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Expression dynamics of hsa-miR-18a-5p and hsa-miR-135b-5p are associated with pathological tumor stage and lymph node status in locally advanced rectal cancer patients undergoing neoadjuvant chemoradiotherapy

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Abstract: Locally advanced rectal cancer (LARC) is characterized by tumor invasion into surrounding tissues and frequent lymph node involvement, often requiring neoadjuvant chemoradiotherapy (nCRT) followed by surgical resection. LARC presents a significant therapeutic challenge because it is typically diagnosed at an advanced stage and shows variable responses to standard nCRT, highlighting the need for predictive biomarkers. microRNAs are considered valuable biomarker candidates due to their biological characteristics. We investigated the expression dynamics of hsa-miR-18a-5p and hsa-miR-135b-5p and their predictive potential for response to nCRT. We demonstrate a significant post-nCRT decrease in tumor expression of hsa-miR-18a-5p. High pre-treatment hsa-miR-18a-5p expression was significantly associated with lower post-treatment pathological stage and absence of lymph node metastasis, indicating potential predictive value. The expression of hsa-miR-135b-5p after therapy was associated with advanced disease stage and positive lymph node status, indicating it may be linked to more aggressive disease after the treatment. Despite these associations with tumor characteristics, neither miRNA showed a significant association with therapy response. Our findings suggest that while hsa-miR-18a-5p and hsa-miR-135b-5p are not predictive of nCRT response, their expression patterns before and after therapy may reflect underlying tumor biology and hold potential for LARC patient stratification.

Keywords: hsa-miR-18a-5p, hsa-miR-135b-5p, neoadjuvant chemoradiotherapy, locally advanced rectal cancer

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in humans, ranking as the third most prevalent cancer in both men and women [1,2]. The incidence of CRC is increasing worldwide, particularly among individuals under the age of 50, with the number of cases rising [3]. Apart from genetic predisposition, the main environmental risk factors for CRC development include lifestyle choices, frequent alcohol consumption, tobacco smoking, and a diet high in fatty foods and red meat [4]. Among CRC patients, rectal cancer is the most common form, accounting for roughly one-third of cases [5].

Locally advanced rectal cancer (LARC), defined as tumor extension beyond the rectal wall and/or lymph node involvement without distant metastasis (T3–T4 with any N, or any T with N+, M0), presents a substantial therapeutic challenge. The standard treatment protocol for LARC patients includes preoperative (neoadjuvant) chemoradiotherapy (nCRT), which aims

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to reduce tumor size and stage. If a complete response is not achieved following nCRT, LARC patients typically undergo surgery involving total mesorectal excision, which may be followed by adjuvant chemotherapy. However, the response to nCRT varies widely and only 10-30% of patients achieve a complete pathologic response, while 40% show a partial response, and approximately 20% develop resistance to the therapy [5,6]. These findings highlight the need for reliable molecular biomarkers capable of predicting treatment response, thereby improving patient stratification and reducing unnecessary therapies and associated toxicities.

miRNAs are small, non-coding, single-stranded RNA molecules, ~21-25 nucleotides in length, that regulate gene expression post-transcriptionally [7,8]. miRNAs regulate a broad spectrum of essential cellular functions, including development, differentiation, growth, and metabolism. Aberrant expression of miRNAs is associated with various complex diseases, including cancer [9]. miRNAs are promising biomarkers for diagnosis, prognosis, and monitoring response to treatment, as well as a source for the development of novel therapeutic strategies [10]. Altered miRNA functions, which are involved in regulating various signaling pathways deregulated in rectal cancer, may serve as potential predictive biomarkers for response to nCRT in LARC patients [11].

Altered miRNA expression can disrupt signaling pathways that are critical for cancer development and therapy resistance. The TGF-beta signaling pathway plays a central role in the progression of CRC [12]. Previous studies have shown that genes involved in the TGF-beta signaling pathway are frequently deregulated in CRC and might have potential predictive value [13]. Thus, miRNAs that target gene members of the TGF-beta pathway may be important predictors of nCRT response. In this study, we focused on two specific miRNAs, hsa-miR-18a-5p and hsa-miR-135b-5p, both of which regulate key genes in the canonical TGF-beta signaling pathway [14] and whose expression is known to be altered in CRC [15]. Hsa-miR-18a-5p has been shown to have a dual role, acting as either a tumor suppressor or an oncogene, depending on the context [16]. In CRC, it inhibits malignant progression, demonstrating tumor suppressor activity [17]. In contrast, hsa-miR-135b-5p has been identified as an oncogene in CRC, promoting malignant transformation [17]. Hsa-miR-135b was shown to have prognostic significance as it was associated with disease-free and rectal cancer-specific survival [18]. Data on the expression of these two candidate miRNAs are limited in the literature, particularly regarding therapy-related expression dynamics and their predictive value for nCRT in LARC.

No specific biomarkers are currently available to predict response to nCRT. Careful selection of LARC patients for nCRT could reduce healthcare costs and, more importantly, improve quality of life by avoiding unnecessary treatments and their associated side effects. This study aimed to investigate hsa-miR-18a-5p and hsa-miR-135b-5p expression levels in tumor samples before and after nCRT and their potential as biomarkers for predicting pathological response to therapy in LARC patients. We hypothesized that changes in the expression of these miRNAs may reflect their role in therapeutic response and potentially predict outcomes, ultimately informing more personalized treatment strategies for LARC.

MATERIALS AND METHODS

Ethics statement

This study was approved by the Ethics Committee of the Faculty of Medicine of the University of Belgrade (approval number 1550/V-2, May 31, 2019). All patients provided written informed consent before inclusion in the study.

Bioinformatic analysis

The interaction of hsa-miR-18a-5p and hsa-miR-135b-5p with their target genes was analyzed using the miRNet v2.0 software, a visual analytics platform for miRNA-centric networks [19]. miRTarBase v9.0 was used as a resource for validated target genes and miRNAs of interest. Pathway enrichment analysis on all target genes of both miRNAs was performed using the KEGG database and the hypergeometric test. A P value less than 0.05 was considered significant.

