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Association of *TCF7L2*, *CASC8* and *GREM1* Polymorphisms in Patients with Colorectal Cancer and Type II Diabetes Mellitus

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Abstract: Background: The aim of the study is to explore the association between the *TCF7L2* rs7903146, *CASC8* rs6983267 and *GREM1* rs16969681 polymorphisms in patients diagnosed with type 2 diabetes mellitus (T2DM) and colorectal cancer. Methods: Sixty individuals were enrolled in this case-control study: thirty with colorectal cancer and type II diabetes mellitus (T2DM) and thirty healthy control individuals. Real-time PCR was used to determine the genotypes of *TCF7L2* rs7903146, *CASC8* rs 6983267 and *GREM1* rs16969681 in patients with CRC and T2DM and in patients without T2DM and CRC. The Hardy–Weinberg equilibrium was determined in the control group for the genotype distribution of every polymorphism. Results: People carrying the TT genotype of rs7903146, rs6983267 and rs1696981 had a significant association with T2DM and CRC. Moreover, the people with the TT genotype of rs1696981 had a greater risk for T2DM and CRC (OR = 7, CI 0.397–23.347). Conclusions: *TCF7L2* rs7903146, *CASC8* rs6983267 and *GREM1* rs16969681 could be risk factors for the association of T2DM with CRC.

Keywords: colorectal; cancer; diabetes; *TCF7L2*; *CASC8*; *GREM1*; rs7903146; rs6983267; rs16969681

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1. Introduction

Colorectal cancer (CRC) is a malignancy with several possible risk factors. It is a heterogeneous disease that can be associated with abnormalities in various molecular pathways, with numerous studies showing that environmental factors and genetic susceptibility place certain individuals at a higher risk of developing CRC [1–3].

Epidemiological studies suggest a link between type II diabetes mellitus (T2DM) and CRC; diabetes promotes CRC carcinogenesis through complex processes and is considered an independent risk factor for cancer in general and for CRC in particular, contributing to a higher mortality rate for these diseases [4,5]. T2DM has been associated with increased CRC risk (20–40%) [6]. CRC and DM have in common some general risk factors such as obesity, which is prevalent in sedentary populations where a Western lifestyle is prevalent [7,8]. The risk factors of DM overlap with those of CRC and, therefore, it can be assumed that the genetic variants underlying DM could also influence susceptibility to CRC.

Other evidence is derived from preclinical study and genome-wide association study (GWAS) data that demonstrate that CRC and complications of T2DM may have common pathogenic pathways, as well as an abnormal microbiota, inflammatory mediators and transformed iron metabolisms, some of them reducing to Wnt/β-catenin signaling and mIR-21 [1,2,9]. GWAS have identified susceptibility genes for CRC and DM, such as *TCF7L2*, *CASC8* and *GREM1* [1,9].

The transcription factor 7-like 2 (*TCF7L2*) gene, 10q25.2–q25.3, is a transcription factor and β-catenin transcription partner in the Wnt signaling pathway that represses gene transcription in the absence of β-catenin after DNA-binding and also promotes miR-21 expression [9–11]. There are studies that have confirmed an association between *TCF7L2* rs7903146 and T2DM [12–15], and a weak association with CRC [16–18]. Another polymorphism, rs6983267, is located in Cancer Susceptibility Candidate 8 Noncoding (CASC8) 8q24.21 and the risk allele promote stronger *TCF7L2* binding, facilitating Wnt signaling [9,19]. A *GREM 1* SNP, 15q13.3, rs1696968, is also associated with CRC susceptibility and facilitates *TCF7L2* binding to DNA, leading to an increase in gene expression [20,21].

The aim of this study is to investigate the association of rs7903146, rs6983267, and rs16969681 polymorphisms with the risk of CRC in patients with T2DM.

2. Materials and Methods

2.1. Study Group

A case–control study was conducted among adult South-Eastern Romanian patients (n = 30) diagnosed with T2DM and with positive colonoscopic results for malignancy, histologically confirmed as CRC and prospectively admitted for elective surgery to the Surgery Department of Constanta County Clinical Emergency Hospital between September 2020 and September 2021. The control subjects (n = 30) included people in good health condition (no medical history and with negative colonoscopic preventive examination results) that were recruited in the same period and were frequency-matched to cases based on number, age and sex. Patients with familial adenomatous polyposis, hereditary nonpolyposis CRC, inflammatory bowel disease, or any cancer personal history were excluded from the study. According to the WHO criteria, T2DM is characterised by an inability of the body to produce insulin properly and by a plasma glucose concentration of $\geq$7.0 mmol/L [22].

CRC lesions were treated using laparoscopic or open surgery. The pathological stage, size and localization of the tumor were recorded. Demographic and clinical data of the participants were recorded, and included age, gender, alcohol consumption and smoking status (according to the National Institute on Alcohol Abuse and Alcoholism [23] and the Center for Disease Control and Prevention [24]), and body mass index (BMI) was calculated. Blood samples (glucose, haemoglobin A1c, uric acid, creatinine, CEA, CA 19-9) were collected after overnight fasting.

2.2. Genomic DNA Purification

Peripheral blood collected in specific vacutainers (with ethylenediaminetetraacetic acid) was used for genomic DNA extraction with the GeneJET Genomic DNA Purification Kit (ThermoScientific Baltics, Vilnius, Lithuania), according to the manufacturer protocol.

The purity and yield of the DNA samples were quantified using ultraviolet absorbance at 260/280 nm using a NanoDrop One™ Spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA), where a ratio of A260/A280 = 1.7–2.0 and A260/A230 > 2 was considered acceptable. The concentration of the DNA samples was measured using a Qubit® 3.0 Fluorometer (Thermo Fisher Scientific, Kuala Lumpur, Malayasia) and a Qubit RNA HR (high-range) Assay Kit.

2.3. Genotyping

SNPs of *TCF7L2* (rs7903146, C/T), *CASC8* (rs6983267, G/T), and *GREM1* (rs1696981, C/T) were identified using a real-time PCR method based on the ready-made TaqMan® Genotyping Master Mix (Applied Biosystems, Waltham, MA, USA) and 20× SNP Genotyping Assay (Applied Biosystems) containing target-specific oligonucleotides labeled with a reporter dye at the 5′ end of each probe; VIC dye was linked to the 5′ end of the Allele 1 probe and FAM dye was linked to the 5′ end of the Allele 2 probe (Table 1). The DNA concentration was set between 1 and 10 ng per 10 µL of RT-PCR reaction. Briefly, each 10 µL of RT-PCR reaction consisted of 5 µL TaqMan Genotyping Master Mix (2×), 0.5 µL

of TaqMan Genotyping Assay mix (20×), and 4.5 µL of DNA. Samples were incubated in a 7500 Fast Real-Time System (Applied Biosystems) with the following cycle conditions: 95 °C for 10 min, 95 °C for 15 s, and 60 °C for 1 min. The last two steps of denaturing and annealing/extension were repeated 40 times. Allelic discrimination was made with the help of 7500 Fast Real-Time PCR software, version 2.3.

Table 1. VIC/FAM Sequences of SNP Genotyping Assay.

SNP ID	VIC/FAM Sequences
rs7903146	TAGAGAGCTAAGCACTTTTTAGATA[C/T]TATATAATTTAATTGCCGTATGAGG
rs6983267	GTCCTTTGAGCTCAGCAGATGAAAG[G/T]CACTGAGAAAAGTACAAAGAATTTT
rs1696981	TTTCTTTTTATCTTGATATCTTGCA[C/T]GCGGCCTAACAAAGGCAATAATAAC

2.4. Statistical Analysis

For statistical analysis, SPSS version 28.0 was used (IBM, Armonk, NY, USA). The results are presented as a median with a range or mean ± standard deviation, with categorical variables expressed as counts. The Hardy–Weinberg equilibrium was determined using GeneCalc software at the level of significance of 0.05. A χ^2 test was used to establish the link between genetic variants and disease status. Odds ratios and 95% CIs were estimated. Multivariate logistic regression analysis was used for association analyses with adjustments for BMI and age. Comparisons of clinical parameters of different genotypes among patients with T2DM, CRC and healthy controls were assessed by one-way analysis of variance and the least significant difference test. $p < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. General Characteristics of Patients

Patients with T2DM and CRC were age-matched (within 5 years) with the control participants. Table 2 summarizes selected characteristics of the patients and controls. The mean age of the CRC and T2DM patients was 69.90 ± 8.36 years, with a mean body mass index (BMI) of 30.75 ± 3.90 kg/m^2. The average age of the control subjects was 63.90 ± 11.9 years, with a mean BMI of 26.31 ± 5.23 kg/m^2. There were no significant differences in mean age, sex, or the numbers of moderate alcohol drinkers between the two groups. There was a significant difference with respect to BMI, current or former smokers, glucose, systolic blood pressure, diastolic blood pressure, creatinine and uric acid in patients with CRC and T2DM than in the controls ($p < 0.05$). The time elapsed between the diagnosis of T2DM and that of CRC was between 1 and 14 years, with a mean of 7.07 ± 3.903.

Table 2. Clinical and biochemical characteristics of subjects with CRC + T2DM and controls.

Variable	CRC + T2DM (*n* = 30)	Controls (*n* = 30)	*p*-Value
Age (years)	69.90 ± 8.36	63.90 ± 11.9	0.250
Sex *			0.193
Male	19 (63.3)	14 (46.7)	
Female	11 (36.7)	16 (53.3)	
BMI (kg/m^2)	30.75 ± 3.90	26.31 ± 5.23	0.036
Current of former smokers *	9 (30)	7 (23.3)	0.049
Moderate alcohol consumption *	6 (20)	7 (23.3)	0.072
Glu (mg/dL)	143.29 ± 14.59	83.33 ± 12.87	<0.001
Blood HbA1c (%)	6.87 ± 0.96	5.16 ± 0.33	<0.001
SBP (mmHg)	136.98 ± 16.84	122.48 ± 11.26	0.042
DBP (mmHg)	81.68 ± 0.97	69.88 ± 0.77	<0.001

Table 2. *Cont.*

Variable	CRC + T2DM (*n* = 30)	Controls (*n* = 30)	*p*-Value
UA (mg/dL)	39.7 ± 16.5	16.9 ± 9.3	0.018
Cr (mg/dL)	1.13 ± 0.11	0.58 ± 0.26	0.029

Variables are expressed as mean ± SD (standard deviation), unless indicated otherwise. * Number of cases with percentages in parentheses. CRC—colorectal cancer; T2DM—type 2 diabetes mellitus; BMI—body mass index; smoker—smoking of ≥10 cigarettes daily; alcohol consumption ≥ 1 drink per day for women and ≥2 drinks per day for men; Glu—glucose; HbA1c—haemoglobin A1c; SBP—systolic blood pressure; DBP—diastolic blood pressure; UA—uric acid; Cr—creatinine.

The anatomopathological characteristics of patients with CRC and T2DM are shown in Table 3. The mean value of the tumor markers is above the normal limit (CEA > 5 ng/mL, CA19-9 > 27 U/mL). The most common site of cancer was the left colon (46.7%) and the rectum (30%). Most of the patients in the study were diagnosed in stage III (40%).

Table 3. Tumor characteristics of patients with CRC and T2DM.

Variable	Patients with CRC and T2DM (*n* = 30)	Percentage (%)
CEA (ng/mL) *	50.51	
CA19-9 (U/mL) *	43.15	
Tumor site		
Right colon	7	23.3
Left colon	14	46.7
Rectum	9	30
Disease stage TNM		
Stage I	8	26.7
Stage II	8	26.7
Stage III	12	40
Stage IV	2	6.7

CRC—colorectal cancer; T2DM—type 2 diabetes mellitus; CEA—cancer embryonic antigen; CA19-9—carbohydrate antigen 19-9; TNM—tumor node metastasis. * values are median.

3.2. Genotype Distribution of rs7903146, rs6983267 and rs1696981 in Case and Control Groups

The genotype distribution in control groups for rs7903146, rs6983267 and rs16969681 were consistent with the Hardy–Weinberg law at the level of significance of 0.05. Univariate and multivariate analyses were performed for the genotype and allele frequencies of CRC in the T2DM patients and the control subjects and are summarized in Table 4 for the selected SNPs (rs7903146, rs6983267, and rs16969681). Compared with the TT genotype, the CC + CT genotypes demonstrated a significant association with the risk of CRC and T2DM in the univariate analysis for rs7903146 ($p = 0.003$) and rs16969681 ($p = 0.009$). This significant association was maintained after adjusting for age and BMI for rs7903146 ($p = 0.021$). Among the various parameters studied, GG + GT/TT were significantly different between the cases and controls for rs6983267 ($p = 0.026$) in the univariate analysis.

Table 4. Univariate and multivariate analyses for CRC and T2DM patients and control subjects.

SNP		Univariate Analysis			Multivariate Analysis	
		CRC + T2DM *n* = 30	Controls *n* = 30	*p* Value	OR [95%CI]	*p* Value
rs7903146	CC + CT	21 (70)	29 (96.7)	0.003	0.080 [0.009–0.685]	0.021
	TT	9 (30)	1 (3.3)			
rs6983267	GG + GT	21 (70)	25 (83.3)	0.026	2.143 [0.622–7.387]	0.227
	TT	9 (30)	5 (16.7)			
rs16969681	CC + CT	22 (73.3)	26 (86.7)	0.009	2.364 [0.627–8.917]	0.204
	TT	8 (26.7)	4 (13.3)			

Variables are expressed as number of cases with percentages in parentheses. SNP—single-nucleotide polymorphism; CRC—colorectal cancer; T2DM—type 2 diabetes mellitus; OR—odds ratio; CI—confidence interval.

Genotype and allele distribution and the analysis of the association of rs7903146 of the *TCF7L2* gene in subjects with CRC and T2DM and in the controls are shown in Table 5. The CC, CT, and TT genotype frequencies were 26.7%, 43.3%, and 30%, respectively, in subjects with CRC and T2DM, and were 60%, 36.7%, and 3.3%, respectively, in the control subjects. The CT and TT genotypes were more frequent in subjects with CRC and T2DM than in the controls. The CC genotype was more frequent in the controls than in the subjects with CRC and T2DM. The risk of T2DM and CRC was lower in the heterozygous (CT) genotype group, with an odds ratio of 0.222 (95% CI 0.065–0.754, $p = 0.039$), than in the homozygous (TT) genotype group, which had an odds ratio of 2.042 (95% CI 0.395–10.553, $p = 0.011$). Moreover, the T allele was more frequent in the cases than in the controls and might significantly increase the occurrence risk of T2DM and CRC compared with the C allele (OR = 3.865, 95%CI = 1.743–8.567).

Table 5. Genotype and allele distribution and analysis of the association of rs7903146 of *TCF7L2* in subjects with CRC + T2DM and controls.

Genotype	CRC + T2DM ($n = 30$)	Controls ($n = 30$)	OR	95% CI	p-Value
CC (%)	8 (26.7%)	18 (60%)		Reference	
CT (%)	13 (43.3%)	11 (36.7%)	0.222	0.065–0.754	0.039
TT (%)	9 (30%)	1 (3.3%)	2.042	0.395–10.553	0.011
C (%)	29 (48.3%)	47 (78.3%)		Reference	
T (%)	31 (51.7%)	13 (21.7%)	3.865	1.743–8.567	0.001

CRC—colorectal cancer; T2DM—type 2 diabetes mellitus; OR—odds ratio; 95%CI—95% confidence interval.

Genotype and allele distribution and the analysis of the association of rs6983267 of the *CASC8* gene in subjects with CRC and T2DM and in the controls are shown in Table 6. The GG, GT, and TT genotype frequencies were 30%, 40%, and 30%, respectively, in subjects with CRC and T2DM, and were 30%, 53.3%, and 16.7%, respectively, in the control subjects. The TT genotype had a lower frequency in the controls than in the subjects with T2DM and CRC. The GT genotype was more frequent in the controls than in subjects with CRC and T2DM and the GG genotype was evenly distributed in the two groups. The results indicate a low-significance association in the homozygous (TT) genotype ($p = 0.052$). There was no significant distribution of the G and T alleles between the two groups, which indicate that the presence of one T allele does not increase susceptibility for T2DM and CRC.

Table 6. Genotype and allele distribution and analysis of the association of rs6983267 of *CASC8* in subjects with CRC + T2DM and controls.

Genotype	CRC + T2DM ($n = 30$)	Controls ($n = 30$)	OR	95% CI	p-Value
GG (%)	9 (30%)	9 (30%)		Reference	
GT (%)	12 (40%)	16 (53.3%)	3.000	0.586–15.362	0.586
TT (%)	9 (30%)	5 (16.7%)	1.333	0.139–12.818	0.052
G (%)	30 (50%)	34 (56.7%)		Reference	
T (%)	30 (50%)	26 (43.3%)	0.765	0.373–1.569	0.464

CRC—colorectal cancer; T2DM—type 2 diabetes mellitus; OR—odds ratio; 95%CI—95% confidence interval.

Genotype and allele distribution and the analysis of the association of rs16969681 of the *GREM1* gene in the subjects with CRC and T2DM and in the controls are shown in Table 7. The CC, CT, and TT genotype frequencies were 50%, 23.3%, and 28.7%, respectively, in subjects with CRC and T2DM, and were 63.3%, 23.3%, and 13.3%, respectively, in the control subjects. The TT genotype was more frequent in subjects with CRC and T2DM than in the controls. The CC genotype was more frequent in the controls than in the subjects with CRC and T2DM and the CT genotype was evenly distributed in the two groups. The results indicate a significant association in the homozygous (TT) genotype ($p = 0.047$). For

rs16969689, there was also no significant distribution of the C and T alleles between the two groups, which indicates that the presence of only one risk allele is not important for susceptibility to T2DM and CRC.

Table 7. Genotype and allele distribution and analysis of the association of rs16969681 of *GREM1* in subjects with CRC + T2DM and controls.

Genotype	CRC + T2DM (n = 30)	Controls (n = 30)	OR	95% CI	p-Value
CC (%)	15 (50%)	19 (63.3%)		Reference	
CT (%)	7 (23.3%)	7 (23.3%)	6.250	0.615–63.538	0.109
TT (%)	8 (28.7%)	4 (13.3%)	7.000	0.397–23.347	0.047
C (%)	37 (61.7%)	45 (75%)		Reference	
T (%)	23 (38.3%)	15 (30%)	0.536	0.245–1.173	0.116

CRC—colorectal cancer; T2DM—type 2 diabetes mellitus; OR—odds ratio; 95%CI—95% confidence interval.

Regardless of the SNP, obesity was associated with CRC and DM. When focusing on people with a BMI $\geq$ 30 kg/m^2 and the TT genotype of the rs6983267 and rs16969681 genes, there was an increased frequency of cases in patients with CRC and T2DM compared to the control group (Figure 1).

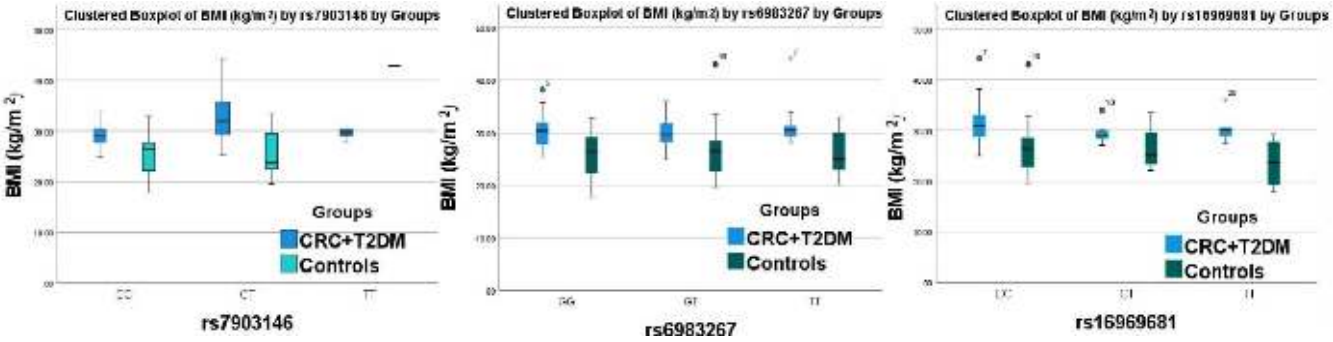

Figure 1. Graphical model represents the genotype distribution by BMI for patients with CRC, T2DM and controls.

4. Discussion

T2DM and CRC are among the most frequent causes of death worldwide [22,24]. The link between DM and cancer has been recognized by the American Diabetes Association (ADA) since the 2010 consensus guidelines and in the 2022 Standards of Medical Care in Diabetes, which state that diabetes is associated with an increased risk of cancer, and CRC is one of them [25,26]. This association may be linked with shared risk factors between T2DM and cancer (older age, obesity and physical activity), but may also be due to diabetes-related factors such as diabetes-related physiology or treatment [26].

Regarding other published studies which investigate genetic links between T2DM and CRC in different multi-ethnic populations [8,27], in our study, all individuals were of Romanian origin, and we selected the SNPs from a GWAS conducted in a Caucasian population, with the association replicated in a GWAS and meta-analysis more recently released than in mentioned studies. From the selected SNPs, only TCF7L2 rs7903146 overlapped with the outlined studies, and is a well-known factor of association between T2DM and CRC. The other two SNPs were selected because of their interaction and binding with *TCF7L2*, and are well-documented SNPs for CRC risk.

A common molecular pathway for T2DM and CRC is the Wnt/β-catenin signaling pathway. In the absence of Wnt signaling, APC attached glycogen synthase kinase 3-β (GSK-3β) and phosphorylated β-catenin to prepare it for ubiquitin–proteosome degradation. In the absence of nuclear β-catenin, a transcription factor from the *TCF* family, such as *TCF7L2*, can interact with transcriptional inhibitors and repress transcription. In CRC, *APC*

is frequently mutated and, in DM, Wnt signaling is activated. Wnt signaling prevents β-catenin degradation, enabling nuclear migration where it promotes the transcription of genes implicated in cell proliferation.

TCF7L2 is a transcription factor in the Wnt signaling pathway. It contains a DNA-binding domain, is implicated in the regulation of cell proliferation and differentiation, and maintains the stability of plasma glucose [28,29]. *TCF7L2* is related with T2DM risk and DM complications such as diabetic nephropathy, and is also a susceptibility locus for CRC [12,15,30]. In the present study, the genotype distribution of the *TCF7L2* rs7903146 polymorphism was correlated with susceptibility for CRC and DM in our population. The data show that the TT genotype frequency, and also the T allele, were significantly higher in patients with CRC and T2DM and the risk of CRC and T2DM was also significantly higher for the TT genotype. A previous study has also shown an association between T2DM and a higher risk of CRC for patients with the risk allele of *TCF7L2* rs7903146, and several of them have shown an independent association with T2DM and CRC, suggesting that the risk allele is likely to have a more important effect on colon tissue than in the pancreatic islet [8,27,28]. The Wnt pathway is frequently implicated in the etiology and pathogenesis of CRC, and *TCF7L2* undergoes changes in CRC.

Another CRC-associated polymorphism, rs6983267, located in chromosome 8q24, is situated at a *TCF7L2* binding site and the risk allele determines an intense binding of *TCF7L2*, facilitating Wnt signaling [9,19]. Our study showed a low-significance association OR 1.33 (CI 0.139–12.818, $p = 0.05$) for the risk genotype with T2DM and CRC. The other studies have shown that rs6983267 within the 8q24 region is one of the strongest genetic risk factors for the development of CRC [31]. Due to interactions with *TCF7L2*, we wanted to investigate the association between CRC and T2DM for this variant. CASC8, rs6983267, is also associated with increased susceptibility to prostate cancer, in addition to CRC, but the molecular mechanism of association is under investigation [31].

The *GREM1* rs16969681 also demonstrated a significant association with the TT genotype and T2DM and CRC, with an OR of 7 (CI 0.397–23.347, $p = 0.047$). GWASs demonstrate that *GREM 1* rs1696968 is an important risk factor for CRC or advanced adenomas in the European population [9]. Other studies propose *GREM1* as an important mediator for diabetic kidney disease, and due to its interaction with *TCF7L2*, which facilitates its DNA binding, it can be considered one of the common genetic factors for T2DM and CRC [9,32]. The *GREM1*, rs16969681, stimulates *TCF7L2* binding to DNA, determining an increase in *GREM1* gene expression. The gene product, Gremlin, activates kidney damage in T2DM and cell migration in CRC [9].

Interestingly, our study showed an increased frequency of association between high BMI and risk genotypes for rs6983267 and rs16969681 in patients with CRC and T2DM. Taken together, our results with the results of other cohort studies suggest that BMI could be an independent risk factor for the association between T2DM and the risk of CRC [8,27,33].

5. Conclusions

In summary, *TCF7L2* rs7903146, *CASC8* rs6983267 and *GREM1* rs1696968 were significantly correlated in our study with T2DM and CRC. There is no other study, to our knowledge, that investigates the association between T2DM and CRC for rs6983267 and rs1696968. In the present study, there are several limitations such as sample size and interaction between genetic and environmental factors that were ignored. Therefore, further research should be conducted to verify this conclusion.

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Observational Study

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TCF7L2, CASC8, and GREM1 polymorphism and colorectal cancer in south-eastern Romanian population

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Abstract

Colorectal cancer (CRC) is a heterogeneous disease with an increasing trend and with multiple epigenetic alterations and different molecular features, a major cause of mortality and morbidity. The Wnt/β-Catenin pathway is involved in multiple aspects of cell dynamics, architecture of developing gastrointestinal tissues, and intestinal tissue homeostasis in adults, but its aberrant activity plays an important role in every aspect of colorectal carcinogenesis. The aim of our study was to investigate the association of the TCF7L2 rs7903146, CASC8 rs6983267, and Gremlin1 (GREM1) rs16969681 polymorphism in patients with CRC without other pathologies. A case-control study conducted on 31 patients diagnosed with CRC and 30 healthy controls age and sex-matched with the patients. Real time PCR was used to determine the genotypes of rs7903146, rs698267, rs1696981. We observed no association between rs6983267 and rs16969681 polymorphism and risk of CRC and low association between TCF7L2, rs7903146, polymorphism and risk of CRC. The recessive model of the TCF7L2 rs7903146 had an OR of 1.6 (95% CI 0.058–4.414, $P < .05$) which means that TT genotype increased the risk and possibility of development of CRC. Our study did not confirm a significant association between TCF7L2 rs7903146, CASC8 rs6983267, and GREM1 rs16969681 with CRC, but emphasizes the possibility of existence of a high risk of CRC development in patients with TT genotype of rs7903146.

Abbreviations: BMI = body mass index, CRC = colorectal cancer, GREM1 = Gremlin1, SNP = single nucleotide polymorphisms, TCF7L2 = transcription factor 7-like 2, TNM = tumor-node-metastasis.

Keywords: CASC8 rs6983267, colorectal cancer, genes, GREM1 rs16969681, TCF7L2 rs7903146

1. Introduction

Colorectal cancer (CRC) is the third most common cancer and the second cause of cancer deaths worldwide,[1,2] which originates from colon epithelium.[3] CRC is initiated by mutations in tumor suppressor genes and oncogenes, and the accumulation of multiple mutations leads to a selective growth advantage for transformed epithelial cells that is modulated by epigenetic changes.[4,5] These tumor-promoting lesions interfere with the regulated activity of the Wnt/β-Catenin pathway and thereby affect proliferation, migration, invasion, and tumor initiation capacity of CRC cells. A major molecular pathway is Wnt signaling activation of the transcription factor β-catenin to promote expression of cell proliferation genes.[6] Aberrant Wnt/β-Catenin pathway activity plays a crucial role in virtually every aspect of colorectal carcinogenesis.[7]

In the healthy gut, these Wnt/β-Catenin pathway functions are executed exclusively via transcription factor 7-like 2 (TCF7L2) gene.[8,9] The TCF7L2 (10q25.2), one of these genes, encodes a transcription factor member of the Wnt signaling pathway.[10] Although biologically plausible, few studies have examined associations between polymorphisms of the TCF7L2 gene and CRC.[8,11,12] Duval et al (1999; 2000) characterized the genomic structure of TCF7L2 in CRC cell lines and demonstrate that long C-terminal end may mediate transcriptional repression.[13,14] Findings by Folsom and colleagues using data from the Atherosclerosis Risk in Communities Study suggest that an association exists.[15] The rs7903146 T allele in TCF7L2 is the strongest genome-wide association studies signal for diabetes risk in different populations across the world and it is associated with insulin synthesis, processing, secretion and action mechanisms.[16]

Genome-wide association studies have revealed that some single nucleotide polymorphisms (SNP) at 8q24, such as rs6983267, located in Cancer Susceptibility Candidate 8 Noncoding, might be effective in susceptibility to various cancers in different

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The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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populations[17,18] and also are strongly associated with CRC and the risk allele gave out a 1 to 3 times risk increases.[16] A number of studies have shown that variations in the TCF7L2 gene, considerably affects risk of type 2 diabetes.[12,19,20] A rare germline duplication upstream of the bone morphogenetic protein antagonist Gremlin1 (GREM1) causes a Mendelian-dominant predisposition to CRC; there are studies that confirm that a common GREM1 polymorphism, rs16969681, is also associated with CRC susceptibility, conferring ~20% differential risk in the general population.[21] Epidemiological studies suggest a link between type 2 diabetes and CRC.[22,23] We evaluated the association of the rs7903146, rs6983267and rs16969681 polymorphism with CRC in patients without other pathologies.

2. Methods

2.1. Study group

A case-control study was conducted among South-Eastern Romanian adult patients (n = 31) with a diagnosis of CRC between September 2020 and 2021. The patients had positive colonoscopic results for malignancy, histologically confirmed as CRC and were recruited from Emergency Hospital of Constanta. Unrelated subjects (controls) (n = 30) were recruited in the same period as the cases from the same hospital and were judged to be in good health according to their colorectal screening examination and medical history. Controls were frequency matched to cases by sex and age. Patients with familial adenomatous polyposis, hereditary nonpolyposis CRC, inflammatory bowel disease or any cancer personal history were excluded from the study. Both colon cancer patients and controls were excluded if they had diabetes or high blood pressure, or if they were under any treatment course.

Cancer lesions were treated appropriately by open or laparoscopic surgery. For each case, the localization and size of the tumor and the pathological stage were recorded. Tumor staging were based on World Health Organisation criteria and the tumor-node-metastasis (TNM) system. According to tumor localization, samples were classified as "right-sided" (localized in the cecum or in the ascending or transverse colon), "left-sided" (set in the descendant or sigmoid colon) and in the rectum. Demographic and clinical data included age, gender, alcohol consumption status (according to National Institute on Alcohol Abuse and Alcoholism[24]), smoking status (according to Center for Disease Control and Prevention[25]), the body weight, height, and blood pressure of participants were recorded, and the body mass index (BMI) was calculated.

2.2. Genotyping

Genomic deoxyribonucleic acid was extracted from paraffin embedded tissue using GeneJET Genomic deoxyribonucleic acid Purification Kit (ThermoScientific), according to manufacturer protocol. SNPs polymorphisms of the TCF7L2 (rs7903146, C/T), cancer susceptibility 21 (rs6983267, G/T), and GREM1 (rs1696981, C/T) were identified using a real-time PCR method based on the TaqMan® Genotyping Master Mix (Applied Biosystems) and 20× SNP Genotyping Assay (Applied Biosystems), using a 7500 Fast Real-Time Systems (Applied Biosystems) according to manufacturer procedure. Allelic discrimination was made with the help of 7500 Fast Real-Time PCR software, version 2.3.

2.3. Statistical analysis

Descriptive statistics were used to describe the profile of study participants. Quantitative variables were described using the mean and standard deviation. Meanwhile, qualitative variables were summarized as frequencies and percentages. Hardy-Weinberg equilibrium was determined using GeneCalc software at the level of significance 0.05. The association between disease status and the genetic variants were tested by Pearson's Chi-square test. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were used in calculating the corresponding χ^2 distribution test. Multivariate logistic regression analysis was used for association analyses with adjustments for age and BMI. Comparisons of clinical parameters of different genotypes among patients with CRC and healthy controls were assessed by 1-way analysis of variance and the least significant difference test. A P value < .05 was considered statistically significant. The SPSS statistical software package for Windows version 28.0 (IBM, Armonk, NY) was used for all statistical analyses.

2.4. Ethical consideration

The study was carried out in accordance with the Declaration of Helsinki on experimentation with human subjects and was approved by the Local Ethics Commission for the Approval of Clinical and Research Developmental Studies (No 5/2020).

3. Results

3.1. General characteristics of study objects

Patients with CRC were matched with control participants by age (within 5 years) and date of diagnosis (within 3 month). The mean age was 66.1 years for CRC patients and 63.9 years for controls. Table 1 summarizes selected characteristics of patients and controls. There were no significant differences in mean age, sex, or the numbers of current smokers, habitual alcohol drinkers, habitual vegetable consumers and habitual exercisers between the 2 groups. Moreover, there was no significant difference with respect to systolic blood pressure and diastolic blood pressure between the 2 groups. However, BMI were higher in patients with CRC than in controls (P = .049).

3.2. Genotype distribution of rs7903146, rs6983267 and rs1696981 in case and control groups

The genotypes distribution in control groups for rs7903146, rs6983267 and rs16969681 were consistent to Hardy Weinberg law at the level of significance 0.05. Genotypic and allelic distributions of the rs7903146, rs6983267, rs16969681 polymorphism in patients with CRC and controls are summarized in Table 2. For rs7903146, the frequencies of CC, CT, and TT genotypes were 48.4, 41.9, and 9.7% in patients with CRC and 60, 36.7, and 3.3% in controls. Comparing with the TT genotype, the CT and CC genotypes demonstrated no significant association with the risk of CRC. There was also no significant correlation with the risk of CRC in dominant models, but a significant correlation was found for recessive models (P = .036).

For rs6983267 SNP, the most frequent genotype was GT (54.8%) in CRC patients and was 53.3% in controls. On the other hand, the TT genotype frequency was significantly higher in CRC cases (22.6%) than in controls (16.7%). The frequencies of CC, CT, and TT genotypes for rs16969681 were 71%, 25.8%, and 1% in CRC patients and 63.3%, 23.3%, and 13.3% in control subjects. For rs6983267 and rs16969681 gene, there was no significant correlation with the risk of CRC in either dominant or recessive models.

When focusing on individuals with BMI ≥ 25 kg/m², for rs7903146 gene, was found to significantly increase the risk of CRC development in individuals with CC + CT genotypes (OR = 2.684, 95% CI 0.254–28.311, P = .037), while for rs6983267 and rs16969681 genes, no significant correlation with the risk of CRC was found (Table 3).

We observed a significant tendency toward higher stage colorectal adenocarcinomas and depth of invasion, based on the

Table 1

Characteristics of patients with CRC and controls.

Variable	Patients with CRC (n = 31)	Controls (n = 30)	*P* value
Age (yrs), mean ± SD	66.1 ± 11.69	63.9 ± 11.99	.583*
Sex			.252**
Male	19 (61.3)	14 (46.7)	
Female	12 (38.7)	16 (53.3)	
Smoking	7 (22.6)	7 (23.3)	.944**
Habitual alcohol use	2 (6.5)	7 (23.3)	.117**
Habitual vegetable consumer	5 (16.1)	9 (30%)	.430**
Habitual exercise	4 (12.9)	12 (40%)	.836**
SBP, mm Hg	146.12 ± 29.33	122.48 ± 11.26	.098*
DBP, mm Hg	71.44 ± 12.16	69.88 ± 0.77	.114*
BMI, kg/m²	28.61 ± 4.66	26.31 ± 5.23	.049**

Number of cases, with percentages in parenthesis.

BMI = body mass index, CRC = colorectal cancer, DBP = diastolic blood pressure, SBP = systolic blood pressure, SD = standard deviation.

**P* value evaluated by analysis of variance.

***P* value evaluated by X^2 test.

Table 2

Multivariate analysis of association between rs7903146, rs6983267, rs16969681 and risk of CRC.

	Variable	Patients with CRC (n = 31)	Controls (n = 30)	OR [95% CI]	*P* value
rs7903146	Genotype				
	TT	3 (9.7)	1 (3.3)	-	-
	CT	13 (41.9)	11 (36.7)	(Reference)	-
	CC	15 (48.4)	18 (60)	1.418 [0.494–4.075]	.516
	Dominant				
	TT	3 (9.7)	1 (3.3)	(Reference)	-
	CC + CT	28 (90.3)	29 (96.7)	3.107 [0.305–31.680]	.317
	Recessive				
	TT + CT	16 (51.6)	12 (40)	(Reference)	-
	CC	15 (48.4)	18 (60)	1.600 [0.0.580–4.414]	*.036*
rs6983267	Genotype				
	TT	7 (22.6)	5 (16.7)	-	-
	GT	17 (54.8)	16 (53.3)	(Reference)	-
	GG	7 (22.6)	9 (30)	1.366 [0.411–4.539]	.610
	Dominant				
	TT	7 (22.6)	5 (16.7)	(Reference)	-
		24 (77.4)	25 (83.3)	1.458 [0.407–5.230]	.561
	GG + GT				
	Recessive				
	TT + GT	24 (77.4)	21 (70)	(Reference)	-
	GG	7 (22.6)	9 (30)	1.469 [0.466–4.633]	.510
rs16969681	Genotype				
	TT	1 (3.2)	4 (13.3)	-	-
	CT	8 (25.8)	7 (23.3)	(Reference)	-
	CC	22 (71)	19 (63.3)	0.987 [0.302–3.230]	.983
	Dominant				
	TT	1 (3.2)	4 (13.3)	(Reference)	-
	CC + CT	30 (96.8)	26 (86.6)	0.217 [0.023–2.063]	.150
	Recessive				
	TT + CT	9 (29)	11 (39.6)	(Reference)	-
	CC	22 (71)	19 (63.3)	0.707 [0.241–2.068]	.525

Variables are expressed as number of cases, with percentages in parenthesis. Values in italics indicate statistical significance ($P < .050$).

