The role of miRNA in colorectal cancer diagnosis: A pilot study

MAŠA ŽDRALEVIĆ[1,7], JANJA RAONIĆ[1,2], NATASA POPOVIC[1], LJILJANA VUČKOVIĆ[1,2], ISIDORA ROVČANIN DRAGOVIĆ[1], BATRIĆ VUKČEVIĆ[1,3], VLADIMIR TODOROVIĆ[1,4], FILIP VUKMIROVIĆ[1,2], FLAVIANA MARZANO[5], APOLLONIA TULLO[5], NICOLETTA GUARAGNELLA[6], SERGIO GIANNATTASIO[5] and MIODRAG RADUNOVIĆ[1,3]

[1]Faculty of Medicine, University of Montenegro; [2]Center for Pathology; [3]Center for Digestive Surgery; [4]Institute for Oncology, Clinical Center of Montenegro, 81000 Podgorica, Montenegro; [5]Institute for Biomembranes, Bioenergetics and Molecular Biotechnologies, National Research Council, I-70126 Bari; [6]Department of Biosciences, Biotechnologies and Environment, University of Bari 'Aldo Moro', I-70126 Bari, Italy

Received February 2, 2023; Accepted April 26, 2023

DOI: 10.3892/ol.2023.13853

Abstract. Despite recent advances in diagnosis and treatment, colorectal cancer (CRC) remains the third most common cancer worldwide, and has both a poor prognosis and a high recurrence rate, thus indicating the need for new, sensitive and specific biomarkers. MicroRNAs (miRNAs/miRs) are important regulators of gene expression, which are involved in numerous biological processes implicated in tumorigenesis. The objective of the present study was to investigate the expression of miRNAs in plasma and tissue samples from patients with CRC, and to examine their potential as CRC biomarkers. Using reverse transcription-quantitative PCR, it was revealed that miR-29a, miR-101, miR-125b, miR-146a and miR-155 were dysregulated in the formalin-fixed paraffin-embedded tissues of patients with CRC, compared with the surrounding healthy tissue, and these miRNAs were associated with several pathological features of the tumor. Bioinformatics analysis of overlapping target genes identified AGE-RAGE signaling as a putative joint regulatory pathway. miR-146a was also upregulated in the plasma of patients with CRC, compared with the healthy control group, and had a fair discriminatory power (area under the curve, 0.7006), with 66.7% sensitivity and 77.8% specificity. To the best of our knowledge, this distinct five-miRNA deregulation pattern in tumor tissue, and upregulation of plasma miR-146a, were shown for the first time in patients with CRC; however, studies on larger patient cohorts are warranted to confirm their potential to be used as CRC diagnostic biomarkers.

Correspondence to: Dr Maša Ždralević, [7]*Present address:* Institute for Advanced Studies, University of Montenegro, Cetinjska 2, 81000 Podgorica, Montenegro
E-mail: masaz@ucg.ac.me

Key words: colorectal cancer, miRNA, plasma, FFPE, biomarkers, miR-146a, liquid biopsy

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer globally (10.0%), and the second most important cause of cancer death (9.4%). More than 1.9 million new CRC cases and 935,000 deaths were estimated to occur in 2020 (1). The overall incidence increased in most European countries over the last decade, especially in low- and middle-income countries (2). It is therefore clear that new and improved treatment options, prevention, and early detection are essential. The most important prognostic factors of CRC known today are the anatomical spread of the disease (determined by TNM classification), and the status of resection margins. However, it has been shown that using solely anatomical spread is an insufficient prognostic factor (3). As a result, a considerable amount of research has been done at the molecular level, and four consensus molecular subtypes (CMS) of CRC were defined (4), with distinct molecular and biological characteristics. CMS classification has led to better understanding of CRC heterogeneity, progression, and response to treatment (5). However, this has not significantly impacted CRC timely diagnosis and prognosis. Fecal occult blood test and colonoscopy are the two main screening tools currently in use, but with suboptimal efficacy, in terms of insufficient diagnostic accuracy, and invasiveness and high costs, respectively. This further supports the need for new, sensitive, and specific diagnostic and prognostic biomarkers, especially considering that more than half of patients with CRC have metastases at the time of diagnosis, and the average survival time of patients with metastases is 24 months (6). In metastatic disease, *KRAS* mutations status is an indispensable biomarker used to select patients who are good candidates for anti-EGFR treatment (7). *KRAS* mutations are present in approximately 50% of patients with metastatic disease and are related to anti-EGFR biological therapy resistance (8).

Research on microRNAs (miRNAs) as new reliable biomarkers of tumor development and progression is emerging. MiRNAs are short, non-coding RNA sequences,

21-25 nucleotides in length that negatively regulate the expression of protein-coding genes (9). MiRNAs inhibit their targets by acting as translational inhibitors of gene expression or by degrading mRNA transcripts (10). The expression levels of most protein coding genes in human cells are subject to some degree of regulation by miRNAs, meaning that miRNAs influence nearly all the developmental processes and diseases in metazoans (11). Deregulation of miRNAs is involved in the initiation and progression of cancer, and aberrant miRNA expression is found in most, if not all cancers (12). It has been shown that miRNA profiling in patients can be used to classify tumors with accuracy and predict outcome (13). Functional studies revealed that miRNAs can act as both oncogenes and tumor suppressors (14); therefore, understanding miRNAs expression levels in cancer patients could provide clues about the affected molecular pathways and help identification of new targets for treatment. MiRNAs high stability in biological fluids, easy extraction and quantification, proven sensitivity and specificity, makes them particularly suitable for biomarkers research (15).

In CRC, a current literature review identified over 230 potential candidate miRNAs in various biological fluids, with some level of association to the pathology (16). However, more detailed mechanistic studies on the significance of these associations are lacking for most deregulated miRNAs. Thus, in order to give further insights into the use of miRNA for diagnosis and treatment of CRC we conducted a pilot study on small, but well-characterized patient cohort in Montenegro for which both FFPE tissue and blood samples were available. We selected a panel of five candidate miRNAs, miR-29a, miR-101, miR-125b, miR-146a, and miR-155, known to be involved in all essential cancer molecular and cellular pathways, with both tumor suppressor and oncogenic roles. More precisely, quite complex gene networks regulated by these miRNAs were shown to be involved in proliferation, pro-inflammatory signaling, apoptosis, angiogenesis, invasion, metastasis, and drug resistance (17-20). Deregulation of these miRNAs with various levels of correlation with clinical parameters has already been shown in other populations (21,22), however, these findings were often inconsistent and even contradictory. Population differences in miRNA expression were shown to have significant biological and pharmacological implications (23). In addition, miRNAs expression is also influenced by external factors, such as physical activity and lifestyle (24). All of the previous statements prompted us to perform this pilot study and analyze expression levels of the five selected miRNAs in Montenegrin patients for the first time. We assessed this panel of miRNAs in both pre-operative plasma and FFPE tissue samples of patients with CRC and healthy control subjects and correlated the results with demographic, clinical and pathological features of the study participants. This experimental setup enabled us to detect whether any of the studied circulatory miRNAs potentially originated from the tumor. We also performed bioinformatics analysis of the putative target genes of these miRNAs, and their joint regulatory pathways.

Materials and methods

Patient recruitment and study design. The study protocol was approved by the Ethical Committee of the Clinical Center of Montenegro (approval no. 03/01-11417/1) and by the Committee for Medical Ethics and Bioethics of the Faculty of Medicine of the University of Montenegro (approval no. 3824/4). All the procedures were conducted in accordance with the Declaration of Helsinki and all participants signed an informed consent before any study procedures were performed. All participants completed a standardized questionnaire, in order to obtain demographic (age, gender), and basic health information, including eventual comorbidities and frequency of habits associated with an increased risk for CRC (BMI, hypertension, hyperlipidemia, diabetes mellitus, history of smoking, coffee and alcohol consumption, and physical activity). Peripheral blood samples were also collected from all participants.

CRC patients were recruited at the Center for Digestive Surgery of the Clinical Center of Montenegro, between November 2019 and November 2021. All patients were subjected to standard clinical practice. Peripheral blood sampling was performed during preoperative preparation on 24 patients who were admitted for surgical resection of colon tumors previously diagnosed by colonoscopy. Patients who received preoperative adjuvant therapy, those who have clinically diagnosed hereditary adenomatous polyposis or hereditary non-polyposis CRC, with a previous medical history of malignancy, poorly controlled systemic disease, and/or current acute disease, were not included in the study. In addition, paired CRC and surrounding normal colon tissue samples were obtained from CRC patients during the surgical treatment.

Healthy volunteers were recruited at the Faculty of Medicine, University of Montenegro. We have recruited 34 age- and sex-matched healthy volunteers. Inclusion criteria for the healthy group of participants were negative history of cancer, uncontrolled chronic systemic disease, including inflammatory bowel disease (IBD), and/or current acute disease.

