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PROGNOSTIC SIGNIFICANCE OF microRNA-100, -125b, AND -200b IN PATIENTS WITH COLORECTAL CANCER

Background. The discovery of new markers for colorectal cancer (CRC) is of paramount importance for improving the diagnosis, prognosis, and treatment of this disease. CRC is the third most common cancer worldwide and the second leading cause of cancer-related deaths. Early detection and treatment are crucial for improving patient outcomes, but current screening methods are not foolproof. Additionally, there is a need for better prognostic markers to identify patients at high risk of recurrence or metastasis, who may benefit from more aggressive treatment. **Objectives:** To analyze the expression profile of miR-100, miR-125b, and miR-200b in the blood serum of CRC patients and assess its correlation with the clinicopathological factors of cancer course. **Materials and Methods.** Twenty blood serum samples from CRC patients were analyzed by the real-time polymerase chain reaction for miR-100, miR-125b, and miR-200b expressions. The results were normalized and then analyzed using statistical tests. **Results.** According to our results, miR-125b and -200b expressions correlate with T (r = –0.51 and 0.6, respectively, $p < 0.05$) and N (r = 0.47 and –0.52, respectively, $p < 0.05$). Also, miR-125b levels were 1.56 times higher and mir-200b – 1.59 times lower in patients with metastases in the regional lymph nodes. **Conclusions.** Observed levels of miR-125b and -200b in correlation with tumor stage and lymph node metastasis among CRC patients demonstrate their potential clinical utility as minimally invasive biomarkers for the prognosis of cancer course. Therefore, further validation studies with larger participant cohorts are necessary.

Keywords: colorectal cancer, microRNA, prognosis, metastases.

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Cancer ranks as the third leading cause of death globally, following only cardiovascular diseases. Within the realm of cancers, colorectal cancer (CRC) stands as the third most common cause of cancer-related fatalities in Ukraine and Western countries, and fifth in Japan [1]. Of all CRCs, approximately 80% are adenocarcinomas originating from the colon and rectum epithelium, with only a mere 2% of tumors being benign. This malignancy is characterized by late clinical presentation, early and aggressive local as well as systemic invasion, and a poor prognosis [2]. Notably, the 5-year survival rate remains under 5% regardless of the treatment administered. The elevated mortality rate associated with this cancer can be attributed to its aggressive nature, the lack of early detection methods, and the limited efficacy of existing treatments [3].

At the time of disease diagnosis, numerous genetic and epigenetic alterations have already occurred. These include changes in gene expression resulting from mutations, deletions, and gene amplifications, as well as shifts in DNA methylation patterns [4]. These alterations are pivotal in the initiation, progression, and sustenance of CRC. Consequently, it is necessary to reveal the molecular mechanisms underpinning tumorigenesis and the advancement of CRC. Developing novel diagnostic and therapeutic approaches is essential to improve outcomes of CRC patients [5].

Furthermore, there is a pressing demand to identify new biomarkers that could elucidate diverse phenotypes and their associations with varying clinical characteristics and prognoses. In this regard, a recently uncovered category of noncoding small RNAs, known as microRNAs (miRNAs), holds promise for advancing cancer research [6].

MicroRNAs, typically 20 to 22 nucleotides in length, constitute a plentiful class of gene regulators that exert a negative influence on gene expression at the posttranscriptional level due to binding to the 3'-untranslated regions of their target mRNAs and subsequently suppressing the expression of the target genes through mRNA degradation or translational repression [7].

Over the past several years, advanced technologies have been used to quantify miRNAs in CRC, and several deregulated miRNAs have been revealed, including miR-21, miR-200b, miR-182, and others [8]. These findings imply that miRNAs may play a role in the development of CRC. The aberrant regulation of miRNAs may be linked to disruptions in the transcription process and its subsequent processing steps. As a result, the transcriptional regulation of miRNAs might represent an aspect of CRC underlying causes that has not been thoroughly explored [9]. However, there have been limited studies that delve into the mechanisms governing miRNA transcription during the onset and progression of CRC. Furthermore, our understanding of the regulatory functions of only a few selected miRNAs in the initiation and advancement of CRC is currently quite limited [10]. There is, therefore, an urgent need for more extensive investigations into the roles and functions of miRNAs. In this study, we explored the expression patterns of frequently deregulated miRNAs in colorectal adenocarcinoma, using blood samples obtained from CRC patients. We also evaluated their potential prognostic significance.

