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Expression of individual members of the TGF-β/SMAD signalling pathway in the progression and survival of patients with colorectal carcinoma

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Current knowledge of tumor biology offers many "targets" for therapeutic intervention. The molecular basis of many processes that play a role in the pathogenesis of colorectal cancer has been identified. One part of colorectal cancer clinical trials is focused on testing substances in a group of patients with tumors in which the TGF-β signalling pathway is hyperactivated. The TGF-β/SMAD signalling pathway members are considered important markers; however, genetic, proteomic, or metabolomic analyses still yield controversial results. According to our results, TGF-βRII, and SMAD4 can be used in monitoring CRC progression. With increasing CRC stage, TGF-βRII expression decreases and SMAD4 expression increases. The patients with TGF-βRII expression lower than 700 pg/ml had a slightly lower survival time (28.103 months) than patients with higher TGF-βRII expression (31.620 months). Conversely, patients with SMAD4 expression lower than 200 pg/ml had a higher survival rate (30.979 months) than patients with higher expression (26.316 months). Regarding TGF-β1 expression, the patient´s survival assessment determined no significant difference between patients with high or low tissue TGF-β1 expression. A personalized approach and consideration of a wide range of factors are important when using these markers in treatment assessment.

Keywords TGF-β/SMAD signalling pathway, Colorectal carcinoma

Based on current data, colorectal cancer (CRC) ranks third globally in terms of cancer incidence. Over 1.9 million new cases and 930,000 fatalities related to CRC were reported in 2020. According to the World Health Organization (WHO), early-diagnosed CRC (without metastatic spread to distant organs) is standardly treated by surgical removal of the tumor (colectomy, proctectomy) together with nearby lymph nodes. After surgery to reduce the risk of cancer recurrence, adjuvant therapy is administered, most commonly in the form of chemotherapy. In metastatic CRC, chemotherapy is the primary line of treatment. Chemotherapy combined with targeted therapy is used in patients with specific genetic mutations (e.g. KRAS, BRAF). Immunotherapy is considered for patients with tumors exhibiting specific genetic markers (e.g., MSI-H).

The current guidelines from the European Society for Medical Oncology (ESMO) state that patients should gradually receive chemotherapy, all three available cytostatics (fluoropyrimidines, oxaliplatin, and irinotecan), and all targeted biological agents (antiVEGF and, in RAS wt patients, antiEGFR). ESMO reports that in patients with localized colon tumors, MSI/MMR status is the only validated molecular marker used in adjuvant decision-making and should be determined in stage II CRC. In stage III, usage of MMR status is limited to detect and identify Lynch syndrome.

However, these therapies are nonspecific and cytotoxic, leading to serious secondary complications in most cases[1]. The combination of therapies is approached considering several factors, of which the tumor margin and CRC progression are particularly relevant. Nevertheless, the use of combination therapy still results in half of

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the patients developing multidrug resistance. Therefore it is essential to establish new therapeutic strategies targeting all clinical stages of CRC[2].

The goal of clinicians in various fields of medicine is to select markers to identify patients who will benefit the most from treatment, with the lowest possible side effects, and thus make healthcare costs more efficient. Patients can obtain new medications or experimental therapies through clinical trials. Clinical trial participation contributes to the advancement of medical knowledge and may present novel treatment alternatives. One arm of the clinical trials is focused on testing substances in which the TGF-β signalling pathway is hyperactivated.

TGF-β signalling may have contrasting roles in tumor development. On the one hand, it can promote tumor proliferation, invasion, epithelial-mesenchymal transport (EMT), angiogenesis, metastasis formation, and immune escape, but on the other hand, it can promote tumor suppression by regulating cell proliferation, apoptosis (cell cycle arrest in G1 phase), and immune cell modulation[3], which is referred to as the so-called "TGF paradox". Thus, TGF-β signalling plays a dual role during cancer. In premalignant cells, it acts as a tumor suppressor, while in tumor cells, it is a promoter. Indeed, once TGF-β signalling is activated, cells may either undergo apoptosis or become more malignant and acquire mesenchymal properties to promote metastasis formation[4]. Many human cancers, including CRC, are resistant to TGF-β mediated inhibition of signalling in both normal epithelial cells and tumor cells. This resistance may be due to mutation, functional inactivation, or reduced expression of individual members of the signalling pathway[5].

In this article, we focused on the role of individual members of the TGF-β signalling pathway, TGF-β receptor, TGF-β protein, and the very important member SMAD4 in the progression and survival of patients with colorectal cancer.

Materials and methods

The analyzed sample consisted of 103 patients admitted to the First Surgical Clinic of the Louis Pasteur University Hospital in Košice for the need for colon or rectal surgery. A tissue sample from each patient was sent for histological examination and based on the findings, the patients were categorized into a group of patients with benign tumors (BTG; 29 patients; 28.16%) and a group of patients with malignant tumors (MTG; 74 patients; 71.84%). The mean age in the patient group was 66 (45–91). The characteristics of all patients are shown in the table (Table 1). Patients with malignant tumors were subsequently divided into CRC stages according to the standard TNM classification.

All selected patients were informed about the conduct and objectives of the study and provided their informed consent (consent for inclusion in the study, consent for collection of biological material, consent for processing of personal data) before they participated in the study, i.e., before the biological material was collected. The study was carried out based on the Declaration of Helsinki, and the protocol was approved by the Ethics Committee, 2020/EC/06042.

The morning before surgery, patients had fasting blood drawn into a BD Vacutainer tube containing separation gel for serum and into a K_2EDTA tube for plasma. Serum was obtained after centrifugation of blood at 3,500 x g/ 4 min/ RT and plasma after centrifugation of blood at 2,000 x g/ 10 min/ 4 °C. Serum and plasma samples were frozen at -80 °C until laboratory analysis.