Plasma samples processing and miRNA quantification by reverse transcription-quantitative PCR (RT-qPCR). MicroRNAs were extracted from plasma using Qiagen miRNeasy Serum/Plasma Advanced kit (cat. No. 217204, Qiagen, Hilden, Germany) essentially as described in (25). Briefly, peripheral venous blood samples were collected in the EDTA-containing Vacutainer tubes, kept on ice, and processed within 1 h from the collection. Plasma was separated from the whole blood by centrifugation at 1,900 x g for 10 min at 4˚C, followed by an additional centrifugation step at 3,000 x g for 15 min at 4˚C. All samples were aliquoted and stored at -80˚C until further analysis.

The miRNA concentration was determined using Qubit microRNA Assay kit (Q32880, Invitrogen, Thermo Fisher Scientific) on a Qubit 3.0 fluorimeter (Q33216, Invitrogen, Thermo Fisher Scientific, USA). Two μl miRNA from each sample were reversely transcribed to cDNA using TaqMan Advanced miRNA cDNA Synthesis kit (A28007, Applied Biosystems, USA) and analyzed with TaqMan Advanced microRNA Assays (A25576, Applied Biosystems, USA) for miR-29a, miR-101, miR-125b, miR-146a, and miR-155. Context sequences of TaqMan probes were as follows: ACUGAUUUC UUUUGGUGUUCAG for miR-29a; CAGUUAUCACAG UGCUGAUGCU for miR-101; UCCCUGAGACCCUAACUU

GUGA for miR-125b; UGAGAACUGAAUUCCAUGGGUU for miR-146a; and UUAAUGCUAAUCGUGAUAGGGGUU for miR-155. RT-qPCR was run on an Applied Biosystems 7300 Real Time PCR system (Applied Biosystems, USA), with the following thermocycling conditions: enzyme activation: 95˚C, 20 sec; denaturation: 95˚C, 3 sec; annealing: 60˚C, 30 sec, for 40 cycles. The expression levels of target miRNAs were normalized by using the mean expression levels of miR-361-5p gene for plasma samples, and miR-186-5p for FFPE tissue samples, selected as the most stable internal control miRNA by the NormFinder algorithm (26). These miRNAs are recommended as endogenous controls due to their relatively constant expression levels across many different sample types. Expression of every target gene was calculated using the $2^{-\Delta\Delta Cq}$ method (27). Every sample was retrotranscribed twice and run in triplicate each time.

FFPE tissue sample processing and pathohistological analysis. Biopsy samples were fixed in 10% buffered formalin and embedded in paraffin. Serial sections, 5 μm thick, were cut using microtome (Leica SM 200R, Austria). After deparaffinization in xylene and hydration in descending order of alcohol, sections were stained with Mayer's hematoxylin and 1% eosin solution, then illuminated and mounted on slides using dibutylphtalate polystyrene xylene (DPX).

Morphological analysis and reporting were conducted by two independent pathologists using CAP protocols (Cancer Reporting Protocols-College of American Pathologists). For each sample tumor site and size, histological type and grade, tumor extent, presence of lymphovascular and perineural invasion, necrosis, mucus production, inflammatory infiltrate density and composition, status of margins, lymph nodes status, and disease stage were estimated. Representative FFPE tissue samples were selected for miRNA and DNA extraction. Representative image of hematoxylin and eosin stained FFPE tissue sample of the patient with CRC is given in Fig. S1A. Red square in the image denotes the area of the tumor, while the rest is healthy colonic mucosa. FFPE tissue samples chosen for miRNA and DNA extraction were selected by pathologists, so that FFPE tissue sections containing no less than 80% of tumor cells were chosen for the analysis as tumor samples, and healthy surrounding tissue of the same patient was used as a control sample (Fig. S1B).

miRNA extraction from FFPE tissues. MiRNA purification from FFPE tissues was performed using miRNeasy FFPE kit (cat. no. 217504, Qiagen, Hilden, Germany), according to the manufacturer's instructions. Two to three 10 μm inner sections were aseptically collected in nuclease-free microcentrifuge tube and deparaffinization solution (cat. no. 19093) was used to remove all paraffin. The hematoxylin and eosin-stained slides were evaluated by pathologist who designated control and tumor samples, ensuring no cancer cells were present in the controls (Fig. S1B).

DNA extraction from FFPE tissues and KRAS mutation analysis. DNA extraction from the FFPE tissue samples was performed using QIAmp DNA FFPE Tissue kit (cat. No. 56404) from Qiagen. Two 10 μm sections of FFPE tissue samples were cut, treated with deparaffinization solution (cat. no. 19093), and DNA was extracted following the manufacturer's instructions. *KRAS* gene mutations were analyzed using RealLine KRAS Detect kit (REF MED20401) (Bioron diagnostics GmbH). Seven mutations of codons 12 and 13 of the *KRAS* gene that are associated with resistance to anti-EGFR therapy were detected with allele specific qPCR.

Target prediction and enrichment analysis. Potential targets of five studied miRNAs were predicted by miRTarBase database (https://mirtarbase.cuhk.edu.cn) (28), and only targets with strong evidence (reporter assay, Western blot, and qPCR) were selected for further analysis. Subsequently, only genes targeted by more than one miRNA were selected and Gene Ontology (GO) functional annotation and KEGG pathway analyses were made using the STRING online resource (https://string-db.org/) (29).

Statistical analysis. All statistical analyses were performed using GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA). The results were considered statistically significant when $P<0.05$. Continuous variables were first tested for normality of distribution by D'Agostino-Pearson and Shapiro-Wilk tests and analyzed with the unpaired t-test or an appropriate non-parametric test (Mann-Whitney). Normally distributed variables of tumor FFPE tissue samples were analyzed with the paired Student's t-test. Categorical variables were analyzed with the Fisher's exact test. Receiver operating characteristic (ROC) curve analysis was performed to evaluate potential diagnostic performance of studied miRNAs. The area under the curve (AUC) was estimated, along with the 95% CI. Correlations between miRNA expression and clinical variables were explored using the Spearman correlation coefficient. Logistic regression analysis was also performed to evaluate the association of the investigated variables with the CRC.

Results

Patient characterization. Out of a total of 24 CRC patients who were initially recruited for this prospective study, six were excluded due to postoperative pathohistological diagnosis of adenoma (n=3), ulcerative colitis (n=1), and two due to sample hemolysis (Fig. 1B), thus 18 plasma samples in total were further analyzed. From the group of 34 healthy volunteers recruited, 18 were further analyzed, as presented in the scheme of the recruitment in the Fig. 1A. Demographic and clinical characteristics of study participants are given in Table I.

There were no significant differences in prevalence of either gender. The range of ages of the participants enrolled in our study is given in the Table I (55.0-87.0 for patients with CRC, and 55.0-77.0 for healthy controls). The median age of patients with CRC was 66 years, and for healthy control subjects it was 65, and this difference was not statistically significant. Body mass index (BMI) was within the overweight range (>25-30) for both groups, with no statistically significant difference between the two. Hypertension was the most prevalent comorbidity in both CRC patients and healthy group, followed by hyperlipidemia and diabetes mellitus. However, their prevalence did not differ significantly among the two groups (Table I). The same applied for physical activity, history of smoking, and alcohol consumption, whereas coffee consumption was significantly