Materials and Methods

Bioinformatics research methods. A comparison of the expression levels of the studied microRNAs in the blood of patients with CRC concerning the indicators in healthy subjects was carried out using CancerMIRNome (http://bioinfo.jialab-ucr.org/CancerMIRNome/) resource [11].

General clinical characteristics of patients with CRC. This study enrolled 20 patients with primary CRC and 10 healthy individuals as a control. All patients were treated in the Prykarpatsky Clinical Oncology Center of the Ivano-

Frankivsk Regional Council during 2020—2022. All patients were diagnosed with adenocarcinoma of the colon/rectum. All tumors were sporadic, as assessed by the personal and family histories. Also, 10 samples of blood serum from age-matching healthy individuals were used as a control. The study was approved by the Hospital Ethical Committee, and all subjects gave their written informed consent to the sample collection and analyses described in the present study.

The main clinicopathological characteristics of the patients are shown in Table 1. Most cases were of the T3 category according to TNM and G2 differentiation grade, metastasis-free (65.0% of patients), with deep tumor invasion, tumor ulceration, and necrotic changes (Table 1).

Depending on the clinical indications, all patients underwent organ-sparing operations or radical resections as well as neoadjuvant, adjuvant, and radiation therapy by the treatment standards of Ukraine [12].

Real-time PCR. RNA was purified from blood serum samples using the mirVana miRNA isolation kit (Thermo Scientific, USA) according to the manufacturer's instructions. The expression patterns of the miRNA species tested and a housekeeping gene, RNU48, were quantitatively assayed using reverse transcription and the real-time reverse transcriptase polymerase chain reaction (RT-PCR).

RNU48 primer sequences were taken from https://www.ncbi.nlm.nih.gov and synthesized by Metabion, Germany: RT primer: 5'-CTCTGACC-3', forward 5'-AGTGATGAT-

Table 1. **Clinicopathological characteristics of patients with CRC**

Index	Number of patients	
	n	%
Total number of patients	20	100
Average age, years (range)	63.95 ± 11.55 (34—85)	
Sex		
Women	12	60
Men	8	40
T according to TNM		
T2	2	10
T3	14	70
T4	3	15
N according to TNM		
N0	13	65
N1	6	30
N2—10	1	5
M according to TNM		
M0	20	100
Differentiation grade		
G2	19	95
G3	1	5
Invasion of adjacent layers		
Absent	6	30
Detected	14	70
Ulceration		
Absent	4	20
Detected	16	
Necrosis		
Absent	5	25
Detected	15	75

Table 2. **Primer sequences for evaluation of microRNA expression in blood serum of patients with CRC**

miRNA	Stem-loop primer	Forward primer
hsa-miR-100-5p (miR-100)	T2 5' - GTTGGCTCTGGTGCAGGGTCCGAGGTATTCG CACCAGAGCCAAC CACAAG - 3'	5' - GTGAACCCGTA-GATCCGAA - 3'
hsa-miR-125b-1-5p (miR-125b)	5' - GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCA CCAGAGCCAAC TCACAA - 3'	5' - GTTTCCCTGAGA-CCCTAAC - 3'
hsa-miR-200b-3p (miR-200b)	5' - GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCA CCAGAGCCAAC TCATCA - 3'	5' - GTTTGGTAATA-CTGCCTGGTAA - 3'

