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Prothrombin 3'end Gene Variants in Patients With Sporadic Colon Adenocarcinoma

MARIJA CUMBO[1], BRANKO TOMIC[1], SOFIJA DUNJIC[1], TAMARA JOVANOVIC[1], MAJA GVOZDENOV[1], IVA PRUNER[1], GORANA ARALICA[2,3], SANJA KAPITANOVIC[4], TAMARA CACEV[4] and VALENTINA DJORDJEVIC[1]

[1]*Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Belgrade, Serbia;*
[2]*University Hospital Dubrava, Zagreb, Croatia;*
[3]*Medical School University of Zagreb, Zagreb, Croatia;*
[4]*Rudjer Boskovic Institute, Zagreb, Croatia*

Abstract. *Background/Aim: Thrombin plays significant roles in various types of cancer. However, the expression levels of prothrombin, the thrombin precursor, in cancer remain unclear. Variants of the 3'end of the prothrombin gene lead to increased prothrombin expression. This study aimed to analyze prothrombin 3'end gene variants in colon tumor and adjacent normal tissue samples. Materials and Methods: The study group consisted of 93 patients suffering from colon adenocarcinoma. The 3'end of the prothrombin gene was analyzed by DNA sequencing. Results: Three variants, all previously associated with increased prothrombin expression were detected. Frequency of the FII 19911G allele was 46.77% and 47.85% in tumor and normal tissue, respectively. For the FII 20210A allele, the detected frequencies were 2.15% and 1.61%, respectively. The frequency of the FII c.1824T allele was 0.54% in both tissues. Four patients showed different genotypes in tumor and normal tissue. Conclusion: Prothrombin 3' end gene variants may play a role in colorectal cancer.*

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females globally (1). Adenocarcinoma, a cancer starting in mucous glands, is the most common type of CRC, accounting for about 95% of CRC cases (2). Approximately 75% of CRC cases are sporadic, and occur without a family history of the disease (3). Despite the extensive research, the molecular mechanisms of CRC are still not fully elucidated.

A close relationship between coagulation and cancer has been recognized for more than a century. In the context of CRC, there is growing incidence that thrombotic disorders are associated with significant mortality and morbidity (4). Studies have shown increased levels of coagulation factors and markers in cancer patients, thus explaining the presence of hypercoagulable state (5, 6). The recent data suggest that coagulation factors, particularly thrombin, may also have important roles in carcinogenesis.

Thrombin, a serine protease, plays a pivotal role in hemostasis, involving both pro- and anti-coagulant activities (7). Besides its central role in hemostasis, evidence has shown that thrombin can affect different processes in malignant cells through protease activated receptors (PAR), including promotion of cell proliferation and invasion (8). In addition to that, thrombin has been shown to promote tumor angiogenesis, which is one of the essential processes in tumor growth and metastasis (9, 10).

The precursor of thrombin, prothrombin, is predominantly synthesized in the liver and secreted into the bloodstream in the form of zymogen (11). The 3' untranslated region of the prothrombin gene has a noncanonical structure, which is susceptible to gain-of-function variants that can lead to increased prothrombin expression, and consequently to increased risk of thrombosis (12).

It is documented that certain colon cancer cells are capable of prothrombin expression (13), but the influence of the 3' end of the prothrombin gene variants on cancer and the complex genotype-phenotype correlations in colon cancer have been very poorly studied.

In this study we aimed to analyse the 3' end of the prothrombin gene in tumor and normal tissues adjacent to the tumor from patients with colon adenocarcinoma.

Correspondence to: Marija Cumbo, MSc, Vojvode Stepe 444A, P.O. Box 23, 11010 Belgrade, Serbia. Tel: +381 113976658, Fax: +381 113975808, e-mail: marijacumbo@imgge.bg.ac.rs

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Table I. *Clinicopathological characteristics of patients with colon adenocarcinoma.*

	No. of cases	% of cases
Total number	93	100.0
Gender		
Male	54	58.1
Female	39	41.9
Dukes stage		
A	18	19.4
B	26	28
C	43	46.2
D	6	6.5
Tumor size†		
≤5 cm	49	59.8
>5 cm	33	40.2
Histological grade (differentiation)‡		
Poor	7	10.1
Moderate	43	62.3
Well	19	27.5

†data available for 82 patients; ‡data available for 69 patients.