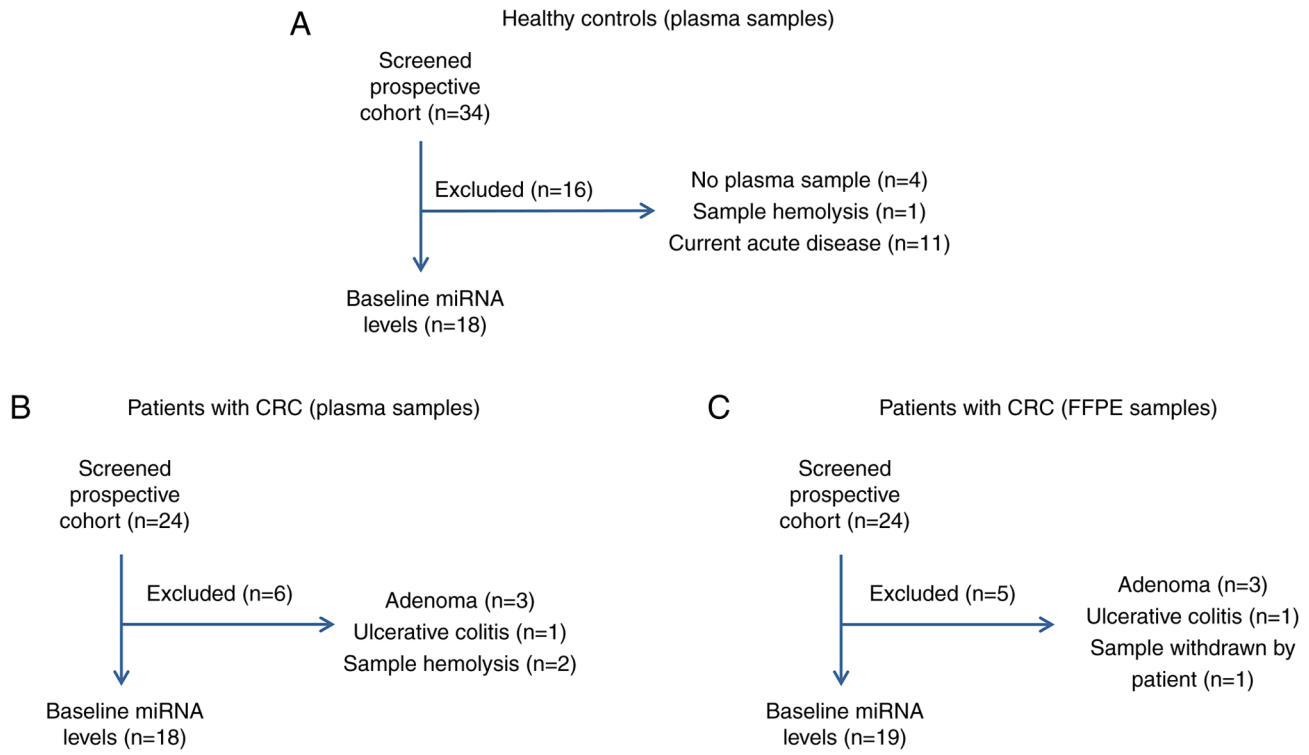

Figure 1. Flow chart of study participant recruitment. Number of screened, excluded and analyzed (A) healthy participants, (B) patients with CRC (with plasma samples) and (C) patients with CRC (with FFPE samples). CRC, colorectal cancer; FFPE, formalin-fixed paraffin-embedded; miRNA, microRNA.

higher in the healthy group, since none of the patients with CRC consumed 3 or more cups of coffee daily. Therefore, patients with CRC and healthy participants proved to have a homogeneous distribution of the demographic and clinical variables included in the questionnaire.

miR-146a is elevated in the plasma of patients with CRC. In order to assess their role in the development of CRC, relative expression of five selected miRNAs: miR-29a, miR-101, miR-125b, miR-146a, and miR-155, was analyzed in plasma samples of patients with CRC and healthy individuals. No statistically significant differences were observed between groups for miR-29a, miR-101, miR-125b, and miR-155 (data not shown). However, expression levels of miR-146a were significantly higher (P=0.0402) in the plasma of patients with CRC, compared to healthy individuals, as shown in Fig. 2A.

miR-146a is one of the most important inflammatory miRNAs, shown to mediate both the innate and adaptive immune response (30). In order to assess the diagnostic potential of miR-146a, we performed ROC analysis (Fig. 2B), which showed AUC of 0,7006 (CI 95%, 0.5252-0.8761), with 66.7% sensitivity and 77.8% specificity at a cutoff value of 1.114.

Correlation of plasma miRNA levels with the demographic and basic health features of the participants. The associations between all five investigated miRNAs levels and patient characteristics are presented in the Table SI. Correlation between expression levels of miR-29a, miR-101, miR-125b, miR-146a, and miR-155, and the following variables was investigated: gender, age, BMI, hypertension, hyperlipidemia, diabetes mellitus, physical activity, history of smoking, and alcohol consumption.

Only two associations were found to be statistically significant, namely, miR-146a expression was significantly higher in male in comparison to female CRC patients (P=0.0441); and lower levels of miR-101 expression were found in patients with a history of smoking (P=0.0307) (Table SI). Given that miR-146a levels were significantly up-regulated in the plasma of CRC patients, we performed a logistic regression analysis on all participants using miR-146a expression, and clinical parameters studied, but the results were not significant (data not shown). This is probably due to both the small sample size and the homogeneity of the clinical parameters investigated between the patients with CRC and healthy participants.

miRNAs deregulated in tissue samples and their correlation with pathological features. Clinical characteristics of the CRC group of patients are given in Table II. After one participant withdrew their tissue samples, there were 19 patients with CRC who were recruited (Fig. 1C).

A majority of patients (89.5%) were TNM stage III; 57.9% had positive nodal status, and tumors were predominantly located in the right colon (68.4%). *KRAS* mutation status was analyzed in all patients with lymph nodes metastasis (n=11), and five of those patients (45.45%) had *KRAS* mutations. The expression levels of miR-29a, miR-101, miR-125b, miR-146a, and miR-155 were analyzed in FFPE tissue samples from the patients with CRC by RT-qPCR and correlated with pathological features, in order to assess their clinical relevance. As shown in the Fig. 3A-E, miR-29a (P<0.0001), miR-125b (P=0.0141), miR-146a (P=0.0068), and miR-155 (P=0.0130) were up-regulated in the tumor tissue, whereas miR-101 was found to be down-regulated (P=0.0007) in the tumor, with respect to the surrounding healthy colon tissue.

Table I. Demographic and clinical characteristics of the study cohorts (plasma samples).

Characteristic	Colon cancer (n=18)	Healthy controls (n=18)	P-value
Sex			>0.999
Female (%)	7 (38.9)	7 (38.9)	
Male (%)	11 (61.1)	11 (61.1)	
Age at diagnosis, years			
Mean ± SD	66.89±9.64	65.44±8.12	0.599
Median (range)	66.0 (55.0-87.0)	65.0 (55.0-77.0)	
Mean ± SD BMI, kg/m^2	26.88±3.67	27.17±3.96	0.7842
Hypertension (%)	9 (50)	8 (44.4)	>0.999
Hyperlipidemia (%)	2 (11.1)	7 (38.9)	0.1212
Diabetes mellitus (%)	3 (16.7)	3 (16.7)	>0.999
Physical activity (%)	16 (88.9)	11 (61.1)	0.1212
History of smoking (%)	10 (55.6)	10 (55.6)	>0.999
Coffee consumption (%)	0 (0)	7 (38.9)	0.0076[a]
Alcohol consumption (%)	5 (27.8)	9 (50)	0.3053

SD, standard deviation; Physical activity, walking ≥30 min at least 5 days per week; History of smoking, current or former smokers; Coffee consumption, consumption of 3 or more cups daily. [a]P<0.05.

Table II. Demographic and clinical characteristics of the study cohort (FFPE samples).

Characteristic	Colon cancer (n=19)
Sex	
Female (%)	7 (36.8)
Male (%)	12 (63.2)
Age at diagnosis	
Mean ± SD	66.84±9.37
Median (range)	66.0 (55.0-87.0)
TNM stage	
I (%)	0 (0)
II (%)	1 (5.3)
III (%)	17 (89.5)
IV (%)	1 (5.3)
Nodal status	
Positive (%)	11 (57.9)
Negative (%)	8 (42.1)
Tumor location	
Left (%)	6 (31.6)
Right (%)	13 (68.4)
KRAS mutation status[a]	
Positive (%)	5 (45.45%)
Negative (%)	6 (54.54%)

[a]Patients with lymph node metastasis.

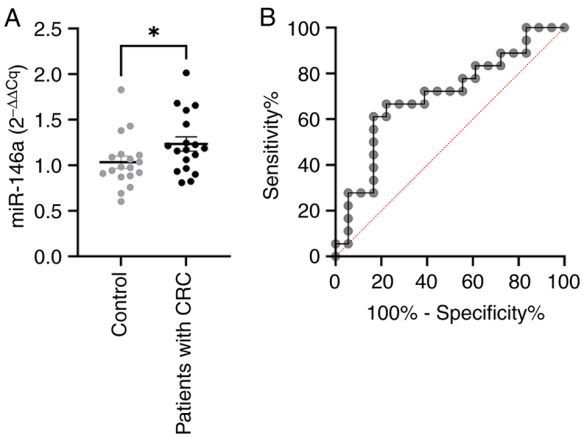

Figure 2. (A) Relative expression and (B) ROC curve of circulatory miR-146a in patients with CRC and healthy control subjects. *P=0.0402. CRC, colorectal cancer; miR, microRNA.

Diagnostic performance of the investigated miRNAs for CRC was evaluated by computing AUC values of the ROC curves for each miRNA. The results presented in the Table III show that miR-29a had highest AUC value (0.8921), with 84.21% sensitivity and 89.47% specificity, while both miR-101 and miR-146a had AUC levels above 0.75.

To investigate whether there is any correlation between CRC tissue and plasma levels, we performed an analysis for each individual miRNA, but no significant correlation was found (data not shown). We have also compared miRNA expression levels in the FFPE samples of the healthy surrounding tissue with miRNA expression levels in CRC plasma samples, and we did not find any significant correlation.