Tumor tissue (primary epithelial tumor) was removed during surgery in the operating room and sent to the Department of Medical and Clinical Biochemistry of UPJŠ University in Košice, where it was weighed, cleared of blood using PBS, and homogenized in extraction buffer on ice (T-PER Tissue Protein Extraction Reagent, ThermoFisher). After a short incubation, tissue homogenates were centrifuged at 18,000 x g/ 20 min/ 4 °C) and the supernatant of each sample was frozen at -80 °C until laboratory analysis.

Criteria	Number of patients			
		Malignant tumor group (MTG)		
	Benign tumor group (BTG)	Stage 1	Stage 2	Stage 3
Sex				
Males	12	11	18	15
Females	17	10	11	9
Tumor localization				
Gut	20	10	16	14
Rectum	9	11	13	10
Finding				
Hemorrhoids	4	-	-	-
Diverticulitis	5	-	-	-
Adenoma	20	-	-	-
Adenocarcinoma	-	21	29	24
Survival				
Living	26	15	19	11
Deceased	3	6	10	13

Table 1. Classification of patients based on individual criteria.

The quantification of proteins in the sample was performed using the standard Bradford method. The absorbance of samples with Bradford reagent was measured spectrophotometrically on a Biophotometer Plus (Eppendorf, Hamburg, Germany) at a wavelength of 450 nm. All samples were measured in duplicate. Based on the equation from the standard curve, the absorbances were converted to total protein concentrations.

Western blot: To confirm the SMAD4 results from ELISA, samples were analyzed by western blot, which was running in 4–12% polyacrylamide gel at 150 V. After electrophoresis, proteins from the gel were transported to the nitrocellulose membrane using (semi-dry transfer) at 15 V. Therefore, the membrane was washed and incubated in primary antibody (SMAD4 Polyclonal Antibody, Invitrogen PA5-34806) overnight at 4ºC. The next day, the membrane was rewashed and incubated in a secondary antibody (Mouse Anti-rabbit IgG mAb, Cell Signaling L27A9) for 1 h at 37ºC. After another thorough washing, blotting substrate (Peroxide Solution and Luminol Enhancer Solution 1:1; Invitrogen WJ335099) was added to the membrane, and then SMAD4 was visualized on the membrane using the iBright™ FL1500 Imaging system (Invitrogen) as a black band on the white background. GAPDH (FL-335):sc-25,778 (Santa Cruz Biotechnology) was used as a control.

ELISA

Expressions of TGF-βRII (Human TGF beta R2 ELISA Kit; Abcam; ab193715), TGF-β1 (Human TGF beta 1 ELISA Kit; Abcam; ab100647), and SMAD 4 (Human SMAD4 ELISA Kit; Abcam; ab253211) were detected in blood serum samples and tissue homogenates by ELISA method. The absorbance of the samples was measured on an ELc808 Microplate Reader (Biotech, USA) at a wavelength of 450 nm.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 8.0.1. Continuous variables were presented as average +/- standard deviation. Categorical variables were expressed as numbers and percentages. The normality of the distribution was assessed using the Shapiro-Wilk test. Our data had a non-normal distribution of values, so the non-parametric Dunn's multiple comparisons test was used to compare the groups. ROC curves were used to determine the reliability and specificity of the selected comparisons. The Kaplan-Meier test in IBM SPSS Statistics 23 software was used to provide comparisons of patient survival.

Gene expression results obtained from the GEPIA database

Gene expression data for individual members of the TGF-β signalling pathway are publicly available in online gene databases. The following data are compiled and modified from http://gepia.cancer-pku.cn/. We searched this database for gene expression of individual members of the TGF-β signalling pathway in patients with CRC and compared it with the expression of these genes in other gastrointestinal tumors. The trend of gene expression for TGF-βRII, TGF-β1, and SMAD4 in CRC was opposite to that in the remaining tumor types (Fig. 1), so we decided to focus on their protein levels.

The results recorded in the Gepia database further demonstrated that no statistically significant differences were observed between the groups for any of the selected genes after stratifying the patients into different clinical stages of CRC (Fig. 2).

Survival curves as a function of the expression of selected genes according to the Gepia database indicate that patients with high TPM (Transcript per million) values for each selected gene have a slightly lower survival time compared to patients with low TPM values (Fig. 3). A statistically significant difference was confirmed only for the TGF-β1 gene ($p = 0.038$; Fig. 3B).

Our results

TGF-βRII

The analysis of tissue TGF-βRII expression in CRC patients stratified by histological findings and TNM classification showed that the highest values were observed in stage 1 patients (938.01 pg/ml; SD 216) and the lowest values were observed in stage 3 CRC patients (405.59 pg/ml; SD 193). The difference between the groups was statistically significant ($p = 0.0005$). Other important data from the graph (Fig. 4I) are presented in the table (Table 2).

Based on the ROC curves we found that the sensitivities and specificities of the individual assays using TGF-βRII expression are very high and statistically significant. The most reliable results are provided by the comparison of groups of patients in stage 1 and stage 2 of CRC (AUC 97.96%; 95% CI 0.9282–1.000) with $p = 0.0005$. Other selected statistically significant data are presented in the table (Table 3). Based on these data, it is more reliable and specific to compare patients in the first stage with patients in the higher stages.

Patient survival was assessed by the Kaplan-Meier test (Fig. 5A), which showed that patients with TGF-βRII expression lower than 700 pg/ml had a slightly lower survival time (28.103 months; 95% CI 0.2313–0.3303) compared to patients with higher TGF-βRII expression (31.620 months; 95% CI 0.2532–0.3791; $p > 0.05$).

The same trend in the change of TGF-βRII expression in CRC patients was also observed using blood serum samples (BTG 219 pg/ml; stage 1: 349 pg/ml; stage 2: 182 pg/ml; stage 3: 65 pg/ml), but due to more significant variations, no statistically significant difference was confirmed between any of these patient groups (supplementary file).

