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Original Article

Overexpression of miR-599 is associated with metastasis in colorectal cancer via inhibition of SATB2

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Abstract: Background: Accumulating evidence supports that aberrantly expressed microRNAs (miRNAs) are associated with tumor development and progression. The aim of this study was to investigate the potential clinical value of miR-599 as a molecular prognostic biomarker and to explore the mechanism underlying its function. Methods: The expression level of miR-599 in CRC tissues and cell lines was determined by quantitative real-time PCR and its clinical value was assessed using the Kaplan-Meier method and the Cox proportional hazards mode. Moreover, the biological functions of miR-599 were investigated *in vitro* by performing cell migration and invasion assays, as well as western blot analysis, on cells in which miR-599 was downregulated through transfection with a specific inhibitor. Finally, luciferase reporter assays were conducted to confirm target associations. Results: MiR-599 expression was significantly higher in CRC tissues than in corresponding non-tumor tissues. Moreover, it was strongly associated with tumor aggressiveness, and multivariate analysis identified it as an independent prognostic factor in CRC patients. The *in vitro* study showed that miR-599 promotes cell migration and invasion, and induces epithelial-mesenchymal transition in CRC cell lines. Furthermore, *SATB2* was identified as a direct target of miR-599 in CRC cells. Conclusions: We for the first time show that miR-599 is upregulated in CRC tissues and cells and might serve as a prognostic marker for this cancer type. Moreover, miR-599 plays a pivotal role in CRC metastasis by negatively regulating SATB2 expression, suggesting that it might represent a potential efficacious therapeutic target in the treatment of CRC.

Keywords: Colorectal cancer, miR-599, metastasis, SATB2, prognosis

Introduction

Colorectal cancer (CRC) is among the most common cancer types and constitutes one of the most frequent causes of cancer-related deaths worldwide [1], with tumor invasion and metastasis being the main causes of mortality among patients [2]. The development of CRC is considered a multistage process involving genetic changes that lead to the activation of oncogenes and the inactivation of tumor suppressor genes [3]. Although various mechanisms underlying these processes have been elucidated and treatment has improved, approximately half of CRC patients still develop metastases after operation [4]. Thus, a deeper understanding of the molecular mechanisms underlying CRC metastasis is required, as it would facilitate the development of novel therapeutic strategies for metastatic CRC patients.

MicroRNAs (miRNAs) are small (20-24 nucleotides in length) endogenous non-coding RNAs that post-transcriptionally regulate the expression of multiple target genes by binding to complementary sequences, mainly in the 3'-untranslated regions (3'-UTRs) [5]. They have been shown to play a critical role in tumor pathogenesis, participating in cell proliferation, metastasis, and differentiation [6-8]. With respect to CRC, several miRNAs involved in its development and progression have been identified. After resection of colorectal liver metastasis, short-term survivors exhibited significantly higher miR-203 levels in tumor tissues compared to long-term survivors [9]. Aberrant miR-296 expression was detected in CRC tissues and cells, in which it could regulate the epithelial-mesenchymal transition (EMT) and exerted an anti-metastatic effect [10]. A study of liver metastatic lesions and primary CRC tumors

showed that the expression of another miRNA, miR-487b, was suppressed in the metastatic tissues, while this miRNA had the ability to inhibit the proliferation and, more evidently, the invasion ability of CRC cells [11]. Recently, the dysregulation of miR-599 was reported to be involved in the carcinogenesis of several carcinomas [12, 13]. However, to date, there is no literature on its role in CRC.

To explore whether miR-599 affects metastasis in CRC, we analyzed its expression signature and discovered that miR-599 expression was markedly increased in CRC tissues and cell lines. Moreover, miR-599 upregulation was a poor prognosis marker in patients with CRC. Subsequent research work revealed that the suppression of miR-599 inhibited the migration, invasion, and EMT of CRC cells *in vitro*. We also searched for potential target genes of miR-599 based on structure complementation and speculated that the mRNA of the *SATB2* gene (special AT-rich sequence-binding protein 2), whose deregulation has been previously shown to be strongly associated with the prognosis of CRC [14], was a direct miR-599 target. Further experimentation using CRC cells confirmed that miR-599 directly bound to the 3'-UTR of *SATB2* and regulated its expression. These findings elucidate the role of miR-599 in CRC and suggest that it may be a potential prognostic biomarker and an effective therapeutic target for the treatment of CRC.