Next, we analyzed miRNA expression levels in correlation with clinical and pathological features of patients with CRC (Table SII). In particular, we compared expression levels of all five investigated miRNAs and the following characteristics: gender, age, histological grade, nodal status, tumor localization, *KRAS* mutation status, number of intratumoral lymphocytes,

Table III. Diagnostic performance of all investigated miRNAs in CRC tissue samples.

FFPE miRNA	AUC	Cutoff point	Sensitivity, %	Specificity, %
miR-29a	0.8921	>1.582	84.21	89.47
miR-101	0.8172	<0.7511	73.68	73.68
miR-125b	0.7313	>1.421	68.42	78.95
miR-146a	0.7535	>1.683	63.16	94.74
miR-155	0.7341	>1.347	73.68	89.47

AUC, area under the receiver operating characteristic curve.

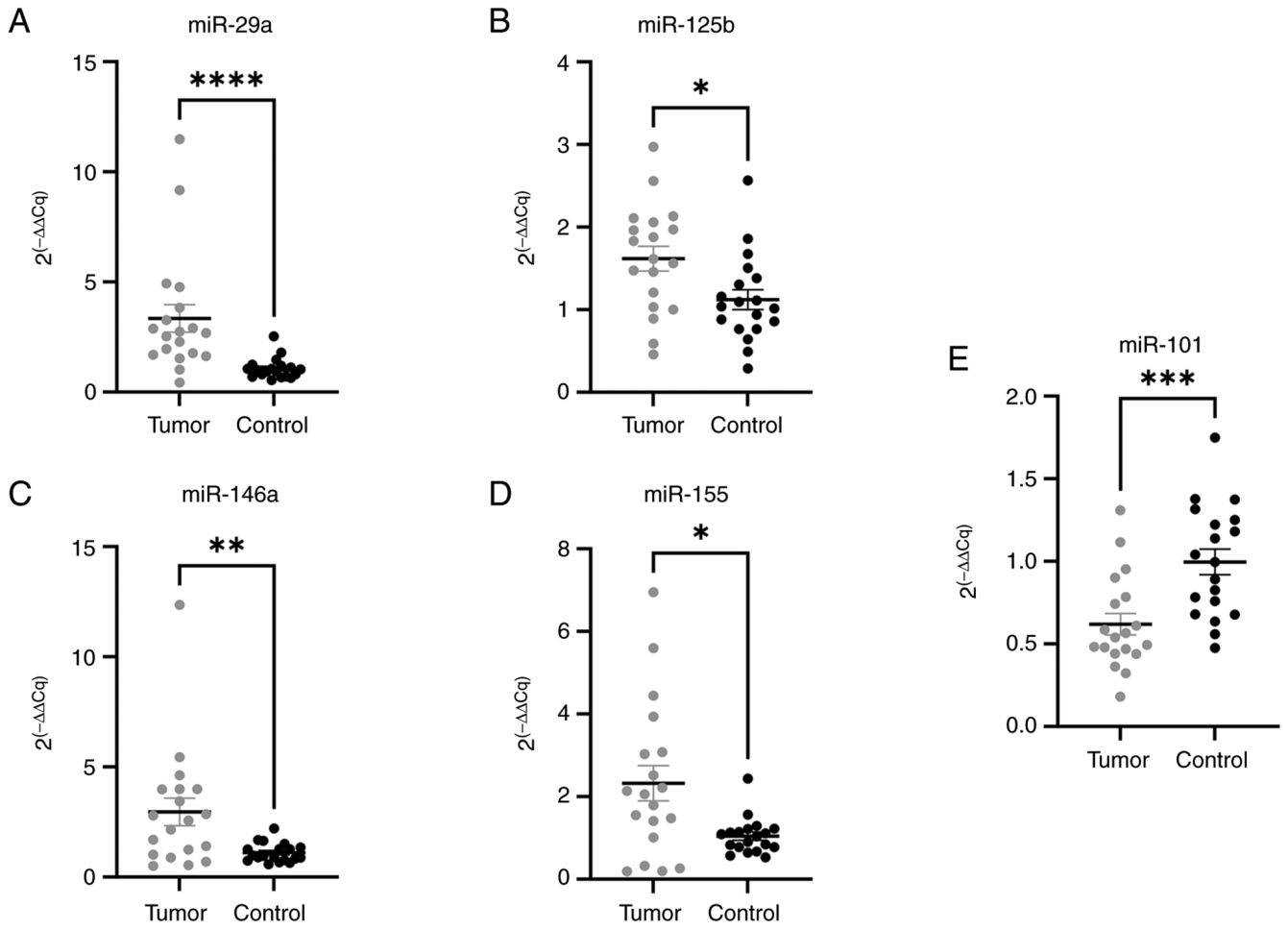

Figure 3. miRNA expression is deregulated in FFPE tissues of CRC patients. Relative expression of (A) miR-29a, (B) miR-125b, (C) miR-146a, (D) miR-155 and (E) miR-101. *P<0.015, **P=0.0068, ***P=0.0007, ****P<0.0001. CRC, colorectal cancer; FFPE, formalin-fixed paraffin-embedded; miR, microRNA.

number of lymphocytes around tumor, tumor stroma quantity, quantity and composition of inflammatory infiltrate, necrosis, mucus, lympho-vascular invasion, and lymph node metastases.

We found that there is a negative correlation between miR-101 levels in tumor tissue and the lymphovascular invasion (r=-0.4901, P=0.0389). We have also analyzed each individual miRNA in two different groups for all investigated demographic and clinical variables, as shown in the Table SI. Significant differences were found for miR-146a, whose expression was significantly higher (P=0.0435) in FFPE CRC tissue samples with higher degree of necrosis; higher expression of miR-155 (P=0.0339) was found in samples with no production of mucus; higher miR-125b expression (P=0.0103) was found in samples without lympho-vascular invasion, and lower miR-101 expression (P=0.0202) was found in samples with positive nodal status. No significant differences were found in expression values of any of the studied miRNA with respect to the *KRAS* mutation status (Table SII).

Putative joint regulatory pathways identified by bioinformatics analysis. In order to better understand the consequences

of this identified five-miRNA deregulation, we performed a bioinformatic analysis on the genes regulated by miR-29a, miR-101, miR-125b, miR-146a, and miR-155. A total of 174 genes were found to be regulated by at least two out of five studied miRNAs (Table SIII), and the most strongly enriched GO processes and KEGG pathways analyzed by the STRING database (31) are presented in the Table IV.

The enrichment analysis revealed that the targets of candidate miRNAs were involved in pathways of several different cancers, including pancreatic, bladder, chronic myeloid leukemia, melanoma, and NSCLC, but also in EGFR tyrosine kinase inhibitor resistance and interestingly enough, the AGE-RAGE signaling pathway (Fig. 4).

Binding of advanced glycation end products (AGEs) to their receptors (RAGEs) activate several different signaling pathways, such as MAPK, p53, PI3K/Akt/mTOR, JAK-STAT, and NF-κB, involved in the proliferation of cancer cells, angiogenesis, and invasion (32), contributing to the progression of cancer.

Discussion

The present pilot study determined the pattern of miRNAs deregulation in CRC patients in Montenegro for the first time, with the aim of contributing new information to the current knowledge of which specific miRNA signature could be used in clinical setting. We have shown that all five of the investigated candidate miRNAs were deregulated in FFPE tissue samples of patients with CRC, compared to the healthy surrounding colonic mucosa (Fig. 3A-E), however, only miR-146a levels were found to be up-regulated in both FFPE tissue and plasma samples of patients with CRC (Figs. 2A and 3C). This finding suggests that circulatory miR-146a probably originate from the tumor, further emphasizing its potential role as a diagnostic biomarker. The discriminatory potential of miR-146a in the plasma was shown to be modest (AUC=0.7006), with a sensitivity and specificity of 66.7 and 77.8%, respectively, at a cutoff value of 1.114. An ideal screening tool should be highly sensitive and specific, safe, affordable, and minimally invasive. The RT-qPCR methodology we used is not expensive, requires venous blood sampling, which is minimally invasive, and is easily repeatable. Our results on deregulation of candidate miRNAs are in agreement with previous studies on their diagnostic ability (21,22,33,34), and the roles of each studied miRNA in CRC, together with previous findings on clinical significance of their differential expression in different populations in comparison to those presented in this study are discussed.

