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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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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

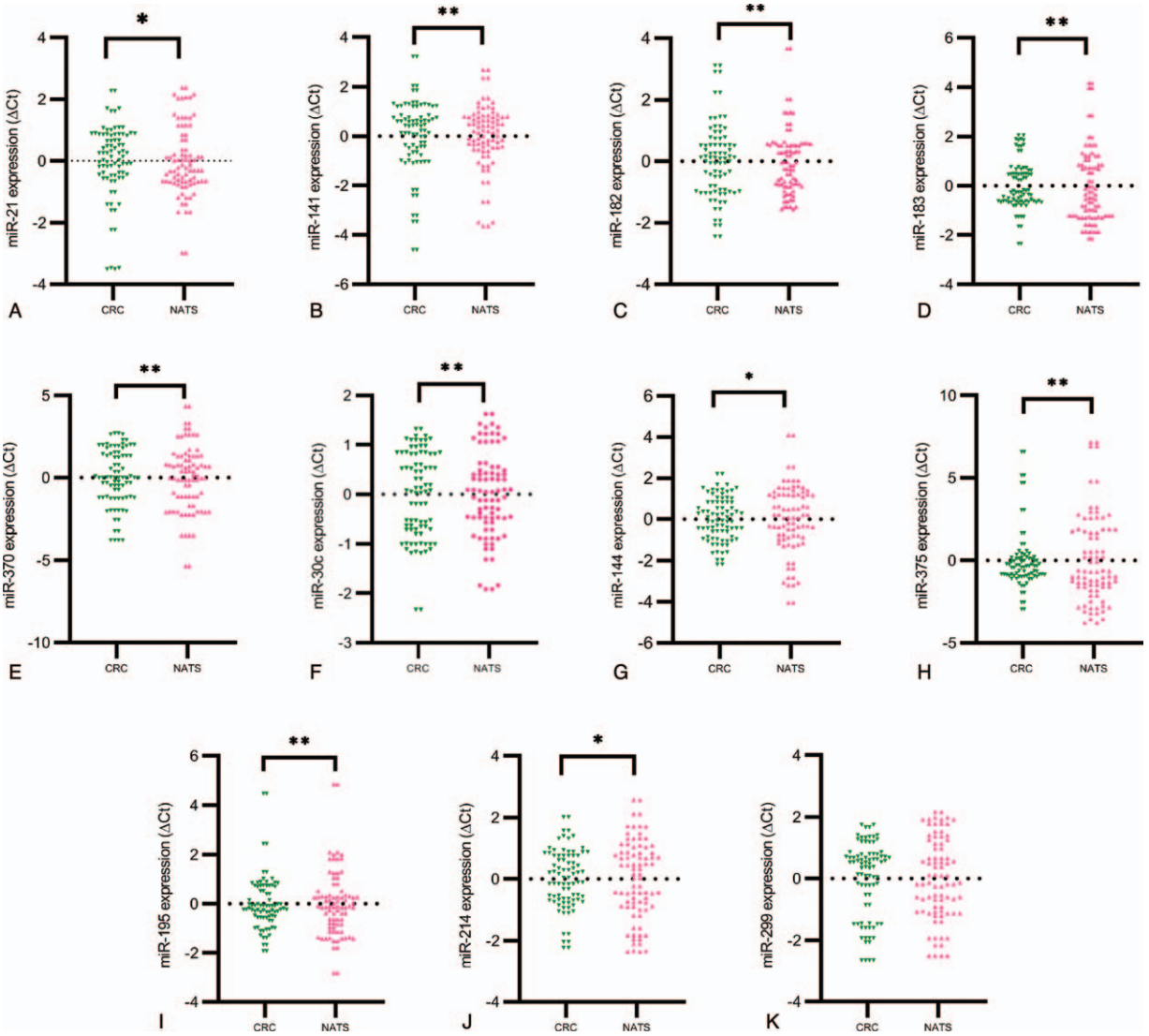

Figure 1. Dots-plot showing the results of deregulated miRNAs' expressions in colorectal cancer tissue (CRC) in relation to normal adjacent tissue samples (NATS). ΔCt values of upregulated miRNAs' expression: (A) miR-21, (B) miR-141, (C) miR-182, (D) miR-183, (E) miR-370. Downregulated miRNAs' expressions 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.

