European Review for Medical and Pharmacological Sciences 2020; 24: 10535-10541

MiR-548b suppresses proliferative capacity of colorectal cancer by binding WNT2

Y. XU[1], Y.-D. ZHONG[2], X.-X. ZHAO[3]

[1]Department of GI Medicine, Linyi Central Hospital, Linyi, China
[2]Department of General Surgery, Zoucheng People's Hospital, Zoucheng, China
[3]Department of Pediatric, Haiyang People's Hospital, Haiyang, China

Yan Xu and Yadong Zhong contributed equally to this work

Abstract. – OBJECTIVE: This study aims to illustrate the role of microRNA-548 (miR-548) in regulating the development of colorectal cancer (CRC) and the involvement of WNT2.

PATIENTS AND METHODS: MiR-548b levels in CRC species and paracancerous ones were detected. The relationship between miR-548b level and clinical parameters of CRC patients was analyzed. After overexpression of miR-548b, the changes in the proliferative and apoptotic capacities of Sw620 and HT29 cells were assessed by Cell Counting Kit-8 (CCK-8), colony formation assay, and flow cytometry, respectively. At last, the involvement of WNT2, the downstream gene of miR-548b, was detected by Luciferase assay and rescue experiments.

RESULTS: Results manifested that miR-548b was lowly expressed in CRC species than paracancerous ones, and *in vitro* level of miR-548b was downregulated in CRC cell lines as well. Compared with CRC patients in T1-T2, miR-548b level was lower in T3-T4 CRC. Moreover, CRC patients with lymphatic metastasis had lower level of miR-548b than those without. Overexpression of miR-548b suppressed proliferative capacity and induced apoptosis in CRC cells. Besides, it was found that WNT2 was the downstream gene of miR-548b, and its level was negatively regulated by miR-548b in CRC. Furthermore, rescue experiments showed that WNT2 was responsible for CRC development regulated by miR-548b.

CONCLUSIONS: MiR-548b is closely linked to tumor stage and lymphatic metastasis of CRC, and it alleviates the malignant development of CRC by targeting WNT2.

Key Words:
MiR-548b, WNT2, CRC, Proliferation, Apoptosis.

Introduction

Under the stimuli of carcinogenic factors, uncontrolled proliferation of tumor cells and tissue infiltration eventually result in tumorigenesis[1-3]. Tumor incidence and mortality have been sharply elevated during the past decades because of lifestyle changes and environmental factors[4,5]. Colorectal cancer (CRC) is a popular malignancy in the gastrointestinal system[6,7]. Its symptoms in the early phase are similar to other gastrointestinal inflammation diseases, leading to a low detective rate in the early stage. The occurrence of CRC involves various factors, including colon polyps, chronic inflammation of the intestinal mucosa, parasites, eating habits, and genetics[8,9]. CRC mainly affects the rectum and sigmoid colon, and adenocarcinoma is the major subtype. Through lymphatic metastasis, blood circulation spread or direct spread, CRC easily metastasizes to distant organs[10,11]. With the enlargement of tumor mass, clinical manifestations of CRC become evident, including changes in bowel habits, bloody stools, constipation, diarrhea, and even alternating diarrhea and constipation. Systemic symptoms, such as anemia and weight loss, appear in the advanced stage of CRC[12,13]. Early detection, timely diagnosis, and radical surgery are the key events to CRC treatment[13].

Clinical studies[14,15] have demonstrated the involvement of microRNAs (miRNAs) in tumorigenesis, so miRNAs may be promising tumor biomarkers. MiRNAs are endogenous non-coding RNAs with 18-25 nucleotides[16,17]. Although the number of miRNAs only accounts for 1% of human genomes, they are capable of regulating more than one third of genes[17,18]. Because of stable distribution and differential expressions in tumors, miRNAs may provide a novel direction in diagnosis and evaluation of tumor diseases[18,19]. Besides, they have been found to be differentially expressed in tumor species, and they are able to regulate tumor-associated genes[19,20]. MiR-548b is a tumor biomarker in hepatocellular carcino-

Corresponding Author: Xuexia Zhao, BM; e-mail: 1293764732@qq.com

ma[21,22]. In this paper, potential influences of miR-548b on CRC cell phenotypes and its underlying mechanism were mainly discussed.