Our bioinformatic analysis identified the AGE-RAGE signaling pathway as one of the putative jointly affected pathways by all five of the investigated miRNAs. AGEs are predominantly formed as a result of chronic hyperglycemic conditions/diabetes or aging (35). The binding of AGEs and activation of their receptor RAGE triggers upregulation of ROS production, and activation of several signaling cascades via phosphatidylinositol-3 kinase (PI3K), MAPK and K-Ras to activate NF-κB (36), ultimately leading to the exacerbation of inflammation and cellular damage (32).

miR-146a is one of the most prominent inflammatory miRNAs, with a key modulatory role both in the innate and adaptive immune response (30). Notably, miR-146a was found to be a NF-κB-dependent gene, whose direct molecular targets are tumor necrosis factor receptor-associated factor 6 (TRAF6), and interleukin 1 receptor associated kinase (IRAK1), the two main adapter proteins downstream of Toll-like receptors (TLR) and cytokine receptors (37), crucial for pro-inflammatory signaling. In this model, miR-146a was proposed to act as a negative feedback regulator of the innate immune response. High miR-146a levels found both in tissue and plasma samples of our cohort of patients with CRC are in line with the role of miR-146a as a suppressor of the immune response. Furthermore, miR-146a is a NF-κB-dependent gene, and NF-κB activation is triggered by the AGE-RAGE signaling pathway, which is one of the jointly regulated pathways of the five miRNAs investigated in this study. The anti-inflammatory and anti-tumorigenic roles of miR-146a in CRC were also demonstrated to occur via modulation of IL-17 signaling *in vivo* (38). miR-146a was shown to have both a tumor suppressive (39), and oncogenic role (40). Finally, miR-146a polymorphisms were shown to be associated with susceptibility to CRC (41). Conflicting findings on the role of miR-146a in not only CRC, but all malignancies, warrants the need for further investigation on its exact function, correlation to clinical parameters and therapeutic potential.

Serum miR-146a was shown to have a significant diagnostic ability in CRC, as a member of a three-miRNA panel, together with miR-30e-3p, and miR-148a-3p (22). A comprehensive meta-analysis regarding the prognostic utility of its expression levels was performed by Li *et al*, who showed that higher miR-146a expression correlated with better survival in solid cancers, especially of the digestive system (42). Our finding of elevated miR-146a expression both in tissue and plasma samples of CRC patients is in agreement with these previous findings. Population-specific genetic variation of miRNA expression and/or function (23) might have also influenced miR-146a levels in the Montenegrin population and should be evaluated. We will continue to monitor our study group over time, to evaluate whether miR-146a plays a role in delaying tumor progression and prolonging overall survival.

miR-29a expression was found to be up-regulated in FFPE tissue samples of the patients with CRC (Fig. 3A), which is in accordance with several studies reporting significantly increased miR-29a expression levels in CRC malignant tissue, plasma, and serum samples (21,43). miR-29a was found to promote both inflammation and CRC development (44), but also, contrary to these findings, to inhibit *in vivo* tumor growth (45). A recent review by Mo and Cao highlights this discrepancy regarding the role of miR-29a in CRC and gives an excellent overview on tissue, circulatory, fecal, and salivary miR-29a as a biomarker for CRC, as well as on the underlying molecular mechanisms of miR-29a, and its role in chemoradiotherapy resistance (17). Taken together, the clinical significance of miR-29a expression levels is still debatable. Conflicting findings could be attributed to the inadequate sample size or analytical procedures used (17). Therefore, more research is needed in order to clarify its exact role, underlying mechanisms, and clinical utility.

We found significantly lower miR-101 expression in FFPE tissue samples of patients with CRC, compared to the normal

Table IV. The most strongly enriched GO processes and KEGG pathways-joint analysis.

A, Biological process (Gene Ontology)

Term or pathway	Description	Count in network	Strength	False discovery rate
GO:0006211	5-methylcytosine catabolic process	3 of 3	2.05	0.00019
GO:1905460	Negative regulation of vascular associated smooth muscle cell apoptotic process	2 of 2	2.05	0.0044
GO:1905075	Positive regulation of tight junction disassembly	2 of 2	2.05	0.0044
GO:1904466	Positive regulation of matrix metallopeptidase secretion	2 of 2	2.05	0.0044
GO:0035622	Intrahepatic bile duct development	2 of 2	2.05	0.0044
GO:0032707	Negative regulation of interleukin-23 production	2 of 2	2.05	0.0044
GO:0014740	Negative regulation of muscle hyperplasia	2 of 2	2.05	0.0044
GO:0003169	Coronary vein morphogenesis	2 of 2	2.05	0.0044
GO:0002384	Hepatic immune response	2 of 2	2.05	0.0044
GO:0061419	Positive regulation of transcription from RNA polymerase II promoter in response to hypoxia	4 of 6	1.87	$2.15x10^{-5}$

B, Molecular function (Gene Ontology)

Term or pathway	Description	Count in network	Strength	False discovery rate
GO:0004517	Nitric-oxide synthase activity	3 of 3	2.05	0.00062
GO:0003886	DNA (cytosine-5-)-methyltransferase activity	3 of 3	2.05	0.00062
GO:0070579	Methylcytosine dioxygenase activity	3 of 4	1.93	0.00098
GO:0034617	Tetrahydrobiopterin binding	3 of 4	1.93	0.00098
GO:0043125	Erb-3 class receptor binding	3 of 4	1.93	0.00098
GO:0003958	NADPH-hemoprotein reductase activity	3 of 6	1.75	0.0021
GO:003958	Type III transforming growth factor beta receptor binding	2 of 4	1.75	0.0282
GO:0070878	Primary miRNA binding	4 of 10	1.65	0.00030
GO:0051525	NFAT protein binding	2 of 5	1.65	0.0375
GO:0046870	Cadmium ion binding	2 of 5	1.65	0.0375

C, KEGG pathways

Term or pathway	Description	Count in network	Strength	False discovery rate
hsa05212	Pancreatic cancer	26 of 73	1.6	$1.37x10^{-29}$
hsa05219	Bladder cancer	13 of 41	1.55	$7.98x10^{-15}$
hsa04933	AGE-RAGE signaling pathway in diabetic complications	28 of 98	1.51	$1.37x10^{-29}$
hsa05220	Chronic myeloid leukemia	20 of 75	1.48	$5.34x\ 0^{-21}$
hsa05218	Melanoma	19 of 72	1.47	$5.88x10^{-20}$
hsa05223	Non-small cell lung cancer	17 of 68	1.45	$1.09x10^{-17}$
hsa01521	EGFR tyrosine kinase inhibitorresistance	19 of 78	1.44	$1.80x10^{-19}$
hsa05213	Endometrial cancer	14 of 57	1.44	$1.22x10^{-14}$
hsa05214	Glioma	17 of 72	1.42	$2.29x10^{-17}$
hsa05222	Small cell lung cancer	21 of 92	1.41	$7.11x10^{-21}$