Materials and methods

Tissue samples

Pairs of primary colorectal cancer tissues and matched non-tumor tissues were obtained from 112 histologically diagnosed CRC patients in the Department of Gastrointestinal Surgery in The first hospital of Hebei Medical University from 2010 to 2013. Patients who had received pre-surgical immunotherapy, chemotherapy, or radiotherapy were excluded. All specimens were frozen in liquid nitrogen immediately after resection and the medical history of all patients was recorded. All experiments were performed in accordance with the guidelines approved by the Ethics Committee of The first hospital of Hebei Medical University, after obtaining informed consent from the patients. The clinicopathological variables taken into account in this study included the gender, age, tumor site, histological grade, and disease stage as defined according to the TNM classification, which combines tumor invasion (T), regional lymph node status (N), and presence or absence of distant metastasis (M) [15]. Patient follow-up data included overall survival (OS), as well as the dates of events and cause of death.

Cell culture and transfection

Human normal colonic epithelial cells (NCM-460) and four colorectal cancer cell lines (LOVO, HCT116, SW480, and SW620) were maintained in low-glucose Dulbecco's modified Eagle's medium (DMEM), or in RPMI1640 medium. Media were supplemented with 10% fetal bovine serum (FBS; Gibco, Gaithersburg, MD, USA), as well as 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich, St Louis, MO, USA). Cells were incubated at 37°C with 5% CO_2. The miR-599 inhibitor, the negative control RNA inhibitor (miR-NC), the *SATB2* siRNA, and the NC siRNA were obtained from Ribobio (Guangzhou, China), and transfected into cells at a final concentration of 50 nM using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA).

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using the TRIzol Reagent (Invitrogen) according to the manufacturer's protocol and reverse-transcribed with the PrimeScript RT-PCR Kit (Takara, Otsu, Japan). The expression of miR-599 was analyzed using the TaqMan Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). QRT-PCR was performed in duplicate using an ABI 7300 Real-Time PCR System (Applied Biosystems) and the SYBR Green PCR Master Mix (Applied Biosystems), with the following reaction conditions: 94°C for 2 min, followed by 40 cycles at 94°C for 10 s, 60°C for 1 min, and 30 s at 72°C. The following primers were used: miR-599 RT, GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACgtttgat; miR-599 forward, GTTGTGTCAGTCTA; miR-599 reverse, GTGCAGGGTCCGAGGT; *U6* forward, CTCGCTTCGGCAGCACA; *U6* reverse, AACGCTTCACGAATTTGCGT; *SATB2* forward, GCAGTTGGACGGCTCTCTT; *SATB2* reverse, CACCTTCCCAGCTTGATTATTCC; *GAPDH* forward, GGTATCGTGGAAGGACTC; *GAPDH* reverse, GGGATGATGTTCTGGAGAG. *U6* and *GAPDH* were used as internal controls. The relative quantification

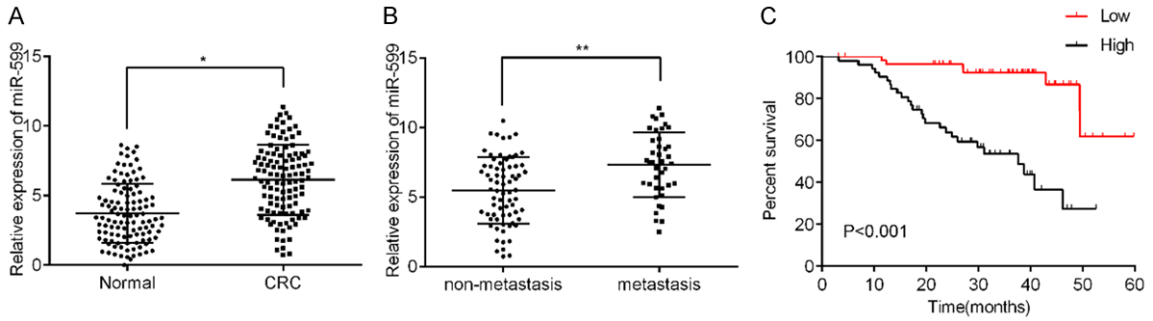

Figure 1. The expression levels of miR-599 in tissue samples and their relevance to prognosis. A: MiR-599 expression in CRC tissues and corresponding non-neoplastic tissues. B: MiR-599 expression in metastatic and non-metastatic tissues of CRC patients. C: Kaplan-Meier survival curve obtained by plotting overall survival vs. miR-599 expression in 112 CRC patients. The *P* value was obtained using a log-rank test; **P* < 0.05 vs. the normal group; ***P* < 0.05 vs. the non-metastasis group.