CI = confidence interval, CRC = colorectal cancer, OR = odds ratio.

T and N factor of the TNM system, in patients with CRC with the rs7903146 and rs16969681, CT genotype (Table 4). For rs6983267, based on T and N factor for TNM system, patients with higher stage of CRC have a higher percent GT genotype. Comparative analysis between colon cancer and rectal cancer in terms of SNPs is not statistically significant in the study.

4. Discussion

CRC is a multigenic disease in which single SNP may only have a modest independent effect, and multiple SNPs may provide a more accurate representation of the risk. The present study explored the interaction of rs7903146, rs6983267, rs16969681 SNPs in 30 controls and 31 cases of CRC. Although a single SNP may have a moderate effect on the development of cancer, several SNPs together can exert a significant influence. Single SNPs (except one, rs7903146) were not associated with CRC risk, underlining the importance of integrating SNP information across genes in a pathway.

TCF7L2 is a gene that can increase the risk of type 2 diabetes.[12,26,27] The function of rs7903146 is under investigation. Surely, it may be another mutation in linkage disequilibrium

Table 3

Multivariate analysis of association between rs7903146, rs6983267, rs16969681 and risk of CRC stratified by BMI.

Genotype	BMI (kg/m²)	CRC (n = 31)	Controls (n = 30)	OR [95% CI]	*P* value
rs7903146					
TT	<25	0	0	-	-
CT + CC	<25	9 (29)	12 (40)	-	NS
TT	≥25	3 (9.7)	1 (3.3)	(Reference)	-
CT + CC	≥25	19 (61.3)	17 (56.7)	2.684 [0.254–28.311]	*.037*
rs6983267					
TT	<25	2 (6.5)	2 (6.6)	(Reference)	-
GT + GG	<25	7 (22.6)	10 (33.4)	1.429 [0.161–12.701]	.748
TT	≥25	5 (16.1)	3 (10)	(Reference)	-
GT + GG	≥25	17 (54.8)	15 (50)	1.471 [0.300–7.218]	.634
rs16969681					
TT	<25	0	2 (6.6)	(Reference)	-
CT + CC	<25	9 (29)	10 (33.4)	1200 [0.932–1.546]	.198
TT	≥25	1 (3.2)	2 (6.6)	(Reference)	-
CT + CC	≥25	21 (67.8)	16 (53.4)	0.381 [0.032–4.581]	.433

Variables are expressed as number of cases, with percentages in parenthesis. Values in italics indicate statistical significance ($P < .050$).
BMI = body mass index, CI = confidence interval, CRC = colorectal cancer, NS = nonsignificant, OR = odds ratio.

with this SNP that affects gene function. Anyway, a causal link between TCF7L2 variation and CRC seems biologically plausible. The TCF7L2 gene, has a central role in the Wnt/β-catenin signaling pathway, which is strongly implicated in colon cancer etiology.[8,11] Findings by Folsom and colleagues using data from the Atherosclerosis Risk in Communities Study suggest that an association between colon cancer and TCF7L2 exists.[28] In their study, the TT genotype of the rs7903146 TCF7L2 gene was associated with a > 2-fold increased risk of colon cancer (hazard rate ratio, 2.15; 95% CI, 1.27–3.64). Our finding was that variation in TCF7L2 SNPs, particularly rs7903146, was low associated with incidence of CRC.

The SNPs rs6983267 and rs16969681 has been investigated by many groups; some researchers examined those association with cancer and a few others studied, those relationship with CRC.[17,18,21,29] In this study, we observed no association between rs6983267 and rs16969681 polymorphism and risk of CRC, with results similar to those in the literature.[17] Findings by Karimi and colleagues suggests that there were no remarkable associations between rs6983267 and susceptibility to esophageal and colon cancers.[17,30,31] Other authors have described that rs6983267 significantly increased the risk of CRC[32–35] and given these differences in outcomes, studies in this regard are needed.

ORs and CIs were calculated for the dominant and recessive inheritance model for each polymorphism. The dominant model is presumed to demonstrate whether the presence of minor allele (T), as a risk allele, increase the risk for CRC, while the recessive model establish the necessity of the presence of 2 copy of the T allele, the TT genotype, in order to increased the CRC risk. Our study revealed that the recessive model of the TCF7L2 rs7903146 had an OR (95% CI) of 1.6 (0.058–4.414). These results may reveal that the TT genotype of rs7903146 increased the risk and possibility of development of CRC.

There are studies that attribute a more specific association between colon cancer alone and TCF7L2 than for grouped CRC.[28] Although we had too few cases of colon cancer to analyze separately, the association with TCF7L2 was not more specific than that of groped CRC.

Obesity is a risk factor for cancer in general and CRC in particular.[36] The association between TCF7L2 and obesity has been a topic of research over time, and the association between the 2 does not appear to be causal related.[37] In this study, only a modest association with rs7903146 CC + CT genotypes was found in patients with BMI ≥ 25 kg/m². Evaluating the SNPs rs6983267 and rs16969681 with BMI, in our study, did not reveal a significant interaction. The results of our study were similar to studies in the literature.

A significant tendency toward higher stage colorectal adenocarcinomas and depth of invasion was seen in CT heterozygotes for rs7903146 and rs16969681 genes, respective GT heterozygotes for rs6983267 gene; indicating maybe that the CT/GT genotype could be a CRC biomarker correlating with stage progression.

4.1. Study limitations

Although the number of subjects was small in this study limiting the statistical power, the finding of this study should be considered. Despite this limitation, the cases and controls were matched by age, sex, smoking status and comorbidities. Nonetheless, most of the findings were similar with those observed from some other populations. Therefore, further research should be conducted to verify this conclusion.

5. Conclusions

The rs7903146 TCF7L2 polymorphism has a low association with development of CRC, and between rs6983267, rs16969681 and CRC, no association was identified in our study. However, our study emphasizes the possibility of existence of a high risk of CRC development in patients with TT genotype of rs7903146. A strategy utilizing biomarkers to stratify patients into appropriate screening programs can potentially prevent CRC and in this regard, future studies should approach epistatic relationships from the SNP level.

Author contributions

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Table 4

Relationship between rs7903146, rs6983267, rs16969681 variants and clinicopathologic features in patients with CRC.

		Genotype - rs7903146			
Variable/category	**n (%)**	**CC**	**CT**	**TT**	***P* value**
Tumor site					.667
Right-sided	8 (25.8)	4 (12.9)	3 (9.7)	1 (3.2)	
Left-sided	9 (29)	4 (12.9)	5 (16.1)	0	
Rectum	14 (45.2)	7 (22.6)	5 (16.1)	2 (6.5)	
TNM (T)					.058
1–2	11 (35.5)	7 (22.6)	3 (9.7)	1 (3.2)	
3–4	20 (64.5)	8 (25.8)	10 (32.2)	2 (6.5)	
TNM (N)					.511
N0	12 (38.7)	6 (19.3)	4 (12.9)	2 (6.5)	
N+	19 (61.3)	9 (29)	9 (29)	1 (3.2)	
TNM (M)					.886
M0	29 (93.5)	14 (45.2)	12 (38.7)	3 (9.7)	
M+	2 (6.5)	1 (3.2)	1 (3.2)	0	
Tumor stage					.511
1–2	2 (38.7)	6 (19.3)	4 (12.9)	2 (6.5)	
3–4	19 (61.3)	9 (29)	9 (29)	1 (3.2)	
			Genotype - rs6983267		
		GG	GT	TT	
Tumor site					.767
Right	8 (25.8)	3 (9.7)	3 (9.7)	2 (6.5)	
Left	9 (29)	1 (3.2)	7 (22.6)	1 (3.2)	
Rectum	14 (45.2)	3 (9.7)	7 (22.6)	4 (12.9)	
TNM (T)					.076
1–2	11 (35.5)	2 (6.5)	4 (12.9)	5 (16.1)	
3–4	20 (64.5)	5 (16.1)	13 (41.9)	2 (6.5)	
TNM (N)					.433
N0	12 (38.7)	4 (12.9)	5 (16.1)	3 (9.7)	
N+	19 (61.3)	3 (9.7)	12 (38.7)	4 (12.9)	
TNM (M)					.548
M0	29 (93.5)	7 (22.6)	16 (51.6)	6 (19.3)	
M+	2 (6.5)	0	1 (3.2)	1 (3.2)	
Tumor stage					.433
1–2	12 (38.7)	4 (12.9)	5 (16.1)	3 (9.7)	
3–4	19 (61.3)	3 (9.7)	12 (38.7)	4 (12.9)	
			Genotype - rs16969681		
		CC	CT	TT	
Tumor site					.638
Right	8 (25.8)	5 (16.1)	2 (6.5)	1 (3.2)	
Left	9 (29)	7 (22.6)	2 (6.5)	0	
Rectum	14 (45.2)	10 (32.2)	4 (12.9)	0	
TNM (T)					.544
1–2	11 (35.5)	9 (29)	2 (6.5)	0	
3–4	20 (64.5)	13 (41.9)	6 (19.3)	1 (3.2)	
TNM (N)					.711
N0	12 (38.7)	9 (29)	3 (9.7)	0	
N+	19 (61.3)	13 (41.9)	5 (16.1)	1 (3.2)	
TNM (M)					.146
M0	29 (93.5)	22 (70.9)	6 (19.3)	1 (3.2)	
M+	2 (6.5)	0	2 (6.5)	0	
Tumor stage					.711
1–2	12 (38.7)	9 (29)	3 (9.7)	0	
3–4	19 (61.3)	13 (41.9)	5 (16.1)	1 (3.2)	

M = metastasis, N = node, TNM = T-tumor.

Writing – review & editing: Anca Florentina Mitroi, Răzvan Cătălin Popescu.

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Observational Study

Medicine

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KRAS, NRAS, BRAF, PIK3CA, and AKT1 signatures in colorectal cancer patients in south-eastern Romania

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Abstract

Somatic mutations in the oncogenes of the epidermal growth factor receptor signaling pathway play vital roles in colorectal carcinogenesis and have been closely linked with clinical resistance to monoclonal therapy. In this study, we have analyzed the mutation frequencies of 5 genes and compared the genetic findings with clinicopathological variables in order to determine diagnostically relevant alterations and compare these findings with those of other studies In our Sanger sequencings, KRAS (exons 2, 3, and 4), NRAS (exons 2, 3, and 4), PIK3CA (exons 9 and 20), BRAF (exon 15), AKT1 (exon 2) genes, and microsatellite instability (MSI) status were analyzed using an ABI 3500 analyzer in a cohort of 58 Romanian colorectal cancer (CRC) patients who underwent surgical resection at Emergency County Clinical Hospital in Constanța, Romania. In our series, mutation rates of KRAS, BRAF, PIK3CA, and AKT1 genes were 39.63%, 8.62%, 6.88%, and 3.44%, respectively. By contrast, we did not find any tumor harboring mutation in the NRAS gene. Notably, the KRAS and PIK3CA mutations were not mutually exclusive, 1 patient harbored 2 mutations in exon2, codon 12 (Gly12Val) of KRAS and exon 20, codon 1047 (His1047Arg) of PIK3CA. The finding of our study are generally consistent with data found in the literature. Regarding to clinicopathological variables, mutation of KRAS was associated with distant metastasis at the time of diagnosis, while mutation of BRAF was significantly associated with MSI-H in contrast with MSI-L/MSS tumors. Moreover, PIK3CA mutation tends to be located in the proximal segment of the colon and to be well/moderately differentiated compared to wild-type tumors. In conclusion, the assessment of these mutations suggests that CRC patients from southeast Romania exhibit a mutation profile similar to other populations. These results could contribute to creating a better method of qualifying patients for molecularly targeted therapies and obtaining better screening strategies.

Abbreviations: AKT1 = v-akt murine thymoma viral oncogene, BRAF = v-RAF murine sarcoma viral oncogene homolog B1, CRC = colorectal cancer, EGFR = epidermal growth factor receptor, KRAS = Kirsten rat sarcoma viral oncogene homolog, MSI = microsatellite instability, NRAS = neuroblastoma rat sarcoma viral oncogene homolog, PCR = polymerase chain reaction, PIK3CA = Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha.

Keywords: AKT, BRAF, colorectal cancer, KRAS, NRAS, PIK3CA

1. Introduction

Colorectal cancer (CRC), which includes malignancies of the colon, rectum, and appendix, is the third most prevalent and deadly cancer worldwide.[1] Romania has seen an increase in CRC over recent decades, consistent with its rapid socio-economic development and Westernization of living standards and dietary habits. As a result, CRC is now the second most common cancer in Romania after lung cancer in men and breast cancer in women.[2]

From a pathophysiological and molecular point of view, CRC is a multifactorial/polygenic neoplastic disease, the consequence of successive accumulations of genetic and epigenetic alterations, including somatic mutations, gene fusions, genetic deletions/amplifications, and epigenetic modifications.[3,4] Accordingly, somatic mutations in key oncogenes including KRAS, NRAS, BRAF, PIK3CA, and AKT1 genes activate multiple signaling pathways downstream of the epidermal growth factor receptor (EGFR). These pathways include RAS/RAF/MAPK signaling, which leads to unrestricted cell growth, and/or the PI3K/PTEN/

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The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. All data generated or analyzed during this study are included in this published article [and its supplementary information files].

The study was carried out in accordance with the Helsinki Declaration and was approved by the Local Ethics Commission for the Approval of Clinical and Research Developmental Studies.

The study was mainly performed at the Research Center for the Morphological and Genetic Study in Malignant Pathology – CEDMOG from Ovidius University.

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AKT/mTOR axis, which plays a major role in increased cell motility and resistance to apoptosis.[5]

The assessment of mutations in pathogenetic genes in gastrointestinal neoplasia is now standard clinical practice. Assessment and understanding the prognostic (i.e., the natural course of the disease) and predictive (i.e., response to therapy) value of these mutations has revolutionized the treatment of CRC patients. For example, chemotherapy and molecularly targeted therapies are given as first-line treatment for metastatic CRCs with wild-type but not mutated KRAS/NRAS.[6] Similarly, anti-EGFR therapies are only effective in a subset of patients with CRC, suggesting these patients harbor mutations in other genes that act downstream of or parallel to the EGFR axis.[7]

To date, numerous studies found KRAS (Kirsten rat sarcoma viral oncogene homolog) and NRAS (neuroblastoma rat sarcoma viral oncogene homolog) are the most commonly altered genes of the RAS oncogene family, occurring in about 20% to 30% of all human cancers. Furthermore, mutations of KRAS and NRAS in CRCs are also frequently, found in 35% to 45% and 1% to 6% of the cases, respectively. Nearly 90% of driver mutations are detected in codons 12 or 13 of exon 2 and to lesser magnitude in codons 59 and 61 of exon 3 or codons 117 and 146 of exon 4.[5,8]

The activation of the BRAF (v-RAF murine sarcoma viral oncogene homolog B1), another constitutive gene of the EGFR pathway, is recognized as a strong predictor of resistance to monoclonal antibody therapy. Additionally in CRC, BRAF gene status has been found to be mutually exclusive from RAS mutations, being persistent in up to 8% to 10% of all CRCs and 90% of these mutations involve substitution to glutamic acid (V600E) from exon 15 (codon T1799A). The mutations result in the constitutively active forms of the protein that lead to a transformation of normal epithelia into serrated adenomas at an early stage of tumorigenesis.[9]

Likewise, mutations in the PIK3CA (Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha) have been associated with a lack of response to anti-EGFR therapy, suggesting that 10% of CRC patients harboring PIK3CA activating mutations, commonly clustered in the 2 hotspot regions (exons 9 and 20).[10] The AKT1 (v-akt murine thymoma viral oncogene) gene represents another component in PI3K signaling pathway. The somatic mutation of this serine-threonine kinase appears in CRC with frequency at 0.7% to 6.0% in exon 2 (E17K), which leads to abnormal activation of AKT1.[11]

The mutations frequency of the aforementioned genes presents wide geographical, racial, and ethnic differences in this type of cancer. Furthermore, most of the studies were carried out in Western countries, and data available for South-East European countries are limited.[12–14] In the same manner, the South-East area of Romania is known as a heterogeneous population with ethnic diversity, where the prevalence of these genetic alterations, especially in patients with CRC has not been explored in detail and often was limited to a few genes and a subset of patients.

In concordance with the background, in this study, we aimed to determine the molecular spectrum of KRAS, NRAS, BRAF, PIK3CA, and AKT1 mutations, and MSI (microsatellite instability) in a series of patients with CRC from the Black Sea coast geographical area of Romania. We compared the genetic findings with clinical and pathological variables in order to determine diagnostically relevant alterations and compare these findings with those of other studies.

2. Materials and Methods

2.1. Case selection

We have conducted an observational study that included 63 fresh tumor samples collected from patients who previously underwent elective surgery or endoscopic colonoscopy for curative or diagnostic purposes at the Emergency County Clinical Hospital in Constanța, Romania. Patients who had poor or insufficient DNA quality of the tumor specimens (n = 5) were excluded. All tissue samples were collected from patients which signed informed consent and preserved in DNA/RNA Shield (Zymo Research, USA) until the total DNA was extracted. The study was conducted in accordance with the Declaration of Helsinki and approved by the Local Ethics Commission for the Approval of Clinical and Research Developmental Studies (06/15.03.2021).

Specimens were processed and evaluated by 2 experienced pathologists according to standard protocols from the Department of Pathology. The tumor staging of the cancer was classified using the tumor-node-metastasis staging system of the American Joint Committee on Cancer guidelines.[15] The individual characteristics of the CRC patients were abstracted from the medical observation sheets and pathology reports, including the following variables: age, sex, tumor location, histological type, degree of histological differentiation, depth of tumor invasion, lymph node involvement, and distant metastasis.

2.2. DNA extraction and quantification

Genomic DNA was extracted and purified from tissue samples by using QAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's protocol.

The quality and yield of the DNA solutions were assessed by measuring the optical density at 260 and 280 nm wavelengths using a NanoDrop One™ Spectrophotometer (Thermo Fisher Scientific, Waltham, MA), where an absorbance ratio A_{260}/A_{280} = 1.8–2.0, and A_{260}/A_{230} > 2 was considered acceptable. Furthermore, the concentration of solutions was measured by fluorescence-based with Qubit® 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA) using the Qubit DNA HR (High-Range) Assay Kit.

2.3. Sanger sequencing of KRAS, NRAS, BRAF, PIK3CA, and AKT1

The MSI status was evaluated using fluorescently labeled polymerase chain reaction (PCR) primers for 5 microsatellite mononucleotide markers (BAT25, BAT26, NR-21, NR-24, and MONO-27) and 2 pentanucleotide markers (Penta D and Penta C). The main protocol was described in detail previously.[16]

Bidirectional sequencing and PCR amplification were conducted in the coding sequences of the following genes: KRAS (exons 2, 3, and 4), NRAS (exons 2, 3, and 4), BRAF (exon 15) PIK3CA (exons 9 and 20), and AKT1 (exon 2). Sequences of primers used in PCR reactions are detailed in Table 1. In the first PCR run, 10 to 15 ng of DNA was amplified using Platinum II Taq Hot-Start DNA Polymerase (Invitrogen). Each reaction consists of 10 µL of Platinum II Taq Hot-Start Master Mix (2×), 0.8 µL of pooled PCR primers (0.2 µM each), 5 µL DNA template, and 4.2 µL water nuclease-free. The following PCR conditions were used: initial denaturation at 94°C for 2 minutes, then 35 cycles for denaturing at 94°C for 15 seconds, annealing at 60°C for 15 seconds, extension at 68°C for 15 seconds, and a cooled at 4°C. For optimal results, primers and unincorporated nucleotides from the PCR templates were purified with ExoSAP-IT Express, being incubated for 5 minutes at 37°C and 1 minute at 80°C.

Sequencing reactions consisted of 3 µL purified PCR product and 1 µL of 3.2 µM forward primer or 1 µL 3.2 µM reverse primer, 2 µL of Big Dye Terminator v3.1 reaction mix, 1 µL of 5× Sequencing Buffer, and 3 µL nuclease-free water. The PCR sequencing plate was conducted on a Biometra thermocycler with the following parameters: 96°C for 1 minute, 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes, and hold at 4°C until purify. Sequencing products obtained were purified prior to sequencing in order to remove unincorporated

Table 1

Detailed information of primers used in Sanger Sequencing analysis.

Nr	Gene		Primer sequences	Length (bp)
1	KRAS (exon 2)	Forward	GAGTGAACATCATGGACCCTGACA	559
		Revers	TTAAGCGTCGATGGAGGAGTTG	
2	KRAS (exon 3)	Forward	CCCACCTATAATGGTGAATATCTTCAAATGAT	232
		Revers	AGTAAAAGGTGCACTGTAATAATCCAGAC	
3	KRAS (exon 4)	Forward	CAGATCTGTATTTATTTCAGTGTTACTTACCT	168
		Revers	GACTCTGAAGATGTACCTATGGTCCTA	
4	NRAS (exon 2)	Forward	TACAGAATATGGGTAAAGATGATCCGACA	246
		Revers	CTGTAGATGTGGCTCGCCAA	
5	NRAS (exon 3)	Forward	ACTTGCTATTATTGATGGCAAATACACAGA	256
		Revers	TAGATGCTTATTTAACCTTGGCAATAGCA	
6	NRAS (exon 4)	Forward	CTCTACCAGAGTTAATCAACTGATGCAA	274
		Revers	ACCCAGCCTAATCTTGTTTTTCTTATGT	
7	PIK3CA (exon 20)	Forward	TGTAAAACGACGGCCAGTCAGAGTAACAGACTAGCTAGAGACAATGA	272
		Revers	CAGGAAACAGCTATGACCGCACTTACCTGTGACTCCATAGAAA	
8	PIK3CA (exon 9)	Forward	TGTAAAACGACGGCCAGTTATTCGACAGCATGCCAATCTCTT	136
		Revers	CAGGAAACAGCTATGACCTCCAGAGTGAGCTTTCATTTTCTCAG	
9	BRAF (exon 15)	Forward	TGTAAAACGACGGCCAGTGTTGAGACCTTCAATGACTTTCTAGT	513
		Revers	CAGGAAACAGCTATGACCTCTGGGCCTACATTGCTAAAATCTA	
10	AKT1 (exon 2)	Forward	TTGTTGCTTGCCAGCCCAGG	501
		Revers	AGCCCGTTTTCAGACACAGCTC	

dye terminators and dNTPs, using BigDye XTerminator Kit, and subsequently, capillary electrophoresis was run on a 3500 Genetic Analyzer (Thermo Fisher Scientific). Electropherograms were analyzed using Sequencing Analysis Software v5.4 (Foster City, CA) and SnapGene® v5.3.2 (San Diego, CA). The electropherogram of each amplicon sequenced was independently read manually by 2 researchers.

2.4. Statistical analysis

Data obtained were analyzed using MedCalc version 19.0.3 software (MedCalc, Ostend, Belgium). The proportions between KRAS, NRAS, BRAF, PIK3CA, and AKT1 mutation status and clinicopathological variables were evaluated using an appropriate chi-square test (χ 2) or Fisher's exact test. The *P* value was considered to be significant if it was less than .05.

3. Results

3.1. Clinical and pathological features of CRC patients

In this study, a total of 58 patients were included, representing the major ethnic groups from the southeast part of Romania. The prevalence of CRC rates was higher in males 53.4% (31/58) compared to females 46.6% (27/58). The mean age of the patient cohort was 68 years (range 45–89). The primary locations of tumors included the rectum, the left side of the colon (respectively sigmoid colon and splenic flexure), and the right side of the colon (cecum, hepatic flexure, and transverse colon). Tumors were significantly higher in the colon (85.6%) where the tumors were equally distributed in the right and left colon, accounting for each 41.4% (24/58) compared to the rectum with 17.2% (10/58).

Regarding the histological subtypes, 89.7% (52/58) of tumors were common adenocarcinomas and 10.3% (6/58) was mucinous carcinoma. In accordance with World Health Organization criteria for Classification of Tumors of the Digestive System, the tumors were graded as follows: 8.6% (5/58) were well-differentiated, 81.1% (47/58) were moderately differentiated, and 10.3% (6/58) were poorly differentiated. As regarding of depth of tumor invasion in the layers of the colon or rectum, T3 represented 65.5% of all cases, followed by T4 and T2 with 17.2% each. The N0, N1, and N2 stages of lymph node involvement had percentages of 39.7%, 36.2%, and 24.1%, respectively. Based on the clinical inspection and medical electronic sheets, 31% of patients had distant metastasis and 69% displayed no metastasis, respectively. Metastases were located in the lungs in 9 patients (50% of patients with metastases), the liver in 6 patients (33.3% of patients with metastases), and other organs in 3 patients (16.7% of patients with metastases). Additionally MSI status was observed in 8.62% (5/58) of CRC patients comparatively with 5.18% (3/58) who display MSI-L and 86.20 (50/58) MSS.

3.2. The KRAS, NRAS, BRAF, PIK3CA, and AKT1 mutations distribution in CRC patients

The prevalences of KRAS, NRAS, BRAF, PIK3CA, and AKT1 mutations in the Romanian CRC patients are summarized in Table 2. Overall, KRAS mutations were detected in 39.63% of cases (23/58), PIK3CA mutations in 6.88% of cases (4/58), BRAF mutations in 8.62% of cases (5/58), and AKT1 in 3.44% of cases (2/58). NRAS mutations were not identified in any of the cases. Altogether, we found 32 patients with driver mutations and 26 patients with the absences of any of the examined mutations. All mutations identified were in the heterozygous state.

Table 2

Aminoacid, base changes, and prevalence of KRAS, NRAS, PIK3CA, BRAF, and AKT1.

Gene	Aminoacid change	Base change	Count	Prevalence (%)
KRAS	G12D	35G > A	5	8.62
	G12V	35G > T	5	8.62
	G13D	38G > A	5	8.62
	G12S	34G > A	3	5.17
	G12R	34G > C	1	1.72
	G12C	34G > T	1	1.72
	G13C	37G > T	1	1.72
	Q61L	182A > T	1	1.72
	K117R	350A > G	1	1.72
NRAS	-	-	-	-
PIK3CA	E545D	1635G > T	1	1.72
	H1047R	3140A > G	2	3.44
	M1040L	3118A > T	1	1.72
BRAF	V600E	1799T > A	5	8.62
AKT1	E17K	49G > A	2	3.44

Regarding KRAS, mutations were detected in 91.3% of cases in exon 2 (21/23), 4.35% in exon 3 (1/23), and 4.35% in exon 4 (1/23). Within KRAS exon 2, 71.5% of the mutations were detected in codon 12, and 28.5% were identified in codon 13. As regards exons 3 and 4 of the KRAS, mutations were detected in codons 61 and 117, respectively. All KRAS mutations are comprised of missense substitution.

The 4 most frequent mutations in exon 2 were the substitution of glycine with aspartate, valine, or serine at codon 12 and the substitution of glycine with aspartate at codon 13. These 4 mutations, G12D, G12V, G12S, and G13D, occur due to substitution at c.35G > A, c.35G > T, c.34G > A, and c.38G > A, which appear in 21.74% (5/23), 21.74% (5/23), 13.03% (3/23), and 21.74% (5/23) of the cases. Other common mutations detected in exon 2 were G12R, G12C, and G13C (Fig. 1A–H). The missense mutations Q61L and K117R have been detected in exons 3 and 4 of KRAS, which occur due to substitution at c.182A > T and c.350 A > G, respectively. Each mutation was noted in a single case, excepting 1 patient who carried 2 mutations, in exons 2 and 4 of the gene.

Mutations in the PIK3CA gene were identified in 75% of the cases in exon 20 (3/4) and 25% in exon 9 (1/4). The mutations detected in exon 20 was the substitution of histidine with arginine at codon 1047 and methionine with leucine at codon 1040. These occur due to transitions of A > G at c.3140 and transversion of A > T at c.3118 (Fig. 1I–J). In exon 9 was noted the substitution of glutamic acid with aspartate in codon 545, as a result of the transversion of G > T at c.1635 (Fig. 1K). The KRAS and PIK3CA mutations were not mutually exclusive, 1 patient harbored 2 mutations in exon2, codon 12 (G12V) of KRAS and exon 20, codon 1047 (H1047R) of PIK3CA.

Reference to the BRAF gene, all 5 mutations detected in this study were represented by the missense substitution of valine with glutamic acid at position 600 (V600E), which appear due to transversion of T > A at the level of c.1799 (Fig. 1L–M). All BRAF mutations were mutually exclusive from KRAS mutations.

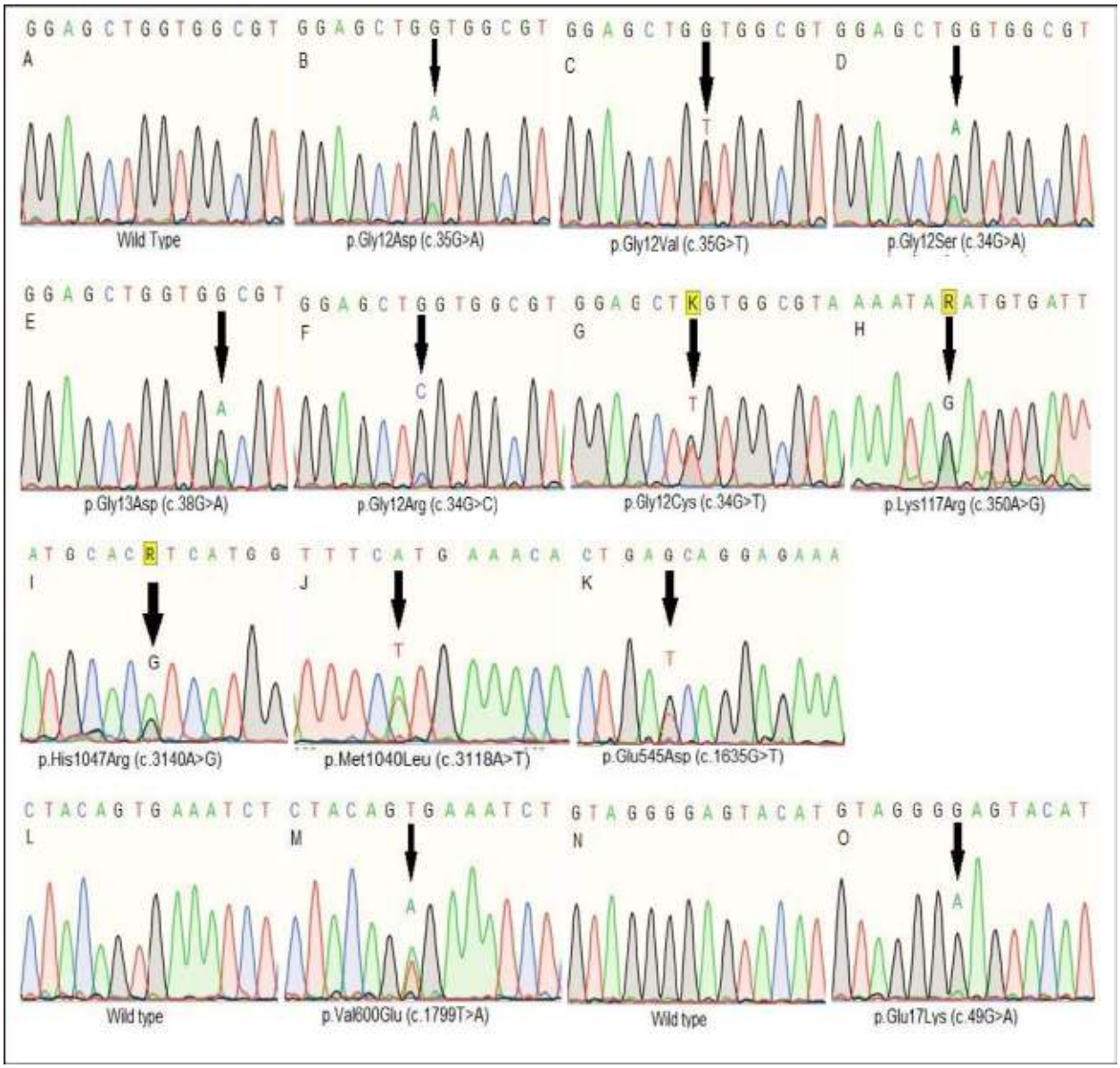

Figure 1. Representative Sanger electropherograms detected in our cohort. (A–K) Wild-type and mutations detected in exons 2, 3, and 4 of KRAS gene. (L–K) Mutations of PIK3CA gene detected in exons 20 and 9. (L–M) Wild-type and mutation detected in exon 15 of BRAF gene. (N–O) Wild-type and mutation detected in exon 2 of AKT1 gene.

The substitution of glutamate with lysine in the AKT1 gene was the single mutation detected across our CRC patients (Fig. 1N–O). Furthermore, all tumors with the AKT1 E17K mutation are found to be negative for KRAS, NRAS, BRAF, or PIK3CA.

3.3. Correlation of gene mutations with clinical and pathological variables

The mutations analysis has been evaluated in relation to several clinical and pathological parameters. No significant correlation was found between the mutational status of any of the 5 genes and gender, age, invasion depth, and lymph node metastasis (Table 3). As an exception, tumors with PIK3CA mutation tend to be located in the proximal segment of the colon ($P = .04$) and to be well and moderately differentiated compared to wild-type tumors ($P = .001$). Moreover, BRAF mutation was significantly associated with MSI-H tumors in contrast with MSI-L/MSS tumors ($P = .001$). The KRAS mutation was significantly correlated with distant metastasis among our cohort ($P = .01$).

4. Discussion

CRC is triggered by activations of intracellular signaling pathways downstream of EGFR, including the RAS-RAF-MAPK and PI3K-PTEN-AKT. The orchestrating of these pathways is achieved by a series of exchange proteins, adaptor proteins, kinases, and phosphatases upheld under tumor suppressors genes and oncogenes regulation.[17] Mutations assessment in the EGFR cascade genes is used not only for selecting the adequate treatment of patients with advanced and recurrent CRC but also for identifying subjects with an unfavorable prognosis. It has been found that patients with mutations in the extended RAS family are non-responsive to therapy with anti-EGFR monoclonal antibodies. Moreover, over 40% of CRC subjects who exhibit wild-type RAS are known to be resistant to monoclonal therapy, thereby highlighting the need for additional biomarkers.[6] A possible explanation for this resistance could be the presence of mutations in other genes of the EGFR cascade, such as BRAF, PIK3CA, or AKT1. While several studies have reported the mutational status of the above-mentioned genes in CRC patients from Western countries, there continues to be a paucity of genotyping data from CRC subjects from the South-East of Europe.[12–14,18] Consequently, the mutation rate of these genetic alterations in the context of Romanian patients was characterized in the present study, after which their prevalence was compared with clinical and pathological variables along with other similar studies.

Our Sanger sequencing reports have indicated that alterations in the KRAS, BRAF, PIK3CA, and AKT1 genes are common events in colorectal tumorigenesis, given that they are mutated in 55.18% (32/58) of the CRC cases.

The KRAS mutations distribution observed in our study was 39.63%, which is in accordance with the average level of KRAS mutations observed in Turkish, Italian, Greek, and Moroccan patients (33.2%–39.5%), but lower than mutation rates observed in the Sloven and Spanish cohorts (46.2%–48%).[12,13,18–21] This evidence suggests that genotyping data can be influenced by a variety of factors such as genetic background, environmental conditions, dietary lifestyle, heterogeneity of populations (different ethnicities), the number of patients enrolled, or methodology of the assay. Furthermore, no substantial differences were observed in the distribution of these mutations among KRAS gene exons in comparison to other studies carried out in the same geographical area. Indeed, the

Table 3

Correlation between KRAS, BRAF, PIK3CA and AKT1 mutations and clinicopathological variables in CRC.