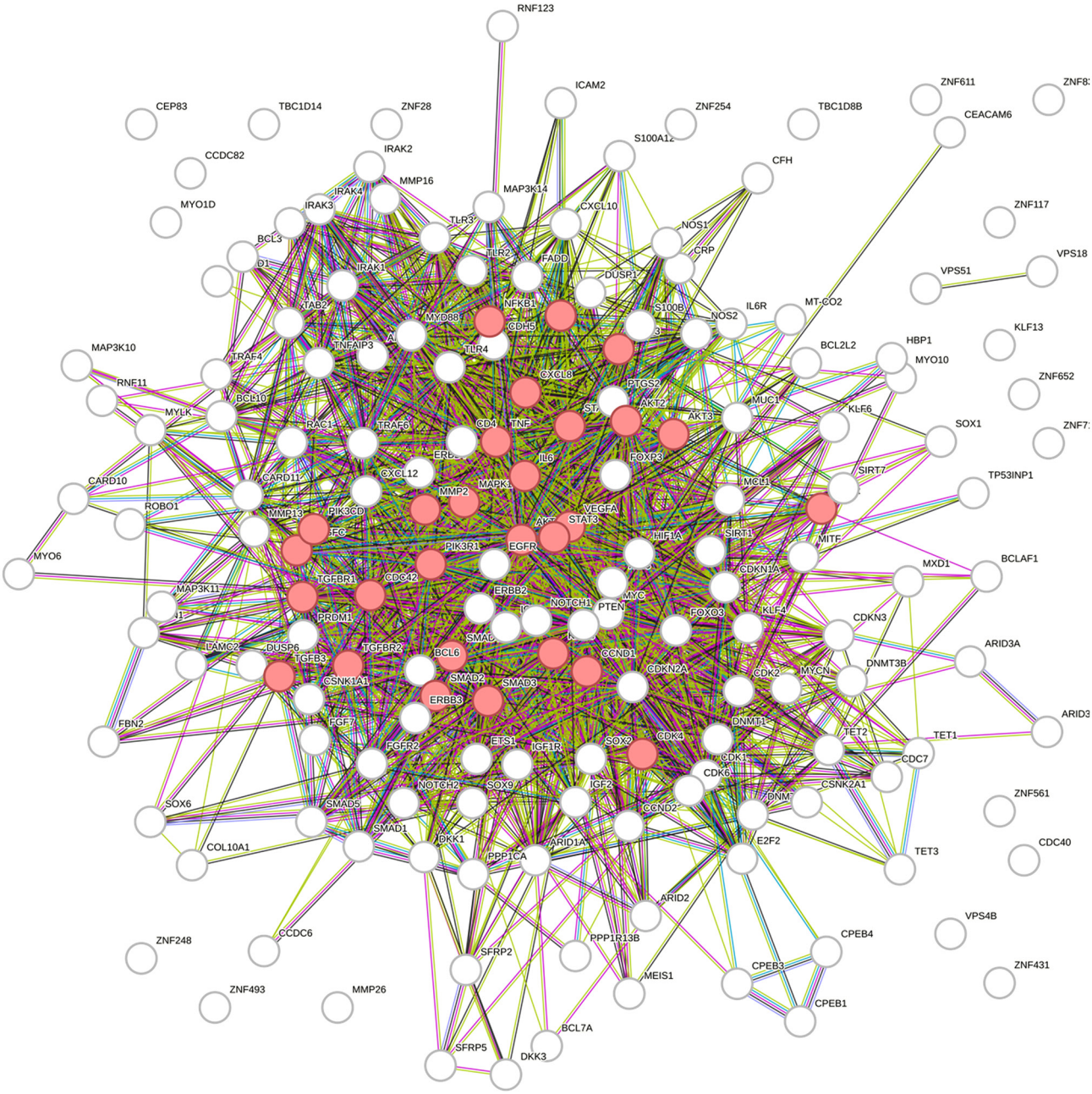

Figure 4. Protein network encoded by jointly regulated genes. Evidenced in red are the proteins involved in the AGE-RAGE signalling pathway (made with the STRING database). Number of nodes: 174; number of edges: 2,206; average node degree: 25.4; avg. local clustering coefficient: 0.602; expected number of edges: 741; PPI enrichment P-value: $<1.0x10^{-16}$.

surrounding colonic mucosa (Fig. 3E), which is in agreement with Chandramouli *et al* (33). Intricate gene networks regulated by miR-101 were shown to be involved in proliferation, apoptosis, angiogenesis, invasion, metastasis, and drug resistance (18). Serum levels of miR-101 were shown to have prognostic significance - low miR-101 levels correlated with advanced cancer stage and a worse overall survival (OS) (46). Also, miR-101 up-regulation is synergistic with chemotherapeutic drugs and promotes their inhibitory effect (47). In our study, lower levels of miR-101 expression were found in patients with history of smoking, compared to those who do not smoke (Table SI), which is in agreement with the study by Gong *et al* (48). This study showed that low doses of cigarette smoke extract induced miR-101 down-regulation in esophageal epithelial and cancer cells (48), targeting cyclooxygenase-2 (*COX-2*), and promoted cancer cell proliferation.

miR-125b was found to be upregulated in both CRC tumors and metastatic sites, compared to adjacent normal tissues, and miR-125b overexpression enhanced CRC cells migration and invasion (49). Also, miR-125b was found to mediate CRC cancer invasion and resistance to 5-FU through enhancing cell autophagy (50). miR-125b expression, together with miR-99a and Let-7c expression correlated with progression-free survival (PFS) and OS of metastatic patients with CRC and could potentially have a role in sensitivity to anti-EGFR monoclonal antibodies therapy (51). miR-125b was identified as one of the hub miRNAs related to CRC prognosis (52), and as one of the combination biomarkers, together with miR-21, miR-29a,

and miR-92a showed a good diagnostic accuracy (53). In the present study, miR-125b was upregulated in the CRC tumor tissues with respect to the surrounding healthy tissue, in agreement with (54). High miR-125b expression was shown to correlate with advanced CRC tumor size, tumor invasion, and poor prognosis (55). Other studies reported lower miR-125b levels in CRC tissues, possibly due to different ethnic origin of the investigated populations (56,57), and its tumor suppressor functions (58). The dual role of miR-125b in malignancies could at least partially be explained by its dependence on the *TP53* mutational status. Namely, miR-125b had anti-cancer effect only in *TP53* mutated colon cancer cells, and not in WT cells (59). miR-125b undoubtedly plays an important role in CRC tumorigenesis and further investigations on its precise role will help to establish its clinical utility.

miR-155 was found to be over-expressed in CRC (60), and to have strong tumorigenic role by promoting CRC cells proliferation, invasion, and metastasis (61). miR-155 is also an important regulator of homeostasis and function of the immune system (62). miR-155 was shown to be involved in mismatch repair and genomic stability in CRC (63), and interestingly enough, in promoting the Warburg phenotype by increasing glucose consumption, lactate production, and HIF-1α levels in human CRC cell lines (64). However, there are also opposing findings showing an oncosuppressive role of miR-155; for example, miR-155 expression was decreased in CRC tissue and cell lines, and inhibited CRC progression and metastasis via silencing collagen triple helix repeat containing 1 (65). Regarding its clinical significance, miR-155 expression was shown to have a potential predictive value for survival of stage II patients with CRC, together with other two lncRNAs and miR-200a (66). Furthermore, an increase in both circulating, and/or tissue miR-155 levels is associated with shorter PFS and OS (67). In addition, re-elevation or sustained elevation of serum miR-155 in postoperative CRC patients treated with chemotherapy is a sign of chemoresistance, and together with miR-200c, and miR-210 levels, a sign of poor prognosis (68). In our study, miR-155 was shown to be upregulated in CRC tissue samples, compared to the adjacent normal tissue (Fig. 3D), which is in agreement with other reports (34,69). Serum miR-155 levels were also higher in patients with CRC compared to the healthy controls, and were independent prognostic factor for PFS, and OS (70). We also observed higher miR-155 expression in CRC tissue samples without the presence of mucus, but failed to find a correlation with TNM staging, invasion, metastasis, and differentiation as in (34,71).

A major limitation of this study that needs to be considered when interpreting its results is a small sample size, thus a follow-up study on a larger prospective cohort of patients is warranted. In general, many potential miRNA biomarkers are still not utilized in the clinical setting, partially due to inconsistent findings. The discovery and validation of a novel miRNA signature in a specific population could potentially aid in the timely diagnosis of patients at risk. Since the development and progression of CRC is a multi-step process which involves mutations in many genes, future combination biomarkers will better reflect the complexity and heterogeneity of CRC. Further analysis of miRNAs in various CRC stages, and the various targets of selected miRNAs is necessary. Circulating miRNAs could potentially be a novel, noninvasive CRC screening tool, which could be utilized along with fecal occult blood testing and colonoscopy. Analysis of specific miRNAs identified in this study, in combination with other CRC diagnostic biomarkers, such as carcinoembryonic antigen, and carbohydrate antigen 19-9 could further increase their sensitivity and specificity.

In conclusion, our study identified statistically significant up-regulation of circulating miR-146a in patients with CRC in the Montenegrin population for the first time. miR-146a was shown to have the potential to be used as a diagnostic biomarker for CRC. This was further corroborated by the finding that its expression was altered in both FFPE tissues and in plasma of patients with CRC. We have also shown for the first time that the distinct five-miRNA (miR-29a, miR-101, miR-125b, miR-146a, and miR-155) pattern of expression is highly involved in CRC carcinogenesis, contributing to the current understanding of which miRNA signature can be potentially used in a clinical setting. Bioinformatics analysis identified the AGE-RAGE signaling axis as a common potentially deregulated pathway. Better understanding of the interaction between the oncogenic effect of the AGE-RAGE axis and the inflammatory tumor microenvironment, and their regulation, could lead to the development of new cancer prevention and treatment methods.

Acknowledgements

The authors would like to thank Dr Katarina Popović (University of Montenegro, Faculty of Medicine) for English language editing.

Funding

This research was funded by the Ministry of Science, Montenegro (grant no. 01-781).

Availability of data and materials

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

Authors' contributions

MŽ, NP, JR, LV, IRD, AT, NG, and SG conceived the study. MŽ, JR, MR, FM, SG, and AT designed the methodology. MŽ, NP, BV, and IRD recruited patients. MŽ, JR, BV, VT, FV and LV performed formal analysis and data curation. MŽ prepared the original draft and data visualization. All authors reviewed and edited the manuscript. MR, VT, and FV performed project administration. MŽ and JR confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Ethical Committee of the Clinical Center of Montenegro (approval no. 03/01-11417/1 on 24.06.2019.) and by the Committee for

Medical Ethics and Bioethics of the Faculty of Medicine of the University of Montenegro (approval no. 3824/4 on 13.12.2018.). Written informed consent was obtained from all study subjects.

Patient consent for publication

Written informed consent has been obtained from all the study subjects to publish this paper.

Competing interests

The authors declare that they have no competing interests.