Table 1. Correlation between the clinicopathological features and expression of miR-599

Clinical Characteristics	miR-599 expression		*P* value
	Low (n = 50)	High (n = 62)	
Age (years)			0.763
< 55	22	29	
≥ 55	28	33	
Gender			0.261
Male	23	32	
Female	27	30	
Tumor site			0.838
Proximal colon	14	19	
Distal colon	12	14	
Rectum	24	29	
Tumor differentiation			0.104
Well/moderate	35	38	
Poor	15	24	
T-stage			0.033*
T1-2	22	20	
T3	18	23	
T4	10	19	
N-stage			0.016*
N0	32	32	
N1-2	18	30	
Distant metastasis			0.001*
N0	33	29	
YES	17	33	

*Statistically significant (P < 0.05).

of miR-599 and *SATB2* expression levels was performed with the $2^{-\Delta\Delta Ct}$ method and values were expressed as fold changes.

Western blot analysis

Total protein was extracted from cells using 1% RIPA Lysis Buffer (Beyotime, Jiangsu, China) and quantified with the Bicinchoninic Acid Protein Assay Kit (Beyotime). After separation by SDS-PAGE, proteins were transferred onto PVDF membranes and probed with specific primary antibodies against SATB2, MMP2, MMP9, E-cadherin, vimentin, and GAPDH (Abcam, Cambridge, MA, USA) overnight at 4°C. Membranes were then washed with TBS-Tween (TBST) and incubated with horseradish peroxidase-labeled secondary antibodies (Abcam) for 2 h. Signals were visualized using an ECL detection reagent (Beyotime) and quantified with the Quantity One v4.4 software (Bio-Rad, Hercules, CA, USA).

Cell migration and invasion assays

CRC cells transfected with the corresponding vectors were seeded into 6-well plates. A cell-scratch spatula was used to create a scratch in the confluent cell monolayer. Plates were washed with PBS to remove cellular debris, followed by incubation at 37°C for 48 h. Immediately (0 h) and 48 h after wounding, plates were examined and photographed under a microscope (Olympus, Tokyo, Japan).

Transwell chambers coated with Matrigel (1:6 dilution) were used for the invasion assays (BD Biosciences, Franklin Lakes, NJ, USA). Following transfection, 5 × 10^4 CRC cells suspended in 100 µL serum-free medium were seeded onto the upper wells, while 500 µL DMEM containing

Table 2. Univariate and multivariate cox regression analysis of risk factors for overall survival

	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age	0.83	0.46-1.39	0.466			
Gender	0.79	0.43-1.07	0.296			
Tumor site	0.94	0.83-1.07	0.343			
Tumor differentiation	1.05	0.87-1.18	0.132			
T-stage	1.63	1.10-3.49	0.027*	1.09	0.79-1.33	0.617
N-stage	2.02	1.37-5.50	0.037*	1.25	0.93-1.57	0.208
Distant metastasis	3.63	1.81-8.29	< 0.001*	1.98	1.14-3.65	0.002*
miR-599 expression	2.35	1.80-3.06	0.001*	1.47	1.06-2.92	0.014*

*Statistically significant (P < 0.05).

10% FBS was used as a chemoattractant in the lower wells. After 24 h, the Matrigel and the upper-surface cells were gently removed, and the invading cells were fixed in 4% paraformaldehyde, stained with 0.1% crystal violet (Sigma-Aldrich), and counted under a microscope (Olympus).

Luciferase reporter assay

The luciferase assay was used to assess whether miR-599 targets the 3'-UTR of the *SATB2* mRNA. A fragment of the 3'-UTR or a scrambled sequence were PCR-amplified and inserted into the pEZX-MT01 vector (GeneCopia, Labomics, Nivelles, Belgium). LoVo cells were plated at a density of 5×10^5 per well in 96-well plates and then co-transfected with the reporter plasmid (pEZX-3'-UTR or the negative control plasmid) and miR-599 or NC mimics using Lipofectamine 2000 (Invitrogen). The reporter activity was measured using the Luc-Pair miR Luciferase Assay Kit (GeneCopia) according to the manufacturer's instructions. All independent experiments were performed in triplicate.