After stratifying CRC patients based on gender, we found that the highest tissue expression of TGF-βRII in males was in the group of patients in the first stage of CRC (1,057.44 pg/ml; SD 226) and the lowest in the group of patients in the third stage of CRC (330.17 pg/ml; SD 220) with $p = 0.0036$. In females, the tissue expression of TGF-βRII was highest in the group of patients with benign tumor (952.52 pg/ml; SD 412) and lowest in the group of patients with stage II CRC (368.01 pg/ml; SD 60) with $p = 0.0078$. Other important data from the graph (Fig. 4I A, B) are presented in the table (Table 2).

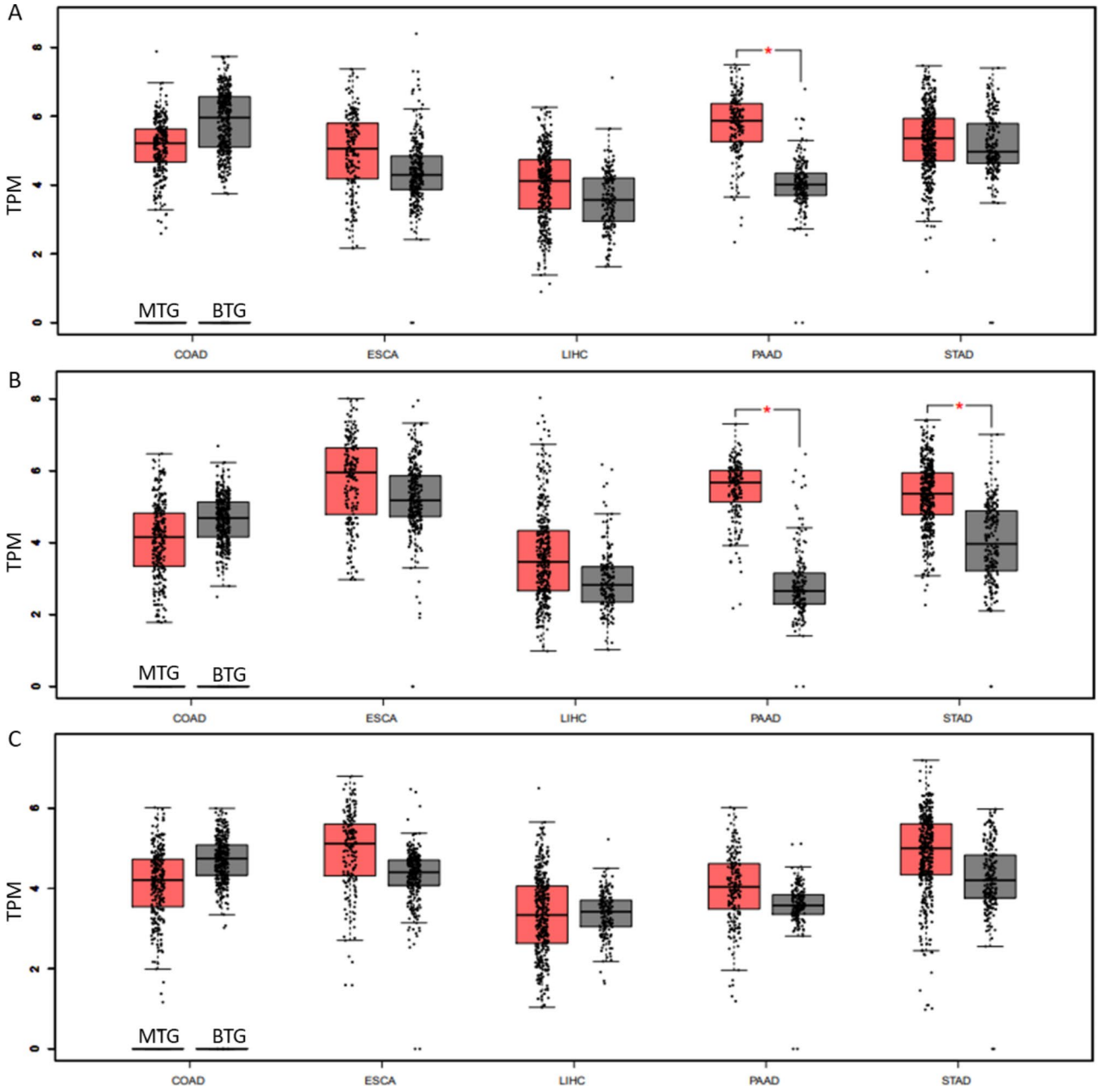

Fig. 1. Expression of genes for TGF-βRII (**A**), TGF-β1 (**B**), SMAD4 (**C**) in tissues of CRC patients compared with other types of GIT tumors (MTG: Malignant tumor group; BTG: Benign tumor group; TPM: Transcript per million; COAD: Colorectal Adenocarcinoma; ESCA: Esophageal carcinoma; LIHC: Liver hepatocellular carcinoma; PAAD: Pancreatic adenocarcinoma; STAD: Stomach adenocarcinoma; *: $p < 0{,}05$).

Based on the ROC curves, we found that the sensitivities and specificities of the individual assays using TGF-βRII expression were high and statistically significant in both sexes, especially in females. The most reliable results are provided by the comparison of groups of female patients in the benign tumor group versus those in stage 2 of CRC (AUC 98.95%; 95% CI 0.9782–1.000) with $p = 0.0082$. Other selected statistically significant data are presented in the table (Table 3).

TGF-β1

Analysis of tissue expression of TGF-β1 ligand in CRC patients of both sexes is divided based on histological findings and TNM classification and showed that the values in all groups were very similar and there was no statistically significant difference between them (Fig. 4II; Table 2).