Patients and Methods

CRC Patients and Species

A total of 43 CRC patients undergoing surgery in Linyi Central Hospital were included and they were not preoperatively treated. The median age of included CRC patients was 64 years old (52-84 years old). Tumor node metastasis (TNM) staging in each CRC patient was determined according to the American Joint Committee on Cancer. CRC species and paracancerous ones (normal intestinal mucosa collected at least 5 cm away from the tumor edge) were stored in liquid nitrogen within 10 min *ex vivo*. Preoperative and postoperative blood samples were collected. This study was approved by the Ethics Committee of Linyi Central Hospital and was conducted after informed consent was obtained from each subject.

Cell Culture

CRC cell lines (Caco2, Sw620, HT29, HCT-8, and HCT-116) and intestinal epithelial cell line (FHC) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) [except for HCT-116 cells that were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; Gibco, Rockville, MD, USA)] containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C. Cell passage was conducted using trypsin at 80-90% confluence.

Transfection

Cells inoculated in 6-well plates were cultured to 30-40% confluence. Then, they were transfected with plasmids constructed by GenePharma (Shanghai, China), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 48 hours later, the cells were collected for the following use.

Cell Proliferation Assay

Cells were inoculated in a 96-well plate with 2×10^3 cells per well. At the appointed time points, absorbance value at 490 nm of each sample was recorded using the CCK-8 kit (Dojindo Molecular Technologies, Kumamoto, Japan) for plotting the viability curves.

Colony Formation Assay

Cells were inoculated in a 6-well plate with 200 cells per well and cultured for 2 weeks. Culture medium was replaced once in the first week and twice in the second week. Next, visible colonies were washed in PBS, fixed in methanol for 20 min and dyed in 0.1% crystal violet for 20 min, which were captured and calculated.

Flow Cytometry

Cells were prepared to suspension at 1×10^6 cells/mL, suspended in 0.5 mL pre-cold binding buffer and induced with 1.25 μL of Annexin V-fluorescein isothiocyanate (FITC) in the dark for 15 min. After centrifugation at 1000×g for 5 min, the precipitant was again suspended in binding buffer and treated with 10 μL of Propidium Iodide (PI) in the dark, followed by apoptosis determination using flow cytometry (FACSCalibur, BD Biosciences, Detroit, MI, USA).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

RNAs extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNAs underwent qRT-PCR using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were the internal references. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. miR-548b: forward: 5'-ACACTCCAGCTGGGCAAAAATCTCAAT-3', reverse: 5'-CTCAACTGGTGTCGTGGAAACTGGTGTC-3', U6: forward: 5'-CTCGCTTCGGCAGCACAGCTTCGGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCGTCGCTTCACGAATT-3', WNT2: forward: 5'-CCAGCCTTTTGGCAGGGTC-3', reverse: 5'-GCATGTCCTGAGAGTCCATG-3', and GAPDH: forward: 5'-GGGCAGTATGTTGT-3', reverse: 5'-GCTATTGGCATTGGTGAA-3'.

Western Blotting

Cells were lysed for isolating cellular protein and electrophoresed. The protein samples were loaded on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 hours. The membranes were reacted with primary and secondary an-

tibodies for indicated time. Band exposure and analyses were finally conducted.

Luciferase Assay

HEK293T cells inoculated in a 24-well plate were co-transfected with miR-548b mimic/NC mimic and WNT2-WT/WNT2-MUT using Lipofectamine 2000 and then lysed for determining relative Luciferase activity 48 h later.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM Corp., Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Differences between groups were analyzed by the *t*-test. Chi-square test was used for analyzing the relationship between miR-548b level and clinical data of CRC. Pearson correlation test was applied for evaluating the relationship between two genes in CRC species. $p<0.05$ suggested that the difference was statistically significant.

Results

Downregulated MiR-548b Was Linked to TNM Staging and Lymphatic Metastasis of CRC

QRT-PCR data showed a lower level of miR-548b in CRC tissues than paracancerous ones (Figure 1A). Compared with FHC cells, miR-548 was downregulated in CRC cell lines (Figure 1B). Included CRC patients were assigned into two groups, according to the median level of miR-548b. By analyzing their clinical data, miR-548b level was detected to be lower in T3-T4 CRC than those in T1-T2. Moreover, CRC patients with lymphatic metastasis had a lower level of miR-548b than those without (Table I).