Study group and biological samples

The study group included 19 patients with LARC, who were enrolled between April 2019 and October

2020. Inclusion criteria were: patients aged over 18 years, histopathologically confirmed adenocarcinoma, and locally advanced tumor as determined by pelvic magnetic resonance imaging (MRI). Exclusion criteria were as follows: the presence of metastatic disease, synchronous CRC, other malignancies, an ASA score over 3, and an unsigned informed consent. All patients underwent nCRT, which consisted of 50.4 Gy radiation delivered in 28 fractions combined with two cycles of chemotherapy (5-fluorouracil at 425 mg/m^2 and leucovorin at 20 mg/m^2) in the first and fifth week of radiation, followed by surgery. All patients were treated at the Clinic for Digestive Surgery – First Surgical Clinic, University Clinical Center of Serbia. The clinical Tumor-Node-Metastasis (cTNM) stage was determined by MRI, confirming LARC. Tumor tissue samples were collected before nCRT using anoscopy or rectoscopy for tumors located in the low (up to 5 cm from the anal verge) and mid rectum (5-10 cm from the anal verge), respectively. Additional samples were collected 8-12 weeks after nCRT, during surgery. The tissue samples were immersed in 500 µl of TRI Reagent™ Solution (Invitrogen, USA) and stored at -80°C until further processing.

Patient characteristics are shown in Table I. Clinicopathological data were obtained from patient medical records. Pathologic Tumor-Node-Metastasis (pTNM) staging was determined according to the 8th release of the American Joint Committee on Cancer (AJCC) from 2017. Pathologic response to nCRT in postoperative tumor tissue samples was estimated by an experienced pathologist according to tumor regression grading (TRG) using the Mandrad scoring system [20]. Patients with TRG1 and TRG2 were classified as responders, while those with TRG3-5 were considered non-responders.

Table 1. Baseline demographic and clinical characteristics

Characetristics		N	%
Sex	female	8	42.1
	male	11	57.9
Age, years (median)	<66	9	47
	>66	10	53
Clinical T stage	T3	13	68.4
	T4	6	31.6
Clinical N stage	N1	5	26.3
	N2	14	73.7
Clinical M stage	M0	19	100
Clinical disease stage	IIIB	5	26.3
	IIIC	14	73.7
Pathological T stage	T1	1	5.3
	T2	4	21
	T3	13	68.4
	T4	1	5.3
Pathological N stage	N0	11	57.9
	N1	7	36.8
	N2	1	5.3
Pathological M stage	M0	19	100
Pathological disease stage	0	1	5.3
	I	2	10.5
	II	8	42.1
	III	8	42.1
Tumor regression grade (TRG)	TRG1	1	5.3
	TRG2	1	5.3
	TRG3	7	36.8
	TRG4	10	52.6
CEA before therapy (IU/mL, mean±standard deviation)	23.376±54.038		
CA 19-9 before therapy (IU/mL, mean±standard deviation)	32.410±81.843		

N – total number of patients

RNA isolation, reverse transcription, and relative quantification of the expression levels of hsa-miR-18a-5p and hsa-miR-135b-5p

Total RNA was isolated from tissue samples collected before and after nCRT using TRI Reagent™ Solution (Invitrogen, USA) according to the manufacturer's instructions. The isolated total RNA was stored at -80°C until further use. RNA concentration and purity were determined using an Ultrospec 3300 Pro spectrophotometer (Amersham Biosciences, UK). For reverse transcription, the TaqMan™ microRNA reverse transcription kit (Thermo Fisher Scientific, USA) was used in combination with a pool of stem-loop primers following the manufacturer's instructions. The thermal cycling conditions for cDNA synthesis were as follows: 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, followed by a hold at 4°C. The synthesized cDNA was stored at -20°C.

Relative quantification of the expression levels of hsa-miR-18a-5p, hsa-miR-135b-5p, and the endogenous control RNU6B was performed using TaqMan™ microRNA gene expression assays (Thermo Fisher Scientific, USA): ID 002422 (hsa-miR-18a-5p), ID

002261 (hsa-miR-135b-5p), and ID 001093 (RNU6B). The temperature profile was as follows: 5 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Amplification was performed in triplicate for each sample. Relative expression levels of the miRNAs were determined using the dCt method ($dCt=Ct_{sample}-Ct_{endogenous\ control}$) and presented as 2^{-dCt}. These values were used for statistical analysis.

Public database analysis

To investigate the expression of hsa-miR-18a-5p and hsa-miR-135b-5p in LARC compared to normal tissue and between nCRT responders and non-responders, the Gene Expression Omnibus (GEO) base of the National Center for Biotechnology Information was searched. Four suitable datasets were identified using the keywords rectal cancer, miRNA, therapy: GSE38389, GSE68204, GSE29298, and GSE98959 [18,21-23]. Two datasets, GSE38389 and GSE68204, contained data on miRNA expression in rectal cancer and normal rectal mucosa. The datasets were analyzed using the interactive web tool GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/) with default settings.

Statistical analysis

Statistical data analysis was performed using SPSS v.20.00 (IBM SPSS Statistics for Windows, Armonk, USA), and a visual representation of the results was created with GraphPad Prism v.9. The Shapiro-Wilk test was used to assess the normality of data distribution. For data that did not follow a normal distribution, non-parametric statistical tests were used. The Wilcoxon rank-sum test was used to compare the mean values of relative expression in tumor tissue before and after nCRT. The predictive potential of the analyzed miRNAs was assessed using receiver operating characteristic (ROC) curves, reporting the area under the curve (AUC) with 95% confidence intervals (CI). Correlations between variables were assessed using Spearman's rank correlation test based on the Rho coefficient and P-value. The association between miRNA expression and overall survival was assessed using Kaplan-Meier survival curves, which were compared with the log-rank test. A P-value of <0.05 was considered statistically significant.