Patient survival assessed by the Kaplan-Meier test (Fig. 5B) determined no significant difference between patients with high or low tissue TGF-β1 expression. The survival time of patients with TGF-β1 expression higher

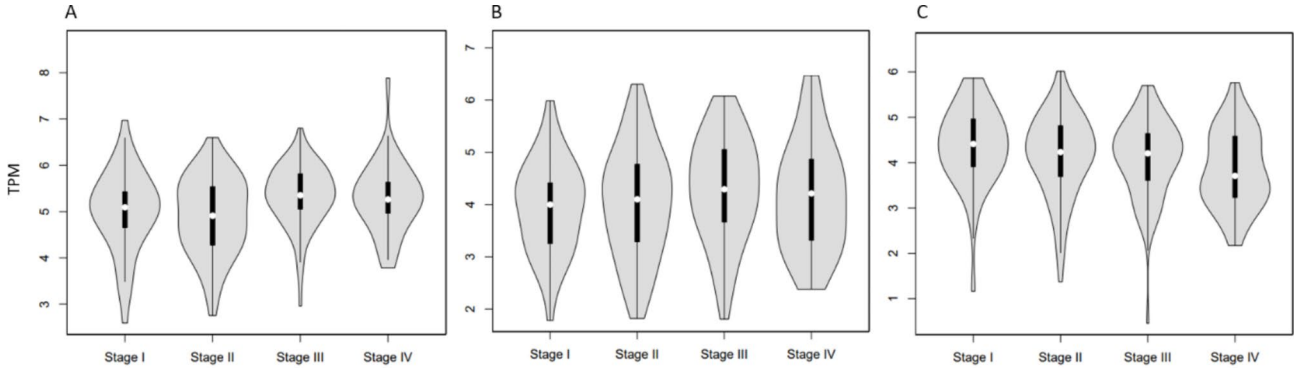

Fig. 2. Gene expression for TGF-βR2 (**A**), TGF-β1 (**B**), and SMAD4 (**C**) in CRC patients divided into different clinical stages (TPM: Transcript per million).

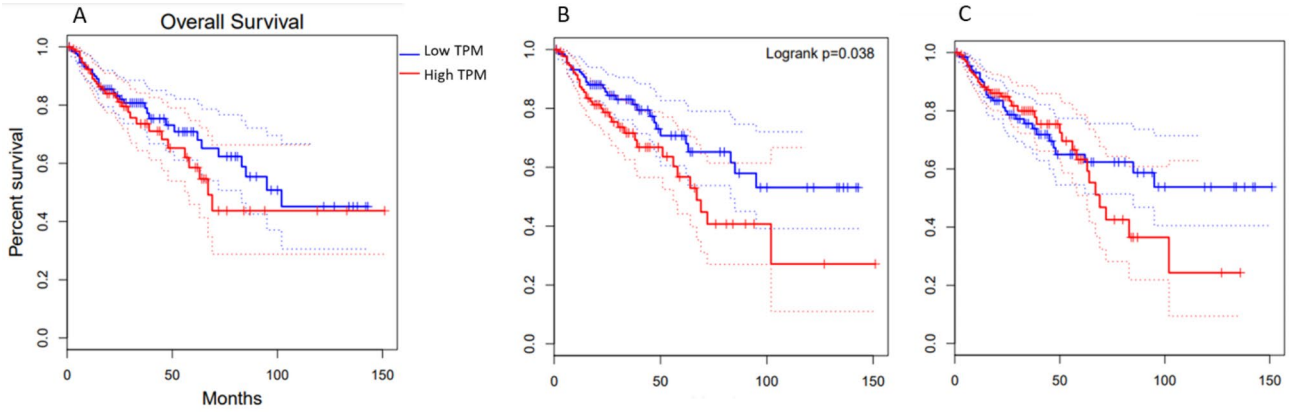

Fig. 3. Survival curves as a function of gene expression for TGF-βRII (**A**), TGF-β1 (**B**), and SMAD4 (**C**) in CRC patients (TPM: Transcript per million).

than 4,000 pg/ml was 29.61 months (95% CI 0.2187–0.3735) and in patients with lower expression was 27.95 months (95% CI 0.21.72–0.3417; $p > 0.05$).

After dividing CRC patients based on gender, we found that the highest tissue TGF-β1 expression in males was in the group of patients with stage 3 CRC (5,669.52 pg/ml; SD 164) and the lowest in the group of patients with benign tumors (3,332.33 pg/ml; SD 721) with $p = 0.0085$. On the other hand, in females, the tissue expression of TGF-β1 was highest in the group of patients with benign tumors (5,073.44 pg/ml; 553) and lowest in the group of patients with stage 2 CRC (3,036.36 pg/ml; SD 1,011) with $p = 0.0126$. In the group of patients with stage 1 CRC, we had a small set of measured samples, which were not sufficient to demonstrate statistically significant differences between groups. Other important data from the graph (Fig. 4II A, B) are shown in the table (Table 2).

Based on the ROC curves, we found that the sensitivities and specificities of the individual assays using tissue TGF-β1 expression were high and statistically significant in both sexes, again, especially in females. As in the case with TGF-βRII, a comparison of groups of female patients in the benign tumor group versus those in stage 2 CRC provided the most reliable results with $p = 0.0062$. Other selected statistically significant data are presented in the table (Table 3).

SMAD4

Within the analysis of the tissue expression of SMAD4 in CRC patients divided on both histological findings and TNM classification, we observed an increase in values with increasing stage, a trend opposite to that reported for TGF-β type II receptor. In this case, the highest values were observed in stage III patients (310.33 pg/ml; SD 166), and, on the contrary, the lowest values were in stage I CRC patients (137.22 pg/ml; SD 56). The difference between the groups was statistically significant ($p = 0.0289$). Other important data from the graph (Fig. 4III) are presented in Table 2.

Based on the ROC curves we found the most reliable results provided by the comparison of the groups of patients in stage 1 and stage 2 of CRC (AUC 89.29%; 95% CI 0.7531–1.000) with $p = 0.0041$. Other selected statistically significant data are presented in the table (Table 3). Based on these data, it is more reliable and specific to compare patients in the first stage with patients in the higher stages.