Clinicopathological variables		No. of patients (%)	KRAS status			BRAF status			PIK3CA status			AKT1 status		
			Mutation	Wild-type	*P*; χ^2	Mutation	Wild-type	*P*; χ^2	Mutation	Wild-type	*P*; χ^2	Mutation	Wild-type	*P*; χ^2
Gender	Male	31 (53.4)	13 (22.4)	18 (31)	.48;	1 (1.7)	30 (51.7)	1.20	2 (3.4)	29 (50)	.70;	1 (1.7)	30 (51.7)	.53;
	Female	27 (46.6)	8 (13.8)	19 (32.8)	0.48	4 (6.9)	23 (39.7)		2 (3.4)	25 (43.1)	0.14	1 (1.7)	26 (44.8)	0.38
Age	< 60	14 (24.1)	4 (6.9)	10 (17.2)	.71;	1 (1.7)	13 (22.4)	.74;	1 (1.7)	13 (22.4)	.57;	1 (1.7)	13 (22.4)	.97;
	≥ 60	44 (75.9)	17 (29.3)	27 (46.6)	1.13	4 (6.9)	40 (69)	0.10	3 (5.2)	41 (70.7)	0.31	1 (1.7)	43 (74.1)	0.001
Tumor site	Right	24 (41.4)	9 (15.5)	15 (25.9)	.90;	3 (5.2)	21 (36.2)	.58;	4 (6.9)	20 (34.5)	**.04**;	NC	24 (41.4)	.23;
	Left	24 (41.4)	9 (15.5)	15 (25.9)	0.20	1 (1.7)	23 (39.7)	1.08	NC	24 (41.4)	6.08	2 (3.4)	22 (37.9)	2.93
	Rectum	10 (17.2)	3 (5.2)	7 (12.1)		1 (1.7)	9 (15.5)		NC	10 (17.2)		NC	10 (17.2)	
Histological type	Adenocarcinoma	52 (89.7)	19 (32.8)	33 (56.9)	.76;	3 (5.2)	49 (84.5)	.13;	4 (6.9)	48 (82.8)	.88;	2 (3.4)	50 (86.2)	.48;
					0.08			2.27			0.02			0.48
	Muc carcinoma	6 (10.3)	2 (3.4)	4 (6.9)		2 (3.4)	4 (6.9)		NC	6 (10.3)		NC	6 (10.3)	
Grading	G1	5 (8.6)	1 (1.7)	4 (6.9)	.38;	NC	5 (8.6)	.06;	3 (5.2)	2 (3.4)	**.001**;	NC	5 (8.6)	.78;
	G2	47 (81.1)	19 (32.8)	28 (48.3)	1.92	3 (5.2)	44 (75.9)	5.42	1 (1.7)	46 (79.3)	24.06	2 (3.4)	45 (77.6)	0.48
	G3	6 (10.3)	1 (4.75)	5 (8.6)		2 (3.4)	4 (6.9)		NC	6 (10.3)		NC	6 (10.3)	
Depth of invasion	T2	10 (17.2)	3 (5.2)	7 (12.1)	.22;	NC	10 (17.2)	.27;	1 (1.7)	9 (15.5)	.62;	1 (1.7)	9 (15.5)	.42;
	T3	38 (65.5)	12 (20.7)	26 (44.8)	2.97	3 (5.2)	35 (60.3)	2.61	3 (5.2)	35 (60.3)	0.95	1 (1.7)	37 (63.8)	1.72
	T4	10 (17.2)	6 (10.3)	4 (6.9)		2 (3.4)	8 (13.8)		NC	10 (17.2)		NC	10 (17.2)	
No. of lymph nodes	N0 (0)	23 (39.7)	6 (10.3)	17 (29.3)	.33;	1 (1.7)	22 (37.9)	.56;	2 (3.4)	21 (36.2)	.87;	2 (3.4)	21 (36.2)	.20;
	N1 (1-3)	21 (36.2)	8 (13.8)	13 (22.4)	2.20	2 (3.4)	19 (32.8)	1.12	1 (1.7)	20 (34.5)	0.26	NC	21 (36.2)	3.15
	N2 (>3)	14 (24.1)	7 (12.1)	7 (12.1)		2 (3.4)	12 (20.7)		1 (1.7)	13 (22.4)		NC	14 (24.1)	
Metasstasis	M0 (No)	40 (69)	10 (17.2)	30 (51.7)	**.01**;	3 (5.2)	37 (63.8)	.95;	4 (6.9)	36 (62.1)	.40;	2 (3.4)	38 (65.5)	.85;
	M1 (Yes)	18 (31)	11 (19)	7 (12.1)	5.52	2 (3.4)	16 (27.6)	0.003	NC	18 (31)	0.69	NC	18 (31)	0.03
MSI status	MSS/MSI-L	53 (91.38)	21 (36.2)	32 (55.2)	.20;	1 (1.7)	52 (89.7)	**.001;**	5 (8.6)	49 (84.5)	.77;	2 (3.4)	51 (87.9)	.40:
	MSI-H	5 (8.62)	NC	5 (8.6)	1.62	4 (6.9)	1 (1.7)	26.11	NC	4 (6.9)	0.08	NC	5 (8.69)	0.70

CRC = colorectal cancer, MSI = microsatellite instability, NC = not calculable.

4 most frequent mutations observed in the present study were in codons 12 and 13 of exon 2, in particular G12D, G12V, G12S, and G13D (21.74%, 21.74%, 13.04% and 21.74%), similar to those reported by Greece (29.4%, 19.3%, 11.27%, and 19.3%).[19] Other common mutations identified were found to occur with a less magnitude in codons 61 and 117 of exons 3 and 4, Q61L, and K117R (4.35% each). This indicates that geographical distribution is not a significant factor in how such mutations are positioned in the codons. Moreover, we found 2 patients with more than 1 mutation, 1 patient carried 2 KRAS mutations (G13D and K117R), whereas the other one had a double point mutation, in exon 20 of PIK3CA (H1047R) and exon 2 of KRAS (G12V). However, no significant associations were found between PIK3CA and KRAS mutations. The coexistence of KRAS mutation with different other gene mutations could be linked to the heterogeneity of the tumor. Likewise, KRAS mutation may influence the activity of kinases from other pathways, suggesting these pathways are synergistically interconnected.[22]

Some studies showed that missense mutations in codon 12 are associated with the mucinous phenotype, while mutations in codon 13 are characterized by a non-mucinous phenotype, localization in the proximal colon, tumor aggressiveness, and increased metastatic potential.[23,24] In this study, no association was observed between histological type and codons 12/13 mutations of KRAS. However, we observed that KRAS mutations tend to be more frequent in males than females (61.9% vs 38.1%) and to be located in the distal and proximal colon compared to the rectum (42.8% vs 14.4%), which is in accordance with studies of Kawazoe et al, but unfortunately with no statistical evidence.[25] Instead, a significant difference was noted in the KRAS mutation frequency with respect to distant metastasis (52% vs 48%; $P = .01$), suggesting that KRAS mutations are associated with a higher incidence of metastatic disease at diagnosis time.[26]

In the framework of the EGFR signaling pathway, alteration of the BRAF gene has important implications in the growth, proliferation, apoptosis, differentiation, and survival of cells in an independent way from the EGFR pathway.[9] Mutations of the BRAF gene affect several sites located in the kinase domain, and over 70% of these alterations imply the V600E codon. Structural studies have shown that valine from position 600 of the kinase domain is essential to keep the Braf protein in an inactive conformation in the absence of the Kras-Braf interaction.[27]

In the present study, BRAF mutation was identified in 8.62% of patients (5/58), nearby value to other studies, namely those conducted in Turkey, Germany, Slovenia, Spain, and UK (5.1%–7.9%), but smaller than identified in Greek patients (14.4%).[13,14,18,19,21,28] The V600E mutation was noted in all BRAF mutant samples. Some reports suggested that BRAF mutations are associated with distinct clinical and pathological features, such as the increased frequency in women, right tumor location, mucinous histology, positivity of Keratin-7, and MSI.[29] In our study, except for the significant association of BRAF mutation with the MSI-H phenotype compared to MSI-L/MSS ($P < .001$), we could not establish a significant association of BRAF with the parameters mentioned above, probably due to the modest number of patients and reduced frequency of these mutations. This finding is consistent with the other data from the literature, which suggests that BRAF mutation, MSI-H status, along with a high CD3 rate, and absence of p53 nuclear expression with Mapsin cytoplasmatic predominance represent the best prognosis for CRC patients. In contrast, in patients who exhibit BRAF mutation, MSS phenotype, the low score of CD3 and p53 (>50%) with Mapsin nuclear predominance, have the worst prognosis.[30]

Activating mutations of the PIK3CA gene are noticed in 7% to 32% of CRC patients.[31] In our study, the frequency of PIK3CA was 6.88%, lower than average reported in Italy and France (13.3%–17.8%), rather closer to rates reported in Singapore and Poland (2.2%–9.6%).[10,12,17] Furthermore, we observed the predominance of mutations at exon 20 compared with exon 9 (5.16% vs 1.72%), which is concordance with Chinese studies, but rather different from the results from Westerns countries.[10] De Roock et al suggest that mutations in exon 20 of PIK3CA might be associated with a low response rate to therapy and outcome.[17] Interestingly, some studies demonstrated that tumors with PIK3CA mutations are characterized by location in the proximal colonic and low grade of histological differentiation.[10] In accordance, PIK3CA mutations in our subjects were exclusively found in proximal colon tumors relative to the distal colon or rectum ($P = .04$). Likewise, PIK3CA mutations were more likely to exhibit well and moderately differentiation ($P = .01$).

AKT1 is an active core of the PI3KCA/AKT/mTOR signaling pathway, controlling diverse cellular processes, including cell survival, proliferation, invasion, and metabolism.[32] To date, AKT1 E17K mutations have been reported in several tumors (colon, lung, and breast) ranging between 1% and 3%.[33] In this study, the sequencing analysis of exon 2 of the AKT1 showed the point mutation of G to A at nucleotide 49 (E17K) in 2 patients (3.44%), which is in concordance with other studies.[34] This missense mutation substitutes glutamic acid with lysine at amino acid position 17, a mutation that affects the Pleckstrin homology domain of the enzyme that is no longer dependent on activation of upstream components of the pathway.[11] Many studies revealed that tumors harboring the AKT1 E17K mutation are generally found to be negative for KRAS and BRAF, which is in concordance with our study. Nevertheless, AKT1 E17K mutations in diverse cancers play a dual role (antitumor functions and oncogenic). The antitumor effect could appear through negative feedback of the AKT pathway, while the oncogenic effect may enhance the migration and metastatic potential of tumor cells.[33]

Particularly, our study has some limitations. First, the relatively small sample size might not provide enough statistical data to explore the relationship between genotyping and clinical and pathological features. Secondly, because our patients were diagnosed recently, follow-up information such as recurrence, and the therapeutic response were not available. Third, hotspot mutations in other exons of the above-mentioned genes were not screened due to financing limitations. Fourthly, MSI-PCR based on PCR amplification of MS regions followed by capillary electrophoresis does not provide indications about MMR genes and requires at least the presence of 20% tumor cells in the sample.

5. Conclusion

In conclusion, our study showed that the occurrence of mutations in the KRAS, BRAF, PIK3CA, and AKT1 genes are common events in CRC patients from the Black Sea coast geographical area of Romania. The results of this study are in concordance with other studies conducted in the same geographical area. These findings have important implications for the personalized treatment of Romanian CRC patients, thereby providing an opportunity to improve healthcare efficiency and resource use in these patients.

Author contributions

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Molecular profiling of the colon cancer in South-Eastern Romania

Results from the MERCUR study

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Abstract

Colorectal cancer is a heterogeneous disease with multiple epigenetic alterations and different molecular features. The molecular classification is based on 2 major distinct pathways: microsatellite stable pathway and the microsatellite instability pathway. Molecular profiling of colorectal cancer provides important information regarding treatment and prognosis. Aim of the study was to assess the frequency of microsatellite instability in colon cancer and the clinicopathological characteristics of the tumors with high level of microsatellite instability (MSI-H) in our region. The secondary outcome was to assess the frequency of v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) mutations in colon cancer.

The study included 129 patients with colon cancer fit for surgery. Demographic data, clinical and pathological data, immunohistochemistry staining pattern (4 mismatch repair proteins were investigated), and *BRAF* gene mutations were assessed. According to microsatellite instability status by polymerase chain reaction, patients were divided into 3 groups: microsatellite stable (MSS) = 108 patients, high level of microsatellite instability (MSI-H) = 15 patients and low level of microsatellite instability (MSI-L) = 6 patients. Different clinicopathological comparisons between MSS and MSI-H patients, and between MSS and MSI-L patients were performed.

Microsatellite instability was found in 16.3% patients: 11.6% had MSI-H and 4.7% had MSI-L. Significantly more patients in the MSI-H group than in the MSS group were female ($P = .01$) and had a family history of colon cancer ($P < .001$). MSI-H and MSI-L groups were associated with the ascending colon location of the tumors, were mostly type G3, T2, and stage I whereas MSS tumors were mostly G2, pT3, and stage III. Overall, *BRAF* mutations were identified in 18/129 patients (13.9%). BRAF mutant tumors were predominantly associated with MSI-H and MSI-L tumors. Immunohistochemistry had a sensitivity of 76% and a specificity of 89% in detecting MSI tumors and an accuracy of 87.6%.

The frequency of microsatellite instability in our study was 16.3%. MSI-H is a distinct molecular phenotype of colon cancer with particular features: female gender, family history of colorectal cancer, a predilection for the ascending colon, poorly differentiated, predominantly T2, and stage I. The frequency of *BRAF* mutations was 13.9% and mutations were more often present in the MSI tumors.

Abbreviations: BMI = body mass index, *BRAF* = v-raf murine sarcoma viral oncogene homolog B1, CRC = colorectal cancer, dMMR = deficient mismatch repair, FFPE tissue = formalin-fixed paraffin-embedded tissue, IHC = immunohistochemistry, MMRP = mismatch repair proteins, MSI = microsatellite instability, MSI-H = high level of microsatellite instability, MSI-L = low level of microsatellite instability, MSS = microsatellite stable, PCR = polymerase chain reaction, PCR = polymerase chain reaction.

Keywords: *BRAF* gene, colon cancer, microsatellite instability, mismatch repair proteins

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1. Introduction

Colorectal cancer (CRC) is the third most common cancer and the second cause of cancer deaths worldwide.[1] CRC is a heterogeneous disease with multiple genetic and epigenetic alterations and different molecular features. Molecular profiling of CRC has the advantage of providing essential information in the pathogenesis of cancer and, also, information about prognosis and therapy.

The molecular classification of CRC is based on 2 major distinct pathways: chromosomal instability or microsatellite stable (MSS) pathway and the microsatellite instability (MSI) pathway; chromosomal instability/MSS accounts for 80% of all CRC and MSI for about 20%.[2] MSI occurs by 2 different mechanisms in sporadic and hereditary CRC: in sporadic cancer, the cause is a hypermethylation of the MLH1 promoter and sometimes sporadic mutations, and in Lynch Syndrome, the cause is a mutation in 1 of the 4 DNA mismatch repair proteins (MMRP).[3]

The DNA mismatch repair system has the role to identify and correct DNA defects; errors in the mechanisms of this system lead to MSI status which is defined by deficient mismatch repair (dMMR) during DNA replication.[3,4] The proteins involved in the mismatch repair system are MLH1, MSH2, MSH6, PMS2; usually, these proteins form a complex represented by a tetramer composed of 2 heterodimers: MLH1/PMS2 and MSH2/MSH6.[5] Their expression is interdependent: if 1 protein is absent, the partner protein is degraded, and the consequence is a dMMR, finally resulting in MSI.[6]

At present, there are 2 methods to detect the status of MSI: by fluorescent based polymerase chain reaction (PCR) assay followed by capillary electrophoresis fragment size analysis and by immunohistochemistry (IHC); IHC detects dMMR and PCR detects MSI. Based on guidelines,[7] a standard set of 5 microsatellites sequences are tested and, according to the number of markers which show instability, tumors are classified as MSI-H (when 2 or more microsatellites are instable), MSI-L (when only 1 microsatellite is instable) and MSS if markers show no expression.[6] The IHC method is based on the detection of expression of MMRP (MLH1, MSH2, MSH6, PMS2) in the tumor cells. In tumors with dMMR, MMRP show a loss of nuclear expression.[8] The majority of the dMMR tumors are characterized by loss of expression of both MMR proteins in a heterodimer (MLH1/PMS2 or MSH2/MSH6), but unusual IHC patterns can also occur, especially in the setting of Lynch syndrome.[9] Fluorescent PCR based assay is considered the gold standard for the detection of MSI in CRC.[10] Several studies showed that the detection of MSI by IHC is similar to the fluorescent PCR based method.[11,12] Although these 2 methods are complementary, they provide different information. There is a trend that all CRC, regardless of age, should be tested for MSI using either IHC, PCR, or both for better results.[6]

Molecular classification provides important information regarding treatment and prognosis. Potential roles of testing MSI in CRC could be: a screening tool for hereditary nonpolyposis colorectal cancer, prediction for chemotherapy response, and, also, a prognostic biomarker.[13] Genetics has an important role in individual risk of developing CRC, but modifiable risk factors (such as diet, lifestyle, obesity, alcohol) also contribute significantly by increasing the individual risk. Advanced research showed that the broad spectrum of genetic, epigenetic, and molecular alterations in CRC is likely to be more extensive than previously reported, thus investigating the underlying genetic phenotype would provide quality data for basic research in the etiopathogeny, prognosis, personalized treatment or even response to treatment, given the opportunity to health workers to apply the concept of "personalized medicine."

Although the tumor stage has the most important role in the prognosis of CRC, the molecular phenotype is also associated with different outcomes. Beside MSI status, *BRAF* (v-raf murine sarcoma viral oncogene homolog B1) gene mutations mainly through mutations at codon 600 (V600E) showed different associations with survival.[14,15] MSS tumors with BRAF mutations have a negative prognostic with poor survival rates,[16,17] but little is known about MSI-H tumors and associated *BRAF* mutations.

The present study aimed to assess the frequency of MSI by the fluorescent PCR based assay in colon cancer and the clinicopathological characteristics of the MSI-H tumors in our region. The secondary outcome was to assess the frequency of *BRAF* mutations in colon cancer according to MSI status.

2. Methods

The present study included all patients diagnosed with CRC and prospectively admitted for elective surgery to the Surgery Department of Constanta County Clinical Emergency Hospital between January 01, 2019 and December 31, 2019. Inclusion criteria: patients with previously histopathological diagnosed colon cancer at colonoscopy with biopsies and scheduled for surgery (stage 0–III), colon location of the tumor (from sigmoid to the cecum), more than 16 years old and fit for surgery, informed written consent. Exclusion criteria: rectal cancers, stage IV tumors, nonelective surgery for colon cancer, unfit for surgery (severe comorbidities which contraindicate surgery), absence of the consent for surgery or for the inclusion in the study, age less than 16 years old.

Out of 163 patients prospectively admitted to the Surgery Department, only 129 patients met the inclusion and exclusion criteria and were enrolled in the study.

Demographic data, clinical data, pathological data, IHC staining pattern, MSI status by fluorescent PCR based assay, and BRAF mutations status were assessed in all patients.

Demographic and clinical data included age, gender, body mass index (BMI) (<18.5 = underweight, 18.5–24.9 = normal weight, 25–29.9 = overweight, ≥ 30 = obese),[18] alcohol consumption status (according to National Institute on Alcohol Abuse and Alcoholism,[19] moderate alcohol consumption is up to 1 drink per day for women and up to 2 drinks per day for men, binge drinking is defined as ≥ 5 drinks (male) and ≥ 4 drinks (female) in about 2 hours, and heavy alcohol use is defined as ≥ 4 drinks on any day for men and ≥ 3 drinks for women), smoking status (according to Center for Disease Control and Prevention,[20] patients were divided into current smoker, former smoker, or never smoker), and family history of CRC (we included patients with available data about 1st-degree relatives with a positive diagnosis of CRC). Patients who met the definitions for moderate and heavy alcohol use were categorized as drinkers and patients who were current and former smokers were categorized as smokers. (We included patients with available data about 1st degree relatives with a positive diagnosis of CRC; other details about relatives were not requested).

Pathological data included details about tumor location, histologic grading (G1 = well-differentiated, G2 = moderate dif-

ferentiated, G3 = poor differentiated), lymphovascular and perineural invasion (present/absent), pTNM classification and tumor stage, IHC staining pattern for MMPR (4 MMPR were investigated: MLH1, MSH2, MSH6, PMS2), MSI status by PCR and BRAF mutations status.

2.1. Mismatch repair proteins immunohistochemistry

Immunohistochemistry for the 4 most common MMRP was performed in all cases using the standard procedure recommended by Vitro, Master Diagnostica. Tumor representative blocks were selected for analysis with normal junction to assess staining results properly. Primary monoclonal antibodies against MLH1 (clone BS29, ready to use, Vitro SA, Master Diagnostica, Spain), MSH2 (clone FE11, ready to use, Vitro SA, Master Diagnostica), MSH6 (clone EP49, ready to use, Vitro SA, Master Diagnostica) and PMS2 (clone BS29, ready to use, Vitro SA, Master Diagnostica) were applied on 4 μm deparaffinized, rehydrate and heat-induced epitope retrieval sections. The reaction was visualized with Master Polymer Plus Detection System (DAB included) and slides were counterstained with hematoxylin.

Non-neoplastic colonic mucosa and appendix were used as internal positive controls. The known MMRP deficient colorectal carcinomas served as external negative controls. Two experienced pathologists evaluated the staining results independently and blindly to the MSI status. Positive expression was defined as nuclear staining within tumor cells, while negative protein expression was defined as a complete absence of nuclear staining within tumor cells with concurrent internal positive controls. If internal non-neoplastic tissues showed invalid negative staining, the procedure was routinely repeated.

2.2. DNA Isolation

Genomic DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol. For DNA isolation, tissue areas up to 250 mm^2, and up to 8 sections with a maximum thickness of 10 μm were used for each case. Hematoxylin and eosin stained sections were used as a reference and the largest tumor area (at least 50% tumor cells) was scraped off with a scalpel under a dissecting microscope.

2.3. BRAF mutation analysis

For identification of *BRAF* mutations we used a method based on PCR and reverse hybridization (Strip Assay, Vienna Lab Diagnostics GmbH, Austria) following the manufacturer's instructions. The assay covers 9 mutations in the BRAF gene (codon 600 and 601: V600A, V600D, V600E, V600G, V600K, V600M, V600R, K601E). Procedure includes a PCR amplification using biotinylated primers hybridization of amplification products to a test strip containing allele-specific oligonucleotide probes immobilized as an array of parallel lines. Bound biotinylated sequences are detected using streptavidin-alkaline phosphatase and color substrates.

2.4. MSI status by fluorescent PCR based analysis

The MSI status was evaluated using fluorescently-labeled microsatellite PCR primers, followed by separation of the amplicons by capillary electrophoresis using a 3500 Genetic Analyzer (Applied Biosystems, Fitchburg, WI), and analysis of data was performed using GeneMapper software, version 5 (Applied Biosystems). Typically, MSI analysis involves comparing allelic profiles of microsatellite markers generated by amplification of DNA from matching normal and test sample, which may be mismatch-repair (MMR) deficient. Alleles that are present in the test sample but not in corresponding normal samples indicate MSI. The MSI assay (MSI Analysis System version 1.2 kit, Promega Corporation) determined 5 quasimonomorphic mononucleotide markers (BAT-25, BAT-26, NR-21, NR-24, and MONO-27) and 2 pentanucleotide markers (Penta C and Penta D). Each PCR amplification assay consists of 2 ng of genomic DNA and 8 μL master mix (5.85 μL Nuclease-Free Water, 1 μL Gold STAR 10X Buffer, 1 μL MSI 10X Primer Pair Mix and 0.15 μl AmpliTaq Gold DNA polymerase). Samples were incubated in a thermocycler with the following parameters: 95°C for 11 minutes, 96°C for 1 minute, then 10 cycles (94°C for 30 seconds, 58°C for 30 seconds, 70°C for 1 minute), followed with 20 cycles (90°C for 30 seconds, 50°C for 30 seconds, 70°C for 1 minute) and final extension for 30 minutes at 60°C. In the capillary electrophoresis analysis, 1 μL of the amplified product was combined and mixed with a loading cocktail (0.5 μL × samples + 9.5 Hi-Di Formamide × sample) and loaded in a PCR thermal cycler (3 minutes at 95°C and cooled at 4°C) before load in the ABI 3500 sequencer.

MSI was defined as a marked alteration in repeat length or as a discrete band below or above the expected allele. Analysis was performed by 2 experienced geneticists who evaluated the results independently and blindly to the IHC results. Following National Cancer Institute guidelines,[10] MSI at more than 2 loci was defined as a high level of microsatellite instability (MSI-H), MSI at a single locus was defined as low level of microsatellite instability (MSI-L), and absence of instability at any of the loci was defined as microsatellite stable group (MSS).

According to MSI status by PCR, patients were divided into 3 groups: MSS = 108 patients, MSI-H = 15 patients and MSI-L = 6 patients. Different comparisons (demographic, clinical, pathological, IHC staining patterns, BRAF mutations status) between MSS and MSI-H patients, and between MSS and MSI-L patients were performed. All analyses were performed with the MSS group as the reference.

2.5. Statistical tests

Statistical analysis was performed using the JASP 0.11.1 statistic software package. Descriptive statistics were used for demographic, clinical, and pathological data: mean+/− standard deviation for continuous variables in the MSI-H group, and median+/− interquartile range in the MSI-H and MSI-L group; frequency was used for categorical variables. Comparisons were performed with the MSS group as the reference. For comparison between variables, the Mann–Whitney *U*-test was used for continuous variables, and the chi-square test or Fisher exact test (when cell count was 0) were used for categorical variables. Sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of IHC for detecting MSI tumors (having PCR method as gold standard for MSI status) were calculated. Results were considered statistically significant if P-value $< .05$.

2.6. Ethical approval

The study was conducted according to good laboratory practice and in accordance with the Declaration of Helsinki and national

and institutional standards. Informed consent was obtained from all patients, and the study was approved by the Local Ethics Commission for the Approval of Clinical and Research Developmental Studies (approval no. 16/2018).

3. Results

Microsatellite instability was found in 21 (16.3%) patients: 15 (11.6%) had MSI-H and 6 (4.7%) had MSI-L. Most of the patients, 108 (83.7%) were MSS.

Demographic and clinical data of the patients according to the MSI status are illustrated in Table 1. There were no significant differences between groups regarding age, alcohol consumption status, and smoking status. Significantly more patients in the MSI-H group than in the MSS group were female. Patients in the MSS group had significantly higher mean BMI and subanalysis between categories of BMI showed that most of the patients in the MSI-H group and MSI-L were normal weighted in contrast with patients in the MSS group which were mostly overweighted. Diabetes and hypertension were more often encountered among patients with MSS tumors. Significant more patients from the MSI-H group than from the MSS group had a family history of colon cancer: 53.3% versus 12%, $P < .001$.

The pathological data of the tumors according to the MSI status is illustrated in Table 2.

Table 1

Demographic and clinical data of the patients according to the MSI status.

Variables	MSS (n = 108)	MSI-H (n = 15)	MSI-L (n = 6)
Age (yr)	66.1 +/− 12.2	67 (57–61)	65.5 (58–68)
Mean +/− SD or median (IQR)	Ref	.81	.75
Gender, n (%)			
Female	50 (46.3%)	12 (80%)	3 (50%)
Male	58 (53.7%)	3 (20%)	3 (50%)
P-value	Ref	.01	.85
BMI			
Mean +/− SD or median (IQR)	26.4 +/− 4.29	19.7 (18.5–22.7)	20.1 (18.7–22.5)
	Ref	.02	.03
Underweight, n (%)	4 (3.7%)	1 (6.6%)	0 (0.0%)
P-value	Ref	.58	>.99
Normal weight, n (%)	20 (18.5%)	7 (46.7%)	4 (66.6%)
P-value	Ref	.01	<.001
Overweight, n (%)	58 (53.8%)	4 (26.6%)	2 (33.3%)
P-value	Ref	.04	.33
Obese, n (%)	26 (24%)	3 (20.1%)	0 (0.0%)
P-value	Ref	.71	.33
Diabetes, n (%)	65 (60.1%)	4 (26.6%)	2 (33.3%)
P-value	Ref	.01	.19
Hypertension, n (%)	60 (55%)	3 (20%)	3 (50%)
P-value	Ref	<.001	.78
Alcohol, n (%)	49 (45.3%)	6 (40%)	5 (83.3%)
P-value	Ref	.69	.06
Smoking, n (%)	72 (66.6%)	8 (53.3%)	4 (66.6%)
P-value	Ref	.31	>.99
Family history of colorectal cancer, n (%)	13 (12%)	8 (53.3%)	0 (0.0%)
P-value	Ref	<.001	>.99

Median and interquartile range (IQR) was used to assess age and BMI in the MSI-H and MSI-L groups. BMI = body mass index, MSI-H = high level of microsatellite instability, MSI-L = low level of microsatellite instability, MSS = microsatellite stable.

Table 2

Pathological data of the tumors according to the MSI status.

Variables	MSS (n = 108)	MSI-H (n = 15)	MSI-L (n = 6)
Location, n (%)			
P-value			
Sigmoid colon	34 (31.4%)	2 (13.3%)	0
	Ref	.14	.17
Descending colon	24 (22.2%)	2 (13.3%)	1 (16.6%)
	Ref	.42	.74
Transverse colon	8 (7.4%)	1 (6.7%)	0
	Ref	.91	> .99
Ascending colon	28 (26%)	8 (53.4%)	4 (66.6%)
	Ref	.02	.03
Cecum	14 (13%)	2 (13.3%)	1 (16.6%)
	Ref	.96	.79
Histologic grading, n (%)			
p-value			
G1	12 (11.1%)	1 (6.7%)	2 (33.3%)
	Ref	.59	.79
G2	74 (68.5%)	5 (33.3%)	2 (33.3%)
	Ref	<.001	.07
G3	22 (20.4%)	9 (60%)	2 (33.3%)
	Ref	<.001	.44
Lymphovascular invasion, n (%)	95 (87.9%)	5 (33.3%)	5 (83.4%)
P-value	Ref	<.001	.73
Perineural invasion, n (%)	77 (71.3%)	5 (33.3%)	4 (66.6%)
P-value	Ref	<.001	.80
pT, n (%)			
P-value			
T1	2 (1.8%)	0 (0.0%)	1 (16.6%)
	Ref	>.99	.1
T2	35 (32.4%)	11 (73.3%)	5 (83.4%)
	Ref	<.001	.01
T3	58 (53.7%)	3 (20.0%)	0 (0.0%)
	Ref	.01	.01
T4	13 (12.1%)	1 (6.7%)	0 (0.0%)
	Ref	.1	>.99
pN, n (%)			
P-value			
N0	67 (62.0%)	8 (53.3%)	4 (66.6%)
	Ref	.51	.81
N1	30 (27.7%)	4 (26.7%)	1 (16.6%)
	Ref	.92	.55
N2	11 (10.3%)	3 (20.0%)	0
	Ref	.26	>.99
Stage, n (%)			
P-value			
I	16 (14.8%)	10 (66.6%)	4 (66.6%)
	Ref	<.001	<.001
II	41 (38%)	4 (26.7%)	2 (33.3%)
	Ref	.39	.81
III	51 (47.2%)	1 (6.7%)	0
	Ref	<.001	.03

MSI-H = high level of microsatellite instability, MSI-L = low level of microsatellite instability, MSS = microsatellite stable, pN = lymph node metastasis, pT = depth of tumor invasion.

Location: MSI-H and MSI-L groups were associated with the ascending colon location of the tumors. There were no significant differences between groups regarding other locations of the tumors.

Histologic grading: MSI-H tumors were mostly type G3 whereas MSS tumors were mostly G2. Regarding MSI-L tumors, there were no significant differences.

Lymphovascular invasion and perineural invasion: Lymphovascular invasion was more common in MSS tumors than in MSI-

H tumors. Similarly, MSS tumors were associated with perineural invasion. MSI-L tumors analysis did not show significant differences in contrast MSI-H tumors regarding lymphovascular and perineural invasion.

TNM classification: MSI-H and MSI-L tumors were predominantly T2 whereas MSS tumors were predominantly T3. There were no tumors classified as Tis. Analysis of lymph nodes involvement (pN) showed no significant differences between groups. All patients included in the study were without distant metastases (M0).

Stage: MSI-H tumors and MSI-L tumors were predominantly stage I and MSS tumors were predominantly stage III. There were no patients with stage 0 tumors.

Of all the 15 patients with MSI-H tumors, only 2 patients had a medical history of cancer: 1 patient had endometrial cancer, and 1 had urinary bladder cancer.

3.1. Frequency of BRAF mutations according to the MSI status

Overall, *BRAF* mutations were identified in 18/129 patients (13.9%). Out of the 9 mutations investigated (codon 600 and 601: V600A, V600D, V600E, V600G, V600K, V600M, V600R, K601E), only V600E was present. BRAF status of the tumors according to the MSI status is illustrated in Table 3. BRAF mutant tumors were significantly associated with MSI-H and MSI-L tumors in contrast with MSS tumors.

Demographic and clinical subanalysis of the MSI – *BRAF* mutant tumors (including both MSI-H and MSI-L) in contrast with MSI *BRAF* non-mutant tumors (illustrated in Table 4) showed an association of *BRAF* mutant status with female gender ($P = .01$) and family history of colon cancer ($P < .001$).

IHC staining data pattern is illustrated in Table 5. According to the IHC staining, we observed 11 (10.2%) patients in the MSS group that had 1 MMPR negative (false MSI tumors). Also, we observed 1 patient from MSI-H who had all MMPR positive (false MSS tumor). Most of the patients from the MSS group had all MMPR positive, and most of the patients from MSI-H had 2 MMPR negative. Regarding MSI-L tumors, 66.7% had all MMPR positive and 33.3% had 1 MMPR negative. The pattern with 3 or all 4 MMPR negative was not encountered in any of the 3 groups. Statistical analysis showed that IHC had a sensitivity of 0.76 (95% confidence interval [CI] 0.52–0.91) and a specificity of 0.89 (95% CI 0.82–0.94) in detecting MSI tumors with a positive predictive value of 0.59 (95% CI 0.44–0.72), a negative predictive value of 0.95 (95% CI 0.90–0.97), a positive likelihood ratio of 7.48 (95% CI 4.07–13.75), a negative likelihood ratio of 0.27 (95% CI 0.12–0.57), and an overall accuracy of 0.87 (95% CI 0.80–0.92).

Table 3

***BRAF* status of the tumors according to the MSI status.**

BRAF STATUS	MSS (n = 108)	MSI-H (n = 15)	MSI-L (n = 6)
Mutant, n (%)	3 (2.8%)	11 (73.3%)	4 (66.6%)
Non-mutant, n (%)	105 (97.2%)	4 (26.6%)	2 (33.3%)
p-value	Ref	<.001	<.001

BRAF = v-raf murine sarcoma viral oncogene homolog B1, MSI-H = high level of microsatellite instability, MSI-L = low level of microsatellite instability, MSS = microsatellite stable.

Table 4

Demographic and clinical data of the MSI patients according to the *BRAF* mutations status.

Variables	MSI *BRAF* mutant (n = 15)	MSI *BRAF* non-mutant (n = 6)	*P*-value
Age (yr)			
Median (IQR)	60 (56–69)	62 (60–67)	.67
Gender, n			
Female	13	2	.01
Male	2	4	
BMI			
Median (IQR)	19 (18.3–22.3)	20.3 (19.5–21.8)	.56
Diabetes, n	1	1	.17
Hypertension, n	2	1	.39
Alcohol, n	4	2	.17
Smoking, n	6	1	.82
Family history of colorectal cancer, n	8	0	<.001

Percentages in the MSI *BRAF* non-mutant group were not calculated due to the small number of patients.

MSI-H = high level of microsatellite instability, MSI-L = low level of microsatellite instability, MSS = microsatellite stable.

4. Discussion

The molecular classification of CRC is closely associated with clinicopathological features of the tumors, prognosis, treatment strategy and response to treatment, both in hereditary CRC and sporadic CRC, and detecting MSI is of paramount importance.[21–23]

In our study, microsatellite instability was found in 16.3% patients: 11.6% had MSI-H and 4.7% had MSI-L. Most of the patients (108) were MSS. Our results are in concordance with previous studies: Salovaara et al[24] found MSI in 12% of the 535 investigated CRCs, similar results being reported also by other authors.[25,26] One of the largest pooled analyses of more than 7600 CRC reported MSI in 16.7% of the cases.[21]

As reported previously, MSI-H tumors are known for having some distinctive features: early age of onset, proximal location, higher frequency of family history of CRC, mucinous type and poorly differentiated phenotype, and lymphocytic infiltration.[27,28] In the present study, we identified significant differences between the clinical characteristics of MSS and MSI-H tumors. MSI-H tumors were predominantly associated with female gender ($P = .14$), 53.3% had a family history of CRC ($P < .01$) and had a median BMI of 19.7 ($P = .02$), while MSS tumors were associated with male gender, 12% had a family history of CRC and the mean BMI was higher (26.4). Obesity is an established risk factor for CRC.[29] Regarding categories of

Table 5

IHC staining data pattern according to the MSI status.

IHC staining pattern, n (%)	MSS (n = 108)	MSI-H (n = 15)	MSI-L (n = 6)
One MMRP –	11 (10.2%)	2 (13.4%)	2 (33.3%)
Two MMRP –	0	12 (80%)	0
Three MMRP –	0	0	0
All 4 MMRP –	0	0	0
All MMRP +	97 (89.8%)	1 (6.6%)	4 (66.7%)

"–" = negative, "+" = positive, IHC = immunohistochemistry, MMRP = mismatch repair protein, MSI-H = high level of microsatellite instability, MSI-L = low level of microsatellite instability, MSS = microsatellite stable.

BMI, there were differences between normal weighted and overweighted patients within the 2 groups: most of the patients in the MSI-H group (46.7%) were normal weighted and only 26.6% were overweighted, in contrast with patients from the MSS group where only 18.5% were normal weighted and the majority (53.8%) were overweighted; obesity was present in both groups, irrespective of the MSI status: 24% of the patients with MSS tumors and 20.1% of the patients with MSI-H were obese. Similar to our results, a large case-control study on obesity,[30] CRC risk, and MSI status, concluded that higher BMI was associated with MSS phenotype. Nakayama et al[31] found also that diabetes is more common in MSS tumors than in MSI-H tumors. Unfortunately, there are no other studies on this topic and MSI status in Romania to compare the results.

Alcohol consumption and smoking are other well-established risk factors for CRC.[29] In our study, these risk factors were present in more than half of the patients in each group irrespective of the MSI status. We also investigated the association between MSI status with diabetes and hypertension and we found that patients with MSS tumors are more likely associated with these 2 diseases than MSI-H patients.

In contrast with other studies[27,28] that showed that MSI-H tumors are associated with early age of CRC onset, the median age of the patients from the MSI-H group in our study was 67 years and was similar to the mean age of the patients from MSS group or MSI-L group. This could be a particularity of the patients with MSI-H phenotype in our country, but because of the small number of patients in this group, larger studies are needed to confirm this characteristic.

