of cancer; it is not commonly regarded as a prognostic factor for survival [12].

TGF-β1 is a strong immunosuppressive cytokine produced by immune and non-immune cells, including tumor cells. Inhibition of TGF-β1 signaling pathway has been reported to prevent progression and metastasis of certain advanced tumors [13, 14]. Together with its receptor TGF-β1 receptor type II, they have a pivotal role in the progression and survival of GC. Also, we found published data that some tumors are refractory to the suppressive effect of TGF-β1 with the secretion of TGF-β1 [15]. Our results indicate a tendency between the expression of TGF-β1, TGF-β1-RII and a shorter post-operative survival in gastric cancer patients which seems in accordance with the aforementioned study.

In addition, a functional single nucleotide variation in position 509th of the promoter region of the *TGF-β1* gene has been intensively studied in GC patients. This SNP has been reported to affect *TGF-β1* expression and consequently affect plasma concentrations of circulating TGF-β1 [16] and has been related to clinicoptahological characteristics of GC patients in several studies [4, 17]. The molecular mechanism underlying the differential expression of TGF-β due to the -509C>T SNP has been elucidated in the study of Shah *et al*. [18]. The authors have demonstrated that the activator protein 1 (AP-1) down regulates the transcriptional activity of the gene binding to the ancestral C-allele in the promoter of *TGF-β1*. Thus, the presence of the variant T-allele at 509th position has been associated with higher plasma concentrations of the TGF-β1 protein, especially among individuals homozygous for the T-allele than in heterozygotes.

Although the studies on the association between the *TGF-β1* -509C>T SNP and GC risk are becoming more prevalent, the results are conflicting and inconclusive. In a meta-analysis conducted by Li *et al.* the authors concluded that *TGF-β1* T-allele is a susceptibility genetic factor for gastric cancer [3, 19]. Furthermore, the study have demonstrated that the TT-genotype was associated with increased GC risk in stages III and IV. In accordance with these data our results indicate that the carriers of the TT-genotype had shorter median survival then patients with TC- and CC-genotype. Additionally, we have found that patients in the III-IV stage of the disease are prevalently homozygous for the T-allele, than patients in stage I-II. Our results therefore support the thesis reported by several authors that once a tumor has developed, TGF-β1 promotes tumor growth, invasion and metastasis, as well as inhibiting immune surveillance.

Conclusively, our preliminary results indicate that the expression of TGF-β1, TGF-β1-RII and the TT-genotype of -509C>T polymorphism of *TGF-β1* are related to shorter survival time and rapid progression for the GC patients suggesting a worse prognosis for those patients.

The authors declare no conflict of interest.

References

1. Allemani C, Weir HK, Carreira H, et al. Global surveillance of cancer survival 1995-2009: analysis of individual data for 25,676,887 patients from 279 population-based registries in 67 countries (CONCORD-2). Lancet 2015; 385: 977-1010.
2. GLOBOCAN 2012. Stomach cancer: estimated incidence, mortality and prevalence worldwide in 2012 [Internet] 2012. [cited 2016 January 15]. Available from: http://globocan.iarc.fr/old/FactSheets/cancers/stomach-new.asp.
3. Li K, Xia F, Zhang K, et al. Association of a tgf-β1-509c/t polymorphism with gastric cancer risk: a meta-analysis. Ann Hum Genet 2013; 77: 1-8.
4. Liu HJ, Zhang QG, Wang YB, et al. TGF-β1-509C/T polymorphism and the risk of ESCC in a Chinese Han population. Int J Clin Exp Med 2015; 8: 11524-11528.
5. Kidd M, Schimmack S, Lawrence B, et al. EGFR/TGFα and TGFβ/CTGF Signaling in neuroendocrine neoplasia: theoretical therapeutic targets. Neuroendocrinology 2013; 97: 35-44.
6. Massague J. TGFbeta in Cancer. Cell 2008; 134: 215-230.
7. Padua D, Massague J. Roles of TGFbeta in metastasis. Cell Res 2009; 19: 89-102.
8. Markowitz SD, Roberts AB. Tumor suppressor activity of the TGF β pathway in human cancers. Cytokine Growth Factor Rev 1996; 7: 93-102.
9. Pourfarzi F, Whelan A, Kaldor J, Malekzadeh R. The role of diet and other environmental factors in the causation of gastric cancer in Iran – A population based study. Int J Cancer 2009; 125: 1953-1960.
10. Wang C, Zhang J, Cai M, et al. DBGC: A Database of Human Gastric Cancer. PLoS One 2015; 10: e0142591.
11. Docea AO, Mitruț P, Grigore D, et al. Immunohistochemical expression of TGF beta (TGF-β), TGF beta receptor 1 (TGF-BR1), and Ki67 in intestinal variant of gastric adenocarcinomas. Rom J Morphol Embryol 2012; 53 (3 Suppl): 683-692.
12. Ananiev J, Gulubova M, Tchernev G, et al. Relation between transforming growth factor-β1 expression, its receptor and clinicopathological factors and survival in HER2-negative gastric cancers. Wien Klin Wochenschr 2011; 123: 668-673.
13. Fu H, Hu Z, Wen J, et al. TGF-beta promotes invasion and metastasis of gastric cancer cells by increasing fascin1 expression via ERK and JNK signal pathways. Acta Biochim Biophys Sin (Shanghai) 2009; 41: 648-656.
14. Taylor A, Verhagen J, Blaser K, et al. Mechanisms of immune suppression by interleukin-10 and transforming growth factor-beta: the role of T regulatory cells. Immunology 2006; 117: 433-442.
15. Hawinkels LJ, Verspaget HW, van Duijn W, et al. Tissue level, activation and cellular localisation of TGF-beta1 and association with survival in gastric cancer patients. Br J Cancer 2007; 97: 398-404.
16. Grainger DJ, Heathcote K, Chiano M, et al. Genetic control of the circulating concentration of transforming growth factor type beta1. Hum Mol Genet 1999; 8: 93-97.
17. Watanabe Y, Kinoshita A, Yamada T, et al. A catalog of 106 single-nucleotide polymorphisms (SNPs) and 11 other types of variations in genes for transforming growth factor-beta1 (TGF-beta1) and its signaling pathway. J Hum Genet 2002; 47: 478-483.
18. Shah R, Hurley CK, Posch PE. A molecular mechanism for the differential regulation of TGF-beta1 expression due to the common SNP -509C-T (c. -1347C > T). Hum Genet 2006; 120: 461-469.
19. Bhayal AC, Prabhakar B, Rao KP, et al. Role of transforming growth factor-β1 -509 C/T promoter polymorphism in gastric cancer in south Indian population. Tumour Biol 2011; 32: 1049-1053.

Address for correspondence

Julian Ananiev, MD, PhD
Department of General and Clinical Pathology,
Medical Faculty, Trakia University
Armeiska Str.11
Stara Zagora, 6000
e-mail: julian.r.ananiev@gmail.com
tel. 00359 883 33 69 89