Overexpression of MiR-548b Suppressed Proliferative Rate and Induced Apoptosis In CRC

MiR-548b mimic was constructed and its transfection efficacy was tested in Sw620 and HT29 cells (Figure 2A). Overexpression of miR-548b markedly decreased viability (Figure 2B) and colony number (Figure 2C), suggesting the suppressed proliferative capacity. In addition, apoptotic rate increased in Sw620 and HT29 cells overexpressing miR-548b (Figure 2D).

Overexpression of MiR-548b Downregulated Genes in the WNT2/β-Catenin Pathway

Notably, protein levels of WNT2, β-catenin were downregulated in CRC cells overexpressing miR-548b (Figure 3A). According to the predicted binding sites in the promoter regions of miR-548b and WNT2, luciferase assay verified their binding relationship as overexpression of miR-548b reduced luciferase activity in wild-type WNT2 vector (Figure 3F). Subsequently, pcDNA-WNT2 was constructed and its transfection efficacy was tested by Western blotting (Figure 3B). Besides, overexpression of WNT2 remarkably downregulated miR-548b level in CRC cells (Figure 3C). A negative correlation was identified between expression levels of miR-548b and WNT2 (Figure 3E). As expected, WNT2 was upregulated in CRC species (Figure 3D).

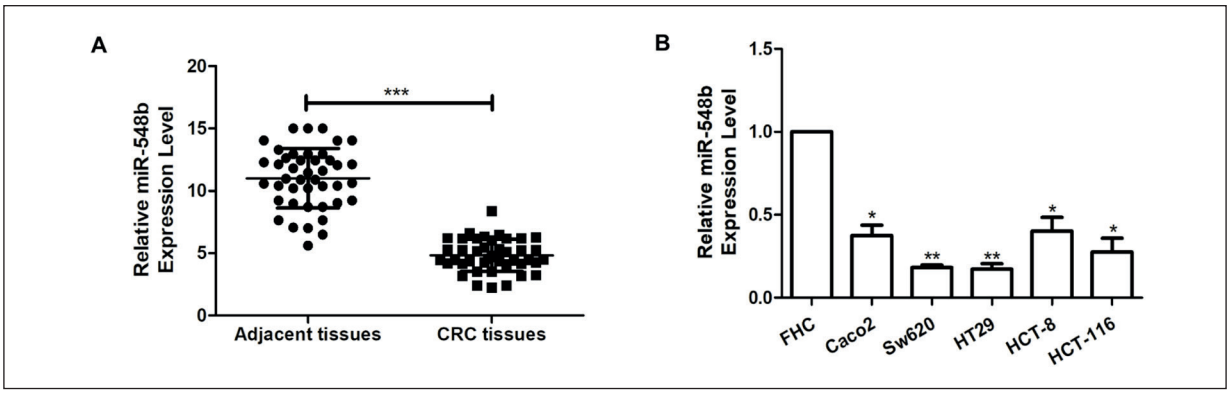

Figure 1. Downregulated miR-548b was linked to TNM staging and lymphatic metastasis of CRC. **A,** MiR-548b levels in CRC species and paracancerous ones. **B,** MiR-548b levels in CRC cell lines. Data are expressed as mean ± SD. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Table I. Association of miR-548b expression with clinicopathologic characteristics of colorectal cancer.

Parameters	No. of cases	MiR-548b expression		*p*-value
		High (%)	Low (%)	
Age (years)				0.834
< 60	17	11	6	
≥ 60	26	16	10	
Gender				0.607
Male	21	14	7	
Female	22	13	9	
T stage				0.011
T1-T2	29	22	7	
T3-T4	14	5	9	
Lymph node metastasis				0.008
No	27	21	6	
Yes	16	6	10	
Distance metastasis				0.405
No	25	17	8	
Yes	18	10	8	

MiR-548b Regulated CRC Cell Phenotypes by Targeting WNT2

Notably, upregulated miR-548b in CRC cells overexpressing miR-548b was reversed by overexpression of WNT2 (Figure 4A). Inhibited viability (Figure 4B) and clonality (Figure 4C) by overexpression of miR-548b in CRC cells were partially reversed after co-overexpression of WNT2. In addition, increased apoptotic rate in CRC cells overexpressing miR-548b was reduced following WNT2 overexpression (Figure 4D).