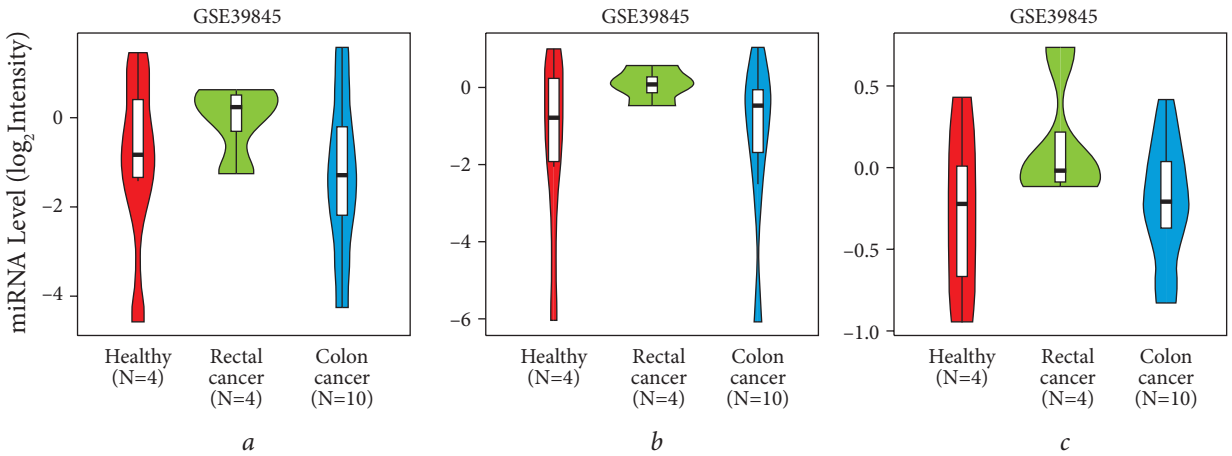

Fig. 1. Indicators of miR-100 (*a*), -125b (*b*), and -200b (*c*) expression in healthy controls, rectal cancer, and colon cancer patients according to the information posted on the CancerMIRNome online platform

GACCCCAGGTAACTC-3', reverse 5'-CTGCG-GTGATGGCATCAG-3'. The relative expression of miRNA was determined by the comparative dCT method [13].

Stem-loop complementary DNA (cDNA) was synthesized using looped reverse transcription primers specific for each miRNA using universal reverse primer 5'-GTGCAGGGTCCGAGGT-3', according to the stem-loop method miRNA RT-PCR [14]. Primer sequences for miRNA detection were obtained using the resource http://genomics.dote.hu:8080/mirnadesigntool/ and synthesized by Metabion (Germany) (Table 2). Luna Universal qPCR Master Mix PCR kit (New England Biolabs, USA) was used according to the manufacturer's protocol. Reverse transcription PCR was performed on a QuantStudio 5 Dx detection system Real-Time PCR System (Thermo Scientific, USA) using a commercial kit for RT-PCR Maxima^{TM} SYBR Green/ROX Master Mix (2X) (Thermo Scientific, USA) according to the manufacturer's protocol. Amplified miRNAs showed specific melting temperature, confirming the accuracy and specificity of the method used. Data were analyzed with the QuantStudio 5 Dx software (Thermo Scientific, USA). The expression of each miRNA was normalized to RNU48 internal control and presented as fold change (dCT).

Statistical analysis. A Mann — Whitney U test was performed to compare differences in miRNA levels between the patients and the controls. Experiments were conducted in triplicate. Correlations between miRNA expression and clinicopathological parameters were analyzed using Pearson's correlation coefficient (r). All statistical analyses were carried out using the GraphPad Prism 4.0 (GraphPad Software, USA). $p < 0.05$ was considered significant.

Results

The comparative analysis of miR-100, -125b, and -200b expression using the online platform CancerMIRNome showed that on the available dataset GSE39845 (Tissue and blood microRNA array profiles of colorectal cancer patients) of 20 subjects there were no differences in studied miRNA expressions in the Selected Circulating miRNome dataset (Fig. 1).

We established that the levels of studied miRNAs vary in healthy controls and CRC patients, but the differences are insignificant (Table 3).

Thus, the results of our analysis of the open database CancerMIRNome as well as our data indicate the lack of prospects for using miR-100, -125b, and -200b as diagnostic markers of CRC.