Statistical analysis

All results were expressed as the mean ± SD. Pearson's χ^2 test was used to analyze the relationship between miR-599 expression and the clinicopathological features of patients with CRC. The significance of differences between groups was assessed with one-way analysis of variance (ANOVA). Pearson correlation was used to determine the relationship between miR-599 and SATB2 expression. $P < 0.05$ was considered to be statistically significant. Survival curves were obtained using the Kaplan-Meier method and compared with the log-rank test. Multivariate survival analysis was performed on all parameters that were found to be significant in the univariate analysis using the Cox regression model. All statistical analyses were performed using SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Graphs were generated with GraphPad Prism v5.0 (GraphPad Software, La Jolla, CA, USA).

Results

Upregulated miR-599 indicates poor prognosis in patients with CRC

We performed qRT-PCR to determine the expression level of miR-599 in sample tissues of patients with CRC. Results showed that miR-599 was expressed at significantly higher levels in CRC tissues compared to adjacent normal colonic tissues ($P < 0.05$; **Figure 1A**). Furthermore, we compared the expression of miR-599 in samples of metastatic and non-metastatic tissues and, unexpectedly, found miR-599 to be upregulated in the former compared to the latter ($P < 0.05$; **Figure 1B**). Therefore, we hypothesized that the level of miR-599 expression was correlated with the outcome of CRC patients and proceeded to analyze the relationship between the clinicopathological characteristics of CRC patients and miR-599 expression (**Table 1**). There was no significant association of miR-599 expression with age, gender, tumor site, or differentiation, whereas miR-599 levels were strongly correlated with tumor invasion ($P = 0.033$), lymph node metastasis ($P = 0.016$), and distant metastasis ($P = 0.001$). Moreover, high miR-599 expression was a poor prognosis marker in CRC patients with a shorter median survival (37.49 ± 2.23 *vs.* 64.52 ± 2.56, $P < 0.001$;

