

## Downregulation of miR-193a-5p correlates with lymph node metastasis and poor prognosis in colorectal cancer

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### Abstract

**AIM:** To investigate the correlation of miR-193a-5p with lymph node metastasis and postoperative survival of colorectal cancer (CRC) patients.

**METHODS:** A total of 304 formalin-fixed, paraffin-embedded specimens (69 paired cancer and normal tissues, 55 primary tumors of stage III CRC and matched lymph nodes, and 56 primary tumors of stage II CRC) were included in this study. The relative expression lev-

els of miR-193a-5p in the normal mucosa, primary cancer, and metastatic lymph node lesions were measured by quantitative real-time reverse transcriptase polymerase chain reaction. We evaluated the association of its expression with colorectal cancer lymph node metastasis, clinicopathological factors, and patient survival.

**RESULTS:** The relative expression level of miR-193a-5p was significantly lower in CRC tissues than in the normal mucosa ( $P = 0.0060$ ). The expression levels of miR-193a-5p were lower in primary CRC tissues with lymph node metastases than in those without metastases ( $P = 0.0006$ ), and decreased expression of miR-193a-5p correlated with advanced lymph node metastatic stage ( $P = 0.0007$ ). Kaplan-Meier analysis showed that patients with low miR-193a-5p expression had decreased disease-free survival (DFS) ( $P = 0.0026$ ) and poor overall survival (OS) ( $P = 0.0003$ ). Interestingly, for the group of patients with lymph node metastases, miR-193a-5p expression was also related to survival. Patients with low miR-193a-5p expression had decreased DFS ( $P = 0.0262$ ) and poor OS ( $P = 0.0230$ ). Moreover, multivariate analysis indicated that downregulation of miR-193a-5p was an independent predictor of poor OS.

**CONCLUSION:** Downregulation of miR-193a-5p correlates with lymph node metastasis and poor survival of CRC. miR-193a-5p may be a useful biomarker for CRC diagnosis, metastasis and prognosis prediction.

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**Key words:** miR-193a-5p; Colorectal cancer; Lymph node metastasis; Prognosis; Biomarker

**Core tip:** We determined the expression of miR-193a-5p in colorectal cancer (CRC) tissues and metastatic lesions in the lymph nodes using quantitative real-time reverse transcriptase polymerase chain reaction. Downregulation of miR-193a-5p correlated with tumor

progression, lymph node metastasis and poor survival in CRC patients. These findings suggest that miR-193a-5p could be used as a molecular biomarker of diagnosis, early-stage metastasis prediction, and prognosis forecast for CRC. This is believed to be the first study to investigate the relationship between miR-193a-5p expression and lymph node metastasis in CRC.

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## INTRODUCTION

Colorectal cancer (CRC) is one of the three leading causes of cancer-related death worldwide, with approximately 1200000 new cases and 600000 deaths annually<sup>[1]</sup>. In China, the incidence and mortality from CRC have increased rapidly in the past several decades<sup>[2]</sup>, with death generally resulting from tumor recurrence or metastasis<sup>[3]</sup>. Metastasis to regional lymph nodes plays a critical role in CRC tumor progression; it affects prognosis<sup>[4]</sup>, and occurs commonly in early-stage metastasis. Moreover, lymph node involvement often promotes further hematogenous metastasis<sup>[5]</sup>. After radical surgery, the 5-year survival rate of patients with early-stage CRC is > 90%, but the 5-year survival rate of advanced-stage patients is < 10%<sup>[6]</sup>. Although some genes related to lymph node metastasis have been reported, the molecular mechanisms of early-stage metastasis in CRC are still unclear<sup>[7-9]</sup>. Thus, identification of biomarkers associated with lymph node metastasis of CRC will benefit clinical evaluation.