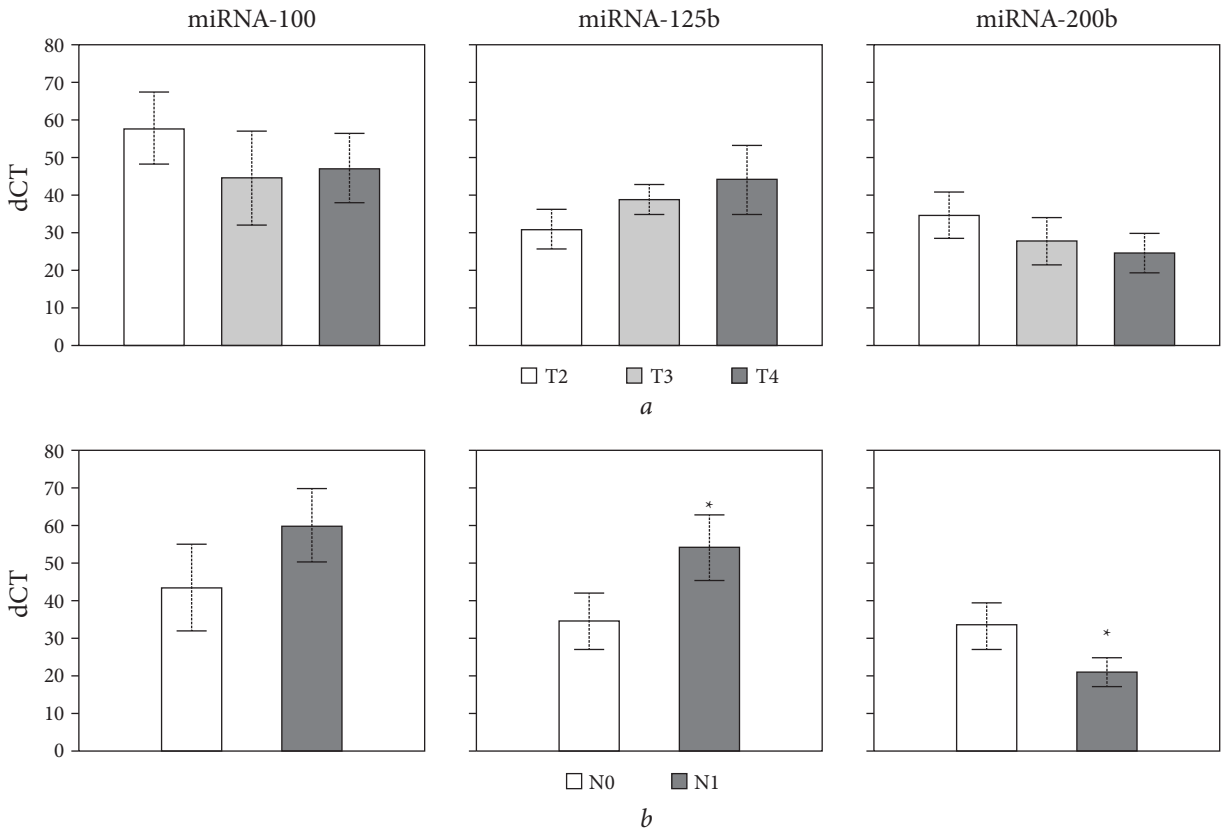

Fig. 2. Indicators of miRNA expression depending on the T (*a*) and N (*b*) status by TMN of patients with CRC. * $p < 0.05$

To better understand the potential roles of these miRNAs in CRC development and progression, we estimated the relationship of the miR-100, -125b, and -200b with various clinical features of CRC (Fig. 2). The number of T2 stage cases was small, however, our results revealed that a low level of miR-125b and a high miR-200b expression correlate with stages T3 and T4 (r = –0.51 and 0.6, respectively, $p < 0.05$).

At the same time, high levels of miR-125b and -200b also correlated with the presence of lymph node metastases (r = 0.47 and –0.52, respectively). Levels of miR-125b were 1.56 times higher in patients with lymph node metastases,

Table 3. **MicroRNA expression in blood serum of patients with CRC compared to healthy controls**

Characteristics		miR-100	miR-125b	miR-200b
Healthy controls	Average level	32.89 ± 21.11	29.96 ± 25.22	29.99 ± 11.54
	Individual fluctuations	11.25—89.36	19.58—76.58	10.78—56.33
	Median	22.59	59.88	34.22
CRC patients	Average level	49.17 ± 30.62	40.08 ± 20.09	26.64 ± 13.46
	Individual fluctuations	18.93—96.63	11.14—72.59	12.4—51.90
	Median	33.61	61.48	22.11
	High level, n = 20	9 (45%)	11 (55%)	12 (60%)
	Low level, n = 20	11 (55%)	9 (45%)	8 (40%)

while miR-200b expression was 1.59 lower in the same patients. No other significant association was found.

We hypothesized that miR-100, -125b, and miR-200b expression levels might affect the prognosis of patients with CRC. In the present study, the average fold changes of miR-100, miR-125b, and miR-200b were 49.17, 40.08, and 26.64, respectively. The average fold change was used as the threshold, and patients were separated into high-expression (above or equal to 49.17-, 40.08-, and 26.64-fold, respectively) and low-expression (below average) groups. Interestingly, all patients were distributed equally by these miRNA expression levels in a ratio close to 1:1 by low/high levels (Table 3). 55% of CRC cases had a low level of miR-100, 45% — low miR-125b, and 40% — low miR-200b expression. However, there was no patients with low levels of all three miRNAs. We evaluated the clinicopathological parameters of our sample within the mentioned groups and found a tendency of lymph node metastasis in patients with low mir-200b expression.