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A and Bray F: Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 71: 209-249, 2021.
2. Arnold M, Sierra MS, Laversanne M, Soerjomataram I, Jemal A and Bray F: Global patterns and trends in colorectal cancer incidence and mortality. Gut 66: 683-691, 2017.
3. Kocarnik JM, Shiovitz S and Phipps AI: Molecular phenotypes of colorectal cancer and potential clinical applications. Gastroenterol Rep (Oxf) 3: 269-276, 2015.
4. Guinney J, Dienstmann R, Wang X, de Reyniès A, Schlicker A, Soneson C, Marisa L, Roepman P, Nyamundanda G, Angelino P, *et al*: The consensus molecular subtypes of colorectal cancer. Nat Med 21: 1350-1356, 2015.
5. Sawayama H, Miyamoto Y, Ogawa K, Yoshida N and Baba H: Investigation of colorectal cancer in accordance with consensus molecular subtype classification. Ann Gastroenterol Surg 4: 528-539, 2020.
6. Yuan ZX, Wang XY, Qin QY, Chen DF, Zhong QH, Wang L and Wang JP: The prognostic role of BRAF mutation in metastatic colorectal cancer receiving anti-EGFR monoclonal antibodies: A meta-analysis. PLoS One 8: e65995, 2013.
7. Afrăsânie VA, Marinca MV, Alexa-Stratulat T, Gafton B, Păduraru M, Adavidoaiei AM, Miron L and Rusu C: KRAS, NRAS, BRAF, HER2 and microsatellite instability in metastatic colorectal cancer-practical implications for the clinician. Radiol Oncol 53: 265-274, 2019.
8. Gong J, Cho M, Sy M, Salgia R and Fakih M: Molecular profiling of metastatic colorectal tumors using next-generation sequencing: A single-institution experience. Oncotarget 8: 42198-42213, 2017.
9. Ullah S, John P and Bhatti A: MicroRNAs with a role in gene regulation and in human diseases. Mol Biol Rep 41: 225-232, 2014.
10. Bagga S, Bracht J, Hunter S, Massirer K, Holtz J, Eachus R and Pasquinelli AE: Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. Cell 122: 553-563, 2005.
11. Bartel DP: Metazoan MicroRNAs. Cell 173: 20-51, 2018.
12. Peng Y and Croce CM: The role of MicroRNAs in human cancer. Signal Transduct Target Ther 1: 15004, 2016.
13. Zhang C, Sun C, Zhao Y, Wang Q, Guo J, Ye B and Yu G: Overview of MicroRNAs as diagnostic and prognostic biomarkers for high-incidence cancers in 2021. Int J Mol Sci 23: 11389, 2022.
14. Garzon R, Fabbri M, Cimmino A, Calin GA and Croce CM: MicroRNA expression and function in cancer. Trends Mol Med 12: 580-587, 2006.
15. Backes C, Meese E and Keller A: Specific miRNA disease biomarkers in blood, serum and plasma: Challenges and prospects. Mol Diagn Ther 20: 509-518, 2016.
16. Jorgensen BG and Ro S: MicroRNAs and 'sponging' competitive endogenous RNAs dysregulated in colorectal cancer: Potential as noninvasive biomarkers and therapeutic targets. Int J Mol Sci 23: 2166, 2022.
17. Mo WY and Cao SQ: MiR-29a-3p: A potential biomarker and therapeutic target in colorectal cancer. Clin Transl Oncol 25: 563-577, 2023.
18. Wang CZ, Deng F, Li H, Wang DD, Zhang W, Ding L and Tang JH: MiR-101: A potential therapeutic target of cancers. Am J Transl Res 10: 3310-3321, 2018.
19. Yin H, Sun Y, Wang X, Park J, Zhang Y, Li M, Yin J, Liu Q and Wei M: Progress on the relationship between miR-125 family and tumorigenesis. Exp Cell Res 339: 252-260, 2015.
20. Wang D, Wang X, Song Y, Si M, Sun Y, Liu X, Cui S, Qu X and Yu X: Exosomal miR-146a-5p and miR-155-5p promote CXCL12/CXCR7-induced metastasis of colorectal cancer by crosstalk with cancer-associated fibroblasts. Cell Death Dis 13: 380, 2022.
21. Yamada A, Horimatsu T, Okugawa Y, Nishida N, Honjo H, Ida H, Kou T, Kusaka T, Sasaki Y, Yagi M, *et al*: Serum miR-21, miR-29a, and miR-125b Are promising biomarkers for the early detection of colorectal neoplasia. Clin Cancer Res 21: 4234-4242, 2015.
22. Peng X, Wang J, Zhang C, Liu K, Zhao L, Chen X, Huang G and Lai Y: A three-miRNA panel in serum as a noninvasive biomarker for colorectal cancer detection. Int J Biol Markers 35: 74-82, 2020.
23. Rawlings-Goss RA, Campbell MC and Tishkoff SA: Global population-specific variation in miRNA associated with cancer risk and clinical biomarkers. BMC Med Genomics 7: 53, 2014.
24. Panico A, Tumolo MR, Leo CG, Donno A, Grassi T, Bagordo F, Serio F, Idolo A, Masi R, Mincarone P and Sabina S: The influence of lifestyle factors on miRNA expression and signal pathways: A review. Epigenomics 13: 145-164, 2021.
25. Rovčanin Dragović I, Popović N, Ždralević M, Radulović L, Vuković T, Marzano F, Tullo A and Radunović M: Inflammation-related microRNAs-146a and -155 are upregulated in mild cognitive impairment subjects among older age population in montenegro. J Alzheimers Dis 90: 625-638, 2022.
26. Andersen CL, Jensen JL and Ørntoft TF: Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64: 5245-5250, 2004.
27. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
28. Huang HY, Lin YCD, Cui S, Huang Y, Tang Y, Xu J, Bao J, Li Y, Wen J, Zuo H, *et al*: miRTarBase update 2022: An informative resource for experimentally validated miRNA-target interactions. Nucleic Acids Res 50 (D1): D222-D230, 2022.
29. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, Simonovic M, Doncheva NT, Morris JH, Bork P, *et al*: STRING v11: Protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res 47 (D1): D607-D613, 2019.
30. Wang H, Li X, Li T, Wang L, Wu X, Liu J, Xu Y and Wei W: Multiple roles of microRNA-146a in immune responses and hepatocellular carcinoma (review). Oncol Lett 18: 5033-5042, 2019.
31. Szklarczyk D, Gable AL, Nastou KC, Lyon D, Kirsch R, Pyysalo S, Doncheva NT, Legeay M, Fang T, Bork P, *et al*: The STRING database in 2021: Customizable protein-protein networks, and functional characterization of user-uploaded gene/measurement sets. Nucleic Acids Res 49 (D1): D605-D612, 2021.
32. El-Far AH, Sroga G, Jaouni SKA and Mousa SA: Role and mechanisms of RAGE-ligand complexes and RAGE-inhibitors in cancer progression. Int J Mol Sci 21: 3613, 2020.
33. Chandramouli A, Onyeagucha BC, Mercado-Pimentel ME, Stankova L, Shahin NA, LaFleur BJ, Heimark RL, Bhattacharyya AK and Nelson MA: MicroRNA-101 (miR-101) post-transcriptionally regulates the expression of EP4 receptor in colon cancers. Cancer Biol Ther 13: 175-183, 2012.
34. Cao H, Huang S, Liu A and Chen Z: Up-regulated expression of miR-155 in human colonic cancer. J Cancer Res Ther 14: 604-607, 2018.
35. Waghela BN, Vaidya FU, Ranjan K, Chhipa AS, Tiwari BS and Pathak C: AGE-RAGE synergy influences programmed cell death signaling to promote cancer. Mol Cell Biochem 476: 585-598, 2021.
36. Snelson M, Lucut E and Coughlan MT: The role of AGE-RAGE signalling as a modulator of gut permeability in diabetes. Int J Mol Sci 23: 1766, 2022.
37. Li Y, Tan S, Shen Y and Guo L: miR-146a-5p negatively regulates the IL-1β-stimulated inflammatory response via downregulation of the IRAK1/TRAF6 signaling pathway in human intestinal epithelial cells. Exp Ther Med 24: 615, 2022.
38. Garo LP, Ajay AK, Fujiwara M, Gabriely G, Raheja R, Kuhn C, Kenyon B, Skillin N, Kadowaki-Saga R, Saxena S and Murugaiyan G: MicroRNA-146a limits tumorigenic inflammation in colorectal cancer. Nat Commun 12: 2419, 2021.