Table II. *Genotype combinations found in patients with colon adenocarcinoma.*

Gene variants in 3' end of prothrombin gene			Number of patients N (%)
FII A19911G	FII G20210A	FII c.1824C>T	
AG	GG	CC	40 (43.0)†
GG	GG	CC	23 (24.7)
AA	GA	CC	1 (1.1)
AG	GA	CC	2 (2.2)‡
AG	GG	CT	1 (1.1)
AA	GG	CC	26 (28.0)

†three patients had different genotypes in tumor tissue compared to normal tissue; ‡one patient had different genotype in tumor tissue compared to normal tissue.

Materials and Methods

DNA samples. Colon tumor and normal adjacent tissue (NAT) samples were obtained from the Croatian Tumor Bank (14). Samples were collected during routine surgery performed in 93 patients with sporadic colon adenocarcinoma, all histopathologically characterized. To determine the proportion of tumor cells, each sample was examined by routine hematoxylin-eosin staining. Fresh tissue samples were frozen in liquid nitrogen and stored at –80˚C until use. DNA extraction from tumor and normal tissue was performed using proteinase K digestion and phenol chloroform extraction (15).

Written informed consent was obtained from all patients included in the study. The study was approved by the Ethics Committee of Clinical Hospital Dubrava, Zagreb (approval was obtained on 27th Apr. 2016) and was performed in accordance with the ethical standards of the Helsinki Declaration.

Sequencing of the 3' end of the prothrombin gene. The 715 bp fragment located within the 3' end of the prothrombin gene (including the last intron and exon, 3' untranslated region and 3' flanking region) was amplified by polymerase chain reaction (PCR) using 5'-GGAAACGAGGGGATGCCTGT-3' and 5'-CCTGCCATCTTTCCT CTCAC-3' primers. Amplification conditions were: initial denaturation at 95˚C for 5 min, 39 cycles at 95˚C for 1 min, 61˚C for 1 min and 72˚C for 1 min, and final elongation at 72˚C for 10 min. Generated fragments were sequenced with 5'-GAATAGCACTGGGAGCATTGA-3' and 5'-TCTAGAAACAGTTGCCTGGC-3' primers using the BigDye™ Terminator Version 3.1 Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol, on a 3130 Genetic Analyzer (Applied Biosystems).

Haplotype analysis. Prothrombin DNA fragment of interest (715 bp) was cloned into the pJET1.2/blunt vector, using the CloneJet PCR Cloning Kit according to the manufacturer's protocol (Thermo Scientific, Waltham, MA, USA). After cloning, plasmids were extracted using Pure Link HQ Mini Plasmid Purification Kit (Invitrogen, Carlsbad, CA, USA). The cloned fragments were then sequenced with 5'-ATAGCACTGGGAGCATTGAAGC-3' and 5'-GGATGGGAAATATGGCTTCTAC-3' primers using the BigDye™ Terminator Version 3.1 Ready Reaction Kit (Applied Biosystems) according to the manufacturer's protocol, on a 3130 Genetic Analyzer (Applied Biosystems).

Results

Analysis of the 3' end of the prothrombin gene on tumor and NAT tissues was performed on samples from 93 patients (54 male and 39 female) with colon adenocarcinoma. Average age of patients was 66 years (range=34-90 years). Clinicopathological data of patients are listed in Table I.

In both, tumor and normal colon tissues, three prothrombin variants were detected: FII A19911G (FII c.*1726-59A>G), FII G20210A (FII c.*97G>A), and FII c.1824C>T.

Frequency of the allele FII 19911G was 46.77% in tumor and 47.85% in NAT, of the allele FII 20210A was 2.15% in tumor and 1.61% in NAT, and of FII c.1824T was 0.54% in both tissues.