MicroRNAs are a class of small noncoding RNA molecules that function by targeting 3'-untranslated regions<sup>[10]</sup>, thus altering gene expression. It has been reported that they are involved in various biological processes, including differentiation and tumorigenesis<sup>[11]</sup>. Some studies have demonstrated that miRNAs also participate in lymph node metastasis of human cancer<sup>[12-15]</sup>. As a member of the miR-193 family, miR-193a plays a critical role in cancer progression. In general, its expression has been reported to decrease in human cancers, and its downregulation correlates with advanced progression<sup>[16,17]</sup>. A recent study reported downregulation of miR-193a-5p expression in an advanced human CRC cell line SW620 established from a metastatic lymph node<sup>[18]</sup>, and it showed that miR-193a-5p may participate in lymph node metastasis of CRC. However, correlative studies following the expression of miR-193a-5p in CRC tissues or metastatic lymph node lesions (MLNLS) have not been reported.

In this study, we investigated the expression of miR-193a-5p in CRC tissues, normal mucosa and MLNLS,

and analyzed the association of its expression with lymph node metastasis and clinical prognosis.

## MATERIALS AND METHODS

### Specimens and clinical data collection

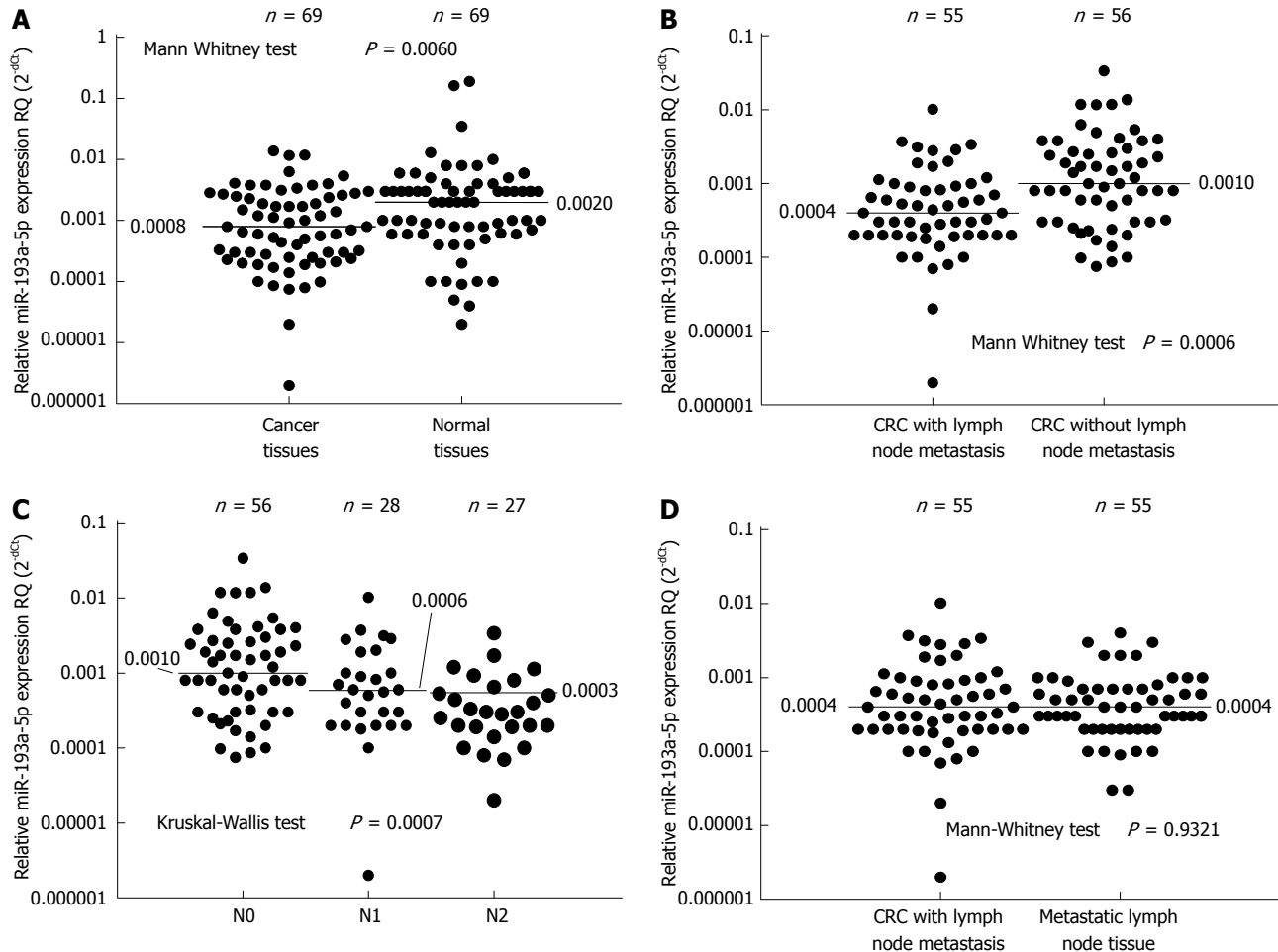
It was difficult to obtain blood samples from the patients, so we chose formalin-fixed, paraffin-embedded (FFPE) specimens for this study. A total of 304 FFPE specimens, including 69 paired cancer and normal tissues, 55 primary tumors of stage III CRC and matched lymph nodes, and 56 primary tumors of stage II CRC, were collected from 111 patients who underwent surgical resection at Beijing Cancer Hospital between January 2006 and October 2007. Patients did not receive any preoperative treatment, in particular, radiotherapy or chemotherapy. After excision, tissue specimens were sectioned for subsequent analysis. The samples were stained with hematoxylin and eosin and examined histopathologically. Sections containing > 90% carcinoma cells were used to prepare total RNA. Complete clinicopathological data were collected for each patient. All patients signed informed consent forms. This study was approved by the Medical Ethics Committee of Beijing Cancer Hospital.