RESULTS

Bioinformatic analysis of hsa-miR-18a-5p and hsa-miR-135b-5p

To support the selection of hsa-miR-18a-5p and hsa-miR-135b-5p for analysis in this study, we performed in silico analysis. The interaction networks between these two miRNAs and their validated target genes were visualized by miRNet v2.0 (Supplementary Fig. S1). A greater number of genes were predicted to be targets of hsa-miR-18a-5p (277 genes) compared to hsa-miR-135b-5p (88 genes). KEGG enrichment analysis confirmed that the most significantly enriched pathways were the colorectal cancer pathway and the TGF-β signaling pathway (Supplementary Table S1). This finding further supports selecting these miRNAs as candidates for evaluating their potential as predictive biomarkers in LARC.

Relative expression of hsa-miR-18a-5p and hsa-miR-135b-5p in rectal cancer tissue before and after nCRT

Next, the relative expression of hsa-miR-18a-5p and hsa-miR-135b-5p was analyzed in rectal cancer tissues before and after nCRT. The mean relative expression of hsa-miR-18a-5p in tumor tissue was 1.006±1.758 before therapy, and 0.255±0.213 after therapy (values are presented as 2^{-dCt}). The relative expression of hsa-miR-18a-5p in tumor tissue before nCRT was 3.93-fold higher than after treatment (P=0.001, Wilcoxon test) (Fig. 1A, B). In contrast, no significant change was observed in the expression of hsa-miR-135b-5p in tumor tissue before vs. after nCRT (P=0.114, Wilcoxon test) (Fig. 1C, D). No significant correlation was observed between pre- and post-nCRT hsa-miR-18a-5p expression in tumor tissue, nor for hsa-miR-135b-5p (Fig. 2). There was a significant negative correlation between the expression of hsa-miR-18a-5p in tumor tissue before nCRT and hsa-miR-135b-5p expression in tumor tissue after nCRT (Spearman's rho=-0.661, P=0.002); Fig. 2.

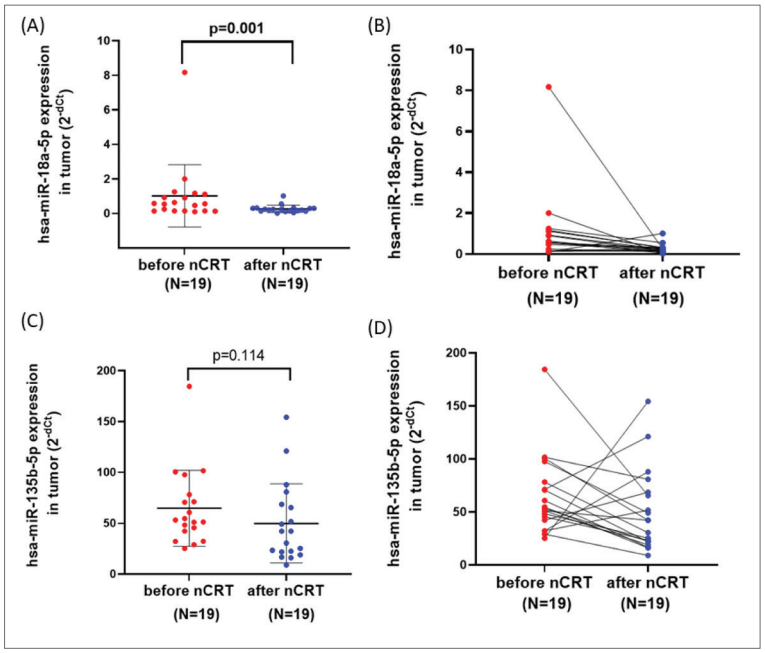


Fig. 1. Relative expression of hsa-miR-18a-5p and hsa-miR-135b-5p in tumor tissue before and after neoadjuvant chemoradiotherapy (nCRT) in patients with locally advanced rectal cancer. **A**, **C** – scatter plot; **B**, **D** – paired samples plot. Data are presented as the mean±standard deviation of the 2^{-dCt} value. Expression of hsa-miR-18a-5p and hsa-miR-135b-5p is normalized to RNU6B endogenous control. N – number of patients

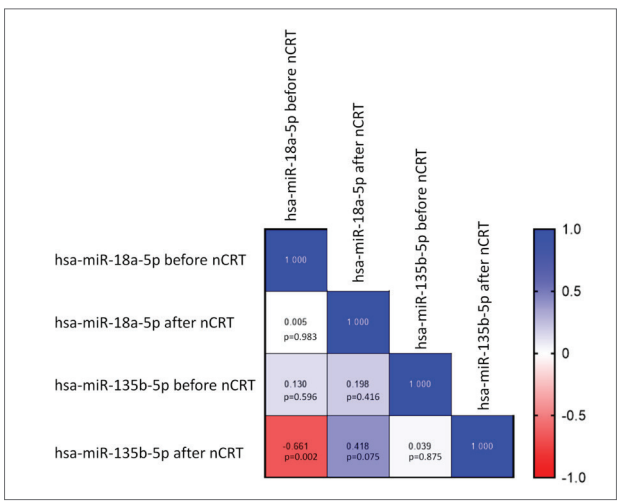


Fig. 2. Correlation matrix of hsa-miR-18a-5p and hsa-miR-135b-5p relative expression in tumor tissue before and after neoadjuvant chemoradiotherapy (nCRT) in patients with locally advanced rectal cancer. Numbers in squares are the correlation coefficient (Spearman rho factor).

Association of hsa-miR-18a-5p and hsa-miR-135b-5p expression with demographic and clinicopathological characteristics and overall survival

The relative expression of hsa-miR-18a-5p and hsa-miR-135b-5p was classified as low or high based on the median values. Among the 19 LARC patients, 10 (52.6%) exhibited decreased pre-treatment expression of hsa-miR-18a-5p and hsa-miR-135b-5p, while 9 (47.4%) showed increased expression of both. Post-nCRT, the distribution was similar, with 10 patients (52.6%) remaining decreased and 9 patients (47.4%) increased for both miRNAs.