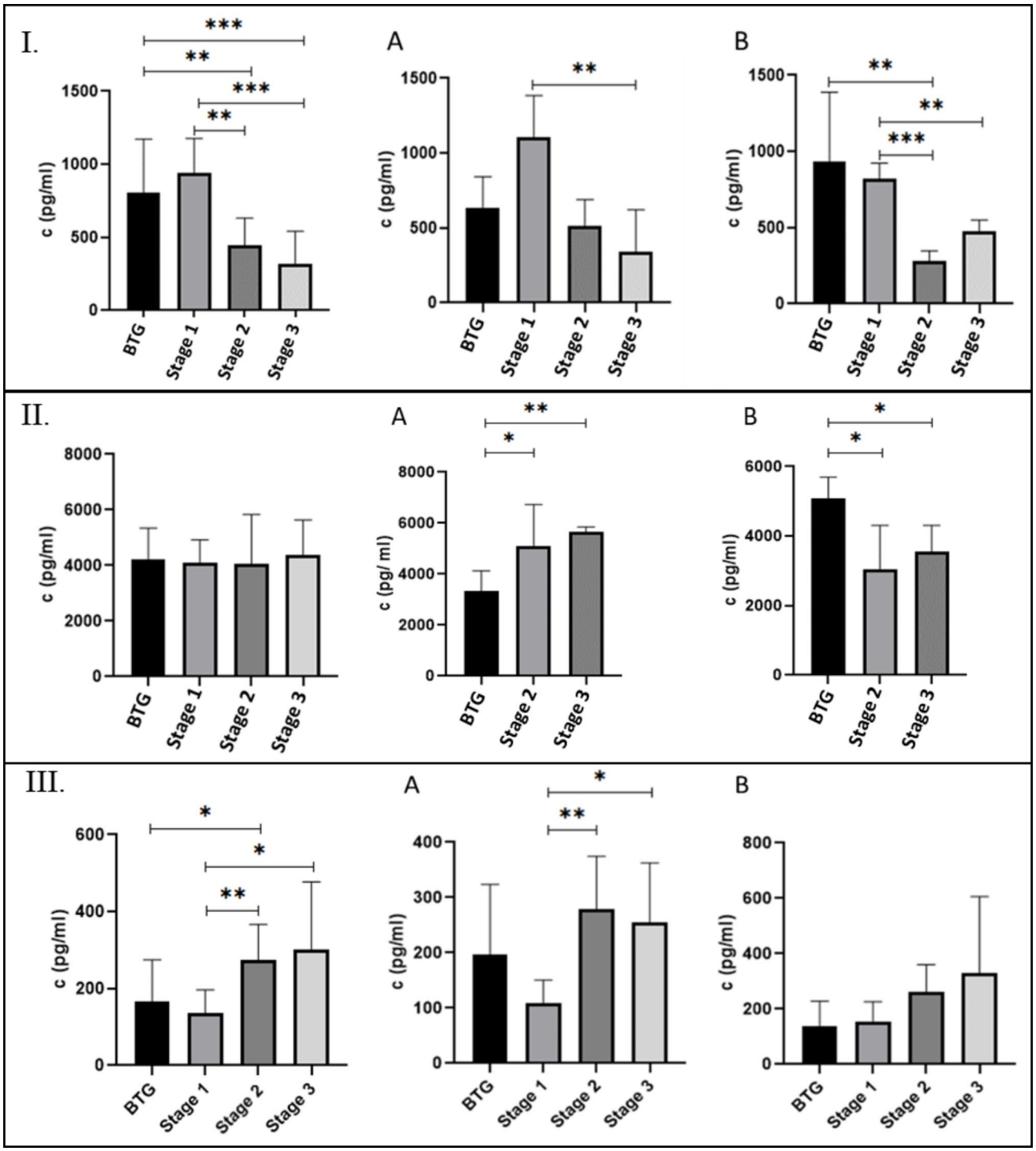

Fig. 4. Expression of tissue TGF-βRII (**I**), TGF-β1 (**II**), and SMAD4 (**III**) in CRC patients divided according to TNM classification markers and gender (A-males and B-females) patients.

Patient survival assessed by the Kaplan-Meier test (Fig. 5C) showed that patients with SMAD4 expression lower than 200 pg/ml had a higher survival rate (30.979 months; 95% CI 0.25974–0.35984) than patients with higher expression (26.316 months; 95% CI 0.19787–0.32845; $p > 0.05$).

The highest SMAD4 expression in stage 3 patients was also confirmed using blood serum samples (BTG 11.67 pg/ml; stage 1 43.89 pg/ml; stage 2 29.65 pg/ml; stage 3 102.23 pg/ml). The only statistically significant difference between serum SMAD4 expression was noted between the BTG and patient groups in III. stage of CRC ($p < 0{,}0001$) (supplementary file).

After categorizing CRC patients based on gender, we found that the highest tissue expression of SMAD4 in males was in the group of patients in the second stage of CRC (278.67 pg/ml; SD 90) and the lowest in the group