### Real-time quantitative reverse transcriptase polymerase chain reaction

Total RNA was extracted from cancer tissues, normal mucosa and paired lymph nodes using the miRNeasy FFPE Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. For real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), we used TaqMan Reverse Transcription Reagents followed by PCR Master Mix (Haoqin Biotechnology, Shanghai, China). All reactions were run in triplicate on an ABI7500 PCR machine using miR-193a-5p specific primers (Haoqin Biotechnology). Following an initial denaturation at 95 °C for 10 min, real-time qRT-PCR cycling parameters included 40 cycles of denaturation at 95 °C for 15 s, annealing at 57 °C for 30 s, and final extension at 72 °C for 30 s. The comparative cycle threshold (Ct) method was used to calculate the expression levels of miR-193a-5p, and U6 small nuclear RNA was used as an internal reference. The relative expression of miR-193a-5p to U6 was determined using the equation  $2^{-\Delta Ct}$ , where  $\Delta Ct = C_{tmiR-193a-5p} - C_{tU6}$ <sup>[19]</sup>.

### Statistical analysis

miR-193a-5p expression in CRC was compared with expression in normal mucosa or lymph nodes using the Mann-Whitney test (for two groups) or the Kruskal-Wallis test (for more than two groups). Measurement data were analyzed using Student's *t* test, while categorical data were studied using the  $\chi^2$  test. We analyzed the post-operative survival rate using the Kaplan-Meier method and performed a log-rank test to assess the differences in survival rates. Multivariate analysis was performed using a Cox regression model. SPSS version 13.0 (SPSS, Chicago,



**Figure 1** Expression level of miR-193a-5p in colorectal cancer, metastatic lymph node and normal tissues. A: Comparison of miR-193a-5p expression between colorectal cancer and normal mucosa in 69 paired samples. miR-193a-5p expression levels were higher in normal tissues than in cancer tissues ( $P = 0.0060$ ); B: Comparison of miR-193a-5p expression in primary colorectal cancer (CRC) tissue with and without lymph node metastasis. miR-193a-5p expression levels were significantly lower in primary CRC with lymph node metastasis than without lymph node metastasis ( $P = 0.0006$ ); C: Correlation of miR-193a-5p expression in primary CRC with lymph node stage (N0/N1/N2). Decreased expression of miR-193a-5p was associated with advanced lymph node stage ( $P = 0.0007$ ); D: Association of miR-193a-5p expression in colorectal cancer with paired metastatic lymph nodes. No significant difference of miR-193a-5p expression was observed between colorectal cancer and paired metastatic lymph nodes ( $P = 0.9321$ ). N: Lymph node stage; N0: Stage N0; N1: Stage N1; N2: Stage N2.