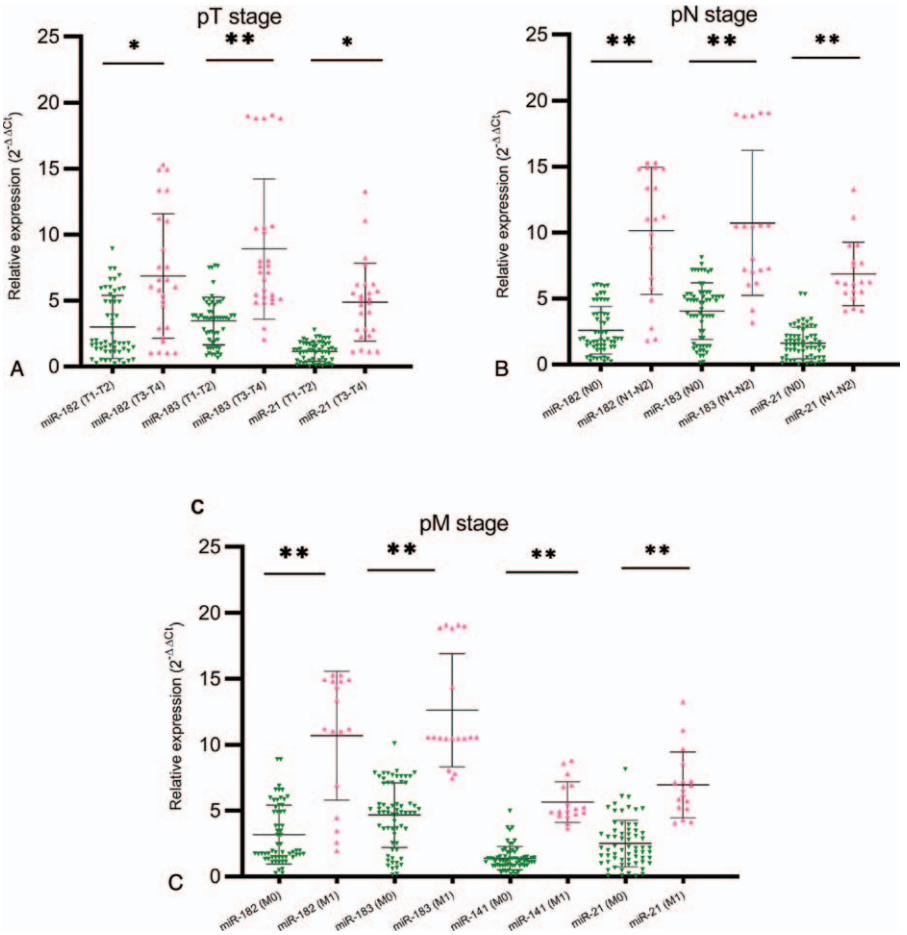

Figure 2. Fold change of the 4 miRNAs genes in Romanian patients with CRC at different clinical stages. The dot plots (using $2^{-\Delta\Delta Ct}$ method) represent mean ± standard deviation of miR-182, miR-183, miR-141, and miR-21 according to the depth of tumor invasion - pT-stage (Fig. A), nodal 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.

