miR-96 affects the proliferation and metastasis of colon cancer by targeting forkhead box F2

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Abstract: Numerous studies have reported that miRNA-96 are involved in the development and progression of various types of human cancers. In the current study, the roles of miR-96 in colon cancer has been investigated. Human colon cancer cell line SW480 cells were transfected with miR-96 inhibitors, and the effect of miR-96 on the proliferation, apoptosis, migration and invasion were examined. Moreover, the targeting relationship between miR-96 and FOXF2 was examined via luciferase reporter assay. Finally, RT-PCR and western blot methods have been applied to detect the effect of miR-96 on the expression of FOXF2 and Wnt/β-catenin signalling. We found that inhibition of the expression of miR-96 could inhibit the proliferation, invasion and migration, and promote the apoptosis of colon cancer cell line SW480 cells. We verified that FOXF2 was the direct target of miR-96 through transfecting miR-96 mimics and luciferase reporter assay. qRT-PCR analysis and Western Blot revealed that inhibition of the expression of miR-96 could affect the expression of FOXF2, GSK-3β and β-catenin on mRNA and protein level. In conclusion, we reported for the first time that down-regulation of miR-96 could inhibit cell proliferation, migration, and invasion of colon cancer through targeting FOXF2 by inhibiting β-catenin signaling pathway.

Key word: miR-96, colon cancer, Wnt/β-catenin, FOXF2

Introduction

Colon cancer, with characteristics of early detection difficult in the early stages, developing from synergistic effect of various factors, and low five-year survival rate in the later stages, is a significant health burden worldwide(1). Metastasis and recurrence of colon cancer was considered as the main cause of lethality (2). Colon cancer develops from various factors (3), however, the underlying mechanism is still unclear. Thus, implement study for colon cancer to explore the pathogenesis of colon cancer has necessary significance for the early diagnosis, timely therapy, and prolonging survival of patients with colon cancer.

Non-coding RNAs have been deemed as a new class of key regulators that plays a key role in the development of many diseases (4). MicroRNAs (miRNAs), with the length of 20–22 nucleotide, are the major categories of small endogenous non-coding RNAs and control almost one-third of all human genes. miRNAs regulate a wide range of biological processes by regulating the expression of the proteins at post-transcriptional level (5-7). Evidence is emerging for the roles of miRNAs in regulating cellular processes, including cell proliferation, apoptosis, migration and differentiation(8-10). On the other hand, the aberrant expression of miRNAs has been considered as a fundamental reason for the development of different types of cancers, such as colon, breast, gastric, liver, and bladder cancers (8, 11-13). miR-96 belongs to the miRNAs family, and it has been recognized as an oncomiR in several types of cancer (14-18). In pancreatic cancer, it has been reported that miR-96 directly target KRAS and showed anti-proliferative, pro-apoptotic, and anti-metastatic properties (19). On the other hand, miR-96 promotes the proliferation of breast cancer cells by targeting FOXO3a (20). In the case of colon cancer, it was found that miR-96 was significantly up-regulated the tumor tissues compared with the adjacent normal tissues (20). However, it remains unclear how miR-96 promote the occurrence and development of colon cancer.

On the basis of these findings, miR-96 was selected for further investigation in the current study. The expression of miR-96 in colon cancer tissue and the adjacent

tissueswere compared, and the effect of miR-96 on the proliferation, apoptosis, migration and invasion of colon cancer cells were examined. Our findings suggested that upregulation of miR-96 plays an important role in promoting carcinogenesis and progression of colon cancer.

Materials and Methods

Cell culture

Colon cancer cell line SW480 cells were maintained in RPMI-1640 medium supplemented with 10% FBS (Tianhang Biotechnology, Zhejiang, China), 100 U/mL penicillin-G and 100 g/ml streptomycin (Beyotime, Shanghai, China) at 37 ℃ in an atmosphere of 5% CO_2.

Quantitative real-time polymerase chain reaction (qRT-PCR)

SW480 cells were collected after 48h of transfection. Total RNAs were isolated form cultured cells using the Trizol (Invitrogen, USA) according to the manufacturer's instructions. The expression levels of miR-96 were quantified using miRNA-specific TaqMan MiRNA Assay Kit(Applied Biosystems). Real-time PCR was performed using the Applied Biosystems 7500 Sequence Detection system. Taking U6 as internal control, miR-96 and U6 were synthesized by the GenePharma (Shanghai, China). Each sample was tested in triplicate, and the reaction conditions were: 95 ℃ for 3 minutes, 40 cycles of 95 ℃ for 10 seconds, 60 ℃ for 10 seconds, and 72 ℃ for 10 seconds.