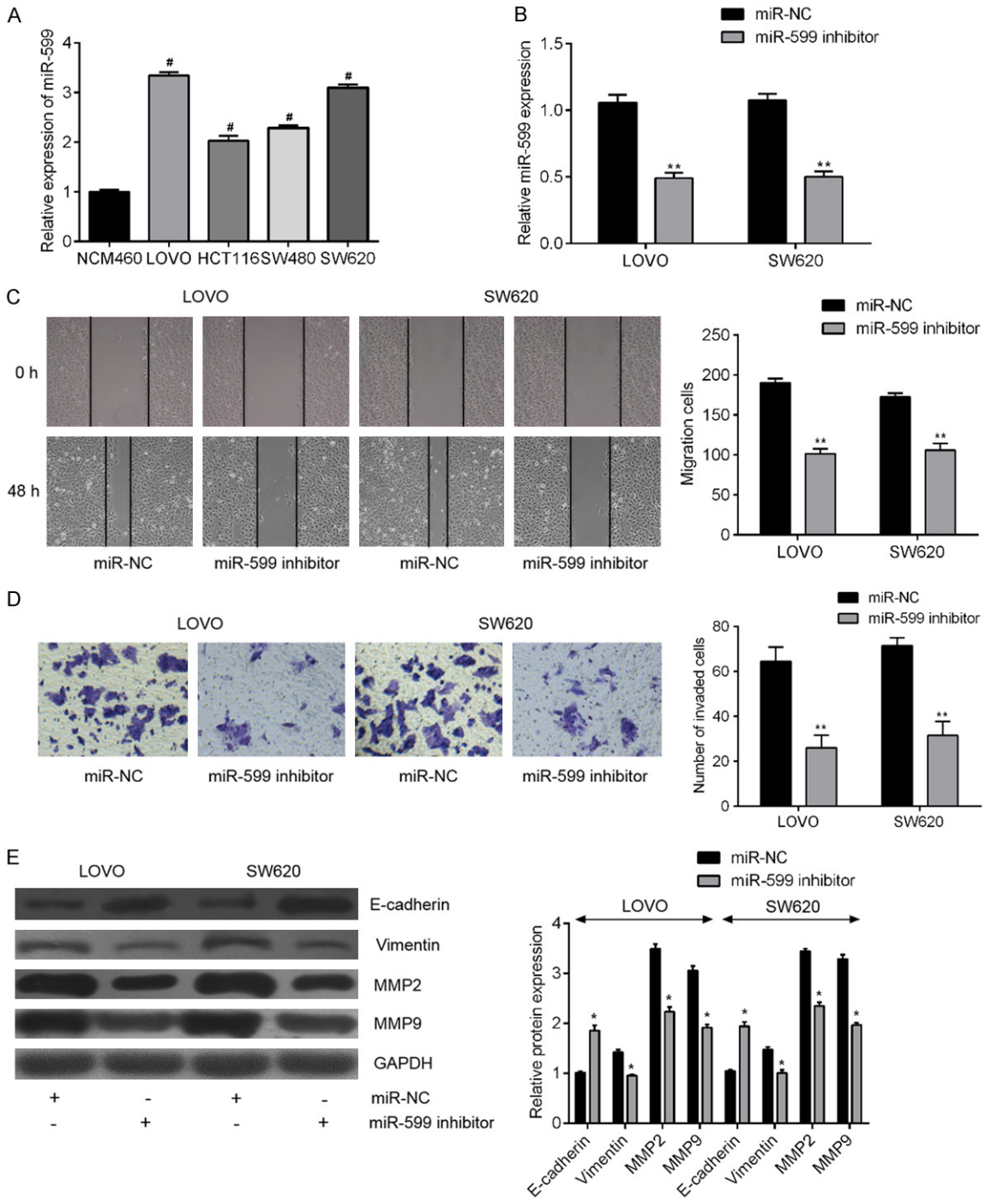

Figure 2. MiR-599 promotes metastasis of CRC cells *in vitro*. A: Relative miR-599 expression levels in four CRC cell lines (LOVO, HCT116, SW480, and SW620) and the human normal colorectal cell line, NCM460. B: Relative expression of miR-599 in LOVO and SW620 cells transfected with a miR-599 inhibitor. C: Wound-healing analysis of LOVO and SW620 cells transfected with the miR-599 inhibitor or miR-NC. D: LOVO and SW620 invading cells stained with 0.1% crystal violet, counted 48 h after transfection. The number of invading cells was determined from three replicate wells and expressed as mean ± SD. E: Western blot analysis of the expression levels of two invasion-related molecules, MMP2 and MMP9, and two epithelial-mesenchymal transition (EMT) markers, E-cadherin and vimentin, after the suppression of miR-599. $^{\#}P < 0.05$ vs. NCM460 cells; $^{*}P < 0.05$; $^{**}P < 0.01$ vs. cells transfected with miR-NC.

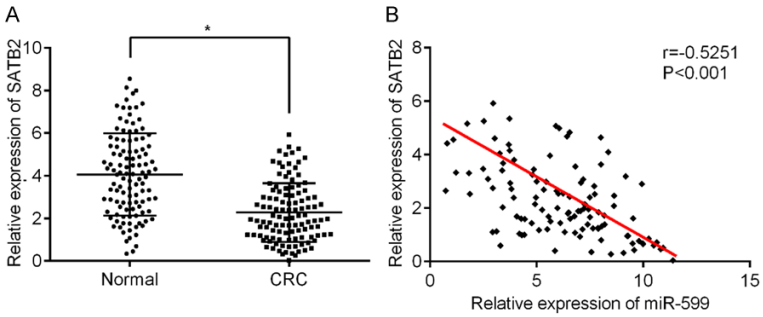

Figure 3. Relationship between SATB2 and miR-599 expression in CRC tissues. A: *SATB2* mRNA expression in CRC tissues and corresponding non-neoplastic tissues. B: The relationship between *SATB2* mRNA levels and miR-599 expression in CRC tissues was investigated by Pearson correlation analysis. *$P < 0.05$ vs. the non-metastasis group.

Figure 1C). Finally, multivariate cox regression analysis indicated that, together with distant metastasis, miR-599 was an independent prognostic factor for CRC (**Table 2**). These findings strongly suggested that miR-599 upregulation may indicate a poor prognosis in CRC patients.