IL, United States) was used for all statistical analyses. We used two-sided  $P$  values and considered  $< 0.05$  to be statistically significant.

## RESULTS

### Expression of miR-193a-5p decreases in primary CRC

To test the effect of miR-193a-5p on tumor progression, the expression levels of miR-193a-5p were measured in cancer tissues and paired normal tissues from 69 patients with CRC. As shown in Figure 1A, miR-193a-5p expression was significantly decreased in cancer tissues to 40% of that in the matched normal mucosa ( $P = 0.0060$ ). These data suggest that miR-193a-5p functions as a tumor suppressor to prevent progression of CRC.

### Expression of miR-193a-5p decreases with early-stage metastasis and with advanced lymph node metastatic stage

To determine the association of miR-193a-5p with early-

stage metastasis, we analyzed the expression level of miR-193a-5p in primary CRC with and without lymph node metastasis. As shown in Figure 1B, miR-193a-5p expression levels in primary CRC tissues with lymph node metastasis were significantly decreased to 40% of those without metastasis ( $P = 0.0006$ ). In addition, downregulation of miR-193a-5p significantly correlated with increasing lymph node stage. miR-193a-5p expression decreased progressively, from 100% (stage N0) to 60% (stage N1) and 30% (stage N2) ( $P = 0.0007$ ) (Figure 1C). These data indicated that low expression of miR-193a-5p was associated with lymph node metastasis in CRC. We also quantified the expression levels of miR-193a-5p in MLNLs, but found no significant differences between primary cancer tissues and MLNLs ( $P = 0.9321$ ) (Figure 1D).

### Decreased expression of miR-193a-5p correlates with venous invasion and lymph node metastasis

To evaluate the relationship between expression levels of miR-193a-5p and clinicopathological factors of CRC, we

**Table 1** Correlation between miR-193a-5p expression and clinicopathological factors of colorectal cancer

Variable	miR-193a-5p expression		P value
	Low (n = 56)	High (n = 55)	
Age (yr)	61.9 ± 13.9	62.3 ± 11.9	0.298
Gender			0.924
Male	28	28	
Female	28	27	
Tumor size			0.768
≤ 5 cm	26	24	
> 5 cm	30	31	
Degree of differentiation			0.053
Well and moderate	38	46	
Poor	18	9	
Venous invasion			0.008 <sup>b</sup>
Negative	43	52	
Positive	13	3	
Depth of invasion			0.484
T1, T2	12	11	
T3, T4	44	44	
Lymph node metastasis			< 0.001 <sup>b</sup>
Absent	19	37	
Present	37	18	
Location			0.921
Colon	27	26	
Rectum	29	29	

<sup>b</sup>P < 0.01 vs control group. CRC: Colorectal cancer; T: Tumor.

classified its expression as low or high according to the median value. As shown in Table 1, the data indicated that decreased expression of miR-193a-5p was significantly associated with venous invasion ( $P = 0.008$ ) and lymph node metastasis ( $P < 0.001$ ) (Table 1). These data indicated that downregulation of miR-193a-5p was associated with malignant behavior of CRC.

### Downregulation of miR-193a-5p is associated with poor prognosis of CRC patients

To assess the correlation of miR-193a-5p with prognosis of CRC patients, we plotted disease-free survival (DFS) and overall survival (OS) curves using the Kaplan-Meier method. As shown in Figure 2, the DFS and OS rates were significantly lower in patients with low miR-193a-5p expression than in those with high miR-193a-5p expression ( $P = 0.0026$ ,  $P = 0.0003$ , respectively). A similar result was found in the group of patients with lymph node metastases. The DFS and OS rates were significantly lower in patients with low miR-193a-5p expression than in those with high miR-193a-5p expression ( $P = 0.0262$ ,  $P = 0.0230$ , respectively). Using univariate analysis of the Cox regression model, we identified four prognostic factors that had statistical significance: depth of invasion ( $P = 0.009$ ), venous invasion ( $P < 0.001$ ), lymph node metastasis ( $P < 0.001$ ), and miR-193a-5p expression levels ( $P = 0.004$ ) (Table 2). Moreover, multivariate analysis indicated that three of these prognostic factors were also independent predictors of poor survival in colorectal cancer: low miR-193a-5p expression levels (log-rank test,  $P = 0.031$ ), increased depth of invasion (log-rank test,