Discussion

CRC is classified into carcinoma of colon and rectum[3-5]. In China, the incidence of CRC ranks the third, which is only second to lung cancer and

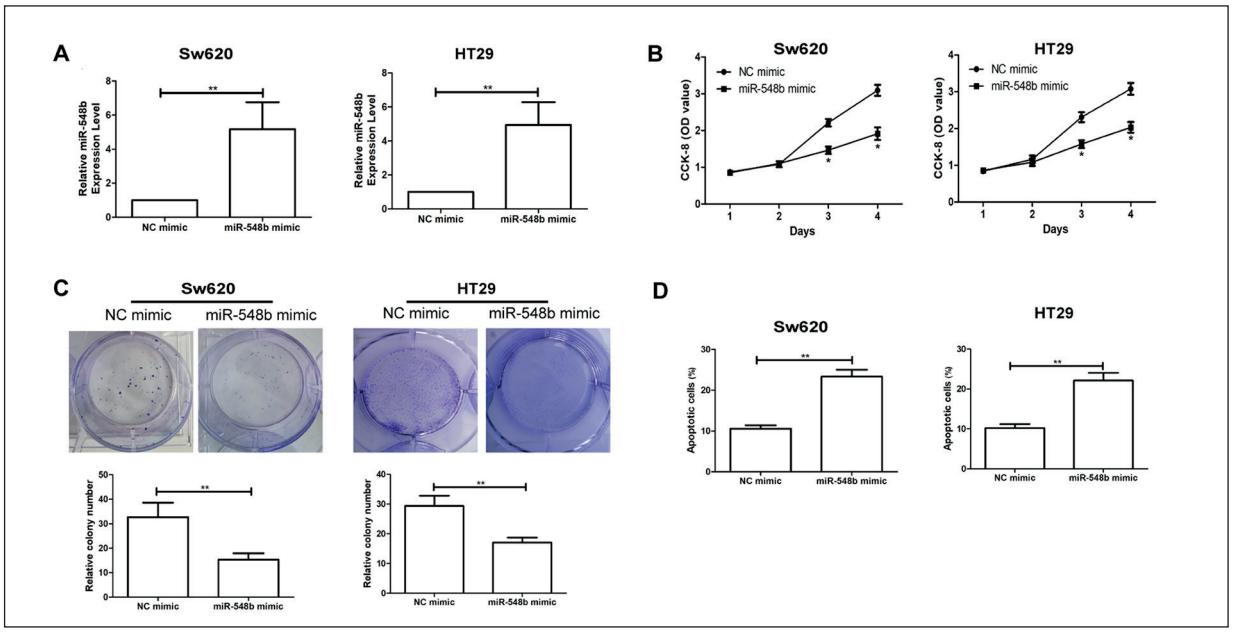

Figure 2. Overexpression of miR-548b suppresses proliferative rate and induces apoptosis in CRC. **A,** Transfection efficacy of miR-548b mimic in Sw620 and HT29 cells. **B,** Viability in Sw620 and HT29 cells transfected with NC mimic or miR-548b mimic. **C,** Colony formation in Sw620 and HT29 cells transfected with NC mimic or miR-548b mimic (magnification: 10×). **D,** Apoptosis in Sw620 and HT29 cells transfected with NC mimic or miR-548b mimic. Data are expressed as mean ± SD. **p*<0.05, ***p*<0.01.