MiR-599 promotes metastasis of CRC cells in vitro

We investigated *in vitro* the role of miR-599 in CRC metastasis. Consistent to previous results, human CRC cell lines, especially LOVO and SW620, displayed relatively higher miR-599 expression levels compared to the normal colonic epithelial cell line, NCM460 (**Figure 2A**). Subsequently, we used an inhibitor to suppress miR-599 expression in LOVO and SW620 cells. QRT-PCR showed that the level of miR-599 in cells transfected with the inhibitor was significantly lower than in cells transfected with miR-NC, confirming the efficiency of the inhibitor (**Figure 2B**). We then examined the effects of miR-599 inhibition on the migration and invasion properties of CRC cells. The results of our wound healing assay showed that the migration ability of LoVo and SW620 cells was significantly reduced by miR-599 downregulation (**Figure 2C**). Likewise, the Transwell invasion assay demonstrated that miR-599 inhibition decreased the invasion ability of both LoVo and SW620 cells (**Figure 2D**). Furthermore, the suppression of miR-599 was followed by a dramatic mesenchymal-epithelial transition (MET)-like transformation of LoVo and SW620 cells, with significant upregulation of E-cadherin and downregulation of vimentin, MMP-2, and MMP9 (**Figure 2E**).

Correlation between the expression of miR-599 and SATB2 in CRC

CRC tissues displayed much lower levels of *SATB2* mRNA compared to corresponding non-neoplastic tissues (**Figure 3A**). Moreover, there was a linear negative correlation between miR-599 and *SATB2* expression ($r = -0.5251$, $P < 0.001$; **Figure 3B**).

miR-599 directly targets the SATB2 3'-UTR in CRC

To validate whether miR-599 targets the 3'-UTR of *SATB2* mRNA, we queried candidate target genes form the human microRNA database and found that *SATB2* was a putative target of miR-599 (**Figure 4A**). To confirm our hypothesis, we performed a luciferase assay and found that the increased expression of miR-599 reduced the luciferase activity in LoVo cells transfected with a reporter plasmid containing the 3'-UTR sequence of the *SATB2* mRNA, while no change was observed in cells transfected with a plasmid containing a scrambled sequence (**Figure 4B**). Subsequently, we analyzed SATB2 expression, at both the mRNA and the protein level, in LoVo cells transfected with the miR-599 inhibitor. Results showed that the downregulation of miR-599 resulted in a significant increase in both the mRNA (**Figure 4C**) and protein (**Figure 4D**) levels of SATB2. Taken together, these findings indicated that miR-599 regulates *SATB2* expression by targeting its 3'-UTR.

Discussion

In recent years, numerous studies have reported on the emerging roles of miRNAs during carcinogenesis. Hsa-miR-599, located at the 8q22.2 locus of the human genome, is a newly identified tumor-related miRNA and only a few studies have investigated its exact role in cancers [13, 16]. Tian et al. found that miR-599 was upregulated in lung cancer cells; their subsequent biological analysis indicated that it affected cell proliferation, migration, and invasion [16]. However, another study reported miR-599 to be downregulated in hepatocellular carcinoma tissues and cell lines, while the restoration of its expression inhibited cell proliferation, migration, and invasion [17]. This dispar-