**Table 2** Univariate analysis of clinicopathological factors for overall survival of colorectal cancer patients

Variable	Cases	HR	95%CI	P value
Gender			0.582-1.874	0.885
Male	56	1		
Female	55	1.004		
Age (yr)			0.820-2.738	0.189
≤ 60	51	1		
> 60	60	1.498		
Tumor size			0.921-3.124	0.090
≤ 5 cm	50	1		
> 5 cm	61	1.696		
Differentiation			0.852-3.098	0.140
Well, moderate	84	1		
Poor	27	1.625		
Venous invasion			2.058-7.781	< 0.001 <sup>b</sup>
Negative	95	1		
Positive	16	4.002		
Depth of invasion			1.494-15.589	0.009 <sup>b</sup>
T1, T2	23	1		
T3, T4	88	4.826		
Location			0.673-1.945	0.619
Rectum	58	1		
Colon	53	1.144		
Lymph node metastasis			3.194-13.937	< 0.001 <sup>b</sup>
Absent	56	1		
Present	55	6.672		
miR-193a-5p expression			1.340-4.653	0.004 <sup>b</sup>
High	55	1		
Low	56	2.497		

<sup>b</sup>P < 0.01 vs control group. T: Tumor.

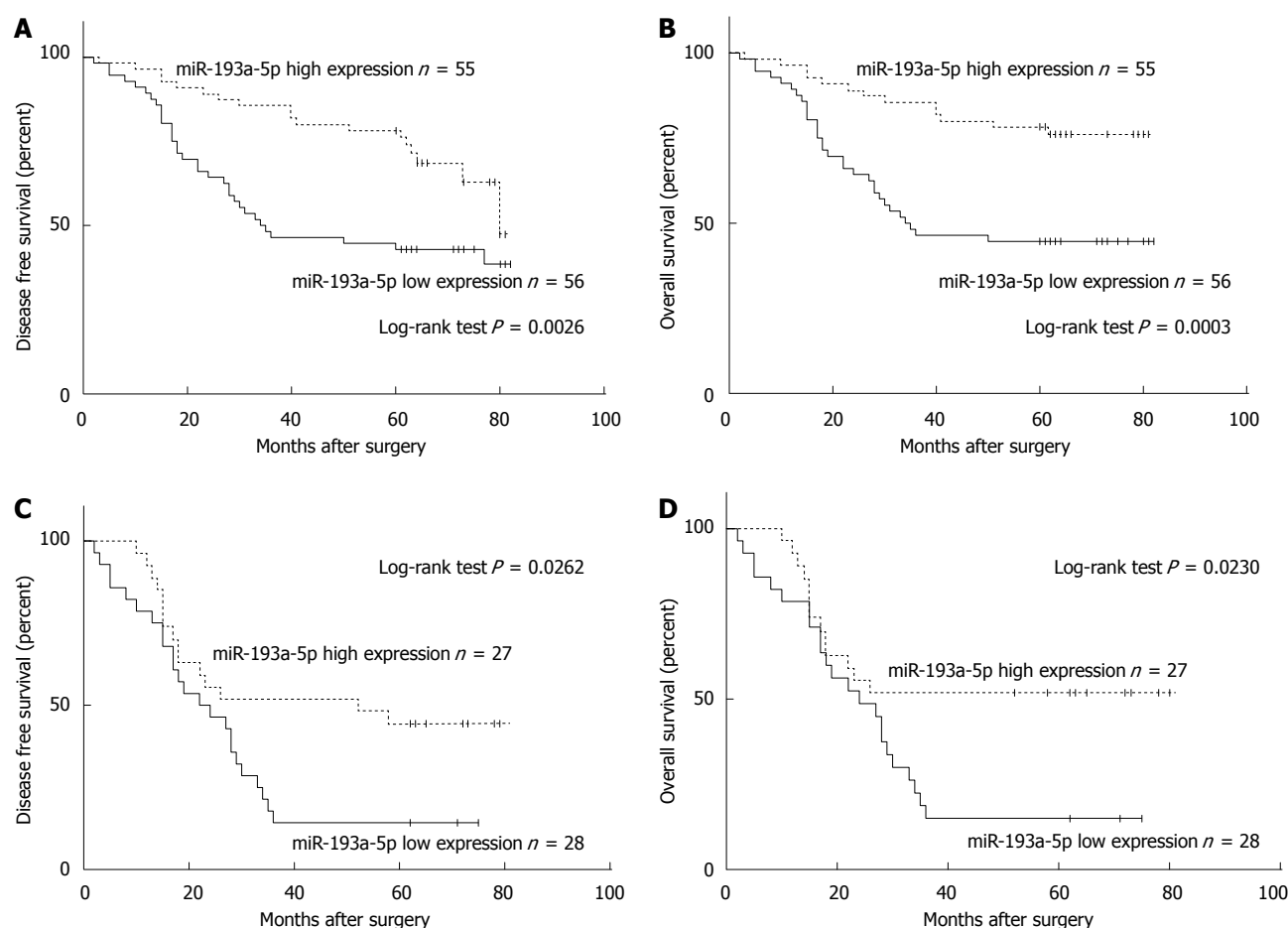
$P = 0.036$ ), and lymph node metastasis (log-rank test,  $P < 0.001$ ) (Table 3). Together, these data indicated that downregulation of miR-193a-5p was correlated with lymph node metastasis and poor survival.