MiRNA transfection

SW480 cells were seeded into 6-well plates and cultured for 24h before transfection. The 50 nmol/L miR-96 inhibitor and negative control sequences were transfected into SW480 cells using lipofectamine 2000 (Invitrogen; USA) in serum-free RPMI 1640 medium for 6 hours. The miR-96 inhibitor and negative control sequences were purchased from RiboBio (RiboBioCo.Ltd, Guangzhou, Guangdong). Lipofectamine 2000 (Invitrogen, USA) was used to transfect miRNAs into cells. Transfection complexes were prepared according to the manufacturer's instructions.

Western blot

SW480 cells were lysis at 4℃ using RIPA lysis buffer (Biomed, China). The protein concentrations were determined using a BCA protein assay kit (Boster, Wuhan, China). Equal amounts of protein lysates (30 μg) were separated by SDS-PAGE (10%, 80 V for 30 min and then 120 V for 60 min). The proteins were transferred onto a PVDF membrane (Millipore Corp, Billerica, MA, USA). Then the PVDF membranes will be incubated in TBS/Tween-20 containing 5% nonfat dry milk at 37 ℃ for 3 h. After blocking, the PVDF membranes were incubated with specific primary antibodies overnight at 4 ℃. Rabbit monoclonal antibodies against FOXF2, β-catenin, GSK-3β, and GAPDH were used at 1:1000. Following incubation with primary antibodies, blots were washed three times in TBS/Tween-20 before incubation at 37 ℃ for about 1 h in goat anti-mouse or goat anti-rabbit horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, USA) conjugating antibody at 1:10000 dilution in TBS/Tween-20 containing 5% non-fat dry milk. After extensive washing in TBS/Tween-20 for another three times, the membranes were detected by the enhanced chemiluminescence system. Proteins were visualized with ECL chemiluminescent kit (ECL-plus, Thermo Fisher Scientific, Waltham, MA, USA). Autoradiographs were scanned using an Image-Pro Plus Imaging analysis software (Media Cybernetics, MD Rockville, USA)

Cell growth measurement

Cell proliferation was evaluated by MTT assay. The medium was changed before the assay. Colon cancer cell lines were seeded in 96-well plates after serum starvation overnight. MTT (Sigma) was dissolved in phosphate buffered saline (PBS) and was added to the culture medium to reach a final concentration of 0.5 mg/mL. After incubation at 37 ℃ for 4 h, the culture media containing MTT were removed, and then DMSO (Sigma, St. Louis, MO,USA) was added into each well and the absorbance at 570 nm was measured by a microplate reader (TECAN M1000, Austria GmbH, Austria). All experiments were performed intriplicates.

Cell apoptosis analysis

The apoptosis was examined by the double staining of PI and Annexin V using a apoptosis detection kit (Beyotime, Shanghai, China) with flow cytometry methods according to the manufacturer's instructions.

Transwell migration assays and wound healing migration

For transwell migration assays and wound-healing assay, SW480 cells were transfected with the miR-96 inhibitor and negative control (NC). Transwell migration assays were performed as reported previously with small modification (21).

Relative luciferase activity assay

3′-UTR of FOXF2 was cloned into a pMIR-REPORT plasmid downstream of luciferase reporter gene. Luciferase activities were assayed using a luciferase assay kit (Promega, Madison, WI, USA), and the target effect was measured as the relative luciferase activity of the reporter vector with target sequence over that without the target sequence.

Statistical analysis

All values were expressed as mean ± standard deviations. Results were subjected to Student's t- test as appropriated with the program SPSS19.0. Differences between two means with $P<0.05$ were considered significant.

Results

Down-regulation of miR-96 enhanced cell apoptosis and reduced the percent proliferation of colon cancer cells in vitro.