Table 2. shows the association between the relative expression of hsa-miR-18a-5p and hsa-miR-135b-5p in tumor tissue before and after nCRT with demographic and clinicopathological characteristics. Hsa-miR-135b-5p expression before nCRT was significantly associated with age (P=0.012), whereas the expression of hsa-miR-18a-5p was associated with pathological disease stage (P=0.009) and lymph nodes metastasis (P=0.009). Specifically, stage I and II LARC patients without lymph node metastases were more likely to have higher hsa-miR-18a-5p expression before therapy. Additionally, hsa-miR-135b-5p expression after therapy was associated with pathological disease stage (P=0.009) and lymph node status (P=0.009). LARC patients with positive lymph node status and advanced disease (III stage) had higher hsa-miR-135b-5p expression after therapy. No significant association was observed between high or low expression of either miRNA and therapy response. There were no differences in overall survival between patients with high and low expression levels of hsa-miR-18a-5p and hsa-135b-5p, both before and after therapy (Supplementary Fig. S2)

Predictive potential of hsa-miR-18a-5p and hsa-miR-135b-5p expression in response to nCRT

The potential of using hsa-miR-18a-5p and hsa-miR-135b-5p as predictive biomarkers for pathological

Table 2. Association between the relative expression of hsa-miR-18a-5p and hsa-miR-135b-5p in rectal tumor tissue before and after nCRT with demographic and clinicopathological characteristics of LARC patients.

Demographic and clinicopathological data		Relative expression before nCRT						Relative expression after nCRT					
		hsa-miR-18a-5p		P	hsa-miR-135b-5p		P	hsa-miR-18a-5p		P	hsa-miR-135b-5p		P
		low N / %	high N / %		low N / %	high N / %		low N / %	high N / %		low N / %	high N / %	
Sex	female	5 / 50	3 / 33.3	0.463	5 / 50	3 / 33.3	0.463	3 / 30	5 / 55.6	0.260	2 / 22.2	6 / 60	0.096
	male	5 / 50	6 / 66.7		5 / 50	6 / 66.7		7 / 70	4 / 44.4		7 / 77.8	4 / 40	
Age (years, median)	<66	7 / 70	3 / 33.3	0.110	8 / 80	2 / 22.2	**0.012**	6 / 60	4 / 44.4	0.498	4 / 44.4	6 / 60	0.498
	>66	3 / 30	6 / 66.7		2 / 20	7 / 77.8		4 / 40	5 / 55.6		5 / 55.6	4 / 40	
Clinical T stage	T3	7 / 70	6 / 66.7	0.876	8 / 80	5 / 55.6	0.252	8 / 80	5 / 55.6	0.252	6 / 66.7	7 / 70	0.876
	T4	3 / 30	3 / 33.3		2 / 20	4 / 44.4		2 / 20	4 / 44.4		3 / 33.3	3 / 30	
Clinical N stage	N1	3 / 30	2 / 22.2	0.701	2 / 20	3 / 33.3	0.510	2 / 20	3 / 33.3	0.510	1 / 11.1	4 / 40	0.153
	N2	7 / 70	7 / 77.8		8 / 80	6 / 66.7		8 / 80	6 / 66.7		8 / 88.9	6 / 60	
Pathological T stage	T1+T2	2 / 20	2 / 33.3	0.510	2 / 20	2 / 33.3	0.510	3 / 30	2 / 22.2	0.701	3 / 33.3	2 / 20	0.510
	T3+T4	8 / 80	6 / 66.7		8 / 80	6 / 66.7		7 / 70	7 / 77.8		6 / 66.7	8 / 80	
Pathological N stage	N0	3 / 30	8 / 88.9	**0.009**	4 / 40	7 / 77.8	0.096	5 / 50	6 / 66.7	0.463	8 / 88.9	3 / 30	**0.009**
	N1+N2	7 / 70	1 / 11.1		6 / 60	2 / 22.2		5 / 50	3 / 33.3		1 / 11.1	7 / 70	
Pathological disease stage	0+I+II	3 / 30	8 / 88.9	**0.009**	4 / 40	7 / 77.8	0.096	5 / 50	6 / 66.7	0.463	8 / 88.9	3 / 30	**0.009**
	III	7 / 70	1 / 11.1		6 / 60	2 / 22.2		5 / 50	3 / 33.3		1 / 11.1	7 / 70	
Response to therapy	responders (TRG1+TRG2)	2 / 20	0 / 0	0.156	1 / 10	1 / 11.1	0.937	1 / 10	1 / 11.1	0.937	1 / 11.1	1 / 10	0.937
	non-responders (TRG3+TRG4)	8 / 80	9 / 100		9 / 90	8 / 88.9		9 / 90	8 / 88.9		8 / 88.9	9 / 90	

N – total number of LARC patients, nCRT – neoadjuvant chemoradiotherapy, TRG – tumor regression grade, P<0.05 is in bold. Low and high levels of relative expression refer to higher or lower than median (hsa-miR-18a-5p: median - 0.554, hsa-miR-135b-5p: median — 53.206. Median values are reported as 2^{-dCt}).