As reported previously,[27,28] MSI-H cancers are usually located in the proximal colon and are poorly differentiated. In our study, 53.4% of the patients from the MSI-H group were located in the ascending colon in contrast with 26% of the patients from the MSS group ($P = .28$). Our results also indicated that tumors with MSI-H phenotype are characterized by poorer differentiation as 60% of them were classified as G3 type. Lymphovascular and perineural invasion were mostly reported in tumors from MSS group (87.9%, 71.3%, respectively) in contrast with tumors from MSI-H (33%, 33%, respectively), suggesting a more extensive disease in patients from MSS group. Regarding the depth of tumor invasion (pT), the majority of the tumors from MSI-H group (73.3%) were limited to muscularis propria (T2), suggesting a less invasive pattern of the tumors in contrast with the MSS tumors which were predominantly invading the subserosa (53.7%). Similar results were reported by Jung et al.[32] In our study, patients from the MSI-H group were predominantly classified as having stage I tumors (66.6%) in contrast with patients from the MSS group who were predominantly stage III (47.2%). This result suggests that patients with MSI-H phenotype have a less advanced disease in contrast with patients with MSS phenotype and can possibly explain the better prognosis of MSI cancers compared with MSS ones. Other studies who investigated the prognostic role of MSI status showed that MSI-H phenotype was an independent prognostic factor, along with stage, tumor grade differentiation, and histology type of the tumor.[32–34]

The distinction between MSI-H phenotype and MSS phenotype was proved in several studies and now it is universally accepted in the literature that MSI-H tumors have a distinct clinicopathologic phenotype and different prognostic and our results are in concordance with literature.[27,28,32–34] In contrast, the MSI-L phenotype was not associated with distinctive features in literature, and usually this type of tumors have similar features to MSS tumors.[33–35] Despite this evidence, some authors tried to demonstrate that MSI-L is a distinct sub-group of sporadic CRC with specific molecular features.[36–38] Interestingly, in our study, some characteristics of the patients with MSI-L phenotype were different from MSS phenotype: 66.6% had tumors located in the ascending colon ($P = .03$), the majority of the patients (83.4%) had a depth of tumor invasion T2 while the majority of the MSS patients had T3. Also, most of the patients with MSI-L phenotype were stage I (66.6%), followed by stage II (33.3%), findings in contrast with features of MSS patients who were predominantly stage III (47.2%). Unfortunately, due to the small number of patients in the MSI-L group, these findings could not be assessed properly and larger studies are needed to confirm these characteristics.

About 10% of the CRC have *BRAF* mutations.[39] The prognostic role of *BRAF* mutations in CRC has been largely investigated and it was demonstrated that mutant tumors are associated with significantly poorer prognosis[15,40] Numerous studies[16,17,41] have shown that patients with MSS phenotype and *BRAF* mutations have a worse prognosis than MSI-H tumors with mutations. In line with the results of previous studies,[39,41] in our study, the frequency of *BRAF* mutations was 13.9%, and our analysis by MSI status showed that *BRAF* mutations were more frequent in the MSI-H group than in the MSS group (73.3% vs 4.6%, $P < .01$). Results from an analysis of a Romanian CRC cohort[42] showed that *BRAF* mutations were detected in 16% of the cases. Contrary to the similarities in age, BMI, presence of diabetes or hypertension in the subanalysis of the MSI patients with *BRAF* mutations vs. without mutations, female gender and family history of CRC were found to differ significantly between the 2 groups, *BRAF* mutations being predominantly associated with female gender ($P = .04$) and family history of CRC ($< .001$). Studies showed that *BRAF*-mutated tumors are often right-sided, more prevalent in women and associated with MSI.[43] Strikingly, among patients with MSS phenotype, we found 5 (4.6%) patients who had *BRAF* mutations, and 4 patients with MSI-L phenotype who had also mutations. According to literature, *BRAF* mutations in MSI-H phenotype has no prognostic effect, but *BRAF*-mutated CRC with MSS phenotype is a distinct molecular phenotype which place patient at risk for poor treatment response and worse prognosis.[43,44]

Regarding the 2 methods for MSI analysis, it has been reported that the IHC method for detection of MSI status has similar results with the PCR method.[11,12] The results obtained from IHC or PCR studies are complementary, but provide different information. The PCR method cannot detect which mismatch repair protein is deficient while IHC provides specific data regarding which protein in the mismatch repair tetramer is deficient.[6] Taking this into account, the PCR method cannot distinguish between sporadic or Lynch syndrome-associated MSI cancer. It is now generally recommended that all CRC patients should be tested for MSI either by PCR, IHC, or both.[8] The decision on which test to use is institution-dependent, but MSI analysis based on PCR remains the gold standard for detecting microsatellite status.[7,10] Using IHC method, tumors displaying loss of 1 or more MMRPs can be classified as deficient MMR and are considered to be MSI-H, whereas those with intact MMRPs can be classified as proficient MMR and are considered to be MSS or MSI-L.[45] Usually, MSI cancers are characterized by 2 negative MMPRs in a heterodimer: in sporadic MSI cancers, loss of MLH1-PMS2 is characteristic, whereas in Lynch syndrome

either heterodimer may be lost, but unusual IHC patterns are reported such as isolated loss of 1 of the 4 MMRPs.[7,8] When all 4 MMPRs are intact, tumors are assumed to be MSS.[7] Lynch syndrome is present in 1% to 2% of the MSI tumors, it is characterized by loss of expression of 1 MMPR, and, in contrast with the sporadic cancers, it is not characterized by BRAF mutations (Lynch tumors have a wild-type BRAF gene).[7,9] In our study, according to the IHC staining pattern only, 97 (89.8%) patients were MSS and 11 (10.2%) patients showed loss of expression of 1 MMPR; taking into account only the IHC data, these 11 patients could be classified as MSI, but PCR showed that these patients are MSS. It is also worth noting that there was 1 patient classified as MSI-H by PCR while all the 4 MMPRs were positively expressed, meaning that this case could be classified as MSS or MSI-L by IHC. This study identified 11 patients that were MSS yet had negative MMPRS by IHC; furthermore, the current study identified 1 patient that was MSI-H despite IHC staining. False-negative rates for IHC were previously reported in the literature.[34,46,47] Despite the close correlation between the 2 methods of detection, our findings among other studies suggest that IHC cannot substitute PCR. Therefore, we believe that cases with all 4 MMPR positive by IHC may be classified as MSS or MSI-L (given the data supporting that MSI-L tumors are similar to MSS tumors and that these 2 phenotypes can be grouped), but when any of the MMPR is deficient, further examination by PCR is necessary to clearly determine the MSI status.

To our knowledge, the current study is one of the few in our country which assessed the MSI status of the CRC by PCR method (additionally to IHC method) and investigated the *BRAF* mutations in all patients.[48] Molecular analyses on CRC were also performed in our region, but they were based on the investigation of microRNAs.[49]

A possible limitation of the study could be the small number of patients in the MSI-H group and MSI-L group (data in the literature are few and limited on these categories of patients) and this topic should be studied in larger analyses to assess more precisely the particular features of these patients and obtain more conclusive results.

MSI tests may be used for diagnosis of suspected Lynch syndrome and also to identify clinical and therapeutical implications of MSI-H phenotype in sporadic CRC. There is extensive data which indicates that molecular testing and analysis should be incorporated into our practice for better management of CRC.

In conclusion, the overall frequency of MSI in our study was 16.3%: 11.6% for MSI-H and 4.7% for MSI-L. MSI-H is a distinct molecular phenotype of colon cancer with particular features: female gender, normal BMI, family history of CRC, a predilection for the ascending colon, poorly differentiated, predominantly T2 and stage I. The frequency of BRAF mutations was 13.9%, mutations were more often present in MSI-H tumors and were associated with female gender and family history of CRC. PCR remains the gold standard for the detection of MSI status in contrast with the IHC method.

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The diagnostic value of miR-92a, -143, and -145 expression levels in patients with colorectal adenocarcinoma from Romania

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Abstract

MicroRNAs (miRNAs) refers to a small, short non-coding RNA of endogenous class. They have shown to have an increasingly altered expression in many types of cancer, including colorectal cancer (CRC).

In the present study, miRNA TaqManMGB and qRT-PCR was used to quantify the expression and clinical significance of 3 mature human miRNA in 82 pairs of colorectal adenocarcinoma tissues and normal adjacent tissue samples (NATS) collected from patients of the south-east part of Romania. Differences between CRC and NATS were analyzed using Wilcoxon test, while correlations between miRNAs expression levels and clinicopathological features were examined using non-parametric tests. In addition, the ability of selected miRNAs to function as biomarkers and, as potential indicators in CRC prognosis was also examined.

When the miRNA expression was compared in CRC related NATS, miR-143, and miR-145 were significantly underexpressed (4.99 ± -1.02 vs -5.66 ± -1.66, $P < .001$; -4.85 ± -0.59 vs -9.27 ± -1.51, $P < .001$, respectively), while the pattern of miR-92a was significantly overexpressed (-5.55 ± -2.83 vs -4.92 ± -2.44, $P < .001$). Moreover, the expression levels of selected miRNAs were identified to be correlated with gradual increases in fold change expression with the depth of tumor invasion, lymph node invasion, and maximal increases with distant metastasis. Furthermore, the receiver operating characteristic analysis demonstrated that potential diagnostic of miR-143, miR-145, and miR-92a in discriminating CRC from NATS, with the area under the curve of 0.74, 0.85, and 0.84 respectively. The Kaplan–Meier and the log-rank test showed that a high level of miR-92a and low levels of miR-143 and miR-145 predicted poor survival rate in our cohorts.

In conclusion, we can summarize that miR-145 and miR-143 are decreased, while miR-92 is increased in CRC compared to NATS, and associated with different stages of CRC pathogenesis. Thus, the expression of selected miRNAs can represent potential diagnostic and prognostic tools in patients with CRC from Romania.

Abbreviations: AUC = area under the curve, CI = 95% interval of confidence, CRC = colorectal cancer, miRNA = microRNA, ncRNAs = non-coding RNAs, NATS = normal adjacent tissue samples, qRT-PCR = real-time quantitative polymerase chain reaction analysis, ROC = receiver operating characteristic, RT = room temperature, TNM = tumor-node-metastasis, UTR = 3' noncoding region, WHO = World Health Organization.

Keywords: colorectal cancer, miR-92a, miR-143, miR-145, real-time quantitative polymerase chain reaction analysis, expression profiling

1. Introduction

Among all ranges of tumors, colorectal cancer (CRC) is one of the most common malignancies and a significant public health burden. Moreover, the incidence rate of CRC is 9.7%, thereby making it the third most common form of cancer worldwide and it is the fourth leading cause of cancer-related mortality.[1] As per the findings of the Global Burden of Cancer Reports in the Romania population, the relative incidence of CRC increased from 8.660 in 2012 to 11.076 in 2018, thus representing the second most common malignancy, after lung/bronchus cancers in

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The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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men and breast cancer in women.[2] CRC is a multifactorial disease characterized by a sequential process associated with alteration of the molecular architecture in oncogene or tumor suppressor gene regulatory networks, mainly due to genomic mutation or epigenetic alterations and the involvement of small noncoding RNA species, named microRNAs (miRNAs) and long noncoding RNAs.[3,4]

In this regard, numerous studies and resources have been devoted to elucidating the molecular mechanisms of the CRC, however, the underlying mechanisms are not yet well understood. Therefore, improving the survival rate of patients with CRC requires a better understanding of tumor biology as well as the development of novel therapeutic and diagnostic strategies. A rapidly developing field of cancer research is the examination of miRNAs genes in CRC carcinogenesis. Molecules that are widespread and differentially expressed in CRC samples when compared to normal non-cancerous samples and may provide new insights into the mechanisms involved.

Functionally, miRNAs represent a novel class of small, non-coding RNAs (ncRNAs), found in plants, animals, and humans that use endogenous RNA interference pathways in order to modulate gene expression networks. Typically, miRNA genes are initially transcribed into the nucleus as longer primary transcripts guided by RNA polymerase II (pri-miRNAs), which are subsequently enzymatically cleaved by the Drosha into small miRNAs precursor (pre-miRNA). These pre-miRNAs are comprised of 70 nucleotides with hairpin stem-loop structures and are translocated into the cytoplasm through the assistance of Exportin-5 to undergo final maturation, within a functional miRNA to approximately 22 nucleotides catalyzed via RNase III endonuclease Dicer.[5] Predominantly, miRNAs exert their functionality in post-transcriptional modulation of gene expression through direct binding to the 3' untranslated region (UTR) of specific messenger RNA targets, thereby leading to cleavage and degradation or suppression of translation.[5] The latest version of the miRBase database contains – 1.917 entries of mature human miRNAs (http://www.mirbase.org), which can be classified into clusters and families based on seed sequence or genomic relatedness, able to regulate the expression of one-third of human protein-coding genes.

It is now widely accepted that the altered functionality of miRNAs plays an important role in various biological and cancer-related processes, such as control of cellular homeostasis, differentiation, cell growth, and apoptosis. In colorectal tumorigenesis, the specific expression patterns of human miRNAs have been used by many researchers to understand the involvement of these regulatory molecules in the diagnosis and prognosis of this type of cancer.[6] Some of these deregulated mature miRNAs, due to high tissue specificity, altered stability, and unique expression in tumor development, might help distinguish CRC from other colon-related diseases, representing a new field of molecular diagnosis and prognosis of CRC. For instance, deregulation of miR-92a, miR-143, and miR-145 have been documented in plenty of pathophysiological events, including neoplastic diseases.[22,24] This suggests that miRNAs clusters function as oncogenic, respectively tumor suppressor genes and their inadequate expression is the result of excessive or deficient processing, and that ultimately leads to altered cellular homeostasis, essential events of tumorigenesis. In light of these data, miRNA genes have relevant biological and biomedical consequences in the detection and evolution of cancer, and their inadequate expression is an almost universal feature in human malignancies.

The present research aimed to investigate the signature of 3 mature human miRNAs involving miR-143, miR-145, and miR-92a in 82 pairs of colorectal adenocarcinoma tissues in the normal adjacent tissue samples (NATS) collected from patients in south-east Romania. The ability of the selected miRNAs to function as potential biomarkers, discriminating between CRC and NATS and, their potential as indicators in CRC prognosis was also examined. The miRNAs were selected based on previous studies on their clinical relevance in complex mechanisms of carcinogenesis.[17–27]

2. Materials and methods

2.1. Case selection

This study included 82 selected patients diagnosed with CRC at the Pathology Department of the Clinical Emergency County Hospital in Constanta, Romania. the Local Ethics Commission for the Approval of Clinical and Research Developmental Studies approved the study and all eligible patients provided written informed consent. Immediately after the surgical resection, CRC tissues and NATS (located at least 5 cm from the tumor site) were stabilized in RNAlater solution (Invitrogen by Thermo Fisher Scientific, USA) and frozen at –80 °C until further processing. A section of each sample (tumor and non-tumor) was stained with hematoxylin and eosin and evaluated by an experienced pathologist. All tumor specimens used in this study were histologically classified as colon or rectum adenocarcinoma. The histological tumor stage and differentiation grade was classified using the tumor-node-metastasis staging system of the American Joint Committee on Cancer, in accordance with standards set by World Health Organization.[7] Subsequently, clinicopathological features of patients were obtained from observation sheets and pathology reports and included age, sex, tumor location, tumor-node-metastasis stage, tumor differentiation degree, and eventual metastasis.

2.2. RNA extraction

Total RNA including miRNA molecules was isolated from the tissue samples using a miRNeasy kit (Qiagen, Germany) closely following the manufacturer's recommendations. We started with 30 mg of tissue which was thoroughly homogenized in 750 μl QIAzol Lysis Reagent for 90 seconds. Thereafter, 140 μL of chloroform was added to tissue homogenate and after 5 minutes incubation at room temperature, the sample was centrifuged for 15 minutes at 12.000 rpm at 4 °C. The upper aqueous phase containing RNA was transferred and precipitated in a new Eppendorf tube by adding 1.5 volumes of 100% ethanol. Approximately 650 μL of the precipitated sample was transferred to a RNeasy Mini column placed in an appropriate collection tube and centrifuged at 12.000 rpm for 1 minute at room temperature. After centrifugation, the filtrate was discarded and then 700 μL wash buffer RW1 was pipetted and centrifuged at 12.000 rpm for 1 minute. Next, 500 μL of wash buffer RPE was pipetted and centrifuged at 12.000 rpm for 1 minute at room temperature. To dry the membrane, the column was centrifuged at maximum speed for 1 minute. Following this, the column was placed in a new tapered collection tube, and 30 μL RNase-free water was added and centrifuged at maximum speed for 1 minute to collect an eluate.

The purity and yield of the RNA solutions were assessed by measuring the optical density at 260/280 nm using a NanoDrop

Table 1

The mature miRNA sequences.

miRNA	Lot ID	Mature miRNA Sequence
hsa-miR-143	P170309-001 H08	GGUGCAGUGCUGCAUCUCUGGU
hsa-miR-145	P170206-005 H09	GGAUUCCUGGAAAUACUGUUCU
hsa-miR-92a	P161221-005 H03	UAUUGCACUUGUCCCGGCCUGU
hsa-miR-26b	P161118-006-G01	UUCAAGUAAUUCAGGAUAGGU
RNU44	P181019-000 G11	UAUUGCACUUGUCCCGGCCUG

One Spectrophotometer (Thermo Fisher Scientific, USA), where a ratio A260/A280 = 2 to 2.1, and A260/A230 > 2 was considered acceptable. The concentration of the samples was measured with Qubit 3.0 Fluorometer (Thermo Fisher Scientific) using the Qubit RNA HR (High-Range) Assay Kit. Furthermore, the RNA integrity number was conducted using the 2200 TapeStation Bioanalyzer (Agilent Technologies GmbH, Germany) with an RNA HS ScreenTape kit.

2.3. Reverse transcription of miRNA to complementary cDNA and qPCR reaction

Selected human miRNAs were reverse-transcribed to complementary DNA (cDNA) using the TaqMan MiRNA Reverse Transcription Kit (Applied Biosystems, San Diego, CA). Each reaction was initiated using an RNA-specific stem-looped for reverse transcription (RT) (Table 1). The RNA concentration was set between 1 to 10 ng per 15 μL of RT reaction. Each 15 μL RT reaction consist of 7 μL master mix (0.15 μL dNTP mix, 1 μL Multiscribe RT enzyme, 1.5 μL 10 x RT Buffer, 0.19 μL RNase inhibitor and 4.16 μL Nuclease-free water), 3 μL primer, and 5 μL RNA sample. Samples were incubated in a thermocycler with the following parameters: 16 °C for 30 minutes, 42 °C for 30 minutes, 85 °C for 5 mintes, and then cooled to 4 °C.

The complementary DNA strand for selected targets of miRNAs was synthesized using a specific sequence TaqManMGB Assay (Applied Biosystems, San Diego, CA). For the 20 μL reaction mix, 10 μL of TaqMan 2 × Universal PCR Master Mix was added to 1.33 μL of the product from the RT reaction, 7.67 μL of RNase-free dH_2O, and 1 μL of TaqMan Small RNA assay (20X). The quantitative real-time polymerase chain reaction analysis (qPCR) was performed in triplicate for each sample using the ABI 7500 Fast qPCR instrument for 40 cycles, where each cycle contained denaturation step at 95 °C for 3 seconds, and an annealing step at 60 °C for 30 seconds, followed by the extension of the primers with cleavage of the probe. Fluorescence was detected at the end of each cycle. A negative control without a template was used with all the qRT-PCR runs.

The average of cycle threshold (Ct) values obtained from triplicates of each miRNAs and endogenous control (miR-26b/RNU44) was calculated using an automatic baseline/threshold setting (7500 Fast Real-Time PCR software, version 2.3, Applied Biosystems, San Diego, CA) in concordance with the equation of Livak, Fold-change (FC) = $2^{-\Delta\Delta Ct}$, where ΔΔCt = ΔCt ($Ct_{miR\ target} - Ct_{miR26b/RUN44})_{tumoral\ tissue}$ − ΔCt ($Ct_{miR\ target} - Ct_{miR26b/RNU44})_{normal\ tissue}$.[8] A fold change value <1 meant that the miRNAs were downregulated, where a value > 1 meant that the miRNAs were upregulated in the CRC relative to NATS. Thus, the results were expressed as FC in comparison with the calibrator sample, which was considered the normal value and assumed to equal 1.

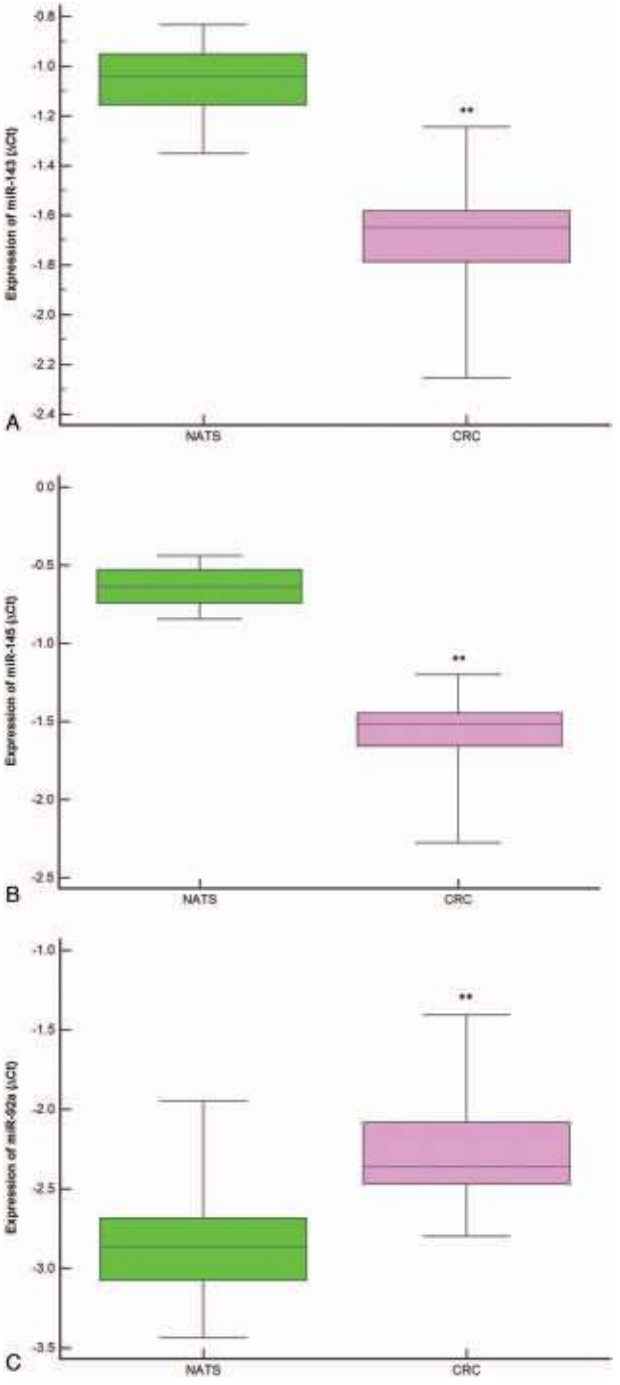

Figure 1. Expression levels of each microRNAs (ΔCt) in colorectal cancer tissue relative to normal adjacent tissue samples are normalized using RNU44 and miR-26b as endogenous controls. The statistically significant difference between colorectal cancer tissue and normal adjacent tissue samples samples was calculated using the Wilcoxon test ($^{**}P < .001$).

2.4. Statistical Analysis

Data obtained were analyzed and graphs were constructed using SPSS version 20.0 software (SPSS, Chicago, IL) and MedCalc version 19.0.3 software (MedCalc, Ostend, Belgium). Differences between CRC and NATS were analyzed by the Wilcoxon test, while correlations between miRNAs expression

Table 2

Analysis of miR-143, miR-145, and miR-92a with the clinicopathologic features of CRC patients.

Clinicopathological variables N (%)	FC[+] miR-143 median (95% CI for median)	FC[+] miR-145 median (95% CI for median)	FC[+] miR-92a median (95% CI for median)
Age (yr)			
≤65–17 yr (20.73)	1.46 (0.81–2.24)	1.75 (1.50–2.66)	1.56 (0.85–1.97)
>65–65 yr (79.26)	1.60 (1.38–3.13)	2.08 (1.58–3.26)	2.00 (1.59–2.47)
Test statistic Z	–1.77	–1.24	–1.85
P-value (2-tailed)	.07[†]	.21[†]	.06[†]
Gender			
Female–38 (46.34)	1.46 (1.15–1.83)	2.13 (1.53–4.65)	1.76 (1.15–2.33)
Male–44 (53.65)	1.60 (1.39–3.13)	1.75 (1.51–2.87)	1.78 (1.56–2.24)
Test statistic Z	–1.04	0.95	–0.47
P-value (2-tailed)	.29[†]	.34[†]	.63[†]
Tumor location			
Proximal colon–30 (36.58)	1.60 (1.41–2.88)	2.08 (1.63–3.16)	2.07 (1.46–2.89)
Distal colon–30 (36.58)	1.54 (0.80–4.62)	2.77 (1.03–4.68)	2.77 (1.03–4.68)
Rectum–22 (26.84)	1.46 (1.06–2.03)	1.58 (1.26–2.07)	1.76 (0.97–3.08)
Test statistic Z	0.02	0.80	0.90
P-value (2-tailed)	.09[‡]	.31[‡]	.34[‡]
Tumor size			
≤5–59 cm (71.95)	1.94 (1.41–2.96)	2.13 (1.66–3.03)	1.73 (1.56–2.22)
>5 cm–23 cm (28.05)	1.41 (1.00–1.58)	1.58 (1.30–2.41)	1.74 (1.57–2.23)
Test statistic Z	1.41	0.98	–0.01
P-value (2-tailed)	.15[†]	.32[†]	1.01[†]
Histological grade			
G1–12 (14.63)	2.48 (0.81–3.13)	1.50 (1.25–3.21)	1.56 (1.27–2.25)
G2–60 (73.17)	1.56 (1.37–2.07)	1.75 (1.57–2.29)	1.76 (1.42–2.15)
G3–10 (12.20)	3.65 (1.05–46.3)	6.30 (1.08–27.06)	2.26 (1.79–5.18)
Test statistic Z	1.55	1.84	1.73
P-value (2-tailed)	.38[‡]	.13[‡]	.07[‡]
Depth of tumor invasion			
T1-T2–14 (17.07)	0.87 (0.66–1.38)	1.53 (0.79–1.89)	0.86 (0.68–1.35)
T3-T4–68 (82.93)	1.60 (1.37–2.96)	3.05 (1.70–3.39)	2.47 (2.11–2.97)
Test statistic Z	3.68	2.97	6.42
P-value (2-tailed)	<.001[†]	<.001[†]	<.001[†]
Nodal status			
N0–37 (45.12)	1.56 (1.15–1.67)	1.58 (1.38–1.88)	1.46 (0.82–1.87)
N1-N2–45 (54.87)	1.94 (1.35–4.63)	3.21 (2.07–9.76)	2.15 (1.77–2.56)
Test statistic Z	2.17	2.49	3.24
P-value (2-tailed)	.02[†]	<.01[†]	<.001[†]
Distant metastasis			
M0–49 (59.75)	1.54 (1.20–1.67)	1.63 (1.50–2.07)	1.58 (1.11–1.76)
M1–33 (40.24)	4.62 (1.23–7.02)	4.70 (1.60–12.47)	2.21 (2.01–2.91)
Test statistic Z	2.27	2.30	2.70
P-value (2-tailed)	.02[†]	.02[†]	<.001[†]

[†] Mann–Whitney *U* Test.

[‡] Kruskal–Wallis H Test.

[+] Fold change expression.

CI = 95% interval of confidence.

levels as well as clinicopathological features were examined using the Mann–Whitney *U* test for 2 independent groups and Kruskal–Wallis H test for three independent groups. Survival rates for each miRNA were estimated using the Kaplan–Meier method and differences between low and high expression were calculated by using log-rank tests. The diagnostic efficacy of selected miRNAs to function as prognostic biomarkers were evaluated by using Receiver operating characteristics (ROC). Similarly, the area under the curve(AUC) was plotted to assess to evaluate the power of selected miRNAs to functions as a diagnostic tool in order to discriminate CRC from NATS. Sensitivity and specificity were then defined by the optimal cut-off point, which refers to the maximized value of the area under the ROC (Youden index). The univariate prognostic analysis revealed the parameters which affected the prognosis of CRC patients, as miRNAs expression levels and clinicopathological characteristics.

3. Results

3.1. Dysregulated expression levels of miRNAs in tumors and NATS from CRC patients

In the present study, 82 patients with colorectal adenocarcinoma along with their NATS, were investigated. This included 30 of proximal colon cases, 30 of distal colon cases, as well as 22 of rectal cases. In order to evaluate the miRNAs expression patterns

in tumor tissue, the quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR) was used. Additionally, it was observed that miR-143 and miR-145 were down-regulated, whereas miR-92a was up-regulated (Fig. 1).

Consequently, the expression levels of miR-143 and miR-145 were found to decrease in 75.60% of CRC cases (–4.99 ± –1.02 vs –5.66 ± –1.66, $P < .001$) and 83% of cases, respectively (–4.85 ± –0.59 vs –9.27 ± –1.51, $P < .001$). On the other hand, the mean FC level expressions of miR-143 and miR-145 in CRC samples were downregulated around 8.21 times less and around 11.66 times less, respectively. In a similar vein, miR-92a expression level was upregulated in 78.00% of cases of CRC as compared to NATS (–5.55 ± –2.83 vs –4.92 ± –2.44, $P < .001$), with its fold increase being 2.32.

3.2. Selected miRNAs expression and association with clinicopathological characteristics

Associations with clinicopathological characteristics were determined in order to explore the clinical relevance of selected miRNAs. Some of these clinicopathological features were grouped, such as histological grade (G1, G2, and G3), pT stage (T1–T2 and T3–T4), pN stage (N0 and N1–N2) and pM stage (M0 and M1). As illustrated in Table 2, the higher expression of miR-92a was found to be closely associated with the depth of tumor invasion ($P = .03$), the involvement of regional lymph node ($P < .001$), and distant metastasis ($P < .001$).

In addition, expression levels of miR-143 and miR-145 were tended to be associated with late-stage tumor invasion ($P = .04$, $P < .001$, respectively), involvement of lymph's node ($P = .02$, $P < .001$, respectively) and with distant metastasis ($P < .001$) as illustrated in Figure 2.

Moreover, no significant statistical differences were observed between selected miRNAs expression levels and gender, age, locations of tumor, tumor size, or tumor differentiation, respectively.

3.3. Correlation between deregulated expression levels of miRNAs and prognosis of CRC patients

In our study, the survival condition of the patients was followed and recorded, beginning from surgery to death or until the date of the last observation (censored data). The follow-up period ranging from 12 to 60 months and was monitored through the Oncology Department. In accordance with the relative expression levels of miR-92a, miR-143, and miR-145, patients were divided based on median values into high-expression groups and low-expression groups. The median survival time in CRC patients with high expression levels of miR-92a was found to be 36 months, which was significantly statistically lower than that median survival time in the low expression group (51 months, $\chi 2 = 10.28$; $P < .01$) (Fig. 3).

In addition, the survival probability of 5 years in the high expression group of miR-143 was 48 months significantly higher than that in the low expression group (36 months, $\chi 2 = 4.43$; $P = .03$). Moreover, the median survival rates of CRC patients in the low expression group of miR-145 was 35 months, significantly lower than that in the high expression group (48 months, $\chi 2 = 7.05$; $P < .01$). According to the univariate analysis of the overall survival rate, the level of miR-92a expression was an independent prognostic indicator for CRC ($P < .01$, Table 3).

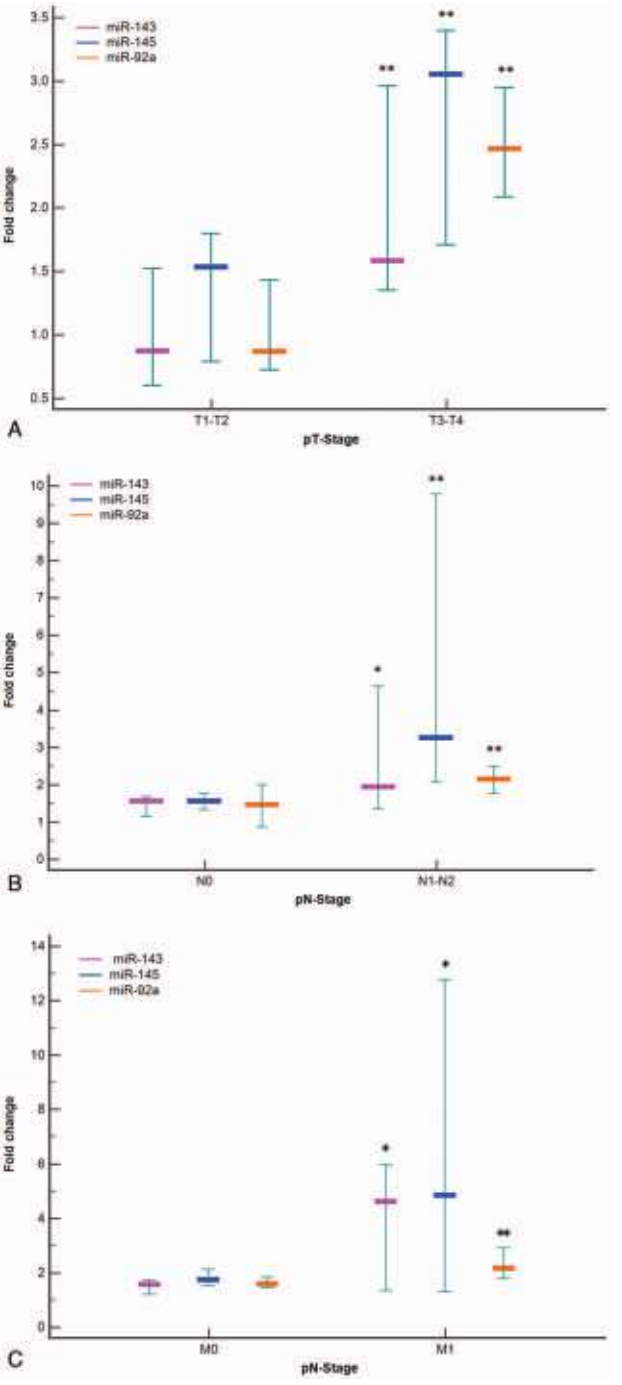

Figure 2. Fold change of miR-143, miR-145, and miR-92a in Romanian patients with colorectal cancer tissue according to different clinical stages. A boxplot showing fold change represented as median and 95% interval of confidence of the median, exhibited that the expressions of analyzed microRNAs increased with increasing pT stage- pT-stage (Fig. A), nodal metastasis - pN-stage (Fig. B), and distant metastasis - pM-stage (Fig. C). All experiments were conducted in triplicate ($^{*}P < .05$, $^{**}P < .001$).

3.4. ROC analysis

Analysis of the ROC curves and AUCs revealed that expression levels of miR-92a, miR-143, and miR-145 could help distinguish tumor tissue from normal adjacent tissues with very good specificity

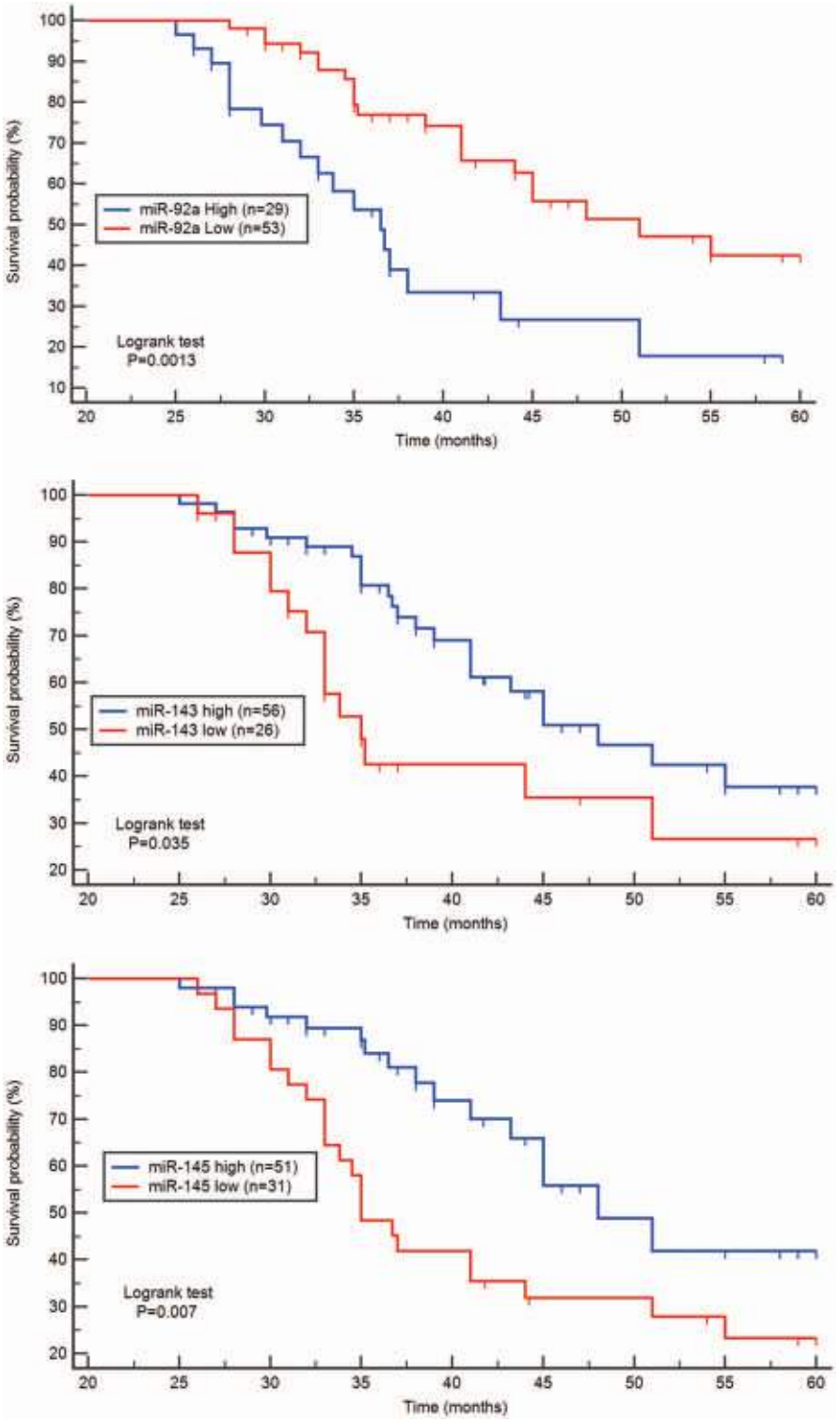

Figure 3. Kaplan–Meier survival curves for CRC patients. CRC patients with high miR-92a expression levels had a significant statistically poorer prognosis than those with low expression ($P < .001$). Moreover, the 5 yr survival probability in the low expression groups of miR-143 and miR-145 had a significant statistically poorer prognosis than in the high expression groups ($P < .01$, $P < .001$ respectively).

and sensitivity (Fig. 4). With regard to the AUC of miR-92a was 0.84 (95% interval of confidence (CI): 0.77 – 0.89, $P < .001$), the specificity was 80.49% and the sensitivity was 71.95% at a cut off value of 0.52. For miR-143 the specificity was 80.49% and the sensitivity was 60.98% at a cut off value of 0.41 and AUC of 0.74% (95% CI: 0.67 – 0.81, $P < .001$). Meanwhile the miR-145 exhibited a specificity of 85.37% and a sensitivity of 82.93% at a cut off value of 0.68 and AUC of 0.85 (95% CI: 0.78 – 0.91; $P < .001$).