To evaluate the biological role of miR-96 expression in the development and progression of colon cancer, we transfected colon cancer cells SW480 with miR-96 inhibitor and examined its effect on cellular proliferation. PT-PCR was used to detect miR-96 mRNA levels. As shown in Fig. 1A, the expression of miR-96 was decreased compared with negative control (NC) group. MTT and flow cytometric assays showed that the inhibition of miR-96 significantly decreased the proliferation and increased the apoptosis colon cancer cells incomparison with the NC group (Figure 1B and 1C, $P<0.01$).

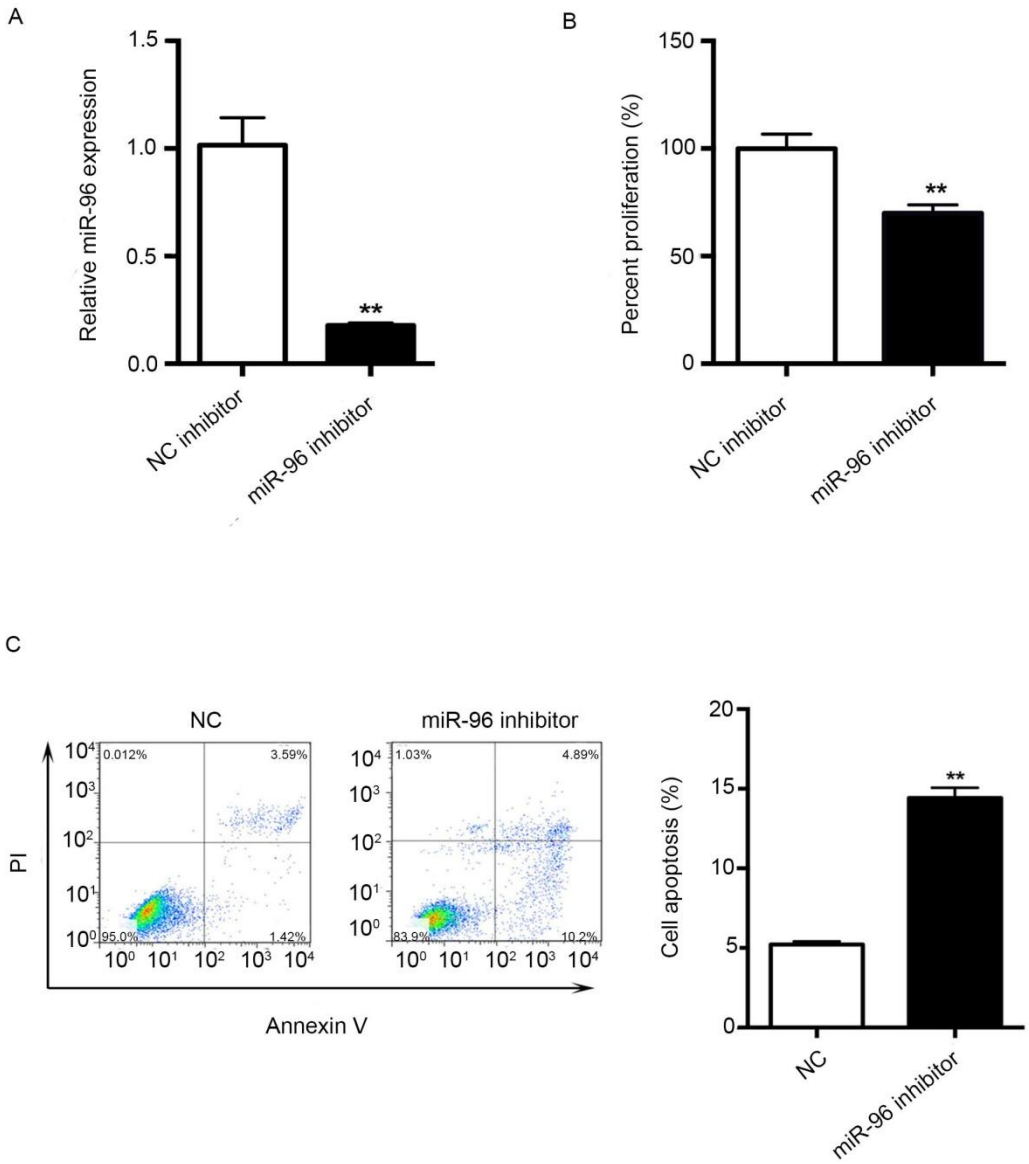

Figure 1. Down-regulation of miR-96 enhanced cell apoptosis and reduced the percent proliferation of colon cancer cells in vitro (**, *P<0.01*,vs NC group)

Inhibition of miR-96 suppressed the migration and invasion of SW480 cells in vitro.