The frequency of the FII 19911AG genotype in tumor tissue and NAT was 41.9% and 46.2%, respectively, while the frequency of the FII 19911GG genotype in tumor tissue and NAT was 25.8% and 24.7%, respectively. The frequency of the heterozygous genotype for FII 20210GA in tumor tissue and NAT was 2.2% and 3.2%, respectively, while the frequency of the FII 20210AA genotype in tumor tissue was 1.1%, but it was not detected in normal tissue. Combinations of genotypes found in patients are shown in Table II.

Differences in genotypes in tumor and normal tissue were detected in four patients. Two patients were non-carriers (tumor tissue)/heterozygous (NAT) and one patient was homozygous (tumor tissue)/heterozygous (NAT) for the FII A19911G variant only. One patient was a carrier of different

Table III. *Clinicopathological data for patients with colon adenocarcinoma carrying different genotypes in tumor tissues compared to NAT.*

	Gene variants in 3' end of prothrombin gene				Tumor characteristics				
	FII A19911G		FII G20210A		Age (years)	Gender	Dukes	Grade	Tumor size (cm)
	Tumor	Normal	Tumor	Normal					
1	AA	AG	GG	GG	75	M	C	3	4
2	AA	AG	GG	GG	68	F	C	2	4
3	GG	AG	GG	GG	71	M	A	2	4
4	AA	AG	AA	GA	76	M	C	/	5

All four patients did not carry the FII c.1824C>T. M: Male; F: female; NAT: normal adjacent tissue.

tissue genotypes for FII G20210A - homozygous (tumor tissue)/heterozygous (NAT), and for the FII A19911G variant - non-carrier (tumor tissue)/heterozygous (NAT). Detected genotypes and clinical characteristics of these patients are given in Table III.

The NAT sample of a patient (number 4 in Table III) with different genotypes in both FII A19911G and FII G20210A variants was used for haplotype analysis. Analysis of the cloned sequence (a total of 16 clones were sequenced and analyzed) showed the presence of four different haplotypes in this tissue: 19911A-20210G, 19911A-20210A, 19911G-20210G and 19911G-20210A.

Discussion

Besides its role in hemostasis, thrombin is known to affect different aspects of tumor biology; proliferation and migration of malignant cells, metastasis and angiogenesis (10). Studies on mice have shown that thrombin has a critical role in colon cancer growth and dissemination through stromal PAR-1 and fibrinogen (16). Analysis of human colon cancer samples determined the presence of Fragment F1+2, byproduct of prothrombin activation to thrombin, thus indirectly indicating the presence of prothrombin in tumor, although its origin still remains unclear (17). Prothrombin expression in colon cancer has been previously examined in few studies (13, 18). Dunjic *et al*. have examined whether cancer-derived cell lines express prothrombin. Results showed that Caco-2 cells, originating from colorectal adenocarcinoma, express prothrombin although in notably lower levels compared to the control liver sample (13). Besides the reported results, prothrombin expression and its regulation in colon cancer are still unclear.

Expression of the prothrombin gene is known to be significantly regulated by its 3' end, which is susceptible to gain-of-function variants that lead to increased prothrombin expression (12). In this study, we examined the presence of 3' end variations of the prothrombin gene in tumor and normal tissue adjacent to tumor in patients with colorectal adenocarcinoma. We detected three variants – FII G20210A, FII A19911G and FII c.1824C>T, in both types of tissues (Tables II and III). All three variants have been previously described and found to be associated with increased prothrombin expression (12, 19, 20).

The FII G20210A gene variant is located in the 3' untranslated region of the prothrombin mRNA, at the position where the pre-mRNA is endonucleolytically cleaved and polyadenylated (12). It causes increased cleavage site recognition, increased 3' end processing and increased mRNA accumulation and protein synthesis (21). In Caucasians, G20210A is present with a frequency of 0.7 to 4%, and an average of about 2% (22). In the Croatian population, its frequency is about 4% in patients with venous thromboembolism (VTE) and 6% in healthy controls (23). In our study, we detected this variant in the heterozygous state in NAT samples in three patients (3.2 %). Among them, two patients were heterozygous carriers in the tumor tissue also, while one was a homozygous carrier. Vossen *et al*. have examined the correlation of clotting factor gene variants and colorectal cancer risk in a large German population-based case-control study, and found that heterozygous carriers of G20210A had a reduced risk compared to non-carriers (24). They considered that this might be due to the influence of the levels of thrombin on carcinogenesis, where low thrombin concentrations could exert protective effects by inducing endothelial barrier protection (24, 25).