TGF-βRII		**BTG**	**Stage 2**	**p**		**Stage 1**	**Stage 2**	**p**
	Average (pg/ml)	803.48	435.86	**0.0032**	Average (pg/ml)	938.01	435.86	**0.0028**
	SD	355	168		SD	216	168	
		BTG	**Stage 3**	**p**		**Stage 1**	**Stage 3**	**p**
	Average (pg/ml)	803.48	405.59	**0.0004**	Average (pg/ml)	938.01	405.59	**0.0005**
	SD	355	193		SD	216	193	
TGF-βRII	**Males**				**Females**			
		Stage 1	**Stage 3**	**p**		**BTG**	**Stage 2**	**p**
	Average (pg/ml)	1,057.44	330.17	**0.0036**	Average (pg/ml)	952.52	368.01	**0.0078**
	SD	226	220		SD	412	60	
						Stage 1	**Stage 2**	**p**
					Average (pg/ml)	819.67	368.01	**0.0006**
					SD	89	60	
						Stage 1	**Stage 3**	**p**
					Average (pg/ml)	819.67	480.67	**0.0087**
					SD	89	60	
TGF-β1		**BTG**	**Stage 1**	**p**		**Stage 2**	**Stage 3**	**p**
	Average (pg/ml)	4,202.67	4,055.66	nsg	Average (pg/ml)	4,065.89	4,609.08	nsg
	SD	1,235	708		SD	1,769	1,354	
TGF-β1	**Males**				**Females**			
		BTG	**Stage 3**	**p**		**BTG**	**Stage 2**	**p**
	Average (pg/ml)	3,332.33	5,669.52	**0.0085**	Average (pg/ml)	5,073.44	3,036.36	**0.0126**
	SD	721	164		SD	553	1 011	
		BTG	**Stage 2**	**p**		**BTG**	**Stage 3**	**p**
	Average (pg/ml)	3,332.33	5,095.21	**0.0273**	Average (pg/ml)	5,073.44	3,548.67	**0.0377**
	SD	721	1 489		SD	553	691	
SMAD4		**BTG**	**Stage 2**	**p**		**Stage 1**	**Stage 3**	**p**
	Average (pg/ml)	157.62	268.59	**0.0032**	Average (pg/ml)	137.22	310.33	**0.0028**
	SD	97	89		SD	56	166	
		Stage 1	**Stage 2**	**p**				
	Average (pg/ml)	137.22	268.59	**0.0004**				
	SD	56	89					
SMAD4	**Males**							
		Stage 1	**Stage 2**	**p**				
	Average (pg/ml)	109.44	278.67	**0.0042**				
	SD	33	90					
		Stage 1	**Stage 3**	**p**				
	Average (pg/ml)	109.44	254.05	**0.0335**				
	SD	33	100					

Table 2. Tissue expression of individual TGF-β markers in CRC patients divided according to TNM classification (BTG: Benign tumor group).

of patients in the first stage of CRC (109.44 pg/ml; SD 33), with $p = 0.0042$. In females, the tissue expression of SMAD4 was highest in the group of patients with stage III CRC (328.33 pg/ml; SD 196) and lowest in the group of patients with benign tumors (139.58 pg/ml; SD 83), but with no statistically significant difference between the groups. Other important data from the graph (Fig. 4III A, B) are presented in the table (Table 2).

ROC curves showed that differentiation of male patients in stage 1 and stage 2 based on SMAD4 expression is sensitive and reliable (AUC 93.33%; 95% CI 0.7825-1.000; $p = 0.0280$), but this is not the case for differentiation of patients in stage 1 and stage 3 (AUC 85.71%; 95% CI 0.5979-1.000; $p = 0.0874$; Table 3). In females, due to non-significant results, ROC curves were not analyzed.

SMAD4 expression was also evaluated by western blot for confirmation. The results from the WB analysis were consistent with the results from the ELISA method, and thus, the highest tissue and serum SMAD4 expression was in patients with stage III CRC (Fig. 6).

Discussion

Our aim in the current study was to investigate biomarkers of signalling TGF-β pathway, which could have a prognostic value in the treatment and monitoring of the progression of colorectal cancer. The TGF-signaling pathway involves the ligand TGF-β1 and the receptors TGF-βRI and TGF-βRII where ligand-receptor

TGF-βRII		**AUC (%)**	**SD**	**95% CI**	**p**
	BTG vs. stage 2	80.95	8.06	0.6516–0.9675	**0.0046**
	BTG vs. stage 3	85.83	8.55	0.6907–1.000	**0.0055**
	Stage 1 vs. stage 2	97.96	2.62	0.9282–1.000	**0.0005**
	Stage 1 vs. stage 3	98.21	2.81	0.9270–1.000	**0.0018**
TGF-βRII	**Males**				
		AUC (%)	**SD**	**95% CI**	**p**
	Stage 1 vs. stage 3	95.83	6.07	83.93–1.000	**0.0247**
	Females				
	BTG vs. stage 2	96.22	6.31	90.34–1.000	**0.0082**
	Stage 1 vs. stage 2	93.47	5.89	82.42–1.000	**0.0209**
	Stage 1 vs. stage 3	94.02	6.77	81.98–1.000	**0.0399**
SMAD4		**AUC (%)**	**SD**	**95% CI**	**p**
	BTG vs. stage 2	78.57	8.56	0.6179–0.9536	**0.0088**
	Stage 1 vs. stage 2	89.29	7.12	0.7531–1.000	**0.0041**
	Stage 1 vs. stage 3	81.82	10.67	0.6090–1.000	**0.0265**
SMAD4	**Males**				
		AUC (%)	**SD**	**95% CI**	**p**
	Stage 1 vs. stage 2	93.33	6.31	0.7825–1.000	**0.0280**
	Stage 1 vs. stage 3	85.71	13.27	0.5975–1.000	**0.0875**
TGF-β1	**Males**				
		AUC (%)	**SD**	**95% CI**	**p**
	BTG vs. stage 2	86.67	9.07	0.8003–1.000	**0.0446**
	BTG vs. stage 3	93.23	5.87	0.8713–1.000	**0.0143**
	Stage 1 vs. stage 3	94.11	5.25	0.8902–1.000	**0.0145**
	Females				
	BTG vs. stage 2	97.15	5.79	0.9157–1.000	**0.0062**
	BTG vs. stage 3	95.02	4.61	0.9052–1.000	**0.0071**

Table 3. Data from ROC curves comparing different groups of CRC patients based on the expression of individual TGF-β markers (BTG: Benign tumor group).