39. Sathyanarayanan A, Chandrasekaran KS and Karunagaran D: microRNA-146a inhibits proliferation, migration and invasion of human cervical and colorectal cancer cells. Biochem Biophys Res Commun 480: 528-533, 2016.
40. Lu D, Yao Q, Zhan C, Le-Meng Z, Liu H, Cai Y, Tu C, Li X, Zou Y and Zhang S: MicroRNA-146a promote cell migration and invasion in human colorectal cancer via carboxypeptidase M/src-FAK pathway. Oncotarget 8: 22674-22684, 2017.
41. Chae YS, Kim JG, Lee SJ, Kang BW, Lee YJ, Park JY, Jeon HS, Park JS and Choi GS: A miR-146a polymorphism (rs2910164) predicts risk of and survival from colorectal cancer. Anticancer Res 33: 3233-3239, 2013.
42. Li MW, Gao L, Dang YW, Li P, Li ZY, Chen G and Luo DZ: Protective potential of miR-146a-5p and its underlying molecular mechanism in diverse cancers: A comprehensive meta-analysis and bioinformatics analysis. Cancer Cell Int 19: 167, 2019.
43. Fellizar A, Refuerzo V, Ramos JD and Albano PM: Expression of specific microRNAs in tissue and plasma in colorectal cancer. J Pathol Transl Med: May 3, 2022 (Epub ahead of print).
44. Wang A, Deng S, Chen X, Yu C, Du Q, Wu Y, Chen G, Hu L, Hu C and Li Y: miR-29a-5p/STAT3 positive feedback loop regulates TETs in colitis-associated colorectal cancer. Inflamm Bowel Dis 26: 524-533, 2020.
45. Wang J, Chen X, Xie C, Sun M, Hu C, Zhang Z, Luan L, Zhou J, Zhou J, Zhu X, *et al*: MicroRNA miR-29a inhibits colon cancer progression by downregulating B7-H3 expression: Potential molecular targets for colon cancer therapy. Mol Biotechnol 63: 849-861, 2021.
46. He D, Yue Z, Li G, Chen L, Feng H and Sun J: Low serum levels of miR-101 are associated with poor prognosis of colorectal cancer patients after curative resection. Med Sci Monit 24: 7475-7481, 2018.
47. Chen LG, Xia YJ and Cui Y: Upregulation of miR-101 enhances the cytotoxic effect of anticancer drugs through inhibition of colon cancer cell proliferation. Oncol Rep 38: 100-108, 2017.
48. Gong J, Chu Y, Xu M, Huo J and Lv L: Esophageal squamous cell carcinoma cell proliferation induced by exposure to low concentration of cigarette smoke extract is mediated via targeting miR-101-3p/COX-2 pathway. Oncol Rep 35: 463-471, 2016.
49. Zhang X, Li T, Han YN, Ge M, Wang P, Sun L, Liu H, Cao T, Nie Y, Fan D, *et al*: miR-125b promotes colorectal cancer migration and invasion by dual-targeting CFTR and CGN. Cancers (Basel) 13: 5710, 2021.
50. Yu X, Shi W, Zhang Y, Wang X, Sun S, Song Z, Liu M, Zeng Q, Cui S and Qu X: CXCL12/CXCR4 axis induced miR-125b promotes invasion and confers 5-fluorouracil resistance through enhancing autophagy in colorectal cancer. Sci Rep 7: 42226, 2017.
51. Cappuzzo F, Sacconi A, Landi L, Ludovini V, Biagioni F, D'Incecco A, Capodanno A, Salvini J, Corgna E, Cupini S, *et al*: MicroRNA signature in metastatic colorectal cancer patients treated with anti-EGFR monoclonal antibodies. Clin Colorectal Cancer 13: 37-45.e4, 2014.
52. Zhou XG, Huang XL, Liang SY, Tang SM, Wu SK, Huang TT, Mo ZN and Wang QY: Identifying miRNA and gene modules of colon cancer associated with pathological stage by weighted gene co-expression network analysis. Onco Targets Ther 11: 2815-2830, 2018.
53. Liu HN, Liu TT, Wu H, Chen YJ, Tseng YJ, Yao C, Weng SQ, Dong L and Shen XZ: Serum microRNA signatures and metabolomics have high diagnostic value in colorectal cancer using two novel methods. Cancer Sci 109: 1185-1194, 2018.
54. Yagi T, Iinuma H, Hayama T, Matsuda K, Nozawa K, Tsukamoto M, Shimada R, Akahane T, Tsuchiya T, Ozawa T and Hashiguchi Y: Plasma exosomal microRNA-125b as a monitoring biomarker of resistance to mFOLFOX6-based chemotherapy in advanced and recurrent colorectal cancer patients. Mol Clin Oncol 11: 416-424, 2019.
55. Nishida N, Yokobori T, Mimori K, Sudo T, Tanaka F, Shibata K, Ishii H, Doki Y, Kuwano H and Mori M: MicroRNA miR-125b is a prognostic marker in human colorectal cancer. Int J Oncol 38: 1437-1443, 2011.
56. Ak S, Tunca B, Tezcan G, Cecener G, Egeli U, Yilmazlar T, Ozturk E and Yerci O: MicroRNA expression patterns of tumors in early-onset colorectal cancer patients. J Surg Res 191: 113-122, 2014.
57. Zeng ZL, Lu JH, Wang Y, Sheng H, Wang YN, Chen ZH, Wu QN, Zheng JB, Chen YX, Yang DD, *et al*: The lncRNA XIST/miR-125b-2-3p axis modulates cell proliferation and chemotherapeutic sensitivity via targeting Wee1 in colorectal cancer. Cancer Med 10: 2423-2441, 2021.
58. Yu M, Yi Z, Chen S, Chen X and Xie X: MIR4435-2HG, miR-125b-5p, and Sema4D axis affects the aggressiveness of colorectal cancer cells. Folia Histochem Cytobiol 60: 191-202, 2022.
59. Cenariu D, Zimta AA, Munteanu R, Onaciu A, Moldovan CS, Jurj A, Raduly L, Moldovan A, Florea A, Budisan L, *et al*: Hsa-miR-125b therapeutic role in colon cancer is dependent on the mutation status of the TP53 gene. Pharmaceutics 13: 664, 2021.
60. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, Visone R, Iorio M, Roldo C, Ferracin M, *et al*: A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci USA 103: 2257-2261, 2006.
61. Qu YL, Wang HF, Sun ZQ, Tang Y, Han XN, Yu XB and Liu K: Up-regulated miR-155-5p promotes cell proliferation, invasion and metastasis in colorectal carcinoma. Int J Clin Exp Pathol 8: 6988-6994, 2015.
62. Rodriguez A, Vigorito E, Clare S, Warren MV, Couttet P, Soond DR, van Dongen S, Grocock RJ, Das PP, Miska EA, *et al*: Requirement of bic/microRNA-155 for normal immune function. Science 316: 608-611, 2007.
63. Valeri N, Gasparini P, Fabbri M, Braconi C, Veronese A, Lovat F, Adair B, Vannini I, Fanini F, Bottoni A, *et al*: Modulation of mismatch repair and genomic stability by miR-155. Proc Natl Acad Sci USA 107: 6982-6987, 2010.
64. Xu Y, Han S, Lei K, Chang X, Wang K, Li Z and Liu J: Anti-Warburg effect of rosmarinic acid via miR-155 in colorectal carcinoma cells. Eur J Cancer Prev 25: 481-489, 2016.
65. Liu J, Chen Z, Xiang J and Gu X: MicroRNA-155 acts as a tumor suppressor in colorectal cancer by targeting CTHRC1 *in vitro*. Oncol Lett 15: 5561-5568, 2018.
66. Wang XJ, Zeng B, Lin S, Chen M and Chi P: An integrated miRNA-lncRNA signature predicts the survival of stage II colon cancer. Ann Clin Lab Sci 49: 730-739, 2019.
67. Ulivi P, Canale M, Passardi A, Marisi G, Valgiusti M, Frassineti GL, Calistri D, Amadori D and Scarpi E: Circulating plasma levels of miR-20b, miR-29b and miR-155 as predictors of bevacizumab efficacy in patients with metastatic colorectal cancer. Int J Mol Sci 19: 307, 2018.
68. Chen J, Wang W, Zhang Y, Chen Y and Hu T: Predicting distant metastasis and chemoresistance using plasma miRNAs. Med Oncol 31: 799, 2014.
69. Hongliang C, Shaojun H, Aihua L and Hua J: Correlation between expression of miR-155 in colon cancer and serum carcinoembryonic antigen level and its contribution to recurrence and metastasis forecast. Saudi Med J 35: 547-553, 2014.
70. Lv ZC, Fan YS, Chen HB and Zhao DW: Investigation of microRNA-155 as a serum diagnostic and prognostic biomarker for colorectal cancer. Tumor Biol 36: 1619-1625, 2015.
71. Zhang GJ, Xiao HX, Tian HP, Liu ZL, Xia SS and Zhou T: Upregulation of microRNA-155 promotes the migration and invasion of colorectal cancer cells through the regulation of claudin-1 expression. Int J Mol Med 31: 1375-1380, 2013.