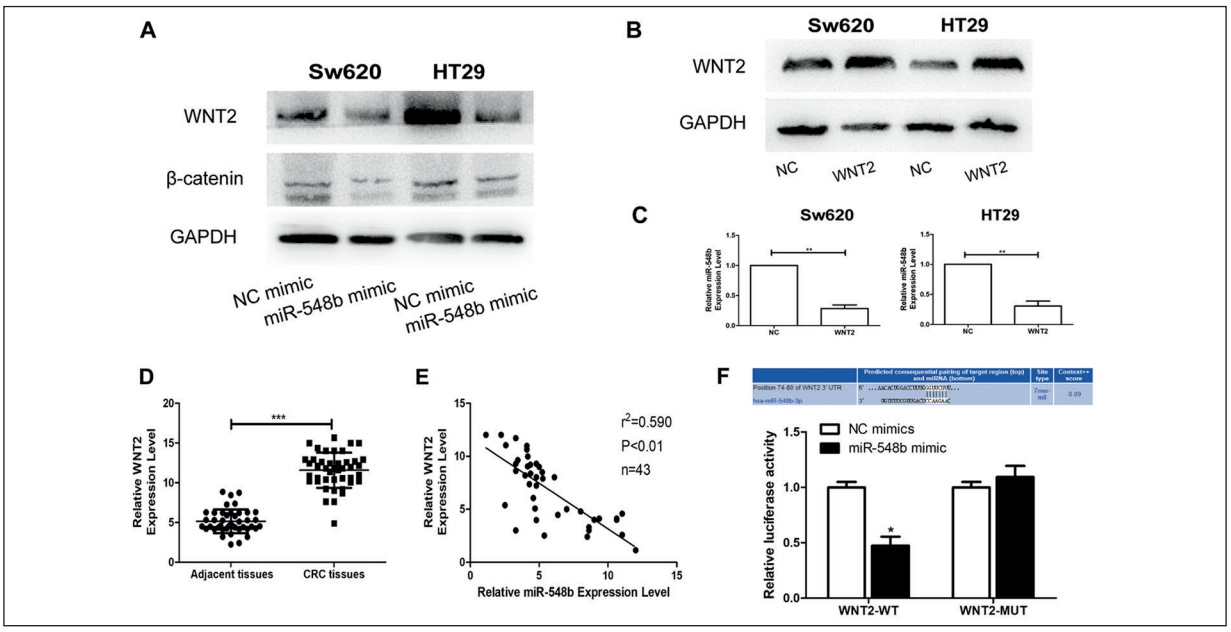

Figure 3. Overexpression of miR-548b downregulates genes in the WNT2/β-catenin pathway. **A,** Protein levels of WNT2, β-catenin in Sw620 and HT29 cells transfected with NC mimic or miR-548b mimic. **B,** Transfection efficacy of pcDNA-WNT2 in Sw620 and HT29 cells. **C,** MiR-548b level in Sw620 and HT29 cells transfected with NC or pcDNA-WNT2. **D,** WNT2 levels in CRC species and paracancerous ones. **E,** A negative correlation between expression levels of miR-548b and WNT2 in CRC species. **F,** Binding sites in the promoter regions of miR-548b and WNT2 (upper). Luciferase activity in cells co-transfected with NC mimic/miR-548b mimic and WNT2-WT/WNT2-MUT (bottom). Data are expressed as mean ± SD. *p<0.05, **p<0.01, ***p<0.001.