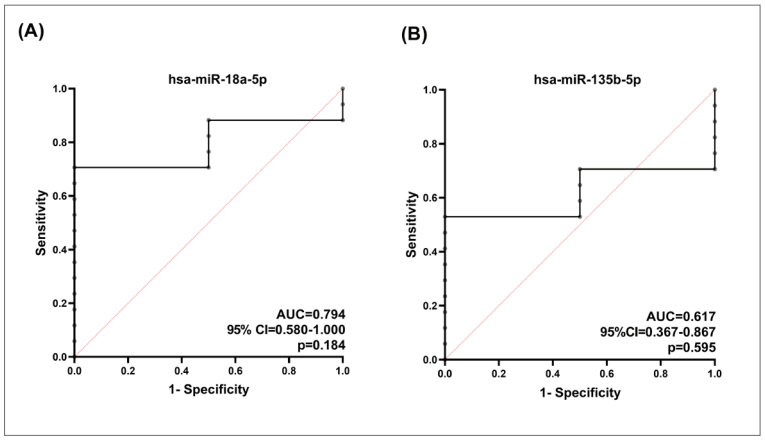
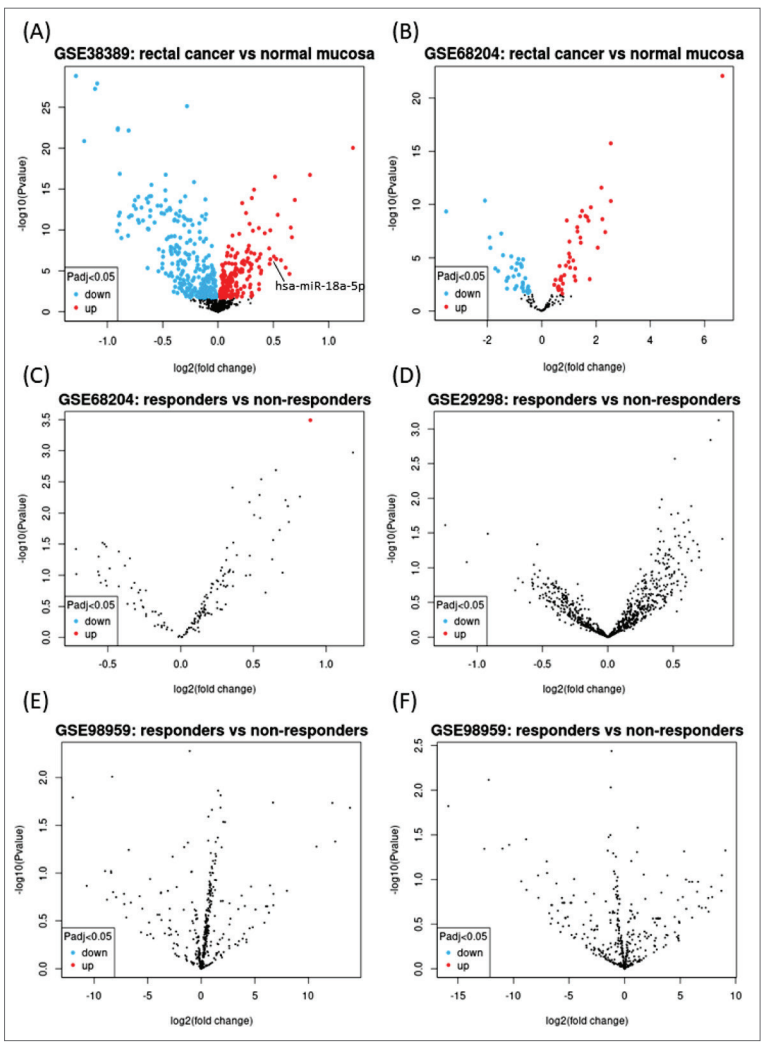


Fig. 3. Receiver operating curve – predictive potential of hsa-miR-18a-5p (**A**) and hsa-miR-135b-5p (**B**) in distinguishing responders from non-responders to neoadjuvant chemoradiotherapy in locally advanced rectal cancer patients. Relative expression of hsa-miR-18a-5p and hsa-miR-135b-5p in tumor tissue before therapy was used in the analysis. AUC – area under the curve, 95% CI – 95% confidence interval.



response to therapy was evaluated using ROC analysis. Neither miRNA was identified as a reliable molecular biomarker for predicting response to nCRT (hsa-miR-18a-5p: AUC=0.794, 95% CI=0.580-1.000, P=0.184; hsa-miR-135b-5p: AUC=0.617, 95% CI=0.367-0.867, P=0.595) (Fig. 3).

Public database analysis

We investigated the expression of hsa-miR-18a-5p and hsa-miR-135b-5p in LARC using four publicly available GEO datasets (GSE38389, GSE68204, GSE29298, and GSE98959) via GEO2R. Detailed information on the analyzed datasets is provided in Supplementary Table S2. In the GSE68204 dataset, neither miRNA showed differential expression between tumor and adjacent normal rectal mucosa (Fig. 4). Interestingly, in the GSE38389 dataset, hsa-miR-18a-5p was moderately overexpressed in tumor tissue compared to normal tissue (LogFC=0.4727, adjusted P=3.10e-06), suggesting potential tumor-related upregulation. However, hsa-miR-135b-5p did not show significant changes in this dataset. In GSE68204, GSE29298, and GSE98959 datasets, consistent with our experimental findings, the expression of both hsa-miR-18a-5p and hsa-miR-135b-5p did not differ between therapy responders and non-responders. These results indicate that while hsa-miR-18a-5p may be upregulated in tumor tissue in

Fig. 4. Volcano plots of differentially expressed miRNAs from analyzed public datasets. Significantly downregulated miRNAs are depicted as blue dots, significantly upregulated miRNAs as red dots, and non-significant miRNAs as black dots. The volcano plots were generated using GEO2R. **E** – results for Pool **A** from the GSE98959 dataset; **F** – results for Pool **B** from the GSE98959 dataset. In all datasets analyzed, responders were classified as TRG1 or TRG2, whereas non-responders were classified as TRG3, TRG4, or TRG5. Padj – adjusted P<0.05 was considered significant.

some datasets, neither hsa-miR-18a-5p nor hsa-miR-135b-5p correlates with therapy response in LARC across the analyzed cohorts.