Table 3

Logistic regression of prognostic values of microRNAs associated with the clinicopathological features in colorectal cancer tissue patients.

Parameters	Subset	HR	95% CI	*P*-value
Age	<65/>65 yr	1.05	1.01–1.09	<.01*
Gender	Female/Male	0.86	0.48–1.51	.60
Location of tumor	Proximal colon/distal colon/rectum	1.09	0.77–1.54	.60
Grading	G1/G2/G3	1.03	0.51–2.08	.91
pT	T1-T2/T3-T4	0.85	0.44–1.63	.62
pN	N0/N1-N2	1.10	0.78–1.55	.55
pM	M0/M1	1.29	0.64–2.60	.46
miR-143	Low/high expression	0.60	0.31–1.18	.14
miR-145	Low/high expression	0.90	0.44–1.82	.77
miR-92a	Low/high expression	0.27	0.13–0.54	<.01*

* *P*-values <.01 were considered significant statistically.
HR = hazard ratio, CI = 95% interval of confidence.

4. Discussion

CRC continues to be a major cause of mortality induced by cancer worldwide. However, since conventional strategies for CRC treatments have not yet been deemed satisfactory, and an ideal therapeutic target should be associated with the causality of disease. Over the past few decades, numerous studies and resources have identified the involvement of ncRNAs in carcinogenesis and tumor progression.[9] Among all types of ncRNAs, miRNAs have received particular attention and have been proposed as useful diagnostic and prognostic biomarkers. This is attributed to the fact that their profile of tumor tissue has been found to have a close association with a tissue of origin, due to their ability to resist degradation by endogenous ribonuclease, as well as their ease of quantitation involving several methods (e.g., qRT-PCR, microarray or sequencing technology).[10–13]

Dysregulated expressions of miRNAs with oncogenic or tumor suppressor activities have been reported in cancer development, metastasis, angiogenesis and drug resistance.[14,15] In CRC, a large variety of miRNAs are up- or down-regulated as compared to normal tissues. MiRNAs that are consistently found to be down-regulated in CRC act as tumor suppressor genes and are accordingly termed "mirsupps." In contrast, miRNAs that are consistently found to be upregulated in CRC act as oncogenes and are referred to as "oncomirs". Against this backdrop, studying the specific function of miRNAs in human carcinogenesis will help characterize new targets for cancer research, diagnosis, and treatment of cancer at the molecular level.

In the present study, we demonstrated that the expression profiles of miRNAs were significantly altered in the selected group of CRC patients from the south-eastern region of Romania. This was done by using the qRT-PCR method and miRNA specific hydrolysis probes TaqManMGB. Moreover, 2 miRNAs, namely, the miR-143 and miR-145, exhibited significantly lower expression, while 1 miRNA, namely the miR-92a, showed increased expression in CRC than in the NATS.

The miR-143 and miR-145 genes are situated in close proximity to each other in a ~1.7 kb region on chromosome 5q32-33. In addition, they are co-transcribed together from a single bicistronic unit, thereby suggesting that they originate from

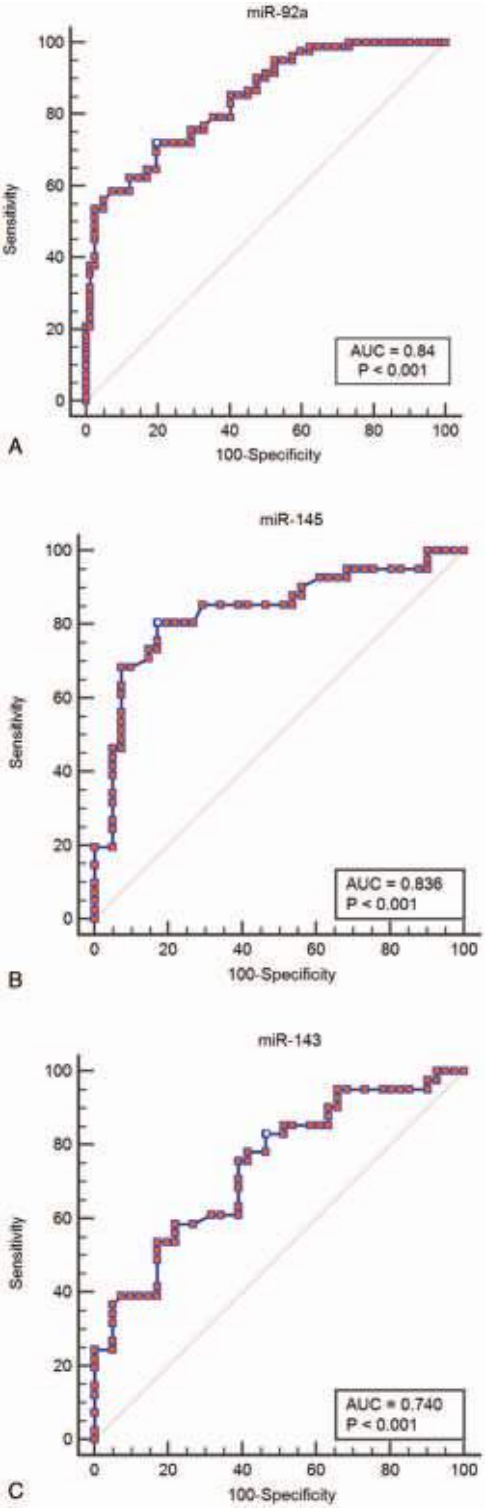

Figure 4. Receiver operating characteristic curves were analyzed to evaluate the miR-92a, miR-143, miR-145 expression levels as potential biomarkers in colorectal cancer tissue detection. area under the curve of upregulated miR-92a (A) expression was 0.84 (sensitivity: 71.95%, specificity: 80.49%; $P < .001$). For downregulated miR-145 (B), and miR-143 (C) expressions, the area under the curves were 0.85 (sensitivity: 82.93%, specificity: 85.37%; $P < .001$), and 0.74 (sensitivity: 60.98%, specificity: 80.49%, $P < .001$).

the same primary transcript and they could be involved in similar functions.[16] Michael et al were the first authors to report the correlation between miRNAs and CRC by uncovering the down-regulation of miR-143 and miR-145 in both precancerous and cancer neoplasia tissues as compared to normal colon epithelium in CRC patients.[17]

In most studies, miR-143 and miR-145 have been presented as independent miRNAs and were not considered as concomitant re-expression genes. On the other hand, co-expression of both miRNAs has been shown to be an early event in the course of cancer development, with anti-oncogenic activities by targeting the same genes, or different genes that regulating the same pathway.[18] Functionally, miR-143/miR-145 cluster expression regulates cell proliferation and differentiation in the Lovo cells by inhibiting the KRAS gene.[19] It is notable that miR-143 and miR-145 could suppress cancer cell proliferation through target sites of the 3'-UTR of insulin-like growth factor 1 receptor and regulate their expression.[20]

In our study, an 8.21-fold decreased expression level of miR-143 in the colorectal samples was observed, as compared to the NATS. However, in the present study in consonance with other authors, we found a significant association between expression of miR-143 and different stages of CRC pathogenesis, including depth of invasion, lymph node metastasis, and distant metastasis.[21,22]

Also, miR-145 expression was decreased 11.66-folds in CRC tissue compared with NATS. Furthermore, we found that miR-145 expression was correlated with a depth of the tumor invasion, lymph node involvement, and distant metastases in CRC patients. Our observation that miR-143 and miR-145 were downregulated in CRC patients was confirmed by other reports.[23–26]

On the other hand, many studies had indicated that dysregulated miR-143, and miR-145 expressions in CRC, are often associated with the CRC progression, and metastasis, which may create a new opportunity to develop the ways of disease mechanism understand, and the potential diagnostic and/or the prognostic biomarkers. Moreover, our obtained results for the expression levels of these miRNAs from CRC samples, are suggesting their use as biomarkers for the diagnostic of the disease with a good performance indicators values (miR-143: 80.49% of specificity and 60.98% of sensitivity; miR-145: 85.37% of specificity and 82.93% of sensitivity, respectively). In addition, the 5 years survival probability time of CRC patients with low expression of miR-143 was shorter than that with the high expression of miR-143. The same difference in survival time was observed in patients with low expression of miR-143 who had a poor prognosis and it can be used as a potential indicator of CRC prognosis.

In CRC research, the role of miR-92a becomes important, as they belong to the miR-17-92 polycistronic cluster, which resides at chromosome 13q13, a region known to be frequently implicated in the proliferation of cancer cells, by suppressing carcinoma cells apoptosis whilst accelerating the progression of the tumor in CRC.[27] Abnormal expression of miR-92a has been known to play a decisive role in various diseases, including breast cancer, CRC, and leukemia.[22,28,29]

Meanwhile, the upregulation of miR-92a plays a pivotal role In CRC pathogenesis, which leads to up-regulation of β-catenin and vimentin, along with the down-regulation of E-cadherin during the regulation of epithelial-mesenchymal transition by specifically targeting phosphatase and tensin homolog, which denotes inhibitory enzyme that is known to block the pathway of PI3K/Akt.[30]

Our study showed that miR-92a was upregulated in CRC, noticing a 2.32-fold increase in colorectal tumors of patients as compared to usual adjacent tissue samples. Furthermore, heightened expression of miR-92a was found to have a significant correlation with the late stage of tumor invasion, lymph node metastasis, and development of distant metastasis in CRC patients.

According to the research carried out by Zhou et al, the upregulation of miR-92a has prognosis prediction in CRC patients; moreover, it was linked to worsening clinical variables and poor rate of survival.[31] Similar conclusions were drawn by our study, which demonstrated that the CRC patients mean survival time was 36 months with a high expression of miR-92a, which is significantly lower as compared to low expression 51 months, respectively. This implies the use of miR-92a as a potentially feasible indicator of prognosis CRC. Meanwhile, Chen et al showed that as an oncomir, diagnostic biomarker miR-92a plays a pivotal role in CRC. In addition, its regulating network may potentially facilitate the mechanism of CRC pathogenesis.[34] When it comes to distinguishing between NATS and CRS, the high specificity (80.49%) and sensitivity (71.95%) of miR-92a expression indicate the potential use of this miRNA as a potential biomarker for CRC diagnosis, which is in alignment with the views of earlier authors.[32,33]

Notably, our study has some limitations. First, because of the small number of cases investigated. The second limitation in agreement with the assumption that abnormal expression levels of selected messenger RNAs were examined only in tissue samples without matching their expression in cell cultures and sera. Therefore, further investigations are needed to confirm our findings.

5. Conclusion

In conclusion, we may summarize that patterns of selected miRNAs are differentially expressed in colorectal adenocarcinoma compared with their normal counterparts with 2 miRNAs decreased (miR-145 and miR-143) and 1 increased (miR-92). Furthermore, the expression levels of selected miRNAs were correlated with gradual increases in fold change expression with different stages of CRC pathogenesis, including depth of tumor invasion, lymph node invasion, and maximal increases with distant metastasis. In addition, ROC analysis and AUC demonstrates the ability of selected miRNAs to discriminating CRC from NATS with good performance of specificity and sensitivity. Prognosis indicators of CRC were also examined using Kaplan–Meier curves and the log-rank test which showed that 5-year overall survival of patients with a high level of miR-92a and low levels of miR-143 and miR-145 are significantly associated with poor prognosis in our cohorts.

Author contributions

All authors contributed equally to this study.
Conceptualization: Mariana Aşchie, Costel Brînzan, Anca Mitroi.
Data curation: Costel Brînzan.
Formal analysis: Costel Brînzan.
Investigation: Costel Brînzan, Anca Mitroi.
Methodology: Costel Brînzan, Anca Mitroi, Georgeta Cozaru.

Software: Costel Brînzan.
Supervision: Mariana Aşchie, Eugen Dumitru.
Validation: Georgeta Cozaru.
Writing – original draft: Costel Brînzan, Georgeta Cozaru.
Writing – review & editing: Costel Brînzan, Anca Mitroi, Eugen Dumitru.

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Molecular expression profiles of selected microRNAs in colorectal adenocarcinoma in patients from south-eastern part of Romania

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Abstract

MicroRNAs (miRNAs) are endogenous, non-coding class of RNAs with functions in the regulation of genes expressions. Dysregulated expressions of miRNAs play important roles in carcinogenesis and cancer progression by targeting various oncogenes and tumor-suppressor genes. miRNAs represent a new field for molecular diagnosis and prognosis of colorectal cancer (CRC) due to their high tissue specificity, their stability, and their dysregulated expression in tumor development.

This study aimed to investigate using the qRT-PCR method the expression profile and prognostic value of 11 mature miRNAs in a cohort of 82 Romanian patients diagnosed with CRC. The relationship between the expression levels of selected miRNAs and clinicopathologic features were evaluated using ANOVA and Pearson test. In addition, the receiver operating characteristic (ROC) and area under the curve (AUC) were used to assess the diagnostic values of the miRNAs to discriminate cancerous from non-cancerous states of the samples.

The expression levels of miR-30c, miR-144, miR-375, miR-214, and miR-195 in CRC tissue were significantly downregulated (all $P < .05$; Paired T-Test) than that in normal adjacent tissue sample (NATS), while the expression of miR-141, miR-182, miR-183, miR-21, and miR-370 in CRC tissue were significantly upregulated (all $P < .001$) than that in NATS. Moreover, the expression levels of miR-182, miR-183, miR-141, and miR-21 were demonstrated to be associated with a gradual increase in fold change expression with depth of tumor invasion (all $P < .05$), lymph node invasion (all $P < .001$), and maximal increase with distant metastasis (all $P < .001$). Moreover, the analysis of ROC curves revealed that AUC (95% CI) of miR-182, miR-183, miR-141, and miR-21 in diagnosis of CRC was 0.76 (0.66–0.87), 0.85 (0.78–0.94), 0.77 (0.62–0.92), 0.83 (0.73–0.90), respectively. The univariate and multivariate Cox-proportional hazard regression for all variables revealed that the nodal status, distant metastasis, miR-21, miR-141, miR-182, and miR-183 were independent prognostic markers of CRC.

In conclusion, altered expressions of miR-21, miR-141, miR-182, and miR-183 in CRC varies at different stages of CRC development and may serve as potential diagnosis molecular biomarkers in Romanian patients with CRC. Further investigations are needed to confirm our findings.

Abbreviations: AJCC = American Joint Committee on Cancer, AUC = area under the curve, Cdc25a = cell division cycle 25 homolog A, CI = 95% interval of confidence, CRC = colorectal cancer, EMT = epithelial to mesenchymal transition, FC = fold change, miRNA = microRNA, miRNAs = microRNAs, mRNA = messenger RNA, NATS = normal adjacent tissue samples, NPV = negative predictive value, PDCD4 = programmed cell death4, PPV = positive predictive value, PTEN = phosphatase and tensin homolog, qRT-PCR = real-time quantitative polymerase chain reaction analysis, RECK = reversion-inducing cysteine-rich protein with kazal motifs, RIN = RNA integrity number, ROC = receiver operating characteristic, RT = room temperature, TNM = tumor-node-metastasis, TPM1 = tropomyosin 1, UTR = 3′ noncoding region, WHO = World Health Organization, ZEB1/2 = E-box-binding homeobox factors.

Keywords: colorectal, depth of tumor invasion, distant metastasis, lymph node, miRNAs, qRT-PCR

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The authors declare no conflict of interests.

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1. Introduction

Colorectal cancer (CRC) is one of the most common types of malignant tumors. Worldwide, CRC poses a major threat to human life and continues to be a significant economic burden.[1] The incidence of CRC is 9.7%, making it the third most common form of cancer after lung and breast cancers and the fourth leading cause of death.[2] According to the results of the Global Burden of Cancer reports, CRC is the second most common malignancy in Romania, after lung cancer in men and breast cancer in women, with 8.660 new cases diagnosed in 2012.[3] Although, in recent years, substantial progress has been made in the prevention, diagnosis, and treatment options as a result of improved clinical management and treatment efficiency. However, CRC remains a public health issue due to the increased prevalence of risk factors associated with Westernization, including unhealthy diets, obesity, and smoking.[2] Colorectal carcinogenesis is linked to the activation of oncogene gene-signaling pathways and the inactivation of tumor suppressor genes, mainly due to genetic mutation and epigenetic changes, including germline or somatic mutation, DNA methylation, histone acetylation, and the involvement of noncoding RNAs, such as those of microRNAs (miRNAs) and long noncoding RNAs.[4]

The discovery of miRNAs took place in the early 1990s when Ambrose et al identified a small RNA that exerted regulatory functions on a specific messenger RNA (mRNA), resulting in the suppression of its action.[5] miRNAs form a class of small, single-stranded, highly conserved, noncoding RNA molecules containing approximately 19 to 24 nucleotides. They bind directly to the 3' noncoding region (UTR) of the target mRNA and act as negative regulators in the expression of the majority of human protein-coding genes.[6]

Currently, a total of 1917 annotated human miRNA precursor genes have been identified, which are processed into ~2654 mature sequences (http://www.mirbase.org), and are able to regulate the expression of one-third of the human genome. miRNAs bind to their mRNA targets by achieving an almost perfect complementarity between the base pairs. A perfect match between base pairs is essential only in the central region of the miRNA and mRNA target to enable the degradation and destabilization or inhibition of mRNA translation and the suppression of the gene expression.[7]

The role of miRNAs in cancer development is well studied, but their biogenesis and mode of action have not yet been fully elucidated. However, it is known that miRNA mediates translation repression and is involved in almost all cellular processes (e.g., proliferation, differentiation, development, cell cycle regulation, metabolism, apoptosis, and carcinogenesis).[8] Pathological alterations in the expression of miRNAs are commonly associated with the occurrence of various diseases, and their expression patterns are used to diagnose various types of cancer, such as breast, lung, pancreatic, and ovarian cancer, as well as colorectal carcinoma.[9–13]

Specific miRNA expressions patterns help distinguish cases of CRC from other colon-related diseases, where they may function either as tumor suppressor or oncogenic genes; however, the mechanisms underlying their potential involvement in proliferation and tumor cell survival are unclear.[14]

In the present study, we aimed to analyze the expression of 11 mature humans miRNA species in colorectal cancer tissues and normal adjacent tissue samples (NATS) collected from 82 Romanian patients and to further explore their association with clinicopathological features. We also examined the ability of selected miRNAs to function as potential biomarkers, discriminating between CRC and NATS states of samples. The miRNAs were selected from a literature review based on their clinical relevance to the complex mechanisms of carcinogenesis.[22–28]

2. Material and methods

2.1. Case selection

Tumor samples with paired adjacent normal tissues (harvested at >5 cm from the cancer tissue) were collected from 82 patients diagnosed with CRC at the Clinical Emergency County Hospital in Constanta, Romania. The study was approved by the Local Ethics Commission for the Approval of Clinical and Research Developmental Studies and informed consent was signed by all patients. Specimens were processed and evaluated by an experienced pathologist according to standard protocols, and only adenocarcinoma types were selected for the miRNA expression analysis. All samples were preserved in RNAlater solution until the total RNA was extracted. The tumor staging of the cancer was classified using the tumor-node-metastasis (TNM) staging system of the American Joint Committee on Cancer (AJCC), in accord with World Health Organization (WHO) standards.[15] The clinicopathological features of the CRC patients were obtained from observation sheets and pathology reports, including age, gender, tumor location, tumor type, tumor size, TNM stage, tumor grade, and eventual metastasis.

2.2. RNA isolation

Small RNA molecules were isolated from the CRC and NATS by using a miRNeasy kit (Qiagen, Germany) according to the manufacturer's instructions. About 30 mg of tissue was homogenized in 700 μlQIAzol lysis buffer for 90 seconds. After 5 minutes of incubation at room temperature (RT), 140 μl chloroform was added and it was centrifuged for 15 minutes at 12.000 rpm at 4°C. The upper aqueous phase was transferred and precipitated in a new Eppendorf tube by the addition of 530 μl 100% ethanol. Approximately 700 μl of the precipitated sample was transferred to a RNeasy Mini column and centrifuged at 12.000 rpm for 1 minute at RT. After centrifugation, the filtrate was discarded and then 700 μl wash buffer 1 was pipetted and centrifuged at 12.000 rpm for 1 minute. Next, 500 μl wash buffer 2 was pipetted and centrifuged at 12.000 rpm for 1 minute at RT. The column was then placed in a new tapered collection tube, and 30 μLRNase-free water was added and centrifuged at maximum speed for 1 minute to collect an eluate.

The purity of the RNA solutions was assessed by measuring the optical density at 260/280 nm using a NanoDrop One Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The concentration of the samples was measured using the Qubit3.0 (Thermo Fisher Scientific, Waltham, MA, USA), and RNA integrity number (RIN) was conducted using 2200 TapeStation Bioanalyzer (Agilent Technologies GmbH, Waldbronn, Germany) with an RNA ScreenTape kit.

2.3. Reverse transcription of miRNA to complementary cDNA and qRT-PCR

miRNA molecules were reverse transcribed to complementary DNA (cDNA) using the TaqMan MicroRNA Reverse Transcrip-

tion Kit (Applied Biosystems, San Diego, CA). Each reaction was initiated using a miRNA-specific stem-looped RT primer, with the aim of prolonging the target of the miRNAs from ~22 bp to over 60 bp (Table 1). The RNA concentration was set between 1 and 10 ng in a final volume of 15 μl of the reaction mixture used. Reverse transcription reagents were combined with total RNA and incubated in a thermocycler with the following parameters: 16°C for 30 minutes, 42°C for 30 minutes, 85°C for 5 minutes, and then cooled to 4°C.

In the second step, the complementary cDNA strand was synthesized using TaqManMicroRNA Assays Inventoried (Applied Biosystems, San Diego, CA). For the 20 μl reaction mix, 10 μl of TaqMan 2 × Universal PCR Master Mix was added to 1.33 μl of the product from the reverse transcription reaction, 7.67 μl of RNase-free dH_2O, and 1 μl of TaqMan microRNA assay. The real-time quantitative polymerase chain reaction analysis (qRT-PCR) was performed in triplicate for each sample using the ABI 7500 Fast qPCR instrument for 40 cycles, where each cycle contained a denaturation step at 95°C for 3 seconds, and an annealing step at 60°C for 30 seconds, followed by the extension of the primers with cleavage of the probe. Fluorescence was detected at the end of each cycle. A negative control without a template was used with all the qRT-PCR runs.

The Ct fluorescent level of each miRNAs was calculated using an automatic baseline/threshold setting (7500 Fast Real-Time PCR software, version 2.3) in concordance with the equation $2^{-\Delta\Delta Ct}$, which represents the fold change (FC) between samples.[16] The miR-26b and miR-92N were selected as reference genes in our experiments, and both were found to be stably expressed in all samples. An FC value < 1 meant that the miRNAs were downregulated. An FC value > 1 meant that the miRNAs were upregulated in the cancer tissue relative to the normal mucosa. Thus, the results were expressed as FC in comparison with the calibrator sample, which was considered the normal value and assumed to equal 1.

2.4. Statistical analysis

Results obtained were analyzed with SPSS version 20 software and GraphPad Prism version 8.0 software. Paired-Samples T Tests were applied to determine the statistical difference of miRNA species between CRC and NATS. Differences between miRNA expression levels and clinicopathological features of colorectal cancer were analyzed using 2 tests (One-Way ANOVA and Pearson correlations), where P value $<.05$ was considered to be statistically significant. Furthermore, univariate and multivariate Cox-proportional hazard regression were performed to determine the prognostic values of selected miRNA expressions and the clinicopathological features in CRC patients. Receiver operating characteristic (ROC) and area under the curve (AUC) were used to evaluate the sensitivity and specificity and to establish the accuracy of the biomarkers in CRC diagnosis. We defined the sensitivity and specificity of the optimal threshold cut-off point as the values that maximized the area under the ROC curve.

Table 1

The mature miRNA sequence.

miRNA	Mature miRNA Sequence
hsa-miR-30c	UGUAAACAUCCUACACUCUCAGC
hsa-miR-182	UUUGGCAAUGGUAGAACUCACACU
hsa-miR-183	UAUGGCACUGGUAGAAUUCACU
hsa-miR-21	UAGCUUAUCAGACUGAUGUUGA
hsa-miR-195	UAGCAGCACAGAAAUAUUGGC
hsa-miR-144	GGAUAUCAUCAUAUACUGUAAG
hsa-miR-141	CAUCUUCCAGUACAGUGUUGGA
hsa-miR-375	UUUGUUCGUUCGGCUCGCGUGA
hsa-miR-370	GCCUGCUGGGGUGGAACCUGGU
hsa-miR-214	UGCCUGUCUACACUUGCUGUGC
hsa-miR-299	UAUGUGGGAUGGUAAACCGCUU
hsa-miR-92N	UAUUGCACUUGUCCCGGCCUG
hsa-miR-26b	UUCAAGUAAUUCAGGAUAGGU

3. Results

3.1. miRNAs expressions in tumor and normal adjacent tissue samples from CRC patients

Among the 82 patients included in our study, 42 patients (51.21%) were males and 40 patients (48.78%) were females; between 48 years and 89 years with a median age of 63.00 years. The tumor site location was the proximal colon for 36.58% of patients (n = 30), the distal colon for 34.14% of cases (n = 28), and the rectum for 29.26% of cases (n = 24).

When the miRNA expressions were compared in the CRC relative to the NATS, 5 miRNAs (miR-21, miR-141, miR-182, miR-183, and miR-370) were found to be overexpressed, and 6 (miR-30c, miR-144, miR-375, miR-214, miR-195, and miR-299) were found to be underexpressed in the CRC samples (Fig. 1). The overexpressions of miR-182, miR-183, and miR-370 and the underexpressions of miR-30c, miR-375, and miR-195 presented the most significant changes in expression.

Among the miRNAs that were overexpressed in the CRC samples, miR-21 was overexpressed in 84% of cases (69/82; $P = .02$), miR-141 in 75% of cases (62/82; $P = .02$), miR-182 in 92% of cases (76/82; $P < .001$), miR-183 in 90% of cases (74/52; $P < .001$), and miR-370 in 86% of cases (71/82; $P < .001$, Table 2). The mean FC level expressions of miR-182, miR-183 and miR-370 in the tumor samples as compared to the NATS were the most significantly overexpressed. Indeed, miR-182 was expressed by about 4.3 times, miR-183 by about 6.1 times, and miR-370 by about 6.0 times, whereas the miR-141 overexpression was only 1.86 times.

Quantification analyses were shown that levels of miR-30c, miR-144, miR-375, miR-214, miR-195, and miR-299 were significantly downregulated in CRC relative to NATS. Thus miR-30c was underexpressed in 75% of cases (62/82; $P < .001$), miR-144 in 75% of cases (62/82; $P = .04$), miR-375 in 78% of cases (64/82; $P < .001$), miR-214 in 70% of cases (58/82; $P = .04$), miR-299 in 73.1% of cases (60/82; $P = .32$), and miR-195 in 90% of cases (74/82; $P < .001$). The mean FC level expressions of miR-375, miR-195, and miR-144 in tumor samples were the most underexpressed. Indeed, miR-375 was downregulated by about 38.1 times, miR-195 by about 4.6 times, and miR-144 was expressed 2.2 times less frequently in the tumor samples as compared to the NATS. The relative expression ratio for miR-299 suggested that it was also underexpressed in CRC by about 1.7 times; however, the statistical analysis did not reveal any significant differences.

3.2. Correlations between expression of miRNAs and clinicopathological features of CRC patients

Expression of selected miRNAs in CRC patients was not significantly correlated with age, gender, tumor size, tumor grade, and tumor locations. Among all miRNAs studied, we

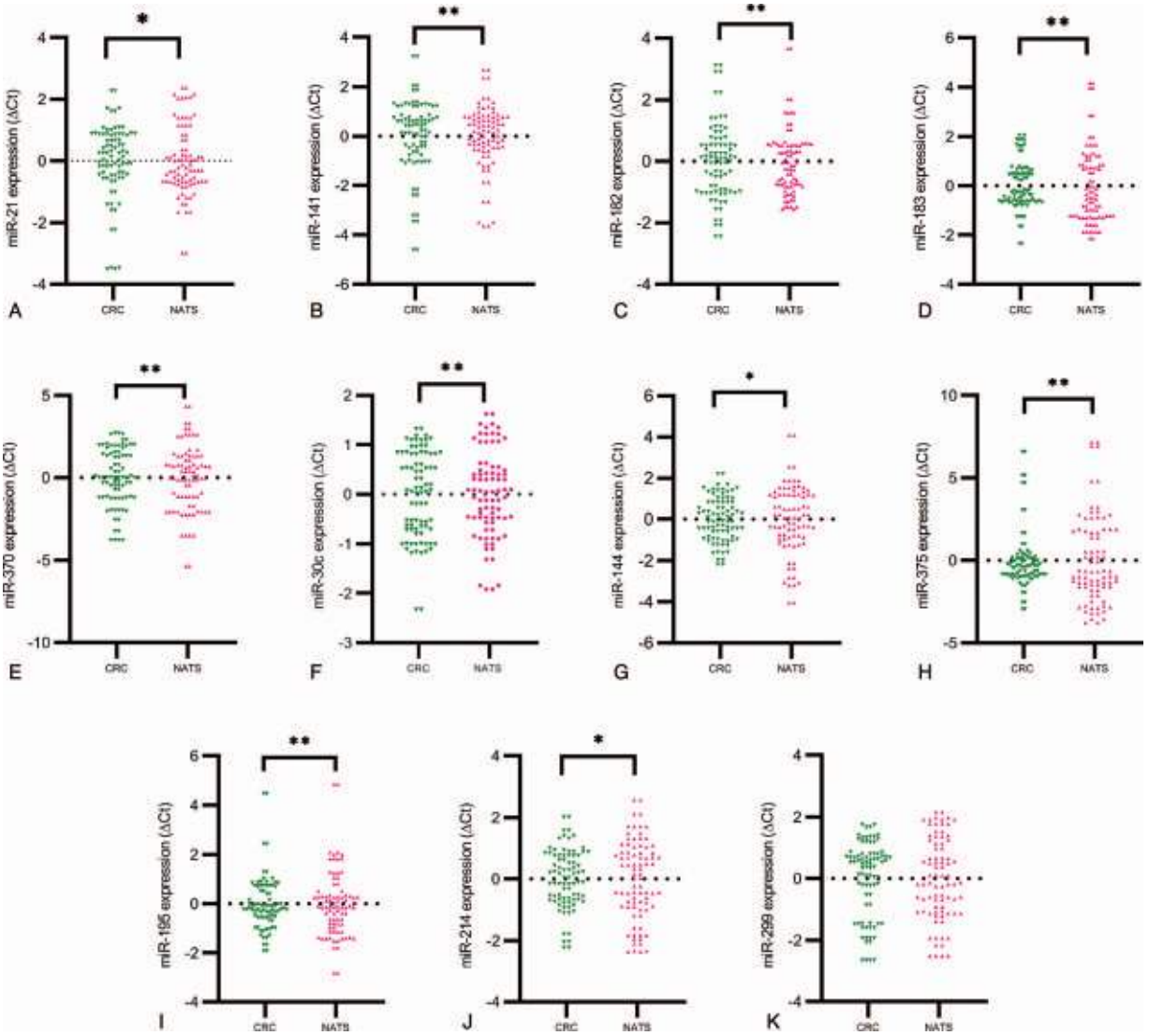

Figure 1. Dots-plot showing the results of deregulated miRNAs' expressions in colorectal cancer tissue (CRC) in relation to normal adjacent tissue samples (NATS). ΔCt values of upregulated miRNAs' expression: (A) miR-21, (B) miR-141, (C) miR-182, (D) miR-183, (E) miR-370. Downregulated miRNAs' expressions levels (ΔCt): (F) miR-30c, (G) miR-144, (H) miR-375, (I) miR-195, (J) miR-214, and (K) miR-299. The statistical difference ranks between the two groups, were calculated using Paired-Samples T-Test, were $*P < .05$, $**P < .001$.

Table 2

MiRNAs overexpressed or underexpressed in CRC relative to NATS.

miRNA species	Relative expression* in NATS§ (Mean ± SD)	95% CI§ of the difference in NATS§		Relative expression* in CRC‡ (Mean ± SD)	95% CI§ of the difference in CRC‡		Fold increase/ decrease in CCR†	*P* value
		Lower	Upper		Lower	Upper		
miR-141	−0.43 ± 0.86	−0.70	0.16	0.01 ± 0.95	−0.28	0.31	1.86	.02
miR-21	−3.11 ± 1.14	−3.48	2.74	−2.25 ± 1.22	−2.65	1.86	3.21	.02
miR-182	7.2 ± 1.07	6.89	7.56	8.69 ± 1.22	8.30	9.07	4.34	<.001
miR-183	7.29 ± 1.99	6.58	7.99	8.93 ± 1.11	8.54	9.33	6.16	<.001
miR-370	8.92 ± 2.05	8.19	9.64	10.5 ± 1.73	9.94	11.1	6.07	<.001
miR-30c	1.55 ± 0.86	1.28	1.82	0.90 ± 0.82	0.62	1.18	1.91	<.001
miR-144	10.0 ± 1.75	9,48	10.5	9.29 ± 1.39	8.86	9.73	2.29	.04
miR-375	2.55 ± 2.64	1.72	3.39	0.52 ± 1.86	−0.06	1.11	38.1	<.001
miR-214	7.61 ± 1.27	7.21	8.02	7.11 ± 0.96	6.81	7.42	1.96	.04
miR-299	10.2 ± 1.27	9.78	10.6	9.85 ± 1.09	9.48	10.21	1.76	.32
miR-195	9.97 ± 1.29	9.56	10.3	8.51 ± 0.80	8.25	8.76	4.65	<.001

* ΔCT;
† $2^{-\Delta\Delta CT}$;
‡ Colorectal cancer;
§ Normal adjacent tissue sample;
†Confidence interval.

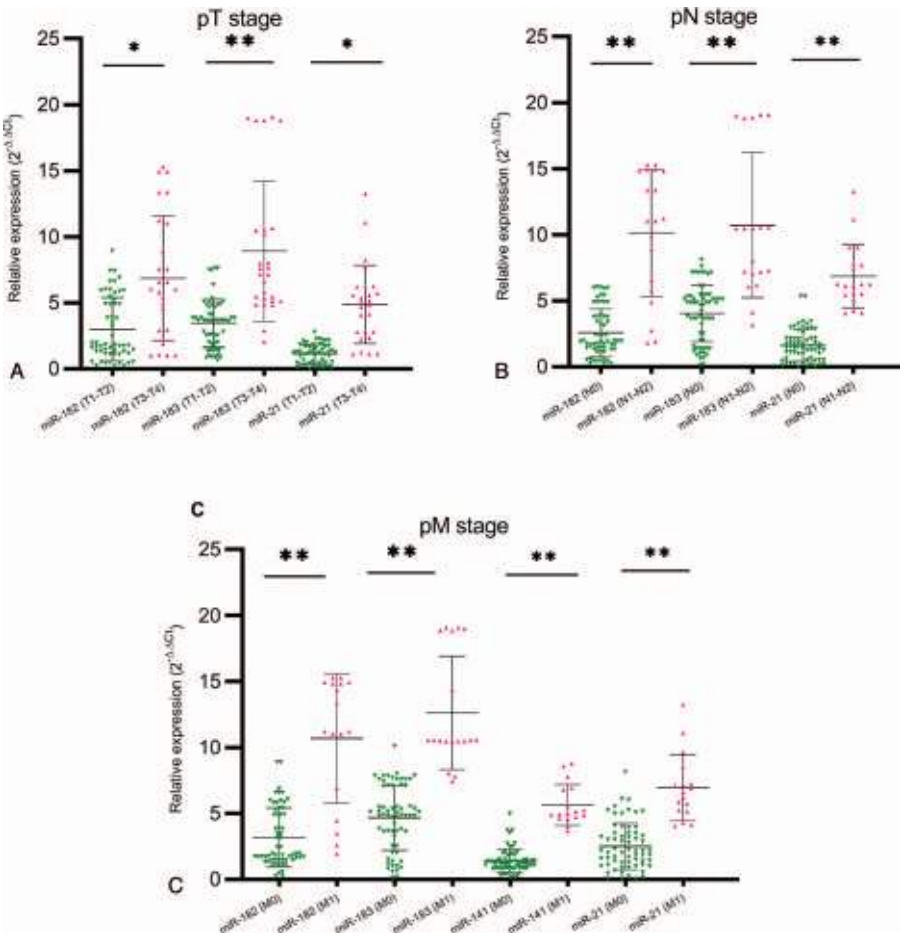

Figure 2. Fold change of the 4 miRNAs genes in Romanian patients with CRC at different clinical stages. The dot plots (using $2^{-\Delta\Delta Ct}$ method) represent mean ± standard deviation of miR-182, miR-183, miR-141, and miR-21 according to the depth of tumor invasion - pT-stage (Fig. A), nodal metastasis - pN-stage (Fig. B), and distant metastasis - pM-stage (Fig. C). All experiments were conducted in triplicate (*$P < .05$**$P < .001$).

found that the miR-183, miR-182, miR-141, and miR-21 levels were positively correlated with some clinicopathological characteristics (Fig. 2 and Table 3).