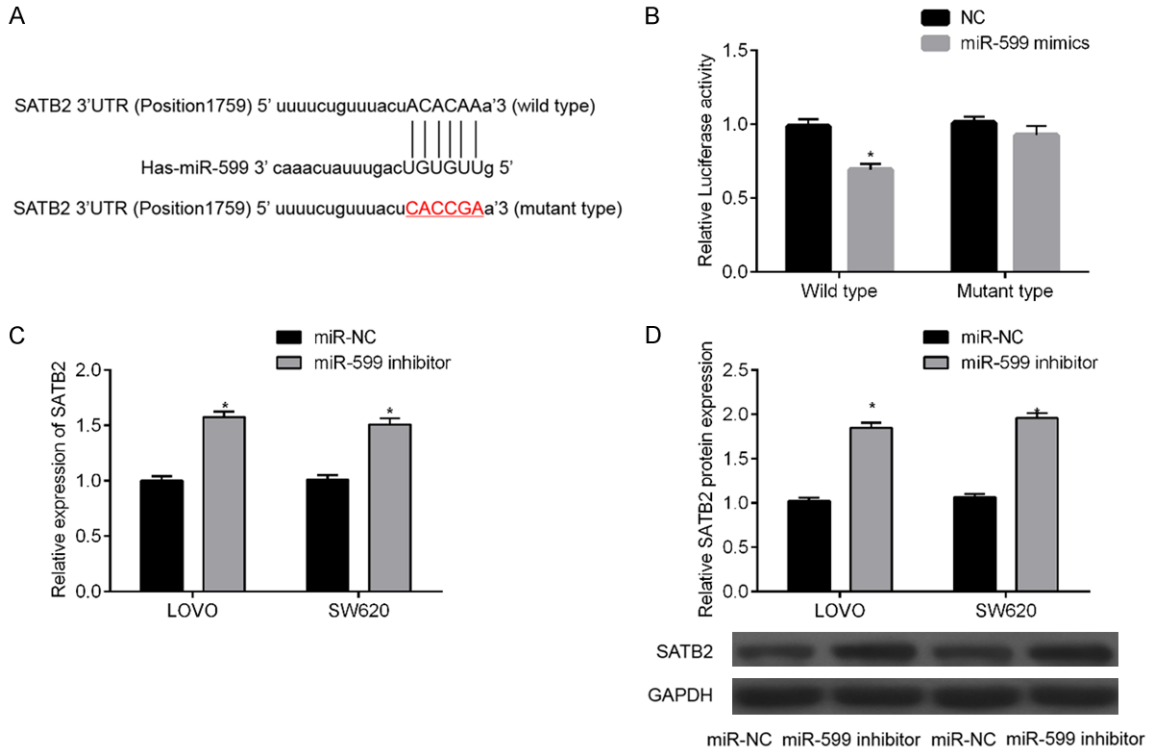

Figure 4. SATB2 is a direct target of miR-599 in CRC. A: The putative target location of miR-599 in the 3'-UTR of SATB2 as predicted by miRBase. B: Luciferase activity of LOVO cells transfected with a reporter plasmid carrying the 3'-UTR of *SATB2* or a scrambled sequence, following the binding of miR-599. C: Relative expression of *SATB2* mRNA in LOVO cells transfected with a miR-599 inhibitor or miR-NC. D: Relative expression of the SATB2 protein as assessed by western blot analysis.

ity suggests that the functional significance of miR-599 in cancer development and progression may be cancer type-specific. However, the exact role of miR-599 in CRC remains to be clarified.

In this study, we first performed a detailed analysis of the expression and function of miR-599 in CRC tissues and cells. Data showed that miR-599 was significantly upregulated in CRC tissues. We also found that miR-599 expression was higher in metastatic compared to non-metastatic tissues, which led us to hypothesize that miR-599 is involved in the metastasis of CRC. Therefore, we investigated the prognostic value of this miRNA. Results showed that the expression of miR-599 was significantly correlated with the TNM status and that the overall survival of patients with low miR-599 expression was higher than the one of the high-miR-599 expression group. Moreover, the Cox proportional hazards mode identified miR-599 as an independent prognostic factor for survival. The above findings encouraged us to further investigate the molecular mechanisms underlying the role of miR-599 in CRC. Our results showed that it regulates the migration, invasion, and EMT of CRC cells. All the above results suggested that miR-599 promotes CRC metastasis and may constitute a novel prognostic marker in CRC.

We also identified *SATB2* as a downstream target of miR-599. SATB2, which was originally discovered as a transcription factor playing an important role in the regulation of bone development and the differentiation of osteoblasts [18], is an important DNA-binding protein involved in transcriptional regulation and chromatin remodeling [18-20]. A more recent study identified it as a highly tissue type-specific protein that is predominantly expressed in the glandular cells of the lower gastrointestinal tract and in CRC [21], while another study reported that the downregulation of SATB2 is associated with metastasis and poor prognosis in CRC [22]. Several miRNAs can reportedly regulate the expression of *SATB2* by directly

targeting its 3'-UTR. It has been shown that miR-182 facilitates tumor growth and metastasis of CRC by suppressing SATB2 expression [23]. MiR-31 is upregulated in CRC and can bind to the 3'-UTR of *SATB2* mRNA, subsequently inhibiting both the mRNA and protein expression of SATB2 [24]. Recently, Sun et al. demonstrated the involvement of a miR-449a-SATB2 negative feedback loop in the development of CRC [25]. Accordingly, the dual-luciferase reporter assays and western blots that we performed suggested that miR-599 could also regulate *SATB2* expression by directly targeting its mRNA. The downregulation of SATB2 seems to be one of a number of important downstream mechanisms through which miR-599 plays a role in CRC.

To sum up, our integrated approach demonstrated that the upregulation of miR-599 is involved in the progression of CRC, as its expression level was significantly associated with the aggressiveness of CRC in the studied patients, indicating a poor prognosis. On the other hand, the suppression of miR-599 upregulated SATB2 expression, inhibited CRC cell migration and invasion, and prevented EMT *in vitro*, thus it may be a valid approach for molecular targeted therapy of CRC.

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Disclosure of conflict of interest

None.

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