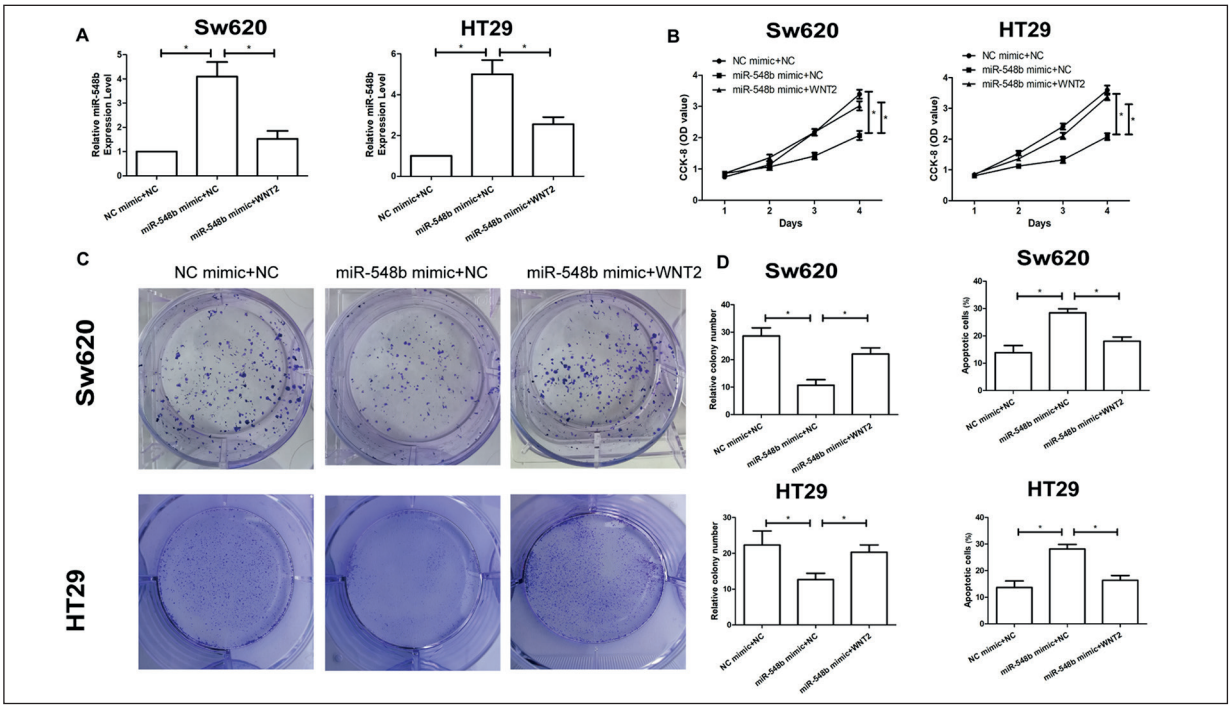

Figure 4. MiR-548b regulates CRC cell phenotypes by targeting WNT2. **A,** MiR-548b level in Sw620 and HT29 cells transfected with NC mimic+NC, miR-548b mimic+NC or miR-548b mimic+pcDNA-WNT2. **B,** Viability in Sw620 and HT29 cells transfected with NC mimic+NC, miR-548b mimic+NC or miR-548b mimic+pcDNA-WNT2. **C,** Colony formation in Sw620 and HT29 cells transfected with NC mimic+NC, miR-548b mimic+NC or miR-548b mimic+pcDNA-WNT2 (magnification: 10×). **D,** Apoptosis in Sw620 and HT29 cells transfected with NC mimic+NC, miR-548b mimic+NC or miR-548b mimic+pcDNA-WNT2. Data are expressed as mean ± SD. *p<0.05.

gastric cancer. Seriously, the mortality of CRC ranks the fifth in cancer death rate[3,6,7]. Most of CRC cases are sporadic, which are aggravated from adenoma to tumor[6,8]. At present, surgery is the preferred strategy for CRC patients[7]. The 5-year survival of CRC in the early phase is up to 90%, which sharply decreases to less than 10% in advanced CRC patients[9-11]. The pathogenesis of CRC is complicated, involving several signaling pathways (i.e., the Wnt pathway)[23].

MiRNAs, extensively expressed in animals and plants, are vital regulators in life activities[14,16]. So far, there are 28,645 discovered miRNAs. A single miRNA has several corresponding target genes and a single gene can be targeted by several miRNAs, and thus a complex regulatory network is formed[15-18]. MiR-548b is downregulated in hepatocellular carcinoma, and it is closely linked to clinical prognosis[21,22]. In this paper, miR-548b was lowly expressed in CRC species than paracancerous ones. By analyzing clinical data of included CRC patients, it was found that miR-548b level was negatively linked to TNM staging and lymphatic metastasis rate. Experimental results uncovered that the overexpression of miR-548b suppressed proliferative capacity and induced apoptosis in CRC cells.

WNT2 was predicted to be the downstream gene binding miR-548b, which was verified by Luciferase assay. Converse to miR-584b, WNT2 was upregulated in CRC species and cell lines. Moreover, WNT2 level was negatively correlated with that of miR-584b. Rescue experiment results showed that the overexpression of WNT2 abolished the regulatory effects of miR-584b on proliferative and apoptotic capacities of CRC cells.

Collectively, the novelty of this study was that a negative feedback loop miR-584b/WNT2 was identified to alleviate the malignant development of CRC in this study, which will provide a new support to explore the target drug for the treatment of CRC.