The FII A19911G, a common gene variant located in the last intron of the prothrombin gene, is associated with slightly increased plasma prothrombin levels (19). This gene variant leads to higher splicing efficiency in the final intron, and also slightly increases the risk of thrombosis in carriers of the G20210A variation (26, 27). In our study, we detected this variant in 40.8% patients in the heterozygous state (one patient being heterozygous carrier of FII G20210A also), and in 24.7% patients in the homozygous state. Currently, there are no data regarding this variation frequency in the

Croatian population. Data for the geographically and genetically close Serbian population (28) have shown that heterozygous and homozygous carriers in the healthy population are represented with frequencies of 50% and 18.3%, respectively (29).

The FII c.1824C>T gene variant is located in the last exon of the prothrombin gene. It is considered as a synonymous variant, since it does not result in a change in the amino acid sequence of the protein. *In vitro* functional assays revealed that the FII c.1824C>T gene variant increased expression levels 1.64 times compared to the wild type, but the exact mechanism of overexpression has not yet been elucidated (20). In our study, we detected this variant only in one patient in the heterozygous state, who was also a heterozygous carrier of A19911G. This is the first report of this variant in the Croatian population.

Analysis of tumor tissue genotypes showed that four patients displayed different genotypes in the tumors compared to NAT (Table III). Since patients were heterozygous carriers in normal tissue and homozygous carriers/non-carriers in the tumor tissue, the transformation to tumor tissue could have been due to loss of heterozygosity (LOH) or *de novo* mutation. Loss of heterozygosity is one the most common features of cancer, with an average of 25-30% of alleles being lost (30). The gene encoding prothrombin is located in chromosome 11p11.2. The LOH on the long arm (31) and short arm (11p11) (32) of chromosome 11 has been previously described in colorectal cancer, but, to our knowledge, there are no data available on LOH regarding the prothrombin gene.

To further examine this, normal tissue of patient with changes in both FII A19911G and FII G20210A variants (Table III), was analyzed for haplotypes. Analysis of haplotypes showed a rather complex picture – there were four different haplotypes in only 16 analyzed clones: 19911A-20210G, 19911A-20210A, 19911G-20210G and 19911G-20210A. Some studies, concerning comparative analysis of different tumors and their normal adjacent tissue, concluded that NAT presents a unique intermediate state between tumor and healthy tissues (33).

To our knowledge, our study is the first to compare genotypes at the 3' end of prothrombin gene in tumor tissue and NAT in patients with colon adenocarcinoma. Though we detected a small number of patients with different tumor and NAT genotypes, which could be due to the size of our study group, existing differences in tissues' genotype in these patients could offer some information about the nature of colon cancer pathogenesis. Nevertheless, further and larger studies should be conducted to elucidate expression of this gene in malignant colon tissue and the relevant mechanisms, as well as to minutely examine its role in colon tumorigenesis. It is expected that the obtained findings will contribute to further elucidation of complex genotype-phenotype correlations in patients suffering from this disease.

Conflicts of Interest

The Authors declare that there is no conflict of interest regarding this study.

Authors' Contributions

Cacev T and Djordjevic V were responsible for study design. Cacev T and Aralica G were responsible for sample and data collection. Jovanovic T, Cumbo M and Dunjic S and were responsible for experimental work. Tomic B, Gvozdenov M and Pruner I were responsible for data management and interpretation of results. Cumbo M, Tomic B and Djordjevic V were responsible for writing the manuscript. Kapitanovic S, Cacev T and Djordjevic V were responsible for supervision of the manuscript.

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