## DISCUSSION

A growing number of studies have shown that miRNAs are involved in tumor progression and carcinogenesis<sup>[20-22]</sup>, and in recent years, there has been an increased focus on miR-193a. Some researchers have reported the downregulation of miR-193a in tumor tissues, with the suggestion that it functions as a tumor suppressor<sup>[17,23-26]</sup>. In agreement with these studies, we found that the expression of miR-193a-5p decreased in primary CRC relative to normal tissues, suggesting that decreased miR-193a-5p expression may correlate with tumor progression and carcinogenesis of CRC.

In contrast, several studies have reported higher expression of miR-193a in tumor cells<sup>[27,28]</sup>. This inconsistency may be explained by the variation in molecular pathways that exist in different types of cancer tissues. In addition, Yong *et al*<sup>[29]</sup> found upregulation of miR-193a expression levels in the blood samples compared with the cancer tissues of CRC patients. The different results are probably due to the different study design. Another reason could be the effect of local tumor microenvironment. It is well known that miRNAs modulate the tumor microenvironment *via* a variety of signaling networks<sup>[30-32]</sup>,





**Figure 2** Kaplan-Meier survival curves of patients with colorectal cancer based on miR-193a-5p expression. A: Kaplan-Meier disease free survival curves of patients with colorectal cancer (CRC) based on miR-193a-5p expression. Patients in the low expression group had significantly lower disease free survival rates than those in the high expression group (log-rank test,  $P = 0.0026$ ); B: Kaplan-Meier overall survival curves of patients with CRC based on miR-193a-5p expression. Patients in the low expression group had significantly lower overall survival rates than those in the high expression group (log-rank test,  $P = 0.0003$ ); C: Kaplan-Meier disease free survival curves of patients with lymph node metastases based on miR-193a-5p expression. Patients in the low expression group had significantly lower disease free survival rates than those in high expression group (log-rank test,  $P = 0.0262$ ); D: Kaplan-Meier overall survival curves of patients with lymph node metastases based on miR-193a-5p expression. Patients in the low expression group had significantly lower overall survival rates than those in high expression group (log-rank test,  $P = 0.0230$ ). N: Lymph node stage; N0: Stage N0; N1: Stage N1; N2: Stage N2.

but in addition, the microenvironment may also exert some effect on miRNA expression. Cismasiu *et al.*<sup>[33]</sup> reported that expression of miR-193 varies by the location of stromal cells, being more specific in blood vessels compared with other cells. We think that the influence of stromal signaling may affect miR-193a expression in cancer tissues and blood.