Discussion

MicroRNAs may directly regulate tissue or organ development and cell differentiation as well as maintain normal functions of many body systems. Thus, disruption in serum miR-100, -125b, and -200b levels in CRC patients may also be affected by other illnesses, physiological conditions, age, and environmental factors, such as diet, pollution, etc. However, we have found some association of mir-125b and -200b with the T and N status by TNM in CRC patients.

Concerning the miR-100 expression, we hoped to establish its association with clinicopathological characteristics of CRC by the results of Chen et al. [15] research, where the authors showed that miR-100 expression in human CRC tissues was significantly lower than in adjacent normal tissues and was associated with larger tumor size, higher incidence of lymph node metastasis and advanced TNM stage. Also, they stated a significant correlation between miR-100 expression and the 5-year overall survival of CRC patients [15].

Another study demonstrated that mutant KRAS CRC cells release protein-laden exosomes, enriched with miR-100 [16]. Although this thesis was not probed in our study, serum miR-100 remains a promising CRC marker due to its involvement in the radiosensitivity of CRC [17].

Regarding miR-125b, Yamada et al. [18] conducted a large-scale study to find miRNAs that could help distinguish early colorectal neoplasia (CRN) patients from healthy controls and stated that the serum levels of miR-21, miR-29a, and miR-125b are very promising biomarkers. The authors faced some limitations because the healthy volunteers in their study were younger than CRN patients. In our study, healthy individuals were of the same age as CRC patients, thus differences in the results may be caused by age-dependent fluctuations of miR-125b levels, but this claim needs accessing separately on a larger sample of individuals.

As for miR-200b expression, Zhu et al. [19] found a correlation between its expression in the serum of CRC patients and liver metastasis, while Toyiama et al. [20] reported no association with clinicopathological characteristics at all. Both studies were limited by a small number of patients in the sample, and there is no available data on the prognostic value of miR-200b in CRC patients. Still, it has been proven that this miRNA is a valuable prognostic marker in many other cancer types, so further research is needed.

In conclusion, we have observed the increased relative expression levels of miR-125b and -200b in correlation with the tumor stage and lymph node metastasis among CRC patients. However, our study was performed on a small number of CRC patients. Therefore, further validation studies on larger cohorts are required to verify the association of serum miR-100, -125b, and -200b with CRC.

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ПРОГНОСТИЧНЕ ЗНАЧЕННЯ МІКРОРНК-100, -125b І -200b У ПАЦІЄНТІВ З КОЛОРЕКТАЛЬНИМ РАКОМ

Вступ. Відкриття нових маркерів колоректального раку (КРР) має ключове значення в покращенні діагностики, прогнозу та лікування цього захворювання. КРР є третім за поширеністю та другим за смертністю типом злоякісних новоутворень у світі. Рання діагностика є вирішальним фактором для покращення прогнозу, але сучасні методи скринінгу є недостатньо надійними. Крім того, існує потреба в кращих прогностичних маркерах для ідентифікації пацієнтів із високим ризиком рецидиву або метастазування, адже вони потребують більш агресивної тактики лікування. **Мета роботи.** Проаналізувати профіль експресії miR-100, miR-125b та miR-200b у сироватці крові хворих на КРР та оцінити їх зв'язок із клініко-патологічними особливостями перебігу захворювання. **Матеріали та методи.** Експресію miR-100, miR-125b і miR-200b оцінювали у двадцяти зразках сироватки крові пацієнтів з КРР за допомогою полімеразної ланцюгової реакції в реальному часі. Результати були нормалізовані, після чого проводився статистичний аналіз із врахуванням нормальності розподілу. **Результати.** Згідно з отриманими даними, експресія miR-125b і -200b корелювала зі категоріями T ($r = –0,51$ і 0,6 відповідно, $p < 0,05$) та N ($r = 0,47$ і –0,52 відповідно). Крім того, у пацієнтів із метастазами в лімфатичні вузли рівень miR-125b був у 1,56 рази вищим, а miR-200b — в 1,59 рази нижчим. порівняно із хворими без метастатичного ураження регіонарних лімфатичних вузлів. **Висновки.** Доведений зв'язок рівнів експресії miR-125b і -200b зі T стадією пухлини та метастазами в лімфатичних вузлах у пацієнтів з КРР демонструє їх потенційну клінічну цінність як малоінвазивних біомаркерів для прогнозу перебігу цього типу онкопатології. Відповідно, необхідними є подальші дослідження з розширеною вибіркою хворих для підтвердження діагностичної ролі досліджених нами мікроРНК.

Ключові слова: колоректальний рак, мікроРНК, прогноз, метастази.