It has been reported that the miR-96 could regulate the cell cycle, ageing and metabolism (22-24). To examine the effect of miR-96 on the migration of colon cancer cells, we performed the capacity of migration and invasion analysis using wound healing and transwell migration assays in SW480 cells. The results showed

that inhibition of miR-96 suppressed the migration and invasion of SW480 cells (Figure 2A and 2B, *p<0.01*).

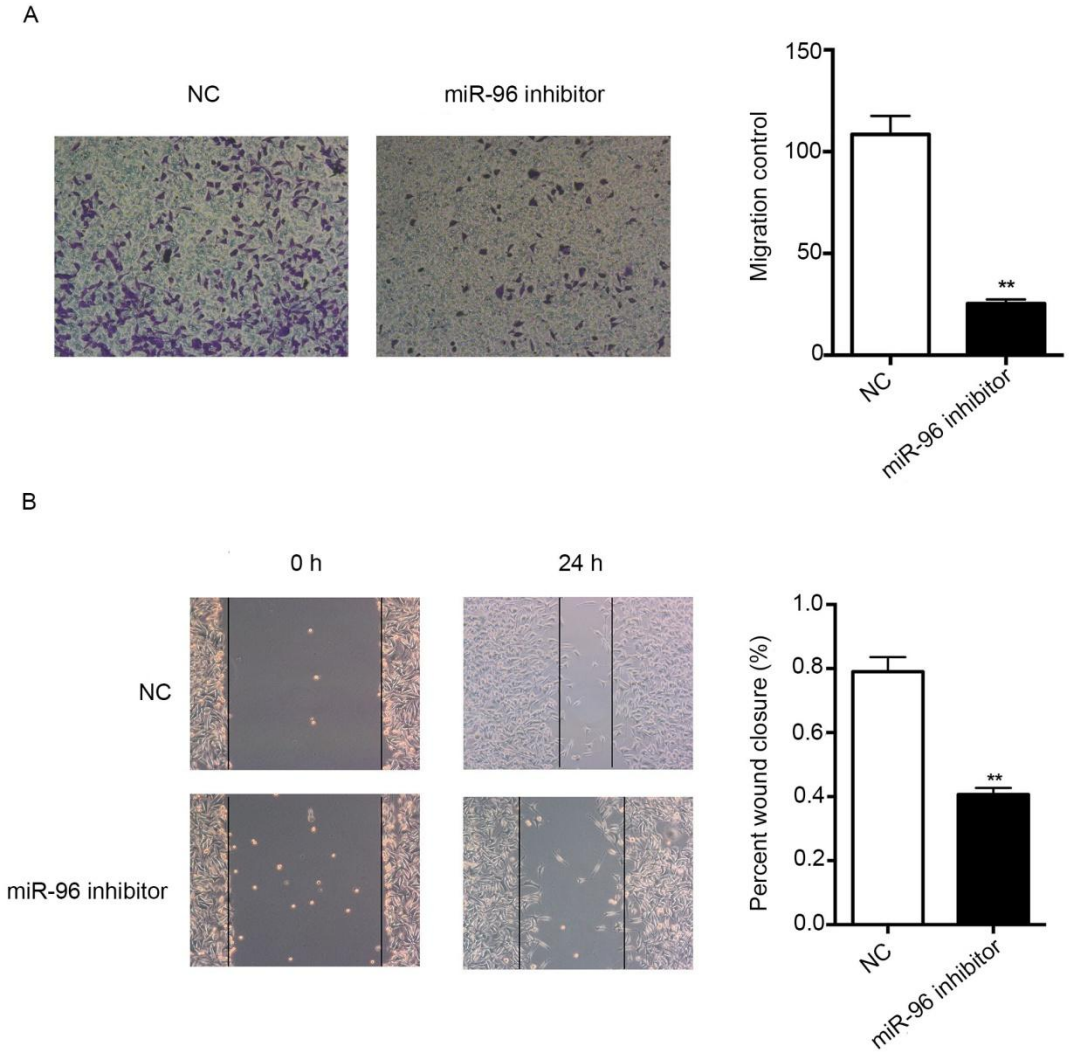

Figure 2. Inhibition of miR-96 suppressed the migration and invasion of SW480 cells in vitro (**, *P<0.01*,vs NC group)

FOXF2 is a target of miR-96 in colon cancer cells.