DISCUSSION

The search for accurate and reliable molecular biomarkers with predictive value in LARC remains an unmet clinical need [24]. Consequently, considerable research efforts have focused on identifying clinically useful biomarkers. Since miRNAs are promising candidates, in this study, we focused on hsa-miR-18a-5p and hsa-miR-135b-5p, their changes in expression due to nCRT, as well as their predictive potential. Previous studies have shown that various staging elements and treatment-related variables influence outcomes in LARC patients treated with nCRT and surgery, with emphasis on the prognostic value of pathological over clinical stage [25,26]. Given these findings and the frequent discrepancies between pre-treatment clinical and post-treatment pathological stages, we evaluated pathological tumor response along with both clinical and pathological stages in relation to hsa-miR-18a-5p and hsa-miR-135b-5p expression.

Our results demonstrated a significant decrease in hsa-miR-18a-5p expression in the tumor tissue of LARC patients following nCRT. This finding suggests that hsa-miR-18a-5p may represent a potential therapeutic target in LARC tumor tissue. The observed decrease in hsa-miR-18a-5p after nCRT may partly reflect reduced tumor cellularity, as post-treatment histologic changes such as fibrosis, stromal remodeling, and inflammatory infiltration can reduce the relative contribution of tumor-derived miRNA within bulk tissue. However, no significant differences in hsa-miR-18a-5p expression were observed when comparing post-treatment tumor tissues with different histologic regression grades (TRG 1-2 versus TRG 3-4), despite the expected differences in residual tumor content. Taken together, these findings indicate that factors beyond tumor cellularity may contribute to the observed decrease in hsa-miR-18a-5p. The expression of hsa-miR-18a-5p was also increased in oral cancer [27]. This supports the role of hsa-miR-18a-5p as an oncomir. However, a comprehensive review of previously published studies has shown that this miRNA may have a dual function, depending on the context [16]. To date, there are no data in the literature about the predictive potential of hsa-miR-18a-5p for nCRT in LARC. Although the expression decreases after nCRT, it does not appear to predict treatment response in LARC patients, which is in accordance with the results from public dataset analysis. In our study, hsa-miR-18a-5p expression before nCRT was significantly associated with pathological disease stage and nodal status. hsa-miR-18a-5p expression may be linked to disease downstaging, as higher pre-treatment levels were associated with lower post-treatment pathological disease stage and absence of lymph node metastasis. This observation suggests a potential predictive role for hsa-miR-18a-5p, despite its expression not being significantly associated with tumor regression grade.

In our study, there was no significant difference in the relative expression of hsa-miR-135b-5p before and after nCRT, but slightly higher levels of expression were observed before nCRT. In other malignancies, hsa-miR-135b-5p is upregulated compared to healthy tissue, as observed in oral cancer [27], suggesting an oncogenic role for this miRNA. hsa-miR-135b was reported to be upregulated in rectal adenocarcinoma tissue in incomplete responders to nCRT compared to healthy mucosa [28].

The significant negative correlation between the expression of hsa-miR-18a-5p in tumor tissue before nCRT and hsa-miR-135b-5p after nCRT suggests an inverse relationship between these two microRNAs during treatment. This finding may reflect a dynamic regulatory mechanism in which changes in hsa-miR-18a-5p expression before nCRT affect hsa-miR-135b-5p expression after treatment, potentially impacting tumor response to therapy. Further studies are needed to elucidate the biological mechanisms underlying this interaction.

The mechanism underlying the effects of chemoradiotherapy on hsa-miR-18a-5p changes is not clear. While some research has investigated miRNA changes following 5-FU and radiation, hsa-miR-18a-5p and hsa-miR-135b-5p remain understudied. One study reported the altered expression of five miRNAs (hsa-miR-223-3p, hsa-miR-20a-5p, hsa-miR-17-5p, hsa-miR-19a-3p, and hsa-miR-7-5p) after 3 months of 5-FU therapy in CRC patients, with levels returning to baseline or increasing after six months in responders

compared to non-responders [29]. In lung cancer cells, hsa-miR-18a-5p increases radiosensitivity by downregulating *ATM* and *HIF-1α* expression [30]. Upregulated hsa-miR-135b-5p in CRC was associated with chemoresistance by targeting ST6GALNAC2 via the PI3K/AKT pathway [31]. Further research is needed to clarify the mechanisms by which chemoradiotherapy affects miRNA expression.

Potential miRNAs, including circulating miRNAs, identified as biomarkers of nCRT response in LARC patients have been previously reviewed [32]. Numerous miRNAs have been found to be differentially regulated between responders and non-responders among rectal cancer patients [5]. Among these, hsa-miR-21 has been confirmed as a predictor of nCRT response in LARC patients [33]. Previous studies reported upregulation of miR-215, miR-190b, and miR-29b-2* in non-responders, whereas let-7e, miR-196b, miR-450a, miR-450b-5p, and miR-99a* were downregulated in responders to nCRT [34]. A panel of 8 miRNAs (hsa-miR-320a, hsa-miR-1260a, hsa-miR-30e-5p, hsa-miR-33a-5p, hsa-miR-338-3p, hsa-miR-130a-5p, hsa-miR-210-3p, hsa-miR-214-3p) in liquid biopsy (plasma) was identified to accurately predict the response to nCRT [35]. Previously, 11 miRNAs (miR-1183, miR-483-5p, miR-622, miR-125a-3p, miR-1224-5p, miR-188-5p, miR-1471, miR-671-5p, miR-1909*, miR-630, miR-765) were shown to be predictors of response to nCRT in LARC patients [22]. Serum miR-199 expression was identified as a predictor of poor pathologic response to nCRT [36]. Even though we have only mentioned a few of the studies carried out so far, it is obvious that the results in the literature are inconsistent. To date, there are no data on the predictive potential of hsa-miR-18a-5p and hsa-miR-135b-5p in LARC. Although not statistically significant, our results contribute to the growing body of knowledge in the search for clinically relevant miRNA biomarkers.