The ANOVA test indicated a tendency of associations between higher expression of miR-182 in CRC tissue relative to NATS in advanced T stages (T3–T4: 6.85 ± 4.70 vs T1–T2: 2.90 ± 2.30; $P = .02$), with the metastasis stage (M1: 10.14 ± 5.76 vs M0: 3.04 ± 2.13; $P < .001$), and nodal status (N1–N2: 10.13 ± 4.82 vs N0: 2.57 ± 1.82; $P < .001$). In addition, miR-183 expression was significantly higher in advanced tumor stages (T3–T4: 8.26 ± 3.58 vs T1–T2: 3.44 ± 2.23; $P < .001$), lymph node metastasis (N1–N2: 10.5 ± 4.01 vs N0: 4.01 ± 2.17; $P < .001$), and in extension of metastases (M1: 12.6 ± 4.29 vs M0: 4.63 ± 2.49; $P < .001$). The miR-141 was upregulated in CRC compared with NATS, and its expression was higher in patients in M1 stage relative to those in M0 stage (M1: 5.63 ± 1.90 vs M0: 1.39 ± 0.89; $P < .001$).

Table 3

Pearson correlations between miRNAs expressions and clinicopathological features in tumor samples at CRC patients.

Clinicopathological features	miR-21	miR-182	miR-183	miR-141
Depth of tumor invasion				
r	0.41	0.34	0.36	0.11
P-value	<.001	.029	.019	.48
Nodal status				
r	0.71	0.62	0.65	0.25
P-value	<.001	<.001	<.001	.10
Distant metastasis				
r	0.54	0.63	0.63	0.36
P-value	<.001	<.001	<.001	.019

The advanced stage of distant metastasis (M1: 7.01 ± 2.22 vs M0: 2.66 ± 3.58; $P < .001$), the late stages of tumor invasion (T3–T4: 4.86 ± 3.91 vs T1–T2: 1.14 ± 0.61; $P = .011$) and lymph node involvement (N1-N2: 7.08 ± 3.83 vs N0: 1.67 ± 1.14; $P < .001$) all presented higher values for miR-21 expression in CRC patients.

Furthermore, in univariate and multivariate analysis (Cox regression), nodal status, distant metastasis, miR-30c, miR-144, miR-375, miR-214, miR-21, miR-195, miR-141, miR-182, miR-183, and miR-370 were independent and significant predictor factors associated with CRC (Table 4).

3.3. ROC curve analysis

ROC curve analyses were performed to determine the sensitivity and specificity of selected miRNAs and used as a discriminatory

Table 4

Logistic regression of prognostic values of miRNAs associated with the clinicopathological features in CRC patients.

Clinical variables	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI*	*P* value	Hazard ratio	*P* value	95% CI*
Age	1.65	0.73–3.98	.32	–	–	–
Gender	1.23	1.07–3.50	.32	–	–	–
Tumor Location	1.52	1.28–3.79	.63	–	–	–
Depth of tumor invasion	1.23	1.26–3.86	.063	–	–	–
Nodal Status	2.83	0.88–7.29	.035	3.86	.012	0.77–3.90
Distant metastasis	6.32	0.79–1.17	.023	3.29	.035	1.00–3.54
Tumor size	2.62	0.10–9.31	.32	2.17	.047	1.29–3.79
miR-30c	3.62	1.02–5.31	.032	4.97	.012	1.02–5.24
miR-144	3.23	0.81–5.06	.028	3.31	.016	0.80–5.51
miR-375	3.22	0.65–5.90	.012	2.06	.035	0.88–5.22
miR-214	3.95	0.54–5.88	.023	4.90	.014	1.00–3.54
miR-21	3.76	1.00–5.20	<.001	1.88	.042	1.29–3.79
miR-195	2.86	0.82–5.56	<.001	2.20	.034	1.24–3.86
miR-141	2.41	0.79–6.17	.035	1.00	.023	0.80–5.51
miR-182	2.32	0.10–7.31	.035	3.98	.023	0.88–5.22
miR-183	2.42	0.73–4.97	.023	4.50	.042	0.76–6.14
miR-370	2.53	1.02–4.31	.011	3.79	.031	1.75–5.62

* Confidence interval

tool to classify tissues in CRC and NATS. Analysis of the ROC curves and AUCs revealed that miR-183, miR-182, miR-141, and miR-21 expressions could be potential diagnostic biomarkers in CRC patients. The AUC of miR-182 was 0.76 (95% interval of confidence - CI: 0.66–0.87; $P < .001$), the specificity was 80.8% and the sensitivity was 66.6%. For miR-183, the AUC was 0.85% (95% CI: 0.78–0.94, $P < .001$), the specificity was 85.0% and the sensitivity was 80.9%. The AUC for miR-141 was 0.77 (95% CI: 0.62–0.92; $P < .001$), the specificity was 75%, and the sensitivity was 84%. The specificity of miR-21 was 87.5%, the sensitivity was 73.8%, and the AUC was 0.83 (95% CI: 0.73–0.90; $P < .001$, Table 5 and Fig. 3). All 4 miRNAs were able to distinguish tumor tissue from normal mucosal tissues with good specificity and sensitivity.

4. Discussion

Now-a-days, the dysregulated expression of miRNAs is observed in almost all types of cancer. This may be attributed to genomic alterations/mutations, inadequate biogenesis of miRNA, transcriptional disorders, or epigenetic silencing.[17] Predominantly, miRNAs play an essential role in the post-transcriptional regulation of gene expression by targeting several oncogenes or tumor suppressor genes that are critical in the pathogenesis of cancer.[18] In CRC, a large variety of miRNAs have been found to be either upregulated or downregulated in tumor tissues as compared to healthy tissues. Upregulated miRNAs in CRC essentially act as oncogenes and are termed "oncomiRs", while downregulated miRNAs act as tumor suppressor genes and are termed "tsmiRNAs".

Wang et al evaluated the expression of 3 miRNAs (miR-34a, miR-155, and miR-200c) in 109 pairs of tumor and non-tumor tissues using qRT-PCR. They found that the selected miRNAs were overexpressed in most cases of CRC.[19] Al-Sheikh et al investigated the expression of 4 mature miRNAs (miR-145, miR-195, miR-29, and miR-92) in the plasma and tissues of a group of 20 patients with CRC using qRT-PCR.[20] In a study conducted by Ahmed et al when compared with 27 healthy control patients, upregulated patterns of miR-92a and downregulated patterns of miR-375 and miR-760 were found in the sera of 64 CRC patients.[21] Similarly, in the present study, we demonstrated that the expression profiles of miRNAs were significantly altered in the selected group of Romanian CRC patients. This was determined using the TaqManMGB qRT-PCR method. Moreover, 5 miRNAs namely miR-21, miR-141, miR-182, miR-183, and miR-370, showed increased expression, while 6 miRNAs, namely miR-30c, miR-144, miR-375, miR-195, miR-214, and miR-299 showed significantly lower expression in the CRC than in the NATS. It should be noted that other studies previously revealed expression profiles of miRNAs species examined in CRC.[22–28] In the present study, we focused our attention on

Table 5

Receiver operating characteristic (ROC) analysis of selected miRNAs in CRC.

miRNAs	AUC	95% CI*	*P* value	Youden J index	Cut-off value	Sensitivity	Specificity	PPV %	NPV %
miR-183	0.85	0.78–0.94	<.001	0.65	>4.70	80.95	85.00	92.68	81.71
miR-182	0.76	0.66–0.87	<.001	0.46	>5.44	66.75	80.89	70.73	87.56
miR-141	0.77	0.62–0.92	<.001	0.59	>2.08	84.00	75.00	58.54	73.17
miR-21	0.83	0.73–0.90	<.001	0.61	>3.68	73.81	87.50	71.95	78.05

AUC = area under the curve, NPV = negative predictive value, PPV = positive predictive value, ROC = receiver operating characteristic.
* Confidence interval; Youden J = sensitivity + specificity − 100.

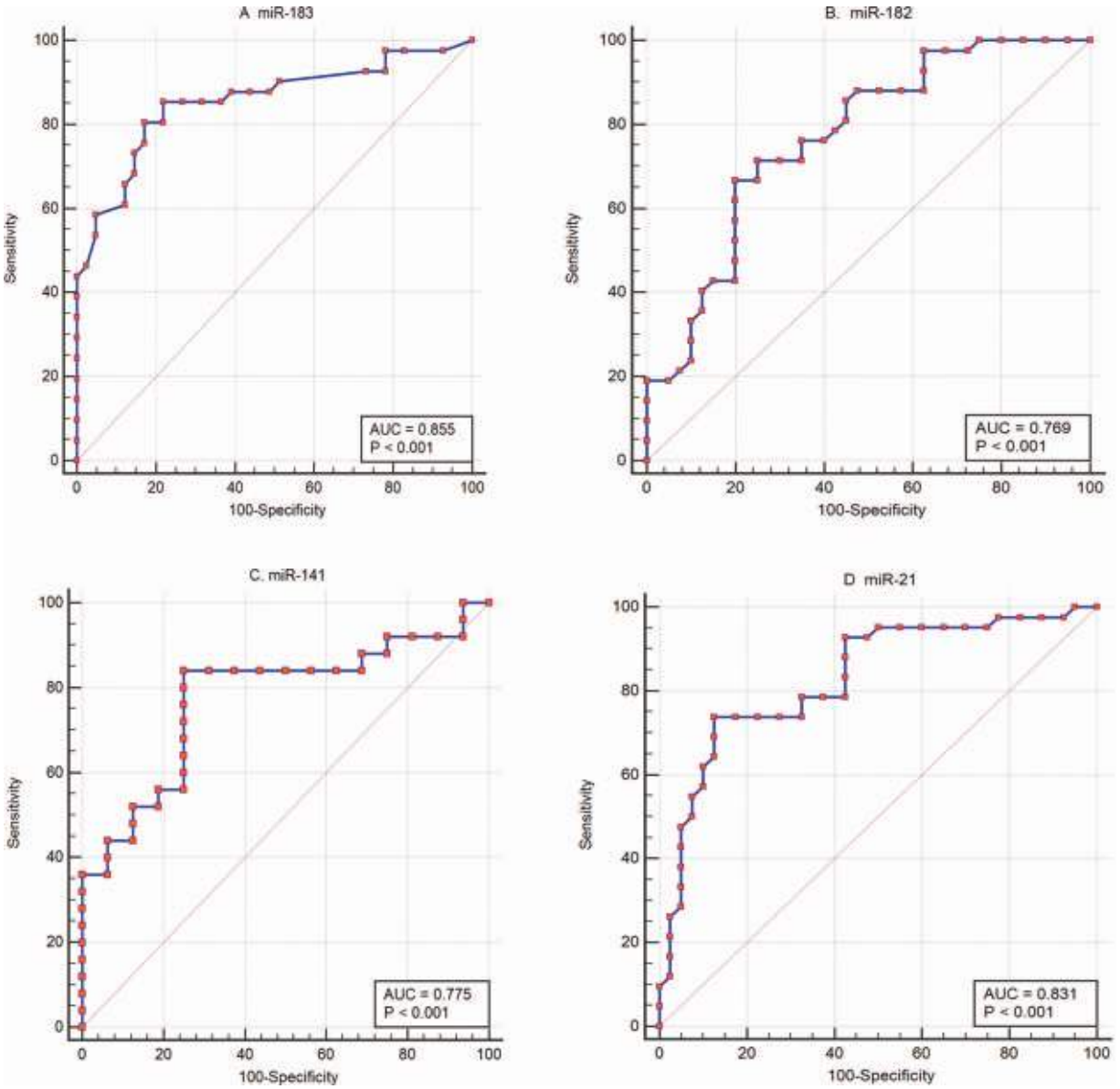

Figure 3. The miR-182, miR-183, miR-141, and miR-21 expressions levels as potential biomarkers in CRC diagnosis. Receiver operating curve (ROC) analyses were generated from 82 patients had a value of the area under the curve (AUC) for miR-183 of 0.85 (sensitivity: 80.95%, specificity: 85%; $P < .001$), for miR-182, miR-141, and miR-21, the AUC were 0.76 (sensitivity: 66.75%, specificity: 80.89%; $P < .001$), 0.77 (sensitivity: 84%, specificity: 75%, $P < .001$), and 0.83 (sensitivity: 73.81%, specificity: 87.50%; $P < .001$), respectively.

miR-21, miR-141, miR-183, and miR-182, which are positively correlated with clinicopathological features. In addition, ROC curves analysis revealed that they could function as potentially useful diagnostic tools in differentiating between colorectal cancer tissue and adjacent non-cancerous tissues.

miR-21 is regarded as an oncomiR and is frequently overexpressed in many types of solid tumors, including CRC.[29] Slaby et al, indicated that the presence of increased levels of miR-21 correlated significantly with clinicopathological features, including lymph node involvement and the development of distant metastases. This indicated a potential role for miR-21 in initiating, progressing and metastasizing CRC.[13] Overexpression of miR-21 may increase cell proliferation, migration, invasion and survival in a variety of cancer cell lines through the targeting and repression of the expression of several tumor suppressor genes. These include programmed cell death4 (PDCD4), phosphatase and tensin homolog (PTEN), cell division cycle 25 homolog A (Cdc25a), reversion-inducing cysteine-rich protein with kazal motifs (RECK), and tropomyosin 1 (TPM1).[30–34] Upregulation of miR-21 has been demonstrated to promote metastasis, invasion and intravasation in CRC cells through the repression of the PTEN/PI-3K/Akt signaling pathway.[35]

Our findings demonstrated that miR-21 levels were significantly upregulated in CRC tissue than in paired NATS, and Pearson statistical analysis revealed a marked correlation between the expression of miR-21 and the depth of tumor invasion ($r - 0.41$; $P < .001$), lymph node metastasis ($r - 0.71$;

$P < .001$), and the development of distant metastasis ($r - 0.54$; $P < .001$). Moreover, the analysis of the ROC curve revealed that the miR-21 expression level could discriminate between CRC and NATS with a sensitivity of 73.8%, specificity of 87.5%, positive predictive value (PPV) of 71.9% and negative predictive value (NPV) of 78% at a cutoff value greater than 3.68 ($P < .001$). All of these are in accordance with prior studies.[36–37]

miR-141 is part of the miR-200 family, which includes the following 4 members: miR-200a, miR-200b, miR-200c, and miR-429. The miR-200 family is organized into 2 clusters with different genomic loci localizations. Cluster 1 (miR-200a, miR-200b, and miR-429) are located on chromosome 1 (1p36.3), whereas cluster 2 (miR-200c and miR-141) are located on chromosome 12 (12p.13.3).[38] Previous studies have demonstrated the role of the miR-200 family in cancer where they are associated with tumorigenesis and the progression of various types of human malignancies.[39–41] Downregulation of miR-141 and miR-200b regulates the epithelial to mesenchymal transition (EMT) by directly targeting the zinc finger E-box-binding homeobox factors (ZEB1/2), which are repressors of E-cadherin and vimentin transcription.[42] miR-141 as part of the miR-200 family is dysregulated in various types of human malignancies. This demonstrates the dual role it can play in carcinogenesis, where it can either act as an oncogene or a tumor suppressor gene. Previous studies have shown that miR-141 and miR-200c are highly expressed in the plasma of patients with CRC ($n = 54$) and that miR-141 levels are increased in patients with liver metastases compared to non-metastatic patients. This suggests that the expression of miR-141 may be used as an indicator of CRC metastasis.[43] In another study, Cheng et al demonstrated that an increased plasma level of miR-141 in 102 patients with stage IV CRC was associated with poor survival. In addition, they found that miR-141 may be a new, useful, non-invasive biomarker in the detection of CRC with distant metastases.[44]

In the present study, miR-141 expression was shown to be significantly upregulated in CRC compared to NATS, and its expression was increased in patients in stage M1 relative to those in stage M0 ($r - 0.36$; $P = .019$). Since increased expression of miR-141 is associated with an epithelial phenotype, it can be assumed that miR-141 expression varies at different stages of carcinogenesis, increasing in primary tumors, and decreasing during the metastatic process when cells acquire mesenchymal characteristics. It may be overexpressed again in metastases where the cells again exhibit epithelial features.[45] Furthermore, the miR-141 expression level at a cutoff value greater than 2.08 ($P < .001$) could discriminate between tumoral tissue and NATS samples among CRC patients with a sensitivity of 84% and specificity of 75%, PPV of 58%, and NPV of 73.1%.

miR-182 and miR-183 belong to the miR-183–96–182 family, which is a highly conserved polycistronic cluster across species and is located within a 5-kb region on chromosome 7q32.2.[46] Either individually, or as a cluster, expression levels of the miR-183 family have been demonstrated to be deregulated in diverse types of malignant tumor. In CRC, the miR-183 family is overexpressed and acts as an oncomiR cluster by promoting cell proliferation, inhibition of apoptosis, accelerated tumor progression and metastasis, with phenotypes that are essential for carcinogenesis.[47] Elevated levels of miR-183 were found by Zhou et al, in 94 CRC specimens relative to their adjacent normal pairs. In relation to clinicopathological features, in the same study, the authors demonstrated that increased expression of miR-183 tends to correlate with lymph node metastasis, depth of tumor invasion, and distant metastasis, suggesting that miR-183 could be considered a promising biomarker for the prognosis or the aggressiveness of CRC.[48]

Similarly, in this study, we found that miR-183 expression was significantly upregulated in CRC relative to NATS, in accordance with prior authors.[49–50] Furthermore, our data showed that increased levels of miR-183 were correlated with advanced clinical stage T ($r - 0.36$; $P = .019$), lymph node involvement ($r - 0.65$; $P < .001$), and distant metastases ($r - 0.63$; $P < .001$) in CRC patients. Moreover, miRNA-183 presented the best diagnostic performance in discriminating CRC tissue from NATS at a cutoff value greater than 4.70 ($P < .001$) with a sensitivity of 80.8% and specificity of 85%, PPV of 92.6%, and NPV of 81.7%.

miR-182 is involved in several key steps of tumorigenesis, including EMT, cell cycle regulation, proliferation, survival, migration, aggressiveness, and drug resistance.[51–52] A study by Hui et al, showed that the expression of miR-182 is higher in CRC compared to adjacent noncancerous tissues, and its overexpression correlates positively with the TNM stage, lymph node metastasis and tumor size, representing an independent prognostic factor for CRC patients.[53] In this study, miR-182 was also found to be upregulated in the CRC samples and the higher expression was positively correlated with the advanced clinical stage T ($r - 0.34$; $P = .029$), with lymph node involvement ($r - 0.62$; $P < .001$) and with distant metastases ($r - 0.63$; $P < .001$). In addition, miR-182 at a cutoff value higher than 5.44 ($P < .001$) could predict patients with tumoral status from those with non-tumoral status among CRC patients with a sensitivity of 66.6% and specificity of 80.8%, PPV of 70.7% and NPV 87.5%. This finding agrees with those of prior authors.[54]

miRNAs have some features that make them attractive as biomarkers of malignancy, offering new opportunities for improving diagnosis, prognosis, and the management of CRC. Their suitability includes the altered expression of miRNAs in malignant vs normal tissue, their ability to resist degradation by endogenous ribonuclease, their ease of quantitation using several methods (e.g., qRT-PCR, microarray, or sequencing technology), and especially their differentiated expression in different types of tumor.[54] Furthermore, the ROC curve analysis demonstrated that miR-21, miR-183, miR-182, and miR-141 are useful tools for differentiating between tumor samples and normal adjacent tissues in CRC patients, with a sensitivity of between 66.7% to 84.0% and a specificity between 75.0% to 87.5%, suggesting the clinical relevance of these biomarkers.

Nevertheless, this study has several limitations. First of all, the small number of patients enrolled. Second, all selected humans miRNAs were based on a review of literature, and perhaps other miRNAs may be more prominently deregulated in Romanian patients. Third, the panels of miRNAs were investigated only in tumor tissue samples, without matching their expression in sera samples so as to ensure dysregulated expression pattern in both tissues and sera. The fourth limitation is represented by normalization strategies based on endogenous miRNAs control. In the present study we used a combination of 2 careful selected controls (miR-2b and miR-92N), which we consider to reduce the effects of intra- and inter-variability in the qRT-PCR assay.

5. Conclusions

In conclusion, the present study demonstrated that the selected miRNAs species were shown to be differently expressed in

colorectal cancer tissue as compared to normal adjacent tissue samples in a cohort of 82 Romanian patients. Furthermore, altered expression levels of 4 miRNAs genes (miR-21, miR-141, miR-182, and miR-183) in CRC varies at different stages of CRC development. In addition, evaluating expression of these 4 genes in tumor tissue could be a valuable tool in the diagnosis of Romanian patients with colorectal cancer. Therefore, further investigations are needed to confirm our findings.

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Clinical Case Report

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A boy with 13.34-Mb interstitial deletion of chromosome 4p15

A new case report and review of the literature

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Abstract

Rationale: To date, >40 cases have been described with interstitial deletions involving the 4p15 region.

Patient concerns and diagnosis: We report a case of a 3-year-old boy with an interstitial de novo deletion of approximately 13.34 Mb in 4p15.1–15.31 having mild developmental delay and multiple minor congenital abnormalities.

Lessons: This case presents a clinical manifestation that is similar but not identical to other reported cases. In this report, we have provided a detailed description of a 3-year-old patient with an interstitial 4p deletion and mildly affected phenotype. We discuss the possible involvement of *SLIT2*, *KCNIP4*, and *LGI2* in cortical development and *RBPJ* in skeletal abnormalities.

Abbreviations: CGH = comparative genome hybridization, CNS= central nervous system, KCNIP4 = potassium channel-interacting protein 4 isoform, LGI2 = leucine-rich glioma inactivated protein 2, OMIM = Online Mendelian Inheritance in Man, PCDH7 = procadherin 7 isoform c precursor, RBPJ = recombination signal-binding protein for kappa J region, SLIT2 = slit homolog 2, SNP = single-nucleotide polymorphism, WHS = Wolf-Hirschhorn syndrome.

Keywords: 4p deletion, developmental delay, minor congenital abnormalities

1. Introduction

Previously interstitial deletions of chromosome 4p have only been rarely described. Deletions encompassing the 4p15 region result in a distinct clinical syndrome, different from Wolf-Hirschhorn syndrome (WHS, Online Mendelian Inheritance in Man 194190). The main clinical features of previously reported cases are mild to moderate mental retardation and multiple minor dysmorphic features such as a long face, up-slanted palpebral fissure with epicanthal folds, large lax lips, pectus excavatum, and tall and thin body habitus [1–8]. To date <40 cases with 4p15 deletions have been reported and in approximately 4 cases the extent of the deletion was ascertained through array comparative genomic hybridization. In this study, we report a 13-Mb interstitial deletion of 4p15.1–15.31 in a patient with mild psychomotor retardation and minor dysmorphic features.

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The authors report no conflicts of interest.

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2. Clinical report

The patient is a 3-year-old boy. He is the first child of a healthy, young nonconsanguineous white couple: the respective family histories of parents were negative for developmental, congenital, genetic or neurologic disorders. The mother was diagnosed with thrombophilia in the second trimester of pregnancy and she had received anticoagulant treatment. The delivery occurred at 40 weeks of gestation and was uneventful. His birth weight was 3600 g and birth length was 51 cm; his Appearance, Pulse, Grimace, Activity, Respiration scores were 9 and 10 at 5 and 10 minutes. His development was slightly retarded. He could hold his head at the age of 6 months and sit at the age of 9 months. He started to walk at the age of 16 months and used single words at the age of 30 months. He was clinically evaluated at the age of 33 months because of unusual physical findings and developmental delay. During his physical examination, a long face with a high forehead, deep-set eyes, puffy eyelids, broad and flat nasal bridge, lateral flaring of the nostrils, long philtrum, and a thick and proeminent lower lip were recorded (Fig. 1). His teeth were normal and his palate was high arched and intact. His skin showed one café-au-lait spot (2,5/3ï¿1/2cm in diameter) on the right thigh. His ears consisted of proeminent and thick lobes, and they were very close to his heade. His height was 97 cm (75th centile), his weight was 16 kg (90th centile), and his head circumference was 48 cm (10th centile). He had pectus excavatum, broad hands and feet, and clinodactyly of the toes. The proband also presented with a left undescended testis and required a surgical intervention for phimosis. His medical history included frequent upper respiratory infections and bronchiolities.

3. Methods

The patient's parents provided an informed consent to publish all clinical information. The report was approved by the local commission for the approval of clinical and reasearch developmental studies.

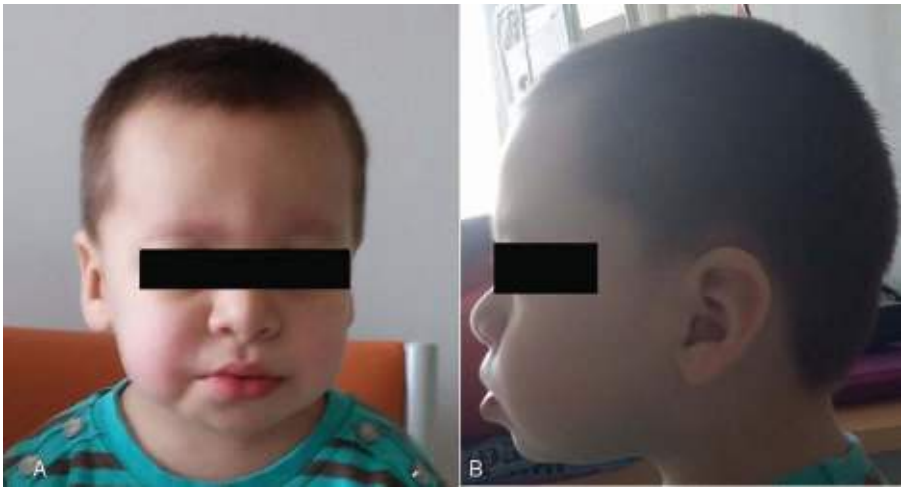

Figure 1. Proband at 3-year-old face (A) and profile (B). Note the long face, high forehead, puffy eyelids, broad and flate nasal bridge, long filtrum, thick and proeminent lower lip.

3.1. Cytogenetic analysis

Cytogenetic analysis was conducted on G-banded metaphases of cultured peripheral lymphocytes in accordance with standard protocols. Metaphases were analyzed at the 400 to 500-band resolution level. The karyotype was described in accordance with the guidelines of the 2016 International System for Human Cytogenetic Nomenclature.

3.2. Array-comparative genome hybridization

For the precise delineation of the deleted region array-comparative genome hybridization (CGH) was conducted using an Agilent Sure Print G3 Human Genome CGH + SNP, 4 × 180K, Microarray Kit in accordance with the manufacturer's protocols. Images were scanned using Sure Scan from Agilent and analyzed using the Feature Extraction and CytoGenomics software programs.

4. Results

The karyotype was 46,XY,del(4)(p15.3p15.1),9qh+ (Fig. 2A). The parental karyotypes were normal. Array-CGH analysis revealed a deletion on chromosome 4p15.1–15.31, which confirmed the karyotype (Fig. 2B). The deleted region was estimated to be 13.34 Mb (chromosome position: 19,108,480–32,448,650) and contained 29 genes (Table 1).

5. Discussion

Interstitial deletion of the short arm of chromosome 4 can lead to several clinical syndromes. Deletions which encompass the 4p16.3 region lead to Wolf-Hirschhorn syndrome, which results in a clinically significant phenotype [8]. Patients with deletion in the 4p15 region present a clinical phenotype different from that of mild and classic Wolf-Hirschhorn syndrome: proximal 4p deletion, characterized by normal growth with psychomotor retardation, multiple minor congenital abnormalities, and a characteristic face. Our patient presented like other reported cases normal growth with slight psychomotor retardation, pectus excavatum, left undescended testis, clinodactyly of the toes, and a somewhat characteristic facial dysmorphism including a long face, deep set eyes, puffy eyelids, flat and broad nasal bridge, long philtrum, and full lips[1–8]. Unlike other reported cases, our

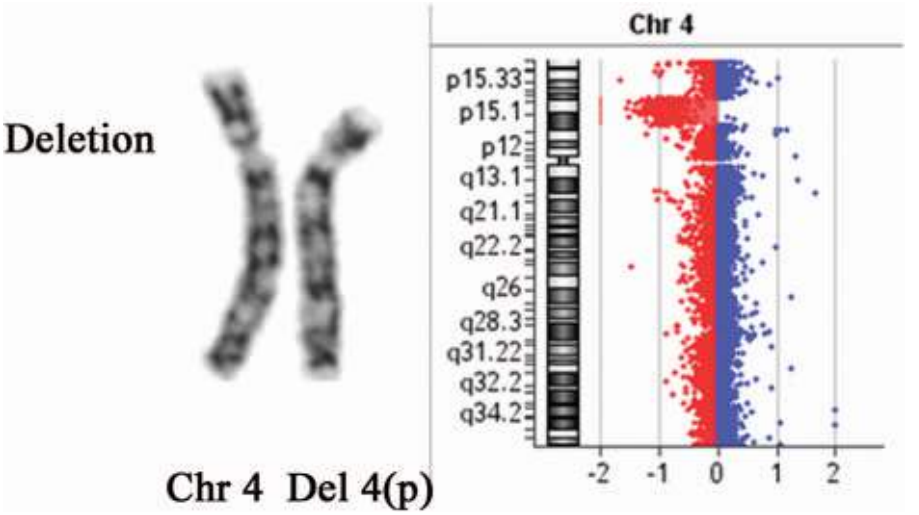

Figure 2. Cytogenetics analysis (A) and array comparative genome hybridization (B) results of the interstitial deletion of 4p15.1–15.31.

Table 1

Genes deleted in the 4p15.1–15.31 region in the presented case.

Gene	OMIM	Protein/transcript name	Function/dysfunction of gene product
SLIT 2	603746	Slit homolog 2	Molecular guidance cue in cellular migration, interact with roundabout homolog receptors
KCNIP4	608182	Potassium channel-interacting protein 4 isoform	Regulatory subunit of Kv4/D (Shal)-type voltage-gated rapidly inactivating A-type potassium chanel
GPR125	612303	G protein-coupled receptor 125	Orphan receptor that may play a role in planar cell polarity pathway
GBA3	606619	Glycosidase beta acid 3	Glycoidase probably involved in intestinal absorption and metabolism of flavnoid glycosides and beta-glycosylceramidase activity
PPARGC1A	604517	Peroxisome proliferative activated receptor gamma	Transcriptional coactivator for steroid receptors and nuclear receptors
DHX15	603403	DEAH box polypeptide 15	Nuclear ATP-dependent helicase
SOD3	185490	Superoxide dismutase 3	Free radical detoxification
LGI2	608301	Leucine-rich glioma inactivated protein 2	May be involved in axonal path finding
SEPSECS	613009	O-phosphoserin tRNA-selenocystein tRNA syntase	Pontocerebellar hypoplasia type 2D (AR)
PI4K2B	612101	Phosphatidylinositol 4-kinase type 2 beta	Phosphatidylinositol 4-kinase type 2 beta
ZCCHC4	611792	Zinc finger CCHC domain-containing protein 4	May be a methyltransferase
ANAPC4	606947	Anaphase-promoting complex subunit 4	Component of anaphase-promoting complex/cyclosome, a cell cycle regulated E3 ubiquitin ligase and the G1 phase of the cell cycle
SLC34A2	604217	Sollute carrier family 34 (sodium, phosphate cotransporter) member 2	Testicular microlithiasis. Pulmonary alveolar microlithiasis (AR)
RBPJ	147183	Recombination signal-binding protein for kappa J region	Adam-Oliver syndrome 3 (AD)
CCKAR	118444	Colecystokinin A receptor	Receptor for cholecystokinin with role in colecystokinin induced regulation of satiety
PCDH7	602988	Procadherin 7 isoform c precursor	Mediation of calcium dependent cell-cell adhesion expressed predominantly in SNC
STIM2	610841	Stromal interaction molecule 2	Regulation of basal cytosolic and endoplasmic reticulum Ca^{2+} concentrations
LOC100505893	—	Hytpothetical protein LOC100505893	Function unknown
MIR 218–1	—	microRNA 218–1	Non-coding RNAs—miRNA
PACRGL	—	PACRG- like protein	Function unknown
NCRNA00099	—	KCNIP4 intronic transcript 1	lincRNA
LOC100505912	—	Hytpothetical protein LOC100505912	Function unknown
MIR573	—	microRNA573	Non-coding RNAs—miRNA
CCDC149	—	Coiled-coil domain containing protein 149	Function unknown
LOC285540	—	Hytpothetical protein LOC100505912	Function unknown
SEL1L3	—	Protein sel-1 homolog 3	Integral component of membrane
C4ORF52	—	Small integral membrane protein 20	Integral component of membrane
TBC1D19	—	TBC1 domain family member 19	GTP-ase activating protein for Rab family protein
MIR4275	—	microRNA573	Noncoding RNAs—miRNA

OMIM = Online Mendelian Inheritance in Man.

patient did not have a thin body habitus, up-slanted palpebral fissures, or midface hypoplasia. Chitayat et al[3] proposed that for proximal 4p syndrome, the minimal deleted segment was represented by (4)(p15.2p15.33); in our patient, this region was not completely deleted.

Moller et al[7] reported a case with the same deleted region at 4p15.1–15.31 in a 38-year old woman with mild mental retardation, divergent strabismus, enlarged lower lip, tooth irregularities, pectus exaecvatum, bilateral genu valgum, hypermobile joints, and late-onset epilepsy with generalized tonic-clonic seizures. In addition, she presented with polymicrogyria adjacent to an arachnoid cyst of the left temporal lobe[7] and the deletion was approximately 15Mb and spanned other chromosome positions. The genes such as *SLIT2* (Slit homolog 2), *KCNIP4* (potassium channel-interacting protein 4 isoform), and *LGI2* (leucine-rich glioma inactivated protein 2) reported by Moller et al of special interest with regard to brain development and epilepsy are also deleted in our case. Another gene, *PCDH7* (procadherin 7 isoform c precursor), which is also deleted in our case, mediates calcium-dependent cell–cell adhesion and is expressed predominantly in CNS, specifically in thalamocortical circuits and the hippocampus, and was reported to be a candidate gene for a common form of epilepsy in a recent study[9]. At present, our patient has not developed epilepsy and does not have congenital brain malformation visible on a brain computerized tomography scan. The presence of developmental delay and intellectual disability in both cases suggests the importance of these genes in cortical development.

Heterozygous missense mutations of *RBPJ* (recombination signal-binding protein for kappa J region) are implicated in Adam Oliver syndrome type 3, a disorder characterized by vertex scalp defect (aplasia cutis congenita) in combination with terminal transverse limb defects[10]. Our patient does not present vertex scalp defect, but has clinodactyily of the toes. *RBPJ*, the principal DNA-binding partner of the Notch intracellular domain, is an evolutionarily conserved protein that coordinates the transcriptional activation of Notch-target genes through the assembly of protein complexes containing coactivators. RBPJ-mediated NOTCH signaling is also important for mesenchyimal cell proliferation, skeletal formation [11] epidermis, and hair follicle development [12] and vascular structure formation.[13] Furthermore, RBPJ- deficient mice have defective cranial bone formation [14]. Therefore, the deletion of the *RBPJ* contributes to or is responsible for other skeletal abnormalities observed in cases of

interstitial 4p deletions such as pectum excavatum and/or long face with high forehead.

In this report, we have provided a description of a 3-year-old patient with interstitial 4p deletion and mildly affected phenotype at this current age. He presented with pre- and postnatal normal growth, mild psychomotor retardation, and multiple minor congenital abnormalities. We therefore emphasize the involvement of *SLIT2, KCNIP4* and *LGI2* in cortical development and that of the *RBPJ* in skeletal abnormalities.

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Role of glutathione-S-transferase gene P1 in the diagnosis of prostate cancer in patients with 'grey level' prostate-specific antigen values

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Abstract. Prostate cancer (PC) represents the second most frequent cancer diagnosis in men and, at the same time, is one of the top six causes of death worldwide. The aim of the present study was to evaluate the diagnostic value of glutathione-S-transferase gene P1 (GST-P1) in patients that fall within the 'grey area' of the prostate-specific antigen (PSA) values. A retrospective observational study on 80 patients with prostate abnormal volumes and PSA values in the range 4-10 ng/ml was performed. The prostate gland was extracted following transrectal ultrasonography, and GST-P1 gene expression was analysed. A histopathological examination was considered the gold standard for PC diagnosis. Among the 53 patients diagnosed with PC, 69.8% (n=37) were GST-P1-positive, whereas, among the 27 patients diagnosed with benign prostatic hyperplasia, 18.5% (n=5) were GST-P1-positive. The sensitivity for diagnosing PC in patients with PSA values between 4 and 10 ng/ml was 69.81%, and the specificity was 81.48%. The positive predictive value was 88.1% [95% confidence interval (CI), 74.37-96.02%] and the negative predictive value was 57.89% (95% CI, 40.82-73.69%). Collectively, these results show the potential of using GST-P1 gene expression in patients who are suspected of having PC, but where the PSA values are inconclusive.

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Abbreviations: PC, prostate cancer; BPH, benign prostatic hyperplasia; GST-P1, glutathione-S-transferase gene P1; PSA, prostate-specific antigen; DRE, digital rectal examination

Key words: prostate cancer, diagnosis, glutathione-S-transferase gene P1, prostate-specific antigen, genetic markers

Introduction

Prostate cancer (PC) is one of the most commonly and frequently diagnosed malignant solid tumours in men. It is the second most diagnosed cancer worldwide, representing one of the major causes of death among men in both industrialized countries and developing countries according to recently published data, with increases in cases of urinary tract carcinomas, such as penile carcinoma, having been identified among the developing countries of Africa, Asia and South America (1,2). The progression of PC worldwide is expected to grow to almost 2.3 million new cases, and 740,000 deaths, by 2040 (1). In Romania, PC is the second most common diagnosed malignancy, with high incidence numbers compared with other neoplastic diseases (3), and the second most common cause of death by cancer in men.