The forkhead box (FOX) family of transcription factors, which with a characteristic of a highly conserved DNA binding domain (25, 26) and tissue-specific expression patterns, critically involved in the regulation of embryogenesis and tissue development is known to cause the progression of many diseases, particularly cancer (27, 28). Forkhead box F2 (FOXF2) is a subfamily of the FOX gene family and it

plays a key role in the development of several types of cancer (29-30). In this study, we explored the correlation between miR-96 with FOXF2 through transfecting miR-96 mimics and luciferase reporter assay test. As depicted in Fig. 3A, miR-96 regulated the expression of the 3'UTR of luciferase carried *FOXF2* (*p<0.01*). After mutation of binding site, the regulatory relationship was disappeared. As predicted, FOXF2 is a target gene of miR-96. To study the expression of FOXF2 in colon cells, we performed the mRNA and protein levels of FOXF2 by PT-PCR and western blot assay. As depicted in Fig. 3B, the inhibition of miR-96 increased the FOXF2 expression in the mRNA and protein levels. The above results verified that down-regulation of miR-96 contributing to high expression of FOXF2 in SW480 cells.

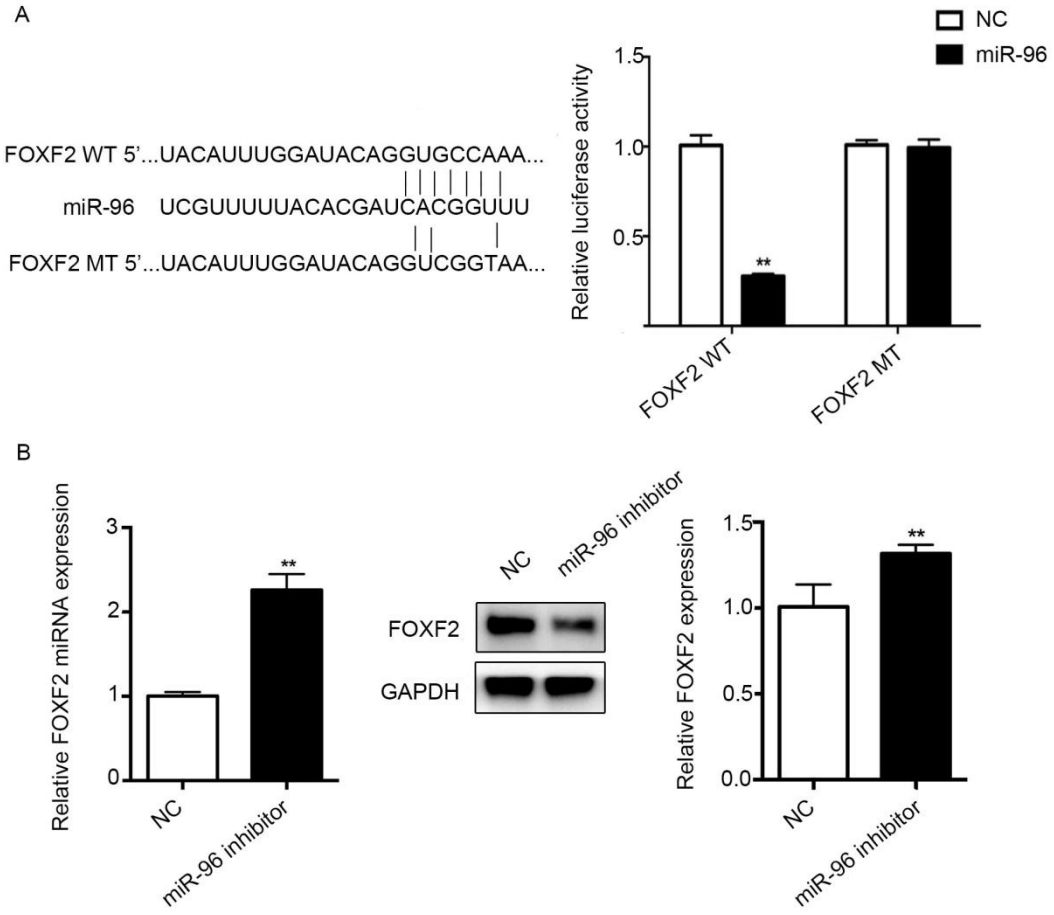

Figure 3. FOXF2 is a target of miR-96 in colon cancer cells (**, *P<0.01*,vs NC group)

MiR-96 regulates FOXF2 for β-catenin signaling.