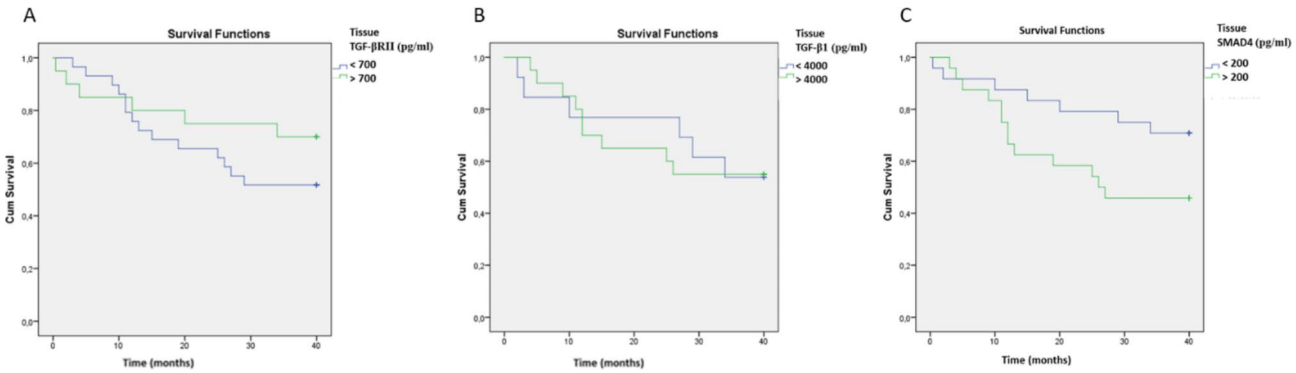

Fig. 5. Kaplan-Meier survival tests based on the expression of individual TGF-β markers.

interactions lead to signal transduction through SMAD members. The analysis of tissue TGF-βRII expression in CRC patients showed that the highest values were observed in stage 1 patients and the lowest values were observed in stage 3 CRC patients. Loss of TGF-β receptor function impairs the tumor-suppressive effects of TGF-β signalling, allowing cancer cells to escape growth inhibition and apoptosis. Early-stage CRC patients may have better survival outcomes if TGF-β signalling remains intact and suppresses the tumor. In our study, we observed reduced survival in patients with low expression of TGF-βRII in tumor tissues. However, we know that a personalized approach is important when starting treatment. We focused on patients in the second stage who succumbed to the disease and divided them by age into 50-year-olds and 68-70-year-olds, the expressions of TGF-βRII were significantly higher in patients 50 years old. The impact of TGF-β receptor alterations on overall survival in CRC patients is influenced by multiple factors, including tumor stage, molecular subtype, treatment strategies, and patient characteristics. The TGF-β signalling pathway in therapeutic intervention in

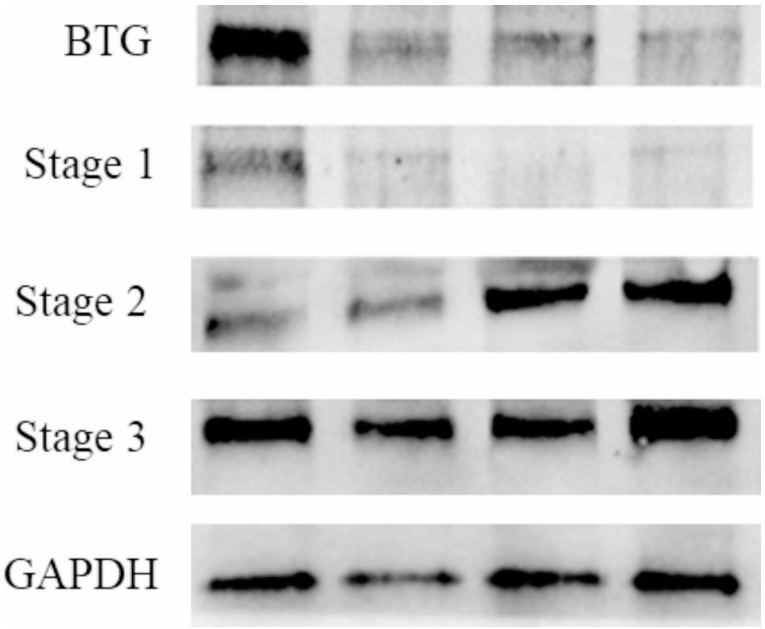

Fig. 6. SMAD4 proteins (60 kDa) confirmed in patients divided according to TNM classification. (BTG: Benign tumor group). *Patients whose SMAD 4 expression most closely reflects the group of patients at the given stage were selected.

advanced CRC remains an area of interest for many scientific groups, although clinical trials investigating TGF-β inhibitors as potential treatments show mixed results.

A very important finding was that these tissue expression patterns were very similarly replicated using blood serum samples. Due to the significant variation, we have not yet been able to confirm statistically significant differences between the patient groups, but we believe that in the future, after expansion of the patient samples and appropriate processing of the blood serum, we will be able to differentiate CRC patients also based on serum TGF-βRII expression. This would be a step towards less invasive collection of biological material, which would significantly increase the chances of incorporating the TGF-βRII marker into routine examinations.

Currently, there are few publications on the analysis of TGF-βRII in patients with colorectal cancer, and even less information is available on the proteomic determination of TGF-βRII in these patients.

TGF-βRII mutations are the most common TGF-β signalling pathway mutations in hypermutated CRC, occurring late during the development of MSI CRC as they are associated with adenoma progression[6]. Preclinical studies show that TGF-βRII mutations are not sufficient to initiate the malignant transformation of intestinal epithelial cells. However, in combination with mutations of other tumor suppressor genes such as PTEN, APC, TP53, and others, TGF-βRII mutations lead to tumor progression. For example, Yu et al[7]. evaluated tumor development in mice with conditional deletion of TGFβ-RII and PTEN. While mutations in TGFβ-RII or PTEN alone did not result in CRC, combined deletion of both genes led to tumors in the small and large intestine in 86% of mice and metastasis in 8% of mice with tumors[8].

Principe et al[9]. reported that TGF-βRII mutation is likely to directly promote inflammation in the tumor microenvironment of CRC. In their work in mouse models of intestinal adenoma, TGF-βRII deficiency was shown to increase the inflammatory burden and promote tumor progression through the production of TNF-α, IL-8, and TGF-β1, as well as suppression of anti-inflammatory cytokines such as IL-10 and IFN-γ. Moreover, inactivating mutations of TGF-βRII in CRC cells contribute to the malignant phenotype through multiple pathways, e.g., WNT-β-catenin or MAPK pathways[10].