During the course of PC diagnosis, several laboratory and clinical tests are routinely performed. Screening tests are frequently used, including the test for prostate-specific antigen (PSA). Despite its low sensitivity, this screening test is widely used (4,5) in detecting PC when a 4 ng/ml cut-off point is used. Furthermore, if the PSA value of the patients falls within 4-10 ng/ml, also known as the 'borderline' or 'grey-level', this poses serious concerns in terms of making the correct diagnosis (6). Therefore, a combination of several other diagnostic tests are recommended, such as digital rectal examination (DRE), prostate health index, the 4k score, IsoPSA™ (Cleveland Diagnostics) and imaging testing (7). Considering all these tests, expanding the pool of biomarkers

that contribute to the early and accurate detection of PC would be of great interest for researchers, medical staff, and people at risk (8).

In the present study, the possibility of using glutathione-S-transferase gene P1 (GST-P1), a genetic marker involved in carcinogen detoxification, antineoplastic product activation and metabolism of chemotherapeutic agents (9), in patients that are in the 'grey area' of the PSA values was evaluated.

Materials and methods

Patient study. This observational, retrospective study was conducted on consecutive patients that presented either for control examination or due to lower urinary tract symptoms (LUTS) at the Urology Clinic of County Hospital of Constanta between January 2018 and January 2020. A total of 80 patients that met the inclusion criteria of having a PSA value between 4 and 10 ng/ml were recruited.

Ultrasound control was conducted in all patients, with the prostatic volume measured by ~~DRE~~ Afiniti 30-Philips Ultrasound Machine with a C9-4v transducer probe. For all patients with abnormal prostate volumes, the PSA level was evaluated using the electrochemiluminescent immunoassay method (Cobas INTEGRA® 411 Analyzer). Transrectal ultrasonography with prostate biopsy was also performed. On the extracted tissue, GST-P1 gene expression was analysed, and histopathological examination was performed to confirm the diagnosis. The histopathological examination (hematoxylin-eosin staining) was considered as being the golden standard for PC diagnosis.

Isolation of genomic DNA from harvested tissue was performed with the aid of a QIAamp DNA mini kit from Qiagen GmbH, which combines the selective property of links on a silicon membrane with a flexible elution volume of 20-100 μl. Isolation of genomic DNA was performed from small amounts of tumour tissue biopsies (<10 mg), which were transferred immediately after harvesting to cryotubes with DNA/RNA shield solution (Zymo Research Corp.) to preserve the integrity of the genetic material. Sodium bisulfite conversion of genomic DNA was performed using an EpiTect Bisulfite Kit (Qiagen GmbH), and subsequently, methylation-specific PCR was performed using a CpG WIZ GST-P1 Amplification Kit (Merck KGaA; see below for further details).

According to the results of the histopathological examination, patients were divided into two groups: Patients with PC and patients with benign tumours, or benign prostatic hyperplasia (BPH; control group). The results from the two groups were compared to identify possible differences in age, prostate volume, PSA value, environment, LUTS and GST-P1 methylation status. The diagnostic accuracy of GST-P1 methylation status in these particular patients for whom the PSA values were inconclusive was evaluated.

The index test (GST-P1 methylation status). The index test (GST-P1 methylation status) can be methylated or unmethylated. Methylation-specific PCR for GST-P1 was performed using a CpG WIZ GSTpi Amplification Kit (Merck KGaA), according to the manufacturer's instructions. Concerning the protocol, the U Primer Set was defined as that which annealed to unmethylated DNA that has undergone a chemical modification, the M Primer Set was that which annealed to methylated DNA, and the W Primer Set was that which served as a control for efficiency of chemical modification. The primer sequence was not provided by the manufacturer, which only specified that the amplified region is defined as the sequence between the 3'-nucleotide of the sense primer and the complement of the 3'-nucleotide of the anti-sense primer for each gene promoter. The nucleotide numbering system was the one used in the GenBank submission, identified as AY324387 for GSTpi. For each experiment, the controls provided by the test were used, namely U control DNA and M control DNA, which were amplified with their corresponding primer set and served as the controls for unmethylated and methylated DNA, respectively, and untreated W genomic control DNA, which was amplified with the W primer set and served as a control for the efficiency of chemical modification. The PCR products were electrophoresed on a 2% agarose gel and visualized with ethidium bromide. Finally, a negative PCR control (i.e., no DNA) was performed for each set of primers (Figs. S1 and S2).

The specificity and sensitivity of the test were determined, to yield positive and negative predictive values of the test. 95% confidence intervals (CI) were calculated to quantify the statistical precision of the measurements (10). For comparing continuous variables, the mean and the standard deviation (mean ± SD) are presented, and comparisons were made using Student's t-test for independent variables. For comparing proportions, in the case of dichotomous variables, the χ^2 test was used. The summary data for these variables are presented as proportions. To determine the relationship between PSA values and the GST-P1 methylation status, a point-biserial correlation was used. This method represents a special case of Pearson's product moment correlation applied to a dichotomous and a continuous variable, as described in IBM documentation for SPSS (v.19.0). $P<0.05$ was considered to indicate a statistically significant difference.

The study received the ethical committee approval (no. 446/30.03.2018) of the Ethical Committee for Clinical Studies of the Emergency County Hospital Constanta. Procedures at all stages of the study were carried out in compliance with the principles of the Declaration of Helsinki. Informed consent forms were received from all participants prior to their enrolment in the study group.

Results

The total number of patients was 80. As the present study was a retrospective study, tests were performed on all of the patients, with no dropouts. The main characteristic of the sample group of patients was that all the participants had PSA values between 4 and 10 ng/ml. The results of the test are detailed in Fig. 1.

Subsequently, the characteristics of patients with PC and those with a benign tumour, or BPH, were analysed (Table I). Patients diagnosed with PC tended to be older (70.02 years; SD=8.7) compared with patients with BPH (64.07 years; SD=8.9), and these patients also came predominantly from urban areas, i.e., a higher percentage of patients from urban areas were diagnosed with PC. All other measured parameters, including prostate volume, LUTS and PSA values, were found not to have statistically significant differences (all P-values ≥0.5). DRE raised the suspicion of PC in 69.8% of the

Table I. Descriptive statistics of the sample (n=80).

Variable	Prostate cancer (n=53)	Benign tumour (n=27)	P-value
Mean age ± SD (years)	70.02±8.70	64.07±8.90	0.005[a,b]
Mean prostate volume ± SD	46.579±13.025	42.226±13.029	0.162[b]
PSA value (ng/ml)	7.08±1.81	7.13±1.87	0.91[b]
Environment (urban/rural)	31/22	8/19	0.015[a,c]
LUTS (present/absent)	22/31	16/11	0.133[c]
Suspicion at digital rectal exam (yes/no)	37/16	8/19	0.001[a,c]
GST-P1 expression (positive/negative)	37/16	5/22	0.001[a,c]

[a]Statistically significant (P<0.05), as highlighted in bold; [b]ANOVA test; [c]Fisher's exact test. LUTS, lower urinary tract symptoms; PSA, prostate-specific antigen; GSTp1, glutathione S-transferase gene P1.

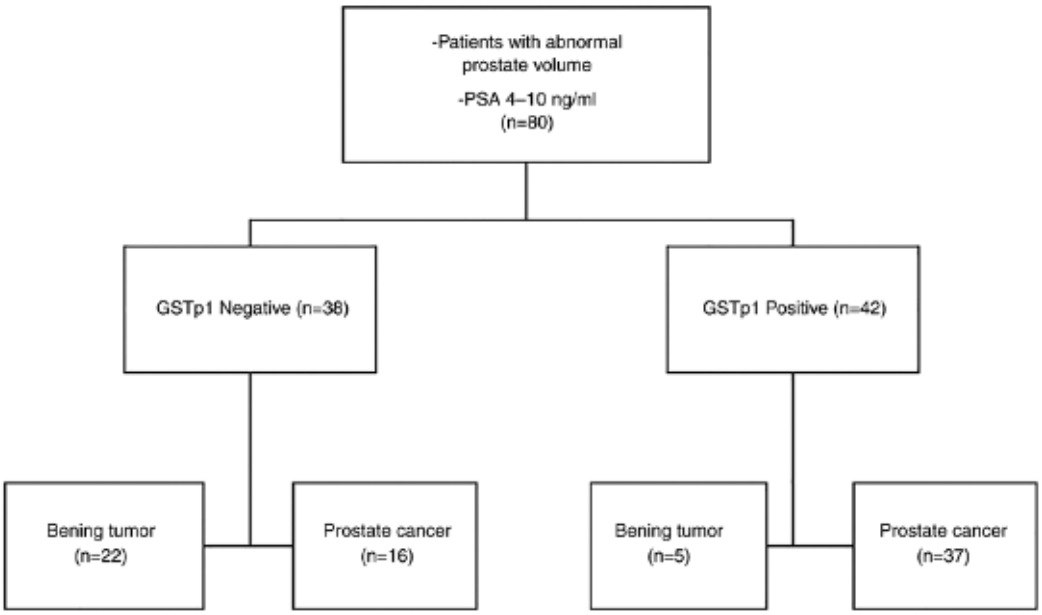

Figure 1. Patient flow chart. PSA, prostate-specific antigen; GSTp1, glutathione S-transferase gene P1.

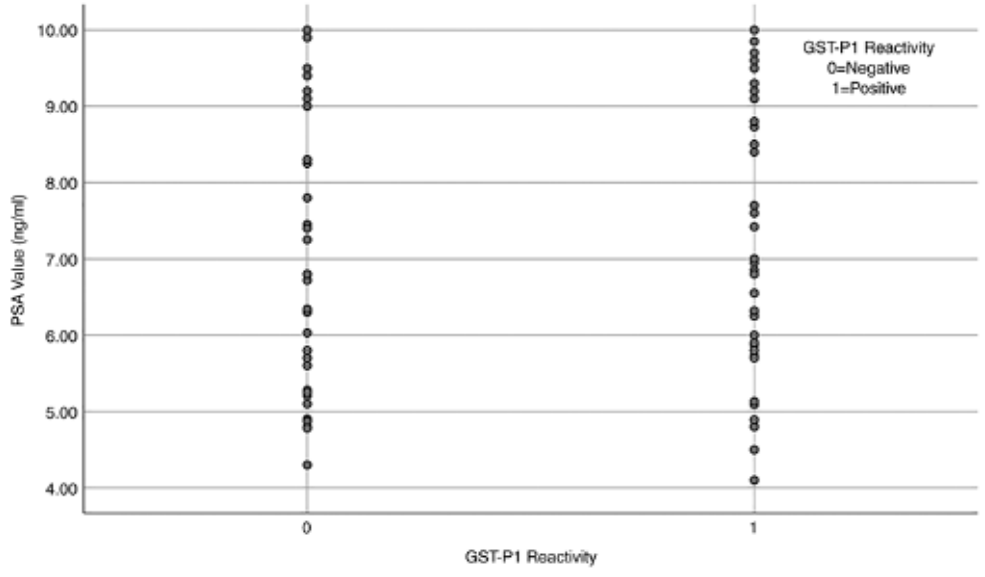

Figure 2. Simple scatter of PSA values by GST-P1 methylation status. GST-P1/PSA correlation curve is shown. PSA, prostate-specific antigen; GST-P1, glutathione S-transferase gene P1.

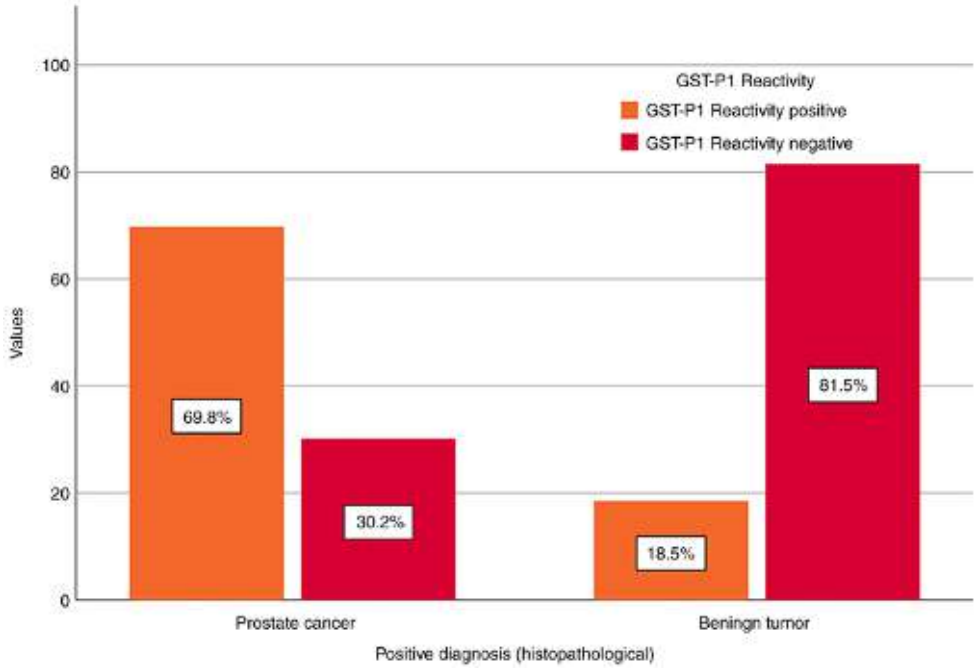

Figure 3. Patients' distribution-GST-P1 reactivity and diagnosis. GST-P1, glutathione S-transferase gene P1.

Table II. Screening test results.

Variable	Value	95% CI
Sensitivity	69.81%	55.66-81.66%
Specificity	81.48%	61.92-93.70%
AUC	0.76	0.65-0.85
Positive likelihood ratio	3.77	1.68-8.48
Negative likelihood ratio	0.37	0.24-0.58
Disease prevalence	66.25%	54.81-76.45%
Positive predictive value	88.10%	74.37-96.02%
Negative predictive value	57.89%	40.82-73.69%

AUC, area under the receiver operating characteristic curve.

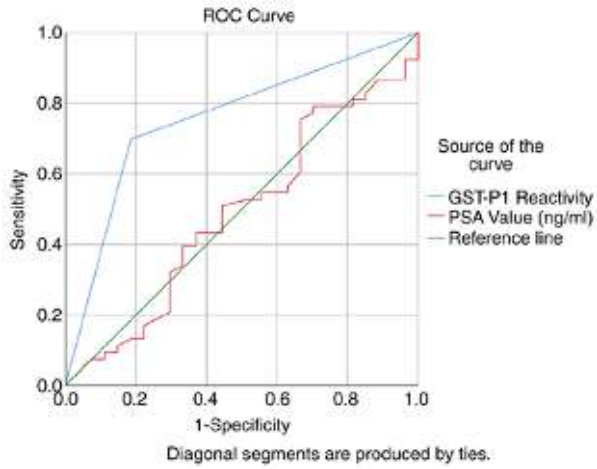

Figure 4. ROC curves for GST-P1 and PSA for diagnosing Prostate Cancer. PSA, prostate-specific antigen; GST-P1, glutathione S-transferase gene P1; ROC, receiver operating characteristic curve.

patients diagnosed with PC, but also raised the suspicion of malign tumour in 29.6% of the patients with a BPH.

A point-biserial correlation analysis was performed to determine the relationship between PSA values and GST-P1 methylation status. A positive correlation was identified, although this was not found to be statistically significant (r_{pb}=0.081; n=80; p=0.473) (Fig. 2).

Furthermore, more detailed attention was paid to the results for GST-P1 reactivity in patients within the grey area of PSA values. Among the 53 patients diagnosed with PC, 69.8% (n=37) were GST-P1-positive, whereas, among the 27 patients diagnosed with BPH, 18.5% (n=5) were GST-P1-positive. The calculated accuracy of the test was 73.75%, as it correctly identified 37 patients with PC and 22 patients with BPH (Fig. 3).

The calculated sensitivity for diagnosing PC in patients with PSA values between 4 and 10 ng/ml was 69.81% (95% CI, 55.66-81.66%), and the specificity was 81.48% (95% CI, 61.92-93.70%) (Table II). At the same time, based on the prevalence given by the study population, the positive predictive value was determined to be 88.1% (95% CI, 74.37-96.02%), and the negative predictive value had a lower value of 57.89% (95% CI, 40.82-73.69%). The receiver operating characteristic (ROC) curve was subsequently drawn for GST-P1 and PSA for the diagnosis of PC (Fig. 4).

Discussion

The present study aimed to evaluate the potential of using the GST-P1 gene as a biomarker for the diagnosis of PC in patients for which the PSA value is inconclusive, i.e., within the 'grey area', defined as values between 4 and 10 ng/ml. The results of the analysis indicate that GST-P1 has good potential to discriminate between patients with PC or BPH.

The calculated sensitivity was 69.81%, whereas the specificity of the test was 81.48%, with a positive predictive value of 88.1% and a negative predictive value of 57.89%. These results suggest that the evaluation of GST-P1 in patients for which the PSA is inconclusive may prove to be useful for diagnosing the presence or absence of PC, allowing for a faster detection time and treatment initiation.

Methylation of the GST-P1 gene represents the most common genetic alteration that is reported in PC (11,12), being observed in >90% of cases of PC, whereas it is seldom observed in benign prostate tissue (13). A recently published systematic review and meta-analysis (14) estimated that the incidence of GST-P1 methylation was higher in patients with PC than in those without, with an odds ratio (OR) of 18.58 (95% CI, 9.6-35.35; P<0.001). The detection of GST-P1 was considered in several studies as a non-invasive diagnostic tool for early detection of PC (15,16), being evaluated within meta-analysis (17). The results tend to vary a lot, and, as determined by Wu *et al* (17), the pooled specificity of GST-P1 was found to be excellent (89%; 95% CI, 80-95%) with a lower sensitivity, of 63% (95% CI, 50-75%). Another meta-analysis that analysed >35 studies which focused on the usefulness of GST-P1 in PC diagnosis (18) concluded that the sensitivity for GST-P1 (on biopsies) was 81.7±8.3%, and the specificity was 95.8±0.6%.

Another recent study suggested that GST-P1 may be involved in the development and progression of various types of cancers, fincluding lung cancer, colorectal cancer, gastric cancer, and even metabolic diseases, with these roles being evaluated in recent works (19).

Although, in general, research conducted previously has been carried out on participants that were evaluated for the presence of PC (and thus the characteristics of the test were applicable to the general population), the particularity of our study was the fact that it was focused solely on patients for which the PSA is inconclusive (within the range of 4-10 ng/ml). This might explain the lower value of the specificity when compared with other studies, and also could account for the higher value of the sensitivity.

Another major difference, which, in the context of screening purposes may be a limitation of our study, refers to the method of measuring the methylation status of GST-P1, which was executed by DNA genomic isolation from the harvested tissue. Previously published studies (16,20-22) have indicated that there is a correlation between the detection of GST-P1 from tissue samples and the methylation status examined from urine samples, within various limits. Other studies showed significant differences in the sensitivity and specificity of GST-P1 for PC, depending on the testing method (23); therefore, new research on the potential of GST-P1 usage as a screening test in patients within the 'grey-area' of PSA values could bring valuable new information for the development of novel methods of identifying patients with PC. Another possible limitation of the present study was the absence of other methods for determining the level of GST-P1 expression (i.e., immunohistochemistry).

The usage of genetic markers for the diagnosis of oncological conditions is increasing, as their potential to serve this purpose is very promising. In the present study, the potential of GST-P1 marker usage was evaluated in the diagnosis of PC in patients for which the PSA values were uncertain (within the 'grey area'). The results indicated a good sensitivity of 69.8% and a good specificity of 81.48%, when compared with the golden standard of diagnosis-histopathological examination. These results have the potential of sustaining the use of this diagnosis method in patients for which the suspicion of PC exists, but the PSA values are inconclusive.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

MS, VB, DOC, AIS, CT and FV contributed to the conception and design of this study. MS, APS, LM, AM, CB and DS were responsible for the data collection and analysis. MS, AIS, APS, CT and DOC oversaw drafting the manuscript. LM, DS, VB, AM, CT, CB and FV revised the manuscript critically for important intellectual content. All authors read and approved the final version of the manuscript. MS and FV confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the Ethics Review Committee of the Clinical County Emergency Hospital 'St. Andrew' Constanta (approval no. 446, approval date: 30.03.2018). The written informed consent was obtained from all subjects. The research was carried out respecting all the international and national regulations and in agreement with the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tissue and Circulating MicroRNA-31, MicroRNA-200b, and MicroRNA-200c Reflects Disease Activity in Crohn's Disease Patients: Results from the BIOMIR Study

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ABSTRACT

Background & Aims: MicroRNAs (miR) have altered expression in multiple autoimmune disorders including inflammatory bowel disease. The aim of the study was to assess the tissue and circulating miR-31, miR-200b, and miR-200c expression levels as potential biomarkers for intestinal disease activity in patients with Crohn's disease (CD).

Methods: The study included 45 patients with histopathological confirmed CD and active disease (defined as fecal calprotectin >50 μg/g and Simple Endoscopic Score (SES) of CD >3), and 21 subjects as controls for the validation cohort. Demographic and clinical data, biomarkers (fecal calprotectin), endoscopy data, the expression levels of miR-31, miR-200b, and miR-200c in tissue and serum were assessed (by RT-PCR). Receiver operating characteristic analysis was performed to assess the miR-31, miR-200b, and miR-200c expression levels as potential biomarkers for active CD.

Results: Mean fecal calprotectin was 1540±890 μg/g. Mean SES-CD was 8.9±4.2. Tissue and circulating miR-31 were significantly correlated with fecal calprotectin (r=0.81, r=0.83, p<0.01) and with SES-CD (r=0.82, r=0.79, p<0.01). The expression level of miR-31 was significantly upregulated in CD tissue cases compared to the control tissue samples (6.24±1.57 vs. 3.70±1.44; p <0.01). Similarly, serum miR-31 expression levels in CD patients were significantly upregulated compared to the control serum samples (0.78±0.42 vs. -2.07±1.00; p<0.01). The expression levels of tissue miR-200b and miR-200c were significantly upregulated in CD tissue cases compared to the control tissue samples (-5.25±0.93 vs. -4.69±0.80, p=0.03 for miR-200b, and -0.86±0.96 vs. 0.39±0.66, p<0.01 for miR-200c). Similarly, serum miR-200b and miR-200c expression levels in CD patients were significantly upregulated compared to the control serum samples (p < 0.05). Receiver operating characteristic analysis revealed that the expression levels of the selected miRNAs could help to discriminate active CD patients from healthy controls with very good specificity and sensitivity.

Conclusions: Tissue and circulating miR-31, miR-200b, and miR-200c reflect disease activity in CD patients and can be used as biomarkers for active disease.

Key words: microRNA – disease activity – Crohn's disease – fecal calprotectin – biomarkers.

Abbreviations: AUC: area under the curve; CD: Crohn's disease; EMT: epithelial-mesenchymal transition; fCal: fecal calprotectin; IBD: inflammatory bowel disease; miR: microRNA; ROC: receiver operating characteristic; SES: Simple Endoscopic Score.

INTRODUCTION

Accurate assessment of disease activity in inflammatory bowel disease (IBD) is of paramount importance in decisions regarding treatment strategies. To apply a treat-to-target strategy, a tight assessment of activity is mandatory. Endoscopy is the gold standard for diagnosis and follow-up of IBD patients and provides the most accurate information, but is invasive, time-consuming, costly, and uncomfortable for patients [1]. Clinical activity is easy to assess but does not always correlate with endoscopic activity [2]. Although fecal calprotectin (fCal) has proved its beneficial role and has substantially improved the clinical care of IBD patients, there is still an urgent need for ideal non-invasive biomarkers that could replace the invasive endoscopy [3]. An objective monitoring biomarker that can assess disease activity could

be extremely useful in the follow-up of patients with IBD [4]. Furthermore, such non-invasive biomarkers could be used also as biomarkers for drug response [5].

MicroRNAs (miRs) are small, non-coding short (ribonucleic acid) RNAs that have a role in the regulation of genes and protein expression [6]. MicroRNAs have altered expression in multiple autoimmune disorders, including IBD, as well in inflammation, chronic degenerative disorders, and cancer [7]. Increasing evidence show that these miRs are involved in the pathogenesis of IBD. Among them, miR-31 has a critical role in inflammation, reduces the inflammatory response and promotes the regeneration of colon epithelium in mice. In vitro studies demonstrated that miR-31 has a direct action on IL-25 and regulates Th1/Th17 cell-mediated mucosal inflammation in colitis and suppresses the immune response; miR-31 also promotes epithelial regeneration in the inflamed epithelium by WNT and Hippo signalling pathways [8]. Advanced research suggests that intestinal epithelial cells and epithelial-mesenchymal transition (EMT) play an important role in the pathogenesis of IBD [9, 10]. Few studies have investigated the role of miRs in chronic inflammation, development of fibrosis and stenosis with promising results. The TGF-beta signalling pathway is the mechanism by which miRs are involved in chronic inflammation and fibrosis in IBD [11]. The miR-200 family has been shown to induce EMT in experimental models and in various human diseases [12,13]. Chen et al. [10] demonstrated that miR-200b promoted proliferation of intestinal epithelial cells by inhibiting TGF-beta1-induced EMT.

This emerging role has led to investigations into miR expression profiles in IBD to understand the pathogenesis of these diseases and led to clinical advances in this area [6]. Several studies showed altered miRs expression profiles in IBD [14-16] and discovered different signatures of miRs associated with susceptibility to IBD, with the risk of clinical and histological exacerbation, or associated with disease remission [17].

The majority of the studies examined miRs in intestinal biopsies from patients with IBD [18]. Besides tissue profiling, miRs were also discovered in serum in a cell-free state suggesting their potential use as non-invasive biomarkers [18]. Extra-cellular miRs are very stable and protected from degradation (packed in vesicles of microparticles, nano proteins, or RNA binding proteins) and are found in most biologic fluids like peripheral blood, serum, urine, plasma, milk, or saliva [18, 19].

Although endoscopy with biopsies remains the gold standard of care and especially in the diagnosis of IBD, the discovery of an accurate non-invasive test is useful in disease monitoring. To improve clinical care, circulating miRs have been investigated in IBD with important and useful results [19].

Also, for accurate results in this field of research, it is important to investigate if the dysregulation of the tissue miRs is similar to the dysregulation of circulating miRs [20].

The study aimed to assess the tissue and circulating miR-31, miR-200b, and miR-200c expression levels as potential biomarkers for intestinal disease activity in patients with Crohn's disease (CD).

METHODS

Study Patients

Out of 86 patients with CD admitted to County Clinical Emergency Hospital of Constanta and Clinical Hospital Colentina of Bucharest between January 01, 2019, and December 31, 2020, 45 patients with histopathological confirmed CD and endoscopic active disease (defined as Simple Endoscopic Score (SES) for CD ≥3) were included in the study. Patients with CD and concomitant infectious (such as *Clostridioides difficile*), inactive endoscopic disease (SES-CD < 3), patients who did not receive a colonoscopy on admission, or who did not express their consent, were excluded (41 patients). The study included also 21 subjects for validation cohort (age and sex-matched healthy volunteer's serum samples served as control samples).

Study Design

Demographic and clinical data, endoscopy data (SES-CD score), biomarkers (fCal), the expression levels of the selected miRs (miR-31, miR-200b, and miR-200c) in tissue and serum were assessed in all the subjects included in the study.

The activity of CD was assessed by endoscopy and fCal.

Endoscopy was performed using a Pentax EPK-i7010. Endoscopic disease severity was assessed by SES-CD; decoding was as following: 0–2 = remission, 3–6 = mild endoscopic activity, 7–15 = moderate endoscopic activity, >15 = severe endoscopic activity [21]. Biopsies for the evaluation of tissue miRs were performed as follows: three fragments from the inflamed areas, and three fragments from the endoscopic normal areas (endoscopic normal areas which served as tissue controls). For fCal determination, patients were trained to collect 3 different samples of at least 2 cm from different locations of the feces and samples were stored in a sterile container; all samples were collected in the morning and were immediately brought to the laboratory for processing; the stool sample was analysed using PETIA (Particle Enhanced Turbidimetric ImmunoAssay). Fecal calprotectine <50 μg/g means inactive disease and >50 μg/g means active disease [22]. Blood samples from the patients with CD and controls were collected for the evaluation of circulating miRs (1.4 ml of serum collected in BD Vacutainer® SSTTM II Advance Tubes).

Extraction and Quantification of MiRNAs from Tissues and Serum

The extraction of total RNA from tissues was carried out using a miRNeasy mini kit (Qiagen) according to manufacturer protocol [23]. Regarding the isolation of RNA from serum exosomes (EVs), we employed miRCURY® Exosome Serum/Plasma kit and miRNeasy Serum/Plasma kit following the manufacturer's instructions. Initially, enrichment of exosomes from serum was done by mixing the 1.4 ml of cleared serum (collected in BD Vacutainer® SSTTM II Advance Tubes) in a microcentrifuge tube with 560 μl Precipitation Buffer A. After overnight incubation at 2–8°C, the precipitate was centrifuged at 1500xg for 30 min at room temperature (RT). The resulting supernatant was discarded, and the formed pellet was resuspended in 240 ul resuspension buffer. Thereafter, 1 ml of QIAzol Lysis Reagent was added to the resuspended RNA

pellet and vortexed before standing 5 min at RT. After adding a 300 µl chloroform, the homogenate was shaken vigorously, left to stand for 5 min at RT, and then centrifuged at 12,000xg for 15 min at 4°C. Following centrifugation, the upper aqueous phase containing RNA was transferred and precipitated in an Eppendorf tube by adding 1.5 volumes of 100% ethanol. Next, 650 µl of the mixture was transferred into a RNeasy MinElute spin column placed in an appropriate collection tube and centrifuged at 12,000 rpm for 1 min at RT. This time the flow-through was discarded and 700 µl wash buffer RWT was pipetted and centrifuged at 12.000 rpm for 1 min. The column was then washed sequentially by centrifugation with RPE buffer and 80% ethanol at the same speed and time as the previous step. To dry the membrane, the column was centrifuged with an open lid at maximum speed for 5 min. Finally, 15 µl of RNase-free water was placed onto the column and incubated for 1 min at RT before centrifugation at maxim speed to elute the RNA.

The purity of isolated RNA was assessed by measuring absorbances at 260 and 280 nm using a NanoDrop One™ Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), where a 260/280 ratio range between 1.9 - 2.1 was considered acceptable. Moreover, the concentration of the samples was measured using the Qubit®3.0 (Thermo Fisher Scientific, Waltham, MA, USA), and RNA integrity number (RIN) was determined using 2200 TapeStation Bioanalyzer (Agilent Technologies GmbH, Waldbronn, Germany).

Reverse Transcription of miRNA to Complementary cDNA and Real-time PCR

Transcription of miR molecules in complementary DNA (cDNA) was achieved using Multiscribe RT enzyme coupled with miRNA-specific stem-looped RT primer (Table 1), reagents of TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, San Diego, CA). A standard amount of 10 ng RNA was loaded in a final volume of 15 µl of the reaction mixture and incubated in a thermocycler at 16 °C for 30 min, 42 °C for 30 min and 85 °C for 5 min to denature the sample.

Quantification level of miR in serum exosomal and tissues was evaluated by quantitative real-time PCR (qRT-PCR) using TaqMan®MicroRNA Assays Inventoried (Applied Biosystems, San Diego, CA). PCR reaction mixture included 10 µl of TaqMan® 2 × Universal PCR Mastermix (no AmpErase UNG), 1.33µl of the product from the reverse transcription reaction, 7.67 µl of RNase-free dH2O, and 1µl of TaqMan® Small RNA assay (20X). All reactions were carried out in duplicate for each sample using the ABI 7500 Fast qPCR instrument in 40 cycles under the following conditions: denaturation at 95 °C for 15 seconds and annealing coupled with extension at 60 °C for 60 seconds. Fluorescence was detected at the end of each cycle and a negative control without a template was used with all the qRT-PCR runs.

The relative gene expression values for the selected miRNAs were normalized against RNU44 in tissue and miR-16 in serum and calculated in concordance with the equation $2^{-\Delta\Delta CT}$, which represents the fold change (FC) between samples [24]. If the FC is positive it means the miRNAs gene is upregulated; if the FC is negative, it means it is downregulated.

Ethics

The study was conducted according to good laboratory practice and in accordance with the Declaration of Helsinki and national and institutional standards. Informed consent was obtained from all patients, and the study was approved by the Local Ethics Commission for the Approval of Clinical and Research Developmental Studies (approval no 16/15.11.2018).

Statistical Analysis

Statistical analysis and graphics were performed using MedCalc® software version 14.8.1. The D'Agostino-Pearson test verified the normality of the data distribution. Parametric tests were used when data were normal distributed.

Data were expressed as a mean and standard deviation, or median and range for quantitative variables or frequency and percentage for qualitative ones.

Independent-samples T-tests were applied to determine the statistical difference of miR species between groups, where a p-value less than 0.05 was considered statistically significant. Receiver operating characteristic (ROC) was employed to evaluate the diagnostic performance of the selected miRNAs as biomarkers useful in discriminate CD cases from control cases. The diagnostic accuracy was asses in terms of area under the curve (AUC), sensitivity (Sn), specificity (Sp), true positive/negative fraction (TP, TN), and false negative/positive fraction (FN, FP), where: sensitivity was calculated as follows = TP/(TP+FN); specificity = TN/(TN + FP).

Pearson rank correlation test was used for correlations between ordinal variables. A p value < 0.05 was considered statistically significant. A value of rho < 0.3 indicated a weak correlation, 0.3-0.7 moderate correlation, and rho > 0.7 indicated a strong correlation [25].

RESULTS

General characteristics are illustrated in Table II. The mean age of the CD patients was 45.5±10.4 years. Out of 45 patients, 29 (64.4%) were female and 16 (35.6%) were male. Regarding location, 10 patients (22.2%) had ileal disease (L1), 5 (11.2%) had colonic disease (L2) and 30 (66.6%) had

Table I. The mature microRNA sequences

microRNA	Lot ID	Mature microRNA sequence
hsa-miR-31	P170311-009 H10	AGGCAAGAUGCUGGCAUAGCU
hsa-miR-200b	P161205-006 D03	CAUCUUACUGGGCAGCAUUGGA
hsa-miR-200c	P161207-003 G05	CGUCUUACCCAGCAGUGUUUGG
hsa-miR-16	P180730-024 E07	UAGCAGCACGUAAAUAUUGGCG
RNU44	P181019-000 G11	UAUUGCACUUGUCCCGGCCUG

ileocolonic disease (L3). Regarding phenotype, 39 patients (86.6%) had inflammatory phenotype (B1) and 6 (13.4%) had stenotic phenotype (B2), and none had penetrant phenotype (B3). Mean fCal was 1540 ±890 µg/g. Mean SES-CD was 8.9± 4.2.

Table II. Characteristics of the patients in the testing cohort

Characteristics	CD	Controls	p
Total (n)	45	21	
Age (mean +/- SD)	45.5±10.4	42±9.8	>0.05
Gender			
Male	16 (35.6%)	9 (42.8%)	>0.05
Location, n (%)			
L1	10 (22.2%)		
L2	5 (11.2%)		
L3	30 (66.6%)		
Behaviour, n (%)			
B1	39 (86.6%)		
B2	6 (13.4%)		
B3	0		
fCal (µg/g)	1540±890		
SES-CD (mean +/- SD)	8.9±4.2		

SD: standard deviation; fCal: fecal calprotectin; SES-CD: Short Endoscopic Score for Crohn's disease.

All selected miRs were significantly correlated with fCal and SES-CD (Table III). MiR-31 and miR-200c showed a strong correlation with SES-CD and fCal, whereas miR-200b showed a moderate correlation.

Table III. Correlations of microRNAs with fecal calprotectin and endoscopic score

MicroRNAs	fCal	SES-CD
Tissue miR-31	r=0.81, p<0.001	r=0.82, p<0.001
Circulating miR-31	r=0.83, p<0.001	r=0.79, p<0.001
Tissue miR-200b	r=0.52, p=0.03	r=0.59, p=0.04
Circulating miR-200b	r=0.55, p=0.04	r=0.51, p =0.03
Tissue miR-200c	r=0.79, p<0.001	r=0.75, p<0.001
Circulating miR-200c	r=0.70, p=0.02	r=0.74, p<0.001

For abbreviations see Table II.

The Expression Level of MiR-31 in Inflammatory Tissues and Serum Samples

The expression of miR-31 in the tissue and plasma of the 45 patients was upregulated. As we presented in Fig. 1A, the mean expression level of miR-31 was found significantly upregulated in CD tissue samples compared to the control tissue samples (6.24±1.57 vs. 3.70±1.44; p<0.001).

Similarly, mean serum miR-31 expression level in CD patients was significantly upregulated than in control volunteers (0.78±0.42 vs. -2.07±1.00; p<0.001, Fig. 1B). The mean FC level of miR-31 in CD tissues and serum samples was upregulated by up to 5.20-fold and 6.23-fold, respectively.

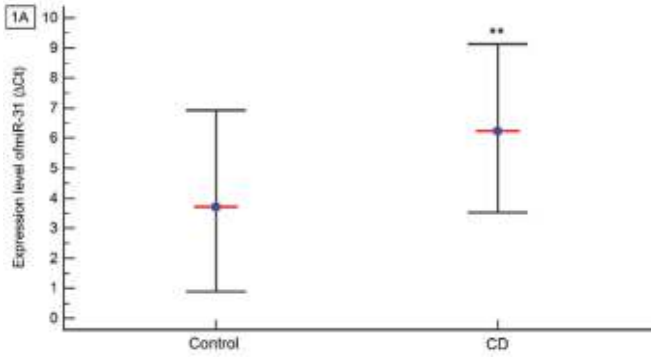

Fig. 1A. The expression level of micrR-31 in tissue samples collected from patients with Crohn's disease and controls.o

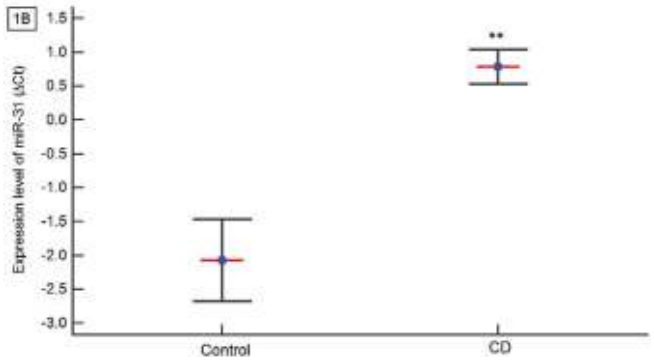

Fig. 1B. The expression level of microRNA-31 in serum samples collected from patients with CD and controls.