Conclusions

In sumary, miR-548b is closely linked to tumor stage and lymphatic metastasis of CRC, and it alleviates the malignant development of CRC by targeting WNT2.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Andersen GB, Tost J. Circulating miRNAs as biomarker in cancer. Recent Results Cancer Res 2020; 215: 277-298.
2) Parakh S, King D, Gan HK, Scott AM. Current development of monoclonal antibodies in cancer therapy. Recent Results Cancer Res 2020; 214: 1-70.
3) Sterea AM, El HY. The role of mitochondrial calcium signaling in the pathophysiology of cancer cells. Adv Exp Med Biol 2020; 1131: 747-770.
4) Yang T, Li X, Montazeri Z, Little J, Farrington SM, Ioannidis J, Dunlop MG, Campbell H, Timofeeva M, Theodoratou E. Gene-environment interactions and colorectal cancer risk: an umbrella review of systematic reviews and meta-analyses of observational studies. Int J Cancer 2019; 145: 2315-2329.
5) Sosa V, Moline T, Somoza R, Paciucci R, Kondoh H, LLeonart ME. Oxidative stress and cancer: an overview. Ageing Res Rev 2013; 12: 376-390.
6) Brody H. Colorectal cancer. Nature 2015; 521: S1. doi: 10.1038/521S1a.
7) Hao JP, Ma A. The ratio of miR-21/miR-24 as a promising diagnostic and poor prognosis biomarker in colorectal cancer. Eur Rev Med Pharmacol Sci 2018; 22: 8649-8656.
8) Gao R, Gao Z, Huang L, Qin H. Gut microbiota and colorectal cancer. Eur J Clin Microbiol Infect Dis 2017; 36: 757-769.
9) Roncucci L, Mariani F. Prevention of colorectal cancer: how many tools do we have in our basket? Eur J Intern Med 2015; 26: 752-756.
10) Gil-Martin E, Egea J, Reiter RJ, Romero A. The emergence of melatonin in oncology: focus on colorectal cancer. Med Res Rev 2019; 39: 2239-2285.
11) Cao Y, Gu J, Deng S, Li J, Wu K, Cai K. Long-term tumour outcomes of self-expanding metal stents as 'bridge to surgery' for the treatment of colorectal cancer with malignant obstruction: a systematic review and meta-analysis. Int J Colorectal Dis 2019; 34: 1827-1838.
12) Kyrochristos ID, Roukos DH. Comprehensive intra-individual genomic and transcriptional heterogeneity: evidence-based colorectal cancer precision medicine. Cancer Treat Rev 2019; 80: 101894.
13) Kastrisiou M, Zarkavelis G, Pentheroudakis G, Magklara A. Clinical application of next-generation sequencing as a liquid biopsy technique in advanced colorectal cancer: a trick or a treat? Cancers (Basel) 2019; 11. pii: E1573
14) Santoni G, Morelli MB, Santoni M, Nabissi M, Marinelli O, Amantini C. Targeting transient receptor potential channels by microRNAs drives tumor development and progression. Adv Exp Med Biol 2020; 1131: 605-623.

15) Andersen GB, Tost J. Circulating miRNAs as biomarker in cancer. Recent Results Cancer Res 2020; 215: 277-298.

16) Budak H, Akpinar BA. Plant miRNAs: biogenesis, organization and origins. Funct Integr Genomics 2015; 15: 523-531.

17) Komal S, Yin JJ, Wang SH, Huang CZ, Tao HL, Dong JZ, Han SN, Zhang LR. MicroRNAs: Emerging biomarkers for atrial fibrillation. J Cardiol 2019; 74: 475-482.

18) Anvarnia A, Mohaddes-Gharamaleki F, Asadi M, Akbari M, Yousefi B, Shanehbandi D. Dysregulated microRNAs in colorectal carcinogenesis: new insight to cell survival and apoptosis regulation. J Cell Physiol 2019; 234: 21683-21693.

19) Soleimani A, Rahmani F, Saeedi N, Ghaffarian R, Khazaei M, Ferns GA, Avan A, Hassanian SM. The potential role of regulatory microRNAs of RAS/MAPK signaling pathway in the pathogenesis of colorectal cancer. J Cell Biochem 2019; 120: 19245-19253.

20) Ma Y. The challenge of microRNA as a biomarker of epilepsy. Curr Neuropharmacol 2018; 16: 37-42.

21) Qiu H, Zhang G, Song B, Jia J. MicroRNA-548b inhibits proliferation and invasion of hepatocellular carcinoma cells by directly targeting specificity protein 1. Exp Ther Med 2019; 18: 2332-2340.

22) Yun Z, Meng F, Jiang P, Yue M, Li S. MicroRNA-548b suppresses aggressive phenotypes of hepatocellular carcinoma by directly targeting high-mobility group box 1 mRNA. Cancer Manag Res 2019; 11: 5821-5834.

23) Bahrami A, Amerizadeh F, ShahidSales S, Khazaei M, Ghayour-Mobarhan M, Sadeghnia HR, Maftouh M, Hassanian SM, Avan A. Therapeutic potential of targeting Wnt/beta-catenin pathway in treatment of colorectal cancer: rational and progress. J Cell Biochem 2017; 118: 1979-1983.