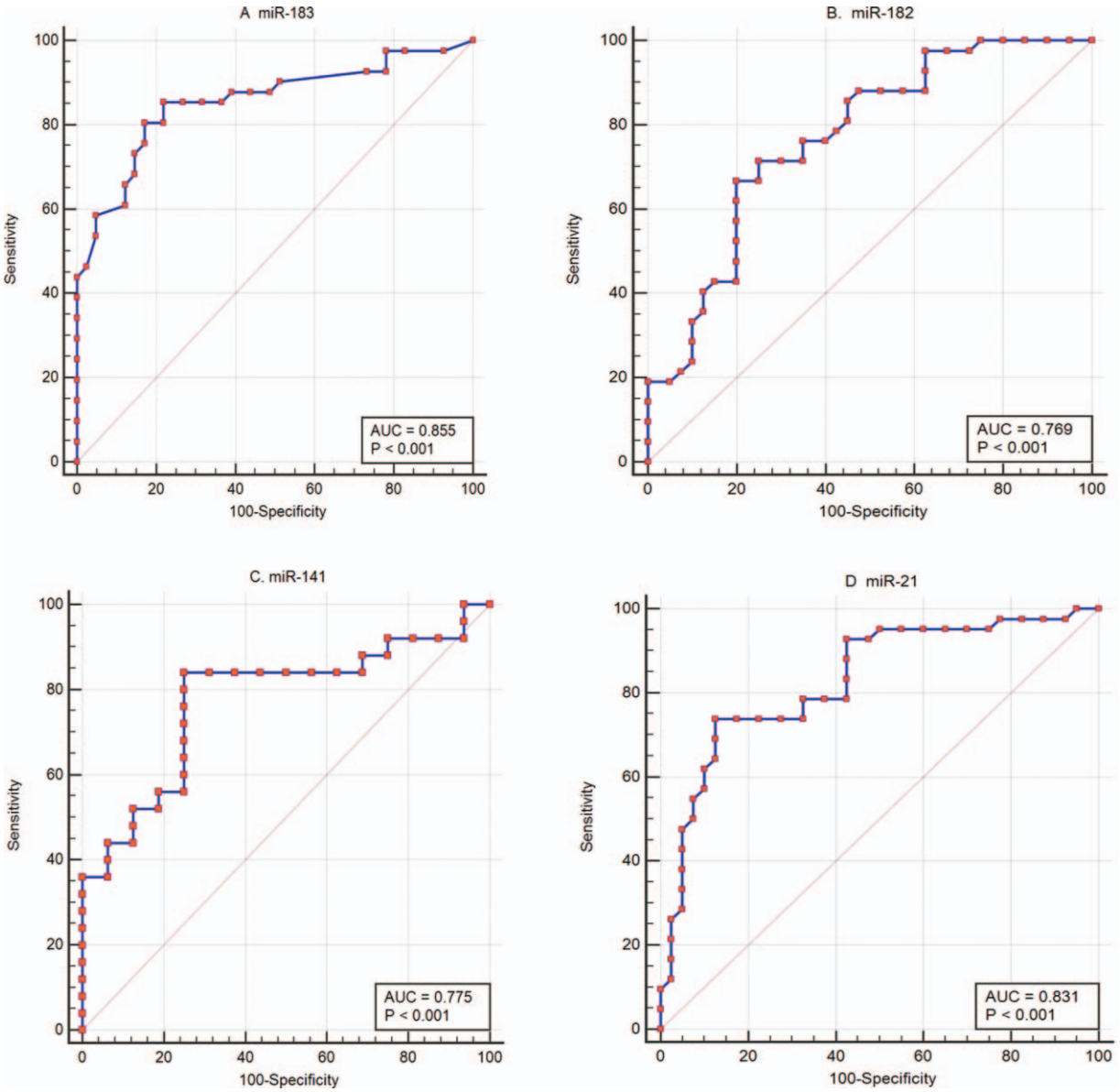

Figure 3. The miR-182, miR-183, miR-141, and miR-21 expressions levels as potential biomarkers in CRC diagnosis. Receiver operating curve (ROC) analyses were generated from 82 patients had a value of the area under the curve (AUC) for miR-183 of 0.85 (sensitivity: 80.95%, specificity: 85%; $P < .001$),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