Recent findings have shown that the varied expression of several miRNAs correlates with CRC lymph node metastasis and prognosis, and that they may be useful as novel biomarkers for clinical prediction of early-stage metastasis and prognosis<sup>[15,34,35]</sup>. Another finding, indicating that decreased expression of miR-193a-5p correlates with early metastasis in other cancer systems<sup>[18]</sup>, suggests that miR-193a-5p is involved in early-stage metastasis of CRC. In this study, we quantified the expression of miR-193a-5p in primary CRC tissues. Our results demonstrated that miR-193a-5p expression was significantly downregulated in cancer tissues with positive lymph nodes relative to those with negative lymph nodes. These findings suggest that downregulation of miR-193a-5p

correlates with lymph node metastasis of CRC and support the previous study of Yu *et al.*<sup>[26]</sup>. He reported that miR-193a-3p and -5p could suppress metastasis in humans. In addition, we also investigated the expression of miR-193a-5p in MLNLs of CRC, but found no significant difference in expression between these and primary cancer tissues. These results indicate that the cancer cells in MLNLs came from the primary cancer lesions and had the same aggressive properties as the primary tumor.

We assessed the correlation between miR-193a-5p expression and clinicopathological factors and prognosis. Our results indicated that miR-193a-5p low expression was associated with venous invasion and lymph node metastasis, suggesting that miR-193a-5p is involved in the progression and early-stage metastasis of CRC. Moreover, using Kaplan-Meier survival curves, we demonstrated that higher miR-193a-5p expression was significantly correlated with increased DFS and OS of patients with CRC postoperatively. Conversely, lower miR-193a-5p expression was associated with poor OS, indicating that low miR-193a-5p expression level may be useful as a marker

**Table 3** Multivariate analysis of clinicopathological factors for overall survival of colorectal cancer patients

Variable	Cases	HR	95%CI	P value
Venous invasion			0.799-3.230	0.183
Negative	95	1		
Positive	16	1.607		
Depth of invasion			1.087-11.793	0.036 <sup>a</sup>
T1, T2	23	1		
T3, T4	88	3.58		
Lymph node metastasis			1.906-9.319	< 0.001 <sup>b</sup>
Absent	56	1		
Present	55	4.214		
miR-193a-5p expression			1.070-3.993	0.031 <sup>a</sup>
High	55	1		
Low	56	2.067		

<sup>a</sup>P < 0.05 *vs* control group; <sup>b</sup>P < 0.01 *vs* control group. T: Tumor.

of poor prognosis in CRC. In addition, we analyzed several clinicopathological factors by the Cox regression method. These results showed that miR-193a-5p is an independent prognostic indicator, suggesting that it could serve as a novel molecular marker of early-stage metastasis and prognosis for patients with CRC. By identifying patients who are at higher risk for early-stage metastasis and have a poor prognosis, they may be screened and followed closely.

The exact molecular mechanisms of miR-193a-5p involvement in early-stage metastasis of CRC have not been elucidated. In some studies, miR-193a-5p has been shown to inhibit tumor cell proliferation and transformation<sup>[36,37]</sup>. It was recently reported that miR-193a-5p suppresses tumor cell activity by targeting the transforming growth factor (TGF), Wnt and mitogen-activated protein kinase pathways<sup>[16]</sup>. Another study found that TGF promotes the progression of prostatic carcinoma by modulating p53 and YY1<sup>[38]</sup>. Other studies have shown that YY1 improves tumor cell growth of CRC by inhibiting p53 and activating the Wnt pathway<sup>[39]</sup>. More recently, Schwitalla *et al.*<sup>[40]</sup> have found that loss of p53 in enterocytes enables invasion and lymph node metastasis of colorectal tumors. We conjecture that miR-193a-5p may inhibit tumorigenesis and lymph node metastasis of CRC by targeting TGF and/or p53 and regulating YY1 expression by inactivation of the Wnt pathway. This may help to explain the downregulation of miR-193a-5p in primary CRC tissues with lymph node metastasis in the current study. The role of TGF in tumor progression is a double-edged sword and its function may depend on cancer progression<sup>[41-43]</sup>. This may partly explain the discordance of tumorigenic and anti-tumor roles of miR-193. We are planning additional studies to identify more precisely the molecular mechanisms of miR-193a-5p involvement in lymph node metastasis of CRC.