Expecting a single biomarker to accurately stratify LARC patients by nCRT response is unrealistic; a combination of multiple biomolecules is more likely to be clinically useful [37]. Therefore, it is crucial to evaluate multiple molecular biomarkers and consider combining them with established markers, such as the blood biomarkers CEA and CA-19, to enhance predictive and prognostic accuracy. In addition, previous studies have combined miR-145 expression with radiomic features of LARC patients and have shown an association with complete clinical response after nCRT [38]. Integrating miRNA profiles with clinical, radiomic, genomic, and, where available, proteomic data could provide deeper biological insight and enhance multimodal prediction of treatment response. Such an approach could strengthen predictive models and should be considered in future studies.

One limitation of our study is the small sample size. Additionally, the low number of patients with favorable pathological responses (TRG1 and TRG2) presents a further constraint. This imbalance complicates robust ROC analysis, which is sensitive to unequal category distributions. Consequently, the predictive potential results from the ROC analysis should be interpreted with caution. The small sample size reflects strict eligibility criteria, the logistical difficulty of obtaining paired pre- and post-nCRT specimens, exacerbated by some patients transferring to other treatment centers after initial biopsy and diagnosis, and the additional difficulties of sample collection during the COVID-19 pandemic. One of the limitations when comparing our study with other studies is that partial responders (TRG2-3) are sometimes classified as TRG3 and considered non-responders, as in our study. In addition to analyzing miRNA candidate expression in tumor tissue before and after nCRT, future studies should include liquid biopsies, such as plasma or serum, to evaluate circulating miRNA biomarkers in LARC patients. Furthermore, we did not compare tumor tissue expression of hsa-miR-18a-5p and hsa-miR-135b-5p with healthy mucosa from the same patients, which is recommended for monitoring nCRT-induced changes in miRNA expression.

Our results show that the expression of hsa-miR-18a-5p significantly decreases in LARC patients after nCRT. Additionally, this study revealed significant associations between miRNA expression and clinicopathological features of LARC. Specifically, higher pre-treatment expression of hsa-miR-18a-5p was linked with a lower post-treatment pathological stage and absence of lymph node metastasis, suggesting a potential predictive role in LARC. Conversely, elevated post-treatment levels of hsa-miR-135b-5p were associated with advanced disease stage and lymph node positivity, indicating a possible connection with residual tumor burden or poor treatment response. However, neither hsa-miR-18a-5p nor hsa-miR-135b-5p showed

a statistically significant association with therapy response, limiting their utility as predictive biomarkers for nCRT efficacy in clinical practice. The role of hsa-miR-18a-5p warrants further investigation as a potential therapeutic target for chemoradiotherapy, given its treatment-related expression changes. These results emphasize the importance of ongoing research into reliable molecular biomarkers for predicting nCRT efficacy in LARC patients.

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Conflict of interest disclosure: The authors declare no conflicts of interest.

Data availability: The data underlying the reported findings have been provided as a raw dataset available here: https://www.serbiosoc.org.rs/NewUploads/Uploads/Rankovic%20et%20al_Research%20Dataset.pdf.

Publicly available GEO datasets were analyzed in this study; this data can be found here: https://www.ncbi.nlm.nih.gov/geo/

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SUPPLEMENTARY MATERIAL

Supplementary Table S1. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enriched in target genes of the hsa-miR-18a-5p and hsa-miR-135b-5p networks

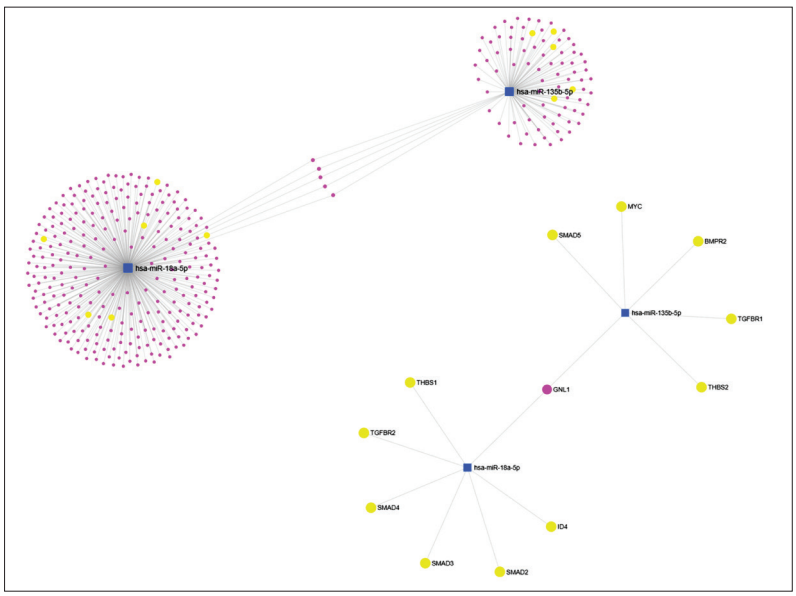
KEGG	Target genes (N)	P	Adjusted P
Pathways in cancer	24	**1.00E-07**	**0.00000595**
Colorectal cancer	10	**1.19e-7**	**0.00000595**
TGF-beta signaling pathway	11	**0.00000295**	**0.0000983**
Chronic myeloid leukemia	9	**0.0000405**	**0.000936**
HTLV-I infection	15	**0.0000468**	**0.000936**
Measles	10	**0.00011**	**0.00176**
Cell cycle	11	**0.000123**	**0.00176**
Pancreatic cancer	8	**0.000172**	**0.00215**
Chagas disease (American trypanosomiasis)	8	**0.000987**	**0.011**
Acute myeloid leukemia	6	**0.00192**	**0.0179**
Jak-STAT signaling pathway	8	**0.00197**	**0.0179**
Small cell lung cancer	7	**0.0024**	**0.02**
MAPK signaling pathway	14	**0.00308**	**0.0237**
Prostate cancer	7	**0.00387**	**0.0276**
Bladder cancer	4	**0.00422**	**0.0281**
p53 signaling pathway	6	**0.00471**	**0.0294**
Osteoclast differentiation	8	**0.00616**	**0.0357**
Focal adhesion	11	**0.00642**	**0.0357**
Hepatitis C	7	**0.00828**	**0.0436**