The Expression Levels of MiR-200b and MiR-200c in Inflammatory Tissues and Serum Samples

The expression levels of miR-200b and miR-200c in the tissue and serum of the 45 patients were upregulated. As we presented in Fig. 2A, the expression level of tissue miR-200b and miR-200c was found significantly upregulated in CD tissue cases compared to control tissue samples: -5.25±0.93 in controls vs. -4.69±0.80 in CD, p=0.03 (for miR-200b), and -0.86±0.96 in controls vs. 0.39±0.66 in CD, p<0.001 (for miR-200c). The mean FC level expressions of miR-200b and miR-200c in CD tissue samples were upregulated by up to 1.65-fold and 2.18-fold, respectively.

Similarly, serum miR-200b and miR-200c expression levels in CD patients were upregulated compared to the

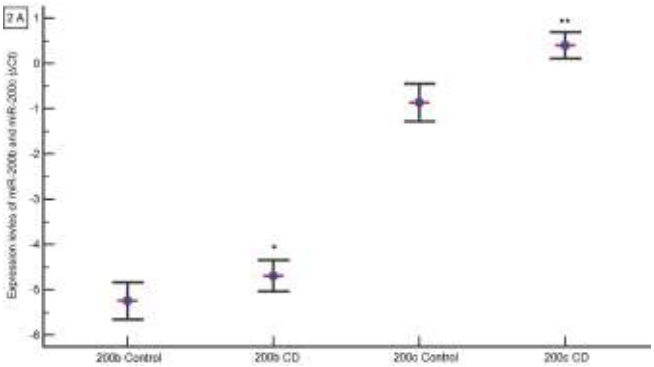

Fig. 2A. The expression level of microRNA-200b and microRNA-200c in tissue samples collected from patients with Crohn's disease and controls.

control volunteers (Fig. 2B). The mean FC level of miR-200b and miR-200c in CD serum samples was upregulated by up to 1.31-fold and 3.16-fold, respectively; however, the statistical analysis revealed significant differences only in the case of miR-200c ($p<0.001$).

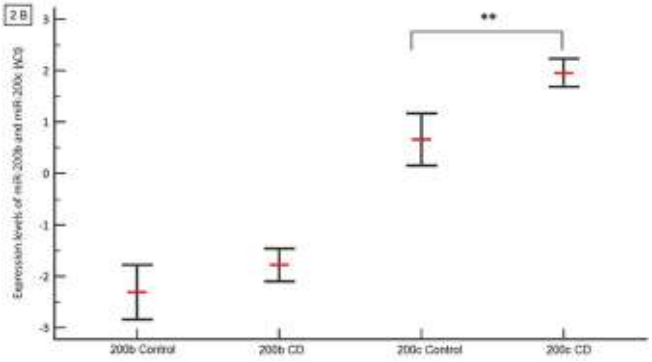

Fig. 2B. The expression level of microRNA-200b and microRNA-200c in serum samples collected from patients with Crohn's disease and controls.

Receiver Operating Characteristic Curve Analysis of the Selected MicroRNAs

Receiver operating characteristic curves were analysed to evaluate the miR-31, miR-200b and miR-200c expression levels as potential biomarkers for active CD. Analysis revealed that the expression levels of tissue miR-31, miR-200b and miR-200c could help to discriminate active CD patients from healthy controls with very good specificity and sensitivity (Table IV and Fig. 3-5).

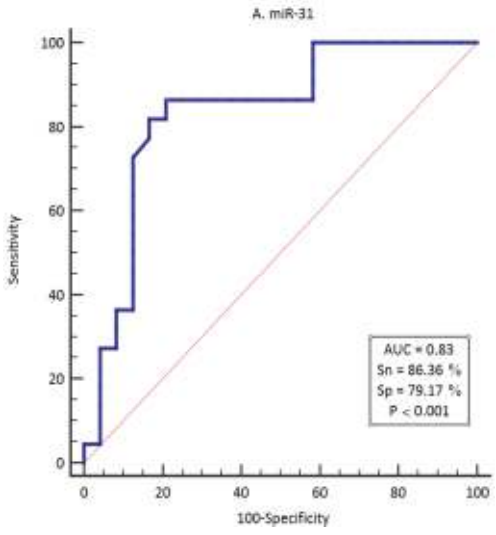

Fig. 3. ROC curve for microRNA-3.

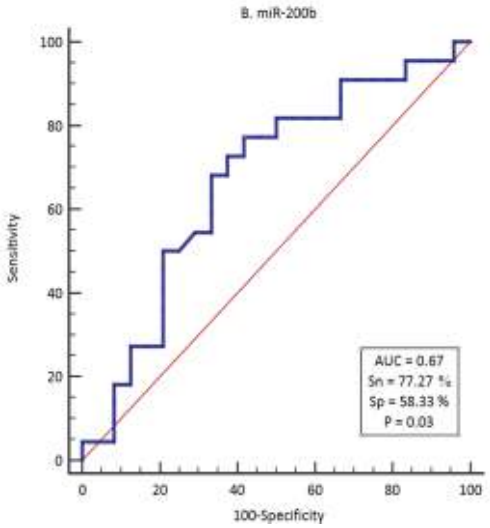

Fig. 4. ROC curve for microRNA-200b.

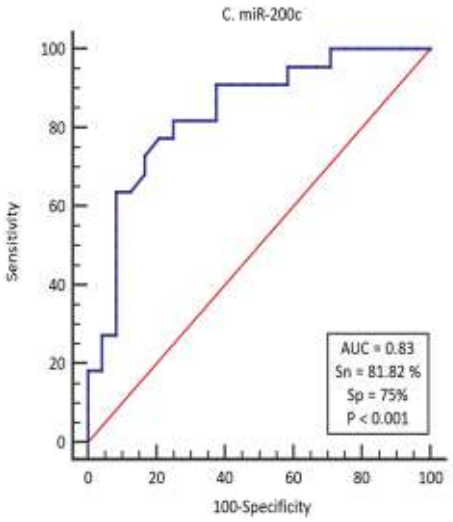

Fig. 5. ROC curve for microRNA-200c.

DISCUSSION

In the present study, we evaluated the feasibility of several tissues and circulating miRs as potential biomarkers for active CD. Our study showed that miR-31, miR-200b, and miR-200c correlates with active CD and these biomarkers could be novel biomarkers for disease monitoring. In the last years, there was a changing paradigm for the management of IBD, aiming to avoid disease progression, to prevent complications, bowel damage, and disability [26]. The possibility to apply the concept of "treat to target" strategies requires accurate and regular

Table IV. Receiver operating characteristic curve analysis of microRNAs-31, microRNAs-200b, microRNAs-200c

microRNA	AUROC (CI, p value)	Sensitivity	Specificity	TP	TN	Cut-off
miR-31	0.83 (0.69 – 0.92, p < 0.01)	86.36%	79.17%	4.15	0.17	0.65
miR-200b	0.67 (0.51 – 0.80, p = 0.03)	77.27%	58.33%	1.85	0.39	0.35
miR-200c	0.83 (0.70 – 0.93, p < 0.01)	81.82%	75%	3.27	0.24	0.56

miR: microRNA; AUROC: area under the curve; CI: confidence interval; TP: true positive fraction ; TN: true negative fraction.

assessment of disease activity [26]. Data showed that early escalation of the treatment based on regular disease monitoring by clinical data and biomarkers (such as fCal) leads to improved outcomes in IBD [26, 27]. Unfortunately, in IBD patients, treatment based only on symptoms failed to stop disease progression. Many studies showed the discordances between clinical symptoms and objective measures of disease activity, especially in CD [2]. A posthoc analysis of the patients with CD from the SONIC trial proved that in half of the patients, clinical activity did not correlate with endoscopic activity [28].

In our study, we tried to objectively assess disease activity by fCal and endoscopy, excluding clinical activity based on symptoms. The CALM trial was the first study to prove that tight disease control using objective biomarkers of inflammation and early treatment escalation in patients with CD leads to better outcomes compared to strategies driven by clinical activity [29]. Fecal calprotectin proved its important role in the monitoring of IBD patients, being a highly sensitive biomarker of endoscopic disease activity and is an adjunctive measure to monitor activity in both CD and ulcerative colitis (UC) [30]. Despite these facts, the optimal cut-off values best predictive of active disease are still being investigated [31-33].

Substantial efforts have been made to identify novel non-invasive biomarkers that could reflect intestinal activity. Despite these efforts, endoscopy with histology remains the „gold standard" and without any doubt, endoscopy is the most accurate instrument for disease activity assessment, but it is invasive, costly, inconvenient for patients, and may be also associated with serious adverse events [2]. Deep remission is the highest target in patients with IBD and this could be achieved only by stringent patient follow-up and regular monitoring. Objective biomarkers of disease activity as treatment targets are an important step towards this direction of achieving deep remission in patients with IBD [26].

Our study showed that miR-31, miR-200b, and miR-200c can differentiate active CD tissues from healthy tissues and the study of their expression levels may be used as a biomarker for the diagnosis of active CD patients. Similarly, in our study, circulating miR-31, miR-200b, and miR-200c follow the course of endoscopic disease activity and were upregulated in the serum of active CD patients in contrast with healthy controls and may be used as non-invasive biomarkers for disease activity in CD.

MicroRNAs, small non-coding molecules, post-transcriptional gene-regulating RNAs, have been investigated in multiple diseases for the past 10 years. It is already proved that miRs are unique stable molecules and protected from degradation, that may become dysregulated early in the course of a disease and that dysregulation can induce alterations in gene expression and promote inflammation or neoplasia [34-37].

MicroRNAs have altered expression in multiple autoimmune disorders, as well in inflammation, chronic degenerative disorders, and cancer [6]. Current evidence shows that miRs have an important functional role in IBD [38]. The miRs investigated in our study were selected based on previous studies. Intensive research in IBD showed that different miRs are involved in inflammation, cell-to-cell communication, cell integrity, immune response, barrier integrity and gut mucosal permeability, apoptosis of epithelial cells, tissue reparation, or malignancy [39, 40]. Even if it is in its infancy, research in this area showed that miRs can be used as a diagnostic and predictive tool for IBD and its complications and predictors of drug response [39]. Wu et al. [14] were the first to discover a differential pattern of miR expression in patients with active UC and controls or UC in remission. Since then, significant scientific progress has been made in the research of miRNAs in IBD. There is increasing data that IBD patients have dysregulated miRs compared to healthy controls.

Several miRs were investigated in tissue samples from patients with CD. For example, miR-23b, miR-106a, and miR-191 were upregulated, and miR-19b and miR-629 were downregulated in CD compared to healthy controls [41]. Another study showed that miR-9, miR-126, miR-130a, miR-181c, and miR-375 were dysregulated in inflamed biopsies from CD compared to healthy controls [42].

Although altered mucosal miR expressions in IBD are an excellent tool for disease activity in IBD, non-invasive assessment of miR in blood samples may become a more interesting and useful tool. As we also demonstrated in our study, circulating miR reflects mucosal changes. One of the advantages of miRs as biomarkers is the measurement which is performed by RT-PCR analysis which is an established standardized and sensitive method for gene expression [43] in contrast with fCal which test assays lack standardization leading to different results [44-46].

Wang et al. [47] investigated miR-223 level expression in 50 serum samples from patients with CD and UC and demonstrated the correlation of miR-223 with endoscopic scores of disease activity, similar to our results. Cordes et al. [43] demonstrated that circulating miR-320a distinguishes active disease from remission and correlates with endoscopic activity scores in both CD and UC. One previous study investigated several circulating miRs in 14 patients with active CD, 5 patients with CD in remission, 13 patients with active UC, 10 patients with UC in remission, and 13 controls and found that most of the investigated miRs (miR-199, miR-362, miR-532, miR-plus-E1271, miRplus-F1065) were differentially expressed only in active CD and miR-340 and miR-149 were dysregulated in both patients with active or inactive CD [48]. Paraskevi et al. [16] studied a profile of multiple miRNAs and found that miR-151, miR-199, and miR-16 were upregulated in IBD compared to controls. Several other studies that investigated mucosal miRs in IBD showed dysregulated miRNAs in IBD versus healthy controls [49-50].

Studies of circulating miRs have demonstrated that they are surrogate biomarkers for the diagnosis and monitoring of IBD and various other diseases [51-53].

MicroRNA-21, miR-155, and miR-31 are among the most investigated miRNAs in IBD [3]. miR-31 was found to be specific for IBD in a study by Zhang et al. [54, 55]. Tian et al. [8] showed that miR-31 is upregulated in tissue samples from patients with IBD and has a positive correlation with biomarkers of active disease. This study clearly demonstrated the molecular pathway of miR-31-5p activation in the inflammatory phase of IBD; in vivo mouse model showed that miR-31-5p activated the Wnt targets proteins and inhibited Hippo signalling target proteins [8,56].

In contrast with a previous study [57] which showed that miRNAs from the miR-200 family were downregulated in surgical specimens of stenotic CD, in our study, miR-200b and miR-200c were upregulated. Chen et al. [10, 58] found miR-200b to be downregulated in the inflamed mucosa and upregulated in the serum of the patients with IBD. Previous studies showed that the miR-200 family induces EMT in experimental models and humans [10, 59, 60]. Fibrosis results after myofibroblasts activation and excessive accumulation of extracellular matrix proteins. The relationship between inflammation and fibrosis and how inflammations lead to fibrosis is poorly understood, but most likely EMT is a key contributor [57]. The discrepancies between the results of our study and previous literature could be explained by this hypothesis: the mentioned studies included patients with severe fibrosis and without inflammation (upon histological examination) and miR-200 family were downregulated; in our study, patients had stenosis but also with active disease and miR-200 family were upregulated. It is worth studying miR-200 only in patients with CD and long-standing stenosis.

MicroRNA-200b was found one of the most important regulators of EMT by targeting ZEB transcription factors and also controlling E-cadherine and vimentin. Chen et al. [10,58] reported that ZEB1 was decreased by miR-200b and concluded that miR-200b plays an important role in the EMT pathogenesis. Furthermore, recent research shows that miR-200b-3p could regulate the gut microbiota and contribute to intestinal homeostasis [61].

Analysis of the ROC curves and AUCs revealed that expression levels of tissue miR-31, miR-200b, and miR-200c could help to discriminate active CD patients from healthy controls with very good specificity and sensitivity. The results of our study support the potential role of the selected miRNAs as biomarkers for active CD.

Regarding the design of our study, the most common design described in studies was to compare patients with IBD versus healthy controls. Other studies, in concordance with the design of our study, have investigated mucosal miR expressions in biopsies from areas with active disease and areas without inflammation with normal endoscopic appearance [18].

The limitations of the study could be the small sample size (45 patients), the mixed phenotype (patients with inflammatory and stricturing disease analysed together), and the absence of healthy control volunteers for tissue samples.

Knowledge about miRNAs involvement in fibrosis is limited, which highlights a growing need for discovering the mechanisms behind the pathogenesis of strictures in IBD. Future directions of IBD research may focus on the role of miRNAs in assessing fibrosis. Our results can be improved in the future with more extensive research which may help solidify differential miRNAs in IBD patients with consideration for specific disease phenotype variation amongst inflammatory, stricturing (with inflammation and without inflammation), and fistulizing phenotypes.

CONCLUSIONS

Tissue and circulating miR-31, miR-200b, and miR-200c reflect disease activity in CD and can be used as biomarkers for active CD.

Conflicts of interest: None to declare.

Authors' contributions: C.T., A.D., L.A. conceived the study, wrote and revised the paper. E.D., B.M., M.S., L.N. performed colonoscopies and tissue samples for analysis. R.P., N.L., M.M.I. data acquisition and database. G.C.C., A.F.M., M.M., E.M., C.B. performed DNA extraction, quantification of miRNAs and RT-PCR in the laboratory and interpreted the results. C.B. and E.D. performed statistics. All authors critically revised the paper and approved the final version.

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The Mutation Profiles of KRAS and BRAF Genes in a Romanian Colorectal Cancer Cohort

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Colorectal cancer (CRC) is one of most commonly diagnosed malignancies and management of CRC differs in according with patient's characteristics, tumor type, differentiation, metastatic extension and KRAS/BRAF mutations. Based on this knowledge, we examined the relationship between KRAS/BRAF mutations in paraffin-embedded tumor specimens and some clinicopathological features at CRC in order to provide reliable results to the oncologists and so to contribute to the best care provided to the patients. A 56 of colorectal cancer samples were analyzed for the KRAS and BRAF mutational status using StripAssay method from ViennaLab, Austria. Assays for identification of KRAS/BRAF mutations were based on polymerase chain reaction (PCR) and reverse-hybridization. KRAS mutations were present in 50% (28 patients) of all analyzed CRC and were located in codons 12, 13 and 61. The most frequent types of mutations were substitution of glycine to valine in codon 12 (c.35G> T; 9/28), followed by glycine to aspartate on codon 13 (c.38G> A; 5/28). BRAF mutations were detected at 9 patients (16%) and in all cases Val600Glu mutation has been observed. In one case we reported a concomitant KRAS/BRAF mutation. According with current data, KRAS and BRAF mutations are associated with a poor patient prognosis in CRC, but KRAS mutation in codon 13 and BRAF appear to have a higher oncogenic potential.

Keywords: colorectal cancer, KRAS, BRAF, mutation, genotype

Colorectal cancer (CRC) is the third most common cancer worldwide accounting for over 9% of all cancer incidences [1, 2]. In Romania CRC is the second most common type of cancer, accounting for 13.3% of all malignancies in men, and 12.6% in women [1]. Average survival of patients with metastases was improved by introduction of anti-EGFR therapy with monoclonal antibodies (Cetuximab and Panitumumab) or small molecules of tyrosine kinase inhibitors (Gefitinib and Erlotinib). Since these agents are only effective in certain subset of patients treated, thus identification and characterization of molecular markers to predict tumor response have been an area of interest. CRC patients with KRAS mutations appear to be relative resistant to treatment with monoclonal antibodies, with lower response rates and poorer survival [3-5]. Ras is a proto-oncogene subfamily that encoding low molecular weight GTP-ase proteins of 21 kDa. Ras proteins are involved in transducing of cellular signals through mitogen activated protein kinase (MAPK) pathway and acts as on-off switch at intersection of multiple upstream signals. Ras molecules play an important role in carcinogenesis processes, and also are involved in fundamental processes such as cell proliferation, differentiation, survival, invasion, and motility [5]. Most common mutant isoform is v-Ki-ras2 Kristensen rat sarcoma viral oncogene homolog (KRAS), which is present in 22% of all human cancers [3]. Identification of KRAS mutations was observed in 35-40% of sporadic CRC cases and over 80% of this were somatic point mutation detected in codon 12 and 13 of exon 2, and less often in codon 61 [3,6]. The v-Raf murine sarcoma viral oncogene homolog B1 (BRAF) gene belongs to RAF (Rapidly Accelerated Fibrosarcoma) family, and has a pivotal role in tumorigenesis [6-8]. The BRAF gene encode cytoplasmic serine/threonine kinase, which play a major role in cell proliferation, survival, differentiation and interfere indirectly with carcinogenesis by constitutive activation of MAPK proliferation pathway in absence of EGFR signals [9,10]. BRAF gene is located on long arm of chromosome 7 (7q34) and has been identified as mutagenic target in cancers such as colorectal, thyroid, gastric, lung or non-Hodgkin's lymphomas. The most common BRAF mutation, found in over 90% of cases it is a unique substitution, of glutamic acid with valine in codon 600 of exon 15 (V600E) [10,11]. Management of CRC, differs in according with patient's characteristics, tumor type, differentiation, metastatic extension and KRAS/BRAF mutations [12,13].

Although there are many articles that address the role of RAS family mutations in colorectal cancer, there are not many published data about KRAS and BRAF mutations in CRC patients in the Romanian population. The aim of this paper is to highlight the profile of KRAS/BRAF gene mutations in patients diagnosed with colorectal cancer and to make genotype-phenotype correlations that can be useful in assessing prognosis at CRC patients.

Experimental part

Material and methods

Cases were selected from the Pathology Department from Clinical Emergency County Hospital in Constanta, Romania. For all analyses, formalin-fixed and paraffin-embedded (FFPE) tissue specimens were used. Fifty-six specimens were processed and diagnosed primarily by an experimented pathologist according to standard protocols. The histological grade of cancer was classified using the tumors, node, and metastasis (TNM) staging system of the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC), according to the standard of the World Health Organization (WHO). Clinical information was substracted from medical records and pathology reports including sex, age at diagnosis, histological subtypes of adenocarcinoma, TNM stage, and

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tumor differentiation was collected. Genetic tests were performed within Centre for Research and Development of Morphological and Genetic Studies of Malignant Pathology (CEDMOG), "Ovidius" University of Constanta. Informed consents for mutations testing were signed by all patients.

For DNA isolation, tissue areas up to 250 mm^2, and up to eight sections with a maximum thickness of 8 ìm were used for each case. Hematoxylin and eosin (H&E) sections were used as a reference and the largest tumor area (at least 50% tumor cells) was scraped off with a scalpel under a dissecting microscope. Genomic DNA was extracted from FFPE cancer tissues by using QIAamp DNA FFPE Tissue Kit (QIAGEN, Germany) according to the manufacturer's protocol. Isolation and purification of DNA derived from FFPE tissues is accomplished in several steps. The first step was deparaffinization of sections by incubation for 10 min in xylene and then washes once in absolute ethanol for 10 min at room temperature. After air drying for 5 minutes at 37°C, tissue sections were completely lysed in presence of proteinase K and ATL lysis buffer at a temperature of 56° C on a heating block for 2-3 hours. In second stage, cell lysate was loaded into QIAamp MinElute column placed in an appropriate collection tube and contaminants were removed by centrifugation, using AW1 and AW2 wash solutions. Elution of DNA from silicon membrane was performed with an AE buffer.

KRAS and BRAF mutations analysis

Method for detecting KRAS and BRAF mutations were based on PCR and reverse-hybridization (*StripAssay ViennaLab, Austria*), and was performed according with manufacturer's instructions. Assay for the identification of KRAS mutations (KRAS XL StripAssay®) covers 29 mutations in the KRAS gene (codon 12, 13, 59, 60, 61, 117 and 146), respectively the assay for BRAF mutations (BRAF 600/601 StripAssay®) covers 9 mutations in the BRAF gene (codon 600 and 601). Procedure includes a PCR amplification step of isolated DNA using biotinylated primers, and a hybridizing step of amplification products to a nitrocellulose strip containing specific allele oligonucleotide probes immobilized as an array of parallel lines. Detection of biotinylated sequences was done using streptavidin-alkaline phosphatase and a chromogenic substrate.

Statistical analysis

Results obtained were statistical analysed using SPSS version 20 software (SPSS, USA). Pearson's correlations were used to determine associations between KRAS/BRAF genes mutation and clinicopathological features. All *p* values below 0.05 were considered statistically significant being calculated by χ^2 test and paired samples t-test.

Results and discussions

Some studies have reported significant differences between the KRAS/BRAF genotypes and clinico-pathological features such as age, gender, tumor location, histopathology, metastasis and tumor grade, while other studies observed no significant effects [14-16]. Among 56 patients included in our study, 28 patients (48.20%) were males and 29 patients (51.80%) were females, their ages ranged between 40 years and 88 years with an age media of 64.89 years. Clinicopathological features at CRC patients and distribution of KRAS/BRAF genes mutation are presented in table 1.

There were no significant differences in the frequency of KRAS mutations based on gender (14 KRAS mutated female vs. 14 KRAS mutated male). The *KRAS* mutation frequencies in European, Asian, and Latin American CRC patients were reported to be 36%, 24.0%, and 40.0%, respectively [1,7,8,17]. In our study, we observed a KRAS mutation rate of 50.00% in colorectal cancers, being a slightly higher frequency than the European average reported in other studies. However, it should be noted that our study sample size was small to draw meaningful

Table 1

CORRELATIONS BETWEEN KRAS/BRAF MUTATION AND CLINICOPATHOLOGICAL FEATURES IN CRC PATIENTS

Variables/ Number/percent (%)	KRAS Mutant (n= 28)	KRAS Wild-type (n=28)	p-value (χ^2-test)	BRAF Mutant (n=9)	BRAF Wild-type (n=47)	p-value (Paired samples t-test)
Sex						
Male	14 (25.00)	13 (23.20)	<0.001**	3 (5.36%)	28 (50.00)	0.32
Female	14 (25.00)	15 (26.80)		6 (10.71%)	19 (33.93)	
p-valne (χ^2-test)	0.70	0.25		0.31	0.18	
Age						
≤65	15 (26.78)	11 (19.64)	0.93	1 (1.79)	24 (42.86)	0.79
>65	13 (23.22)	17 (30.36)		8 (14.28)	23 (41.07%)	
p-value (χ^2-test)	1.00	0.70		0.02*	0.88	
Tumor location						
Proximal colon	9 (15.07)	11 (19.64)	0.74	3 (5.36)	17 (30.36)	0.51
Distal colon	10 (17.86)	10 (17.86)		5 (8.93)	15 (26.78)	
Rectum	9 (15.07)	7 (12.50)		1 (1.79)	15 (26.78)	
p-value (χ^2-test)	0.96	0.62		0.25	0.91	
Histological type						
Adenocarcinoma	14 (25.0)	18 (32.10)	0.11	2 (3.60)	30 (53.60)	0.35
Other carcinoma*	14 (25.0)	10 (17.90)		7 (12.50)	17 (30.40)	
p-value (χ^2-test)	1.00	0.13		0.096	0.058	
Depth of tumor invasion						
T1-T2	1 (1.79)	5 (8.92)	0.63	1 (1.79)	5 (8.93)	0.10
T3-T4	27 (48.21)	23 (41.08)		8 (14.28)	42 (75.00)	
p-value (χ^2-test)	<0.001**	0.001**		0.02*	<0.001**	

Nodal status						
N0	6 (10.70)	17 (30.40)	0.54	1 (1.79)	22 (39.28)	0.005**
N1-N2	22 (39.30)	11 (19.60)		8 (14.28)	25 (44.65)	
p-value (χ^2-test)	0.002**	0.25		0.02*	0.66	
Distant metastasis						
M0	20 (35.71)	22 (39.29)	0.77	6 (10.71)	36 (64.29)	0.53
M1	8 (14.30)	6 (10.70)		3 (5.36)	11 (19.64)	
p value (χ^2-test)	0.023*	0.002**		0.31	<0.001**	
Tumor grade						
G1	2 (3.60)	2 (3.60)	0.69	1 (1.80)	3 (5.40)	0.78
G2	22 (39.30)	21(37.50)		6 (10.70)	37 (66.10)	
G3	4 (7.10)	5 (8.90)		2 (3.60)	7 (12.50)	
p-value (χ^2 test)	<0.001**	<0.001**		0.09	<0.001**	

*Results obtained were statistically significant ($p<0.05$);**Results obtained were statistically significant ($p<0.01$). #Other carcinoma: mucinous adenocarcinoma, signet ring cell, adenosquamous.

Table 1
CONTINUATED

conclusions regarding these variations. A total of 9 patients (16%) were BRAF mutant, which is in according with previous reports [18-20]. Yaeger R. et al., showed that BRAF mutation confers a poor prognosis in metastatic CRC patients. Frequency of BRAF mutations was higher on female patients than male patients (10.71% vs. 5.36%) [18]. Regarding age, KRAS mutations were more common in the age group under 65 years (26.78% vs. 23.22%), and BRAF mutations in those over 65 years (14.28% vs. 1.79%). KRAS/BRAF mutations were more commonly found at patients with tumor location in the distal colon. CRC mutant cases had a higher T stage (T3-T4: 48.21% and 14.28% vs. T1-T2: 1.79% and 1.79%), more frequently a moderate tumor grade and N1-N2 nodal status (39.30% vs. 10.70% for KRAS mutations, respectively 14.28% vs. 1.79%, for BRAF mutations) without distant metastases (M0-35.71% vs. M1-14.30% for KRAS mutations, respectively M0-10.71% vs. M1-5.36%, for BRAF mutations).

The distribution of the KRAS/BRAF mutations identified in CRC patients is shown in figure 1 and the correlations KRAS/BRAF genotype and clinicopathological features in table 2.

The genetic basis for the mutation distribution among KRAS and BRAF isoforms gene is not fully understood, but the specialists support the idea that KRAS and BRAF have different roles in complex process of tumorigenesis, and molecular genetic changes may be more accurate markers than clinicopathological features to evaluate the prognosis of cases with early from medium stage in CRC [21-23]. Both KRAS and BRAF mutations are classified as leading

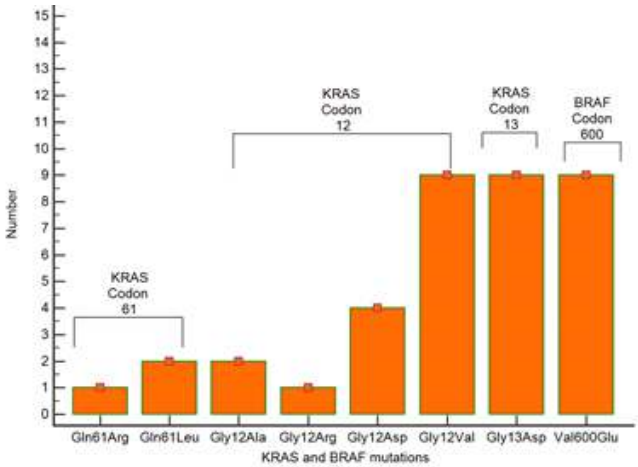

Fig.1. Type of KRAS/BRAF mutations in CRC patients: distribution of the mutant *KRAS* (G> T) and (G> C) and (A> T) transversions and KRAS (G> A) transitions.

mutations, since they are both mutated prior to malignant conversion and are therefore primary genetic events in CRC carcinogenesis [24]. Grimmond et al. (1992), have shown that critical regions of the KRAS gene for activation include codons 12, 13, 59, 61, and 63. In a similar study, K. Kimura et al. (2007) [25-26], showed that mutation in KRAS gene is associated with CRC and appear most often in codons 12 (28%), and 13 (8%), and less frequently in codon 61 of exon 2. Distribution of KRAS mutations in our study is similar to the studies mentioned, but their frequency is different, thus: CRC cases have associated

Table 2
CORRELATIONS GENOTYPE-FENOTYPE IN KRAS/BRAF MUTANT CRC

Clinico-pathological features	KRAS mutations							BRAF mutations	Concomitant mutations
	G12V	G12D	G12A	G12R	G13D	Q61L	Q61R	V600E	G12V (KRAS) V600E (BRAF)
Sex									
Male	-0.088	-0.129	0.007	0.140	0.064	0.140	0.199	-0.130	-0.130
p	0.521	0.344	0.960	0.304	0.638	0.304	0.141	0.338	0.339
Female	0.088	0.129	-0.007	-0.140	-0.064	-0.140	-0.199	0.130	0.130
p value	0.521	0.344	0.960	0.304	0.638	0.304	0.141	0.338	.0339
Age									
≤65 years	0.123	-0.258	0.207	-0.126	0.080	0.145	0.207	-0.212	0.145
p	0.334	0.055	0.126	0.357	0.557	0.287	0.126	0.116	0.287
>65 years	-0.132	0.258	-0.207	0.126	-0.080	-0.145	-0.207	0.212	-0.145
p	0.334	0.055	0.126	0.357	0.557	0.287	0.126	0.116	0.287
Tumor location									
Proximal colon	-0.198	0.083	0.258	-0.101	-0.022	-0.101	-0.143	-0.022	0.181
p	0.144	0.545	0.055	0.461	0.874	0.461	0.292	0.874	0.182
Distal colon	-0.091	0.083	-0.143	0.181	0.080	-0.101	0.057	0.181	-0.101
p	0.503	0.543	0.292	0.182	0.559	0.461	0.674	0.181	0.461
Rectum	0.307	-0.175	-0.122	-0.085	-0.062	0.213	0.091	-0.169	-0.085
p	0.022*	0.196	0.372	0.532	0.653	0.115	0.503	0.213	0.532

Histological type									
Adenocarcinoma	0.059	-0.169	0.222	-0.117	0.014	0.156	-0.167	-0.292	-0.150
p	0.668	0.212	0.100	0.391	0.918	0.252	0.220	0.029*	0.269
Other carcinoma*	-0.044	0.180	-0.214	0.121	0.002	-0.150	0.173	0.309	0.156
p	0.704	0.184	0.113	0.374	0.990	0.269	0.203	0.021*	0.252
Depth of tumor invasion									
T1-T2	0.024	-0.096	-0.067	-0.047	-0.152	-0.047	-0.067	0.006	-0.047
p	0.863	0.481	0.625	0.732	0.265	0.732	0.625	0.967	0.732
T3-T4	-0.024	0.096	0.067	0.047	0.152	0.047	0.067	-0.006	0.047
p	0.863	0.481	0.625	0.732	0.265	0.732	0.625	0.967	0.732
Nodal status									
N0	-0.224	0.061	0.239	0.168	-0.352	-0.108	-0.155	-0.252	-0.108
p	0.097	0.656	0.076	0.217	0.008**	0.426	0.255	0.061	0.426
N1-N2	0.237	-0.050	-0.231	-0.162	0.365	0.113	0.161	0.266	0.113
p	0.079	0.713	0.087	0.234	0.006**	0.409	0.237	0.047*	0.409
Distant metastasis									
M0	-0.214	-0.145	0.116	0.082	0.045	0.082	-0.101	-0.174	0.082
p	0.113	0.285	0.393	0.550	0.741	0.550	0.459	0.198	0.550
M1	0.236	0.160	-0.111	-0.078	-0.140	-0.078	0.111	0.197	-0.078
p	0.080	0.238	0.415	0.568	0.302	0.568	0.415	0.147	0.568
Tumor grade									
G1	-0.097	-0.066	-0.046	0.567	-0.104	-0.032	-0.046	0.112	-0.032
p	0.476	0.629	0.738	<0.001**	0.445	0.814	0.738	0.412	0.814
G2	-0.017	-0.012	0.106	-0.245	0.010	0.074	0.106	-0.105	0.074
p	0.899	0.932	0.438	0.069	0.940	0.587	0.438	0.442	0.587
G3	-0.040	0.067	-0.084	-0.059	0.073	-0.059	-0.084	0.073	-0.059
p	0.771	0.621	0.537	0.666	0.591	0.666	0.537	0.591	0.666

Table 2
CONTINUATED

*Results obtained were statistically significant ($p<0.05$); **Results obtained were statistically significant ($p<0.01$); *Other carcinoma: mucinous adenocarcinoma, signet ring cell, adenosquamous.

KRAS mutations in codon 12 in 57.14% (16/28 cases), codon 13 in 32.14% (9/28 cases), and 10.72% (3/28 cases) in codon 61 (Fig. 1). Allelic mutations result in aminoacid changes, namely Gly to Asp, Ala, Arg, Ser, Val, or Cys in codon 12 and Gly to Asp in codon 13 that lead to conformational changes in the KRAS protein [16]. In CRC, the main mutation of KRAS is representing by substitution of Gly to Asp and that has been found to occur in codon 12. Mutation from GGT (Gly) to GTT (Val) in codon 12 has been observed more frequently in primary metastatic of CRC, suggesting that this mutation may confer a more aggressive phenotype [27,28]. Compared to these data, the most common mutation found in our study was substitution of glycine to valine in codon 12 (c.35G> T; 9/28), followed by glycine to aspartate in codon 13 (c.38G> A; 5/28), and in the same proportion glycine to aspartate in codon 12, respectively in codon 13 (c.35G> A; 4/28) (fig. 1).

The 28 KRAS mutations were equally represented by transitions and transversions. In codon 12, the mutant KRAS (G> T) and (G> C) and (A> T) transversions were the most common mutations, being found in 11 cases, compared to only 4 cases with transitions (G> A). All mutations in codon 13 were transitions (G> A). The CRC with KRAS/BRAF gene mutations were diagnosed in equal proportions as adenocarcinomas and other carcinomas (mucinous adenocarcinoma, signet ring cell, adenosquamous). In a study published by W. S. Samowitz et al., in 2000 it appears that mutations in codon 13, resulting in the substitution of Gly with Asp, observed in CRC has been shown to be associated with reduced survival rates [29]. Similar to these results, we identified KRAS mutations in codon 13 at patients diagnosed with poorly or moderately differentiated CRC, and one case presented concomitant Gly12Val mutation in codon 12 of the KRAS gene and the Val600Glu mutation in the BRAF gene. Regarding the clinical-pathological features, this case did not differ particularly from the other cases diagnosed with only one type of mutation. Possible mechanism of having coexistent KRAS and BRAF mutation is unknown as its frequency is very low and it is not clear whether or not these tumors have a different biology and natural history than KRAS or BRAF mutant tumors or which of two mutations is dominant oncogene driving tumor proliferation [30]. According with current data, KRAS and BRAF mutations are associated with a poor patient prognosis in CRC [33-34] but BRAF has been shown to be a more potent oncogene than KRAS. In our study, a significant positive correlations were observed between tumor location or nodal status for patients and type of KRAS mutations (Gly12Val, $r = 0.307$, $p < 0.05$; Gly13Asp, ($r = 0.365$, $p < 0.01$), while BRAF V600E mutation had a significant positive correlations with advanced stage (N1-N2) of lymph node metastasis ($r = 0.266$, $p < 0.05$) (table 2). However valid conclusions cannot be drawn regarding these findings variations due to the limitations of this study.

Conclusions

Our preliminary findings suggest a fairly high frequency of KRAS/BRAF mutations in colorectal cancer, but a larger study with sufficient numbers would be required to derive meaningful results to determine whether these variations are valid to make genotype-phenotype correlations in mutant KRAS / BRAF colorectal cancers.

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