Fan et al. developed a trifunctional fusion protein, DR30206, composed of Bevacizumab (an antibody against VEGF), and a variable domain of heavy chain of heavy chain antibody (VHH) against PD-L1 and the extracellular domain (ECD) protein of TGF-β receptor II (TGF-β RII), which are fused to the N- and C-terminus of Bevacizumab, respectively. Their findings suggest there is a great potential for DR30206 to become therapeutic for the treatment of multiple cancer types, especially lung cancer, colon adenocarcinoma, and breast carcinoma[11].

The TGF-β1 protein is one of the three known isoforms of the TGF-β ligand, which binds to TGF-β receptors and triggers the canonical signalling pathway. This protein is only mentioned in a very small number of scientific studies focusing on CRC as it is too controversial. TGF-β1 changes from an inhibitor of tumor cell growth to a stimulator of growth and invasion during the progression of human colon cancer[12,13]. Intense TGF-β1 staining significantly correlated with disease progression to metastasis and was independent of nodal status and degree of differentiation of the primary tumor. Patients with high levels of TGF-β1 protein in CRC in the primary lesion are more likely to have disease recurrence than patients whose tumors showed low levels[14].

In our work, there were no significant differences in TGF-β1 expression in patient survival between groups of patients divided according to higher or lower expression levels and TGF-β1 expression did not vary across clinical stages.

Although TGF-β signalling in cancer cells is defective most often due to the inactivation of TGF-βRII, in sporadic CRC, pathway defects are also associated with inactivating mutations of SMAD2, SMAD3, and SMAD4. These mutations are thought to reduce the stability of their proteins or prevent the formation of SMAD complexes involved in transcriptional reactions[6]. Loss of SMAD4 protein expression occurs in approximately 20–40% of human CRCs. Although loss of heterozygosity may be a major cause of SMAD4 loss in CRC, other proposed mechanisms contribute to the SMAD4 defect in posttranscriptional or posttranslational regulation: ubiquitylation, sumoylation, and microRNA interference[15]. It is SMAD4 that is thought to be a key element in changing the function of TGF-β from a tumor suppressor to a promoter of EMT induction and metastasis formation[16]. At the same time, although the loss of SMAD4 inhibits canonical TGF-β signalling, it has been shown to induce BMP signalling which switches from tumor suppression to increased epithelial-mesenchymal processes, invasion, and promotion of metastasis. Loss of SMAD4 has also been shown to induce alternative ERK pathways that induce colon cancer cell migration and invasion in vitro, increase liver metastasis in vivo, and shorten survival in mice with metastatic tumors[10].

In our study, SMAD4 expression was lowest in stage I and highest in stage III CRC patients using both tissue and blood serum samples. The most reliable results were provided by comparing patients in stage I and stage II CRC, thus suggesting a role for SMAD4 as a marker of transition from stage I to stage II. However, this was only significantly confirmed in male patients, who showed a significant difference in SMAD4 expression between stage I and stage II. Since our results did not correlate with published studies, we decided to confirm SMAD4 expression by Western blotting, but reached the same conclusions as with ELISA, namely that SMAD4 expression increases with increasing CRC stage. Our results also do not support the conclusions of studies describing that patients with low SMAD4 expression have a shorter survival time[17–20]. We confirmed a higher survival time in patients with SMAD4 expression below 200 pg/ml in tissue samples, but in this case, no statistically significant difference was confirmed. We propose that low SMAD4 expression in the early stages of CRC inhibits TGF-β signalling, which has anti-tumor effects during this period. Expression begins to increase between stage I and stage II when signalling switches to the tumor promoter. Expressed SMAD4 promotes CRC progression by forming SMAD2/3/4 complexes that can cross the nuclear membrane and influence the expression of other pro-tumorigenic proteins. However, due to many controversial results, no clinical trials of drugs inhibiting individual members of the TGF-β pathway have targeted SMAD4. A better understanding of the role of SMAD4, not only within the TGF-β pathway but especially within other cancer-related molecular pathways, is essential for further progress in this field. At the same time, it is critical to identify a group of patients in whom SMAD4 expression could be exploited.

Conclusion

Overall, the immunosuppressive effect of TGF-β in the tumor microenvironment appears to be of critical importance for CRC progression, and thus therapeutic inhibition of TGF-β is often considered a promising strategy for CRC treatment. Although previous studies targeting TGF-β in CRC have provided only limited results, mainly due to systemic effects, current data suggest that inhibition of the TGF-β pathway in selected patients or combination with immune checkpoint blockade could be a suitable strategy. An appropriate selection of biochemical markers important in the modulation of the TGF- β signalling pathway could help in the adjustment of treatment strategies to increase the expected response to treatment and subsequently minimize its toxicity. The proposed panel of markers to be investigated should include not only mutations of selected genes but also changes in gene expression at the mRNA or protein level, to be able to create a comprehensive profile of each patient and to take advantage of personalized medicine.

In clinical practice, the identification of SMAD4 and TGF-βRII alterations by non-invasive blood sampling (e.g. using circulating tumor DNA) may help in early diagnosis and monitoring of cancer progression. The absence or mutation of these markers might indicate a specific cancer subtype or signal a transition to a more advanced stage. The detection of these mutations in tumors using genomic profiling can help stratify patients into high-risk groups, potentially guiding treatment decisions. Patients with SMAD4 and TGF-βRII alterations may require more aggressive therapy and closer monitoring. Targeting TGF-βRII and SMAD4 in metastasis-prone cancers may help reduce the spread of cancer cells by inhibiting epithelial-mesenchymal processes. Future therapies may focus on blocking TGF-β-induced EMT to prevent metastatic disease.

Data availability

Raw data used during the current study are available from the corresponding author on reasonable request for non-commercial use.

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JM and IV designed and wrote the article. MS and MM supervised the work and critically revised the manuscript. JM, IV, PB and JK, LH reviewed and edited the manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

Ethical approval
The study was conducted by the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of 2020/EK/06042.

Informed consent
Informed consent was obtained from all subjects involved in the study.

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