N – number of genes, P<0.05 is shown in bold

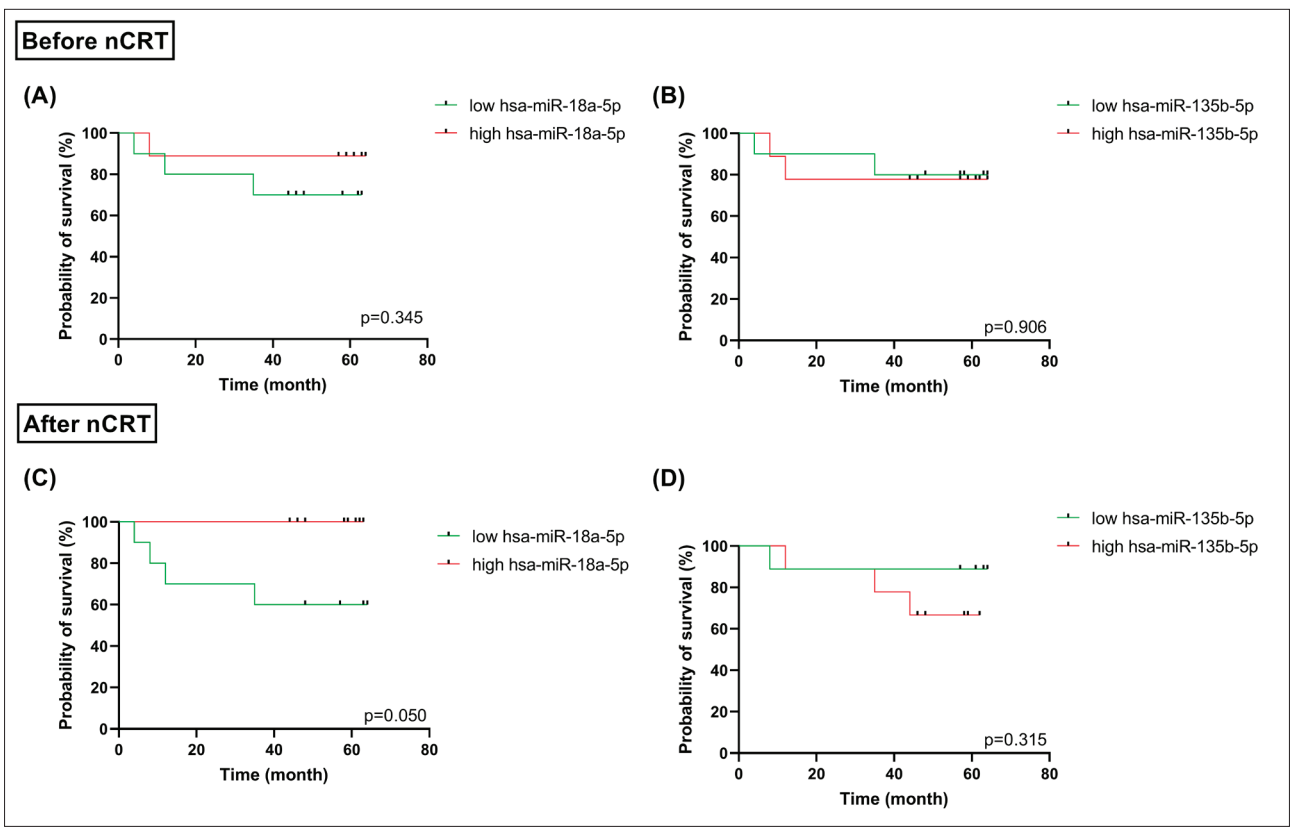
Supplementary Table S2. Detailed information on publicly available datasets from the National Centre for Biotechnology Information Gene Expression Omnibus database.

Dataset	Total number of samples	Number of responders (TRG1+2)	Number of non-responders (TRG3+4+5)	Normal samples	Method used	Reference	Comment
GSE38389	140	/	/	71 normal rectal mucosa 69 rectal cancer	LNA™ (Locked Nucleic Acid) enhanced miRCURY™ microarrays (Exiqon, Vedbaek, Denmark)	[18]	2 technical replicates were considered in the analysis
GSE68204	45	16	21	8	Agilent-021827 Human miRNA Microarray (V3)	[21]	Platform GLP10850 contains data on nCRT responders and non-responders
GSE29298	38	25	13	/	Microarray	[22]	/
GSE98959	22	8 (Pool A) 10 (Pool B)	14 (Pool A) 12 (Pool B)	/	TaqMan® OpenArray® MicroRNA Plates (Life Technologies, USA)	[23]	The dataset comprises two sample pools, each containing 22 samples: Pool A (8 responders and 14 non-responders) and Pool B (10 responders and 12 non-responders)

In all datasets analyzed, responders were classified as TRG1 or TRG2, and non-responders as TRG3, TRG4, or TRG5.



Supplementary Fig. S1. hsa-miR-18a-5p and hsa-miR-135b-5p gene interaction network. miRNAs are shown as blue squares, target genes as purple and yellow circles. The yellow circles represent genes involved in a significantly enriched TGF-beta signaling pathway.



Supplementary Fig. S2. Kaplan-Meier curves of overall survival in patients with locally advanced rectal cancer depending on hsa-miR-18a-5p and hsa-miR-135b-5p expression levels before and after neoadjuvant chemoradiotherapy. **A**, **B** – before neoadjuvant chemoradiotherapy (nCRT); **C**, **D** – after nCRT. Low and high levels of relative expression refer to higher/lower than the median (hsa-miR-18a-5p: median – 0.554, hsa-miR-135b-5p: median – 53.206; median values are reported as 2^{-dCt}).