In conclusion, our studies confirm that miR-193a-5p expression is downregulated in CRC. In addition, downregulation of miR-193a-5p is correlated with lymph node metastasis and poor prognosis. We also found that miR-193a-5p is an independent prognostic factor. Identifica-

tion is economically feasible if it is applied clinically to CRC patients because just a trace of total RNA can be tested for miR-193a-5p. Together, these results suggest that miR-193a-5p is a useful novel biomarker of early-stage metastasis diagnosis and prognosis, and a promising therapeutic target in the treatment of CRC.

## COMMENTS

### Background

Colorectal cancer (CRC) is one of the three leading causes of cancer-related death worldwide. In general, patients die from tumor recurrence or metastasis. Metastasis to regional lymph nodes plays a critical role in CRC progression and occurs commonly in early-stage metastasis. MicroRNAs are a class of small noncoding RNA molecules that are involved in various biological processes, including tumorigenesis. Some studies have demonstrated that miRNAs also participate in lymph node metastasis of human cancer. A recent study has shown that miR-193a-5p may participate in lymph node metastasis of CRC. However, correlative studies following the expression of miR-193a-5p in CRC tissues or metastatic lymph node lesions (MLNLs) have not been reported.

### Research frontiers

Various studies have shown that miRNAs may be involved in lymph node metastasis of CRC. Although they analyzed their correlation with lymph node metastasis as a clinicopathological factor, no prior study has investigated miRNA expression in MLNLs. Although miR-193a-5p has shown variant expression in an advanced human CRC cell line SW620 established from a metastatic lymph node, no further study has been conducted.

### Innovations and breakthroughs

In this study, the authors found that downregulation of miR-193a-5p was correlated with tumor progression, lymph node metastasis and poor survival of CRC patients. The findings of the study are novel and of clinical significance. It is a promising strategy to use miR-193a-5p as a molecular biomarker for diagnosis, early-stage metastasis prediction, and prognosis forecast for CRC.

### Applications

The authors found that low expression of miR-193a-5p was associated with lymph node metastasis and poor prognosis of CRC patients. Therefore, by identifying patients who are at higher risk for early-stage metastasis and who have poor prognosis, they may be screened and followed closely. This study could provide a clue for clinical application of miRNAs to prevent early metastasis, resulting in good outcomes for CRC patients.

### Terminology

U6 is one of the small nuclear ribonucleic acids (snRNAs); it is also commonly referred to as U-RNA and often is used as an internal reference to normalize miRNA expression because of its stable expression. Tumor microenvironment is the cellular environment in which the tumor exists, including surrounding blood vessels, immune cells, fibroblasts, other cells, signaling molecules, and the extracellular matrix. As mentioned in discussion, different components of the tumor microenvironment interact with miR-193a-5p and further induce its variant expression.

### Peer review

In this manuscript, the authors examined the expression of miR-193a-5p in colorectal cancer tissues and the metastatic lymph node using quantitative polymerase chain reaction. It was found that down-regulation of miR-193a-5p correlated with tumor progression, lymph node metastasis and poor survival in CRC patients. The manuscript is nicely written and the study design is well constructed. The topic is innovative and of future clinical value and is indeed within the scope of the journal. This article may open the door wide for the future use of miR-193a-5p as a molecular biomarker for diagnosis and prognosis in colorectal cancer patients.

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