Activation of Wnt/β-catenin signaling promotes cell proliferation and growth, which plays critical roles in the regulation of initiation and progress of various cancers (31-33), including colon cancer. β-catenin entering nuclear combines with TCF/LEF transcription factors, which cause expression of target genes, such as GSK-3β, c-Myc and cyclin D1 (34). The final objects are to promote cell proliferation and control characteristics of the malignant phenotype (35). In order to confirm whether miR-96 regulates FOXF2 for Wnt/β-catenin signaling, Western Blot was done to examine expression of β-catenin and GSK-3β. We found that miR-96 could regulate the expression of β-catenin and GSK-3β in protein level (Fig. 4, $p<0.01$).

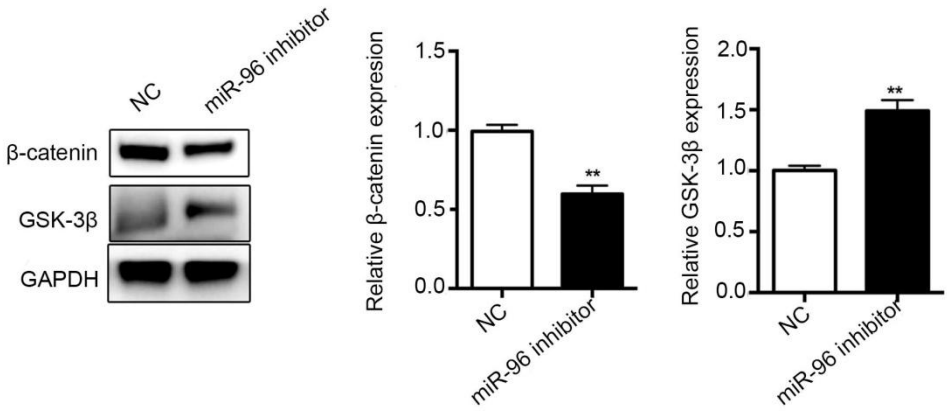

Figure 4. miR-96 regulates FOXF2 for β-catenin signaling (**, $P<0.01$,vs NC group)

Discussion

Studies from the recent years have proved the roles of miRNA in human cancers(36), and previous research demonstrated that miR-96 was associated with the development many cancers. In this study, we found that inhibition of the expression of miR-96 could reduce the proliferation, migration, and invasion of colon cancer cells via targeting FOXF2 *in vitro*, suggesting that miR-96 may serve as an oncomiR in colon cancer.

The FOX family of transcription factors have been proved to be associated with cancers through various mechanisms. Evidence showed that in prostate cancer the expression of FOXF2 was down-regulated(37). However, little was known about the

correlation between FOXF2 expression and miR-96 in colon cancer. In this study, we explored the correlation between FOXF2 mRNA expression and miR-96. We verify that FOXF2 is the direct target of miR-96 through transfecting miR-96 mimics and luciferase reporter assay test. qRT-PCR analysis and Western Blot revealed that inhibition of the expression of miR-96 could upregulated the expression of FOXF2.

Wnt/β-catenin signaling pathway is a highly evolutionarily conserved pathway with β-catenin as the main molecule. Wnt signaling is involved virtually in every aspect of embryonic development and also controls homeostatic self-renewal in various adult tissues (38). β-Catenin is a key component of the Wnt signaling pathway (39). Some evidence proved that the constitutive activation of Wnt/β-catenin is associated with many kinds of cancer, including colon cancer. In our present study, to understand the mechanism of miR-96 in regulating the proliferation and migration of colon cancer cells, we analysed the expression of β-catenin and GSK-3β after down-regulating miR-96. The results indicated that inhibition of miR-96 could regulate the expression of β-catenin and GSK-3β in protein level. We hypothesized that aberrant expression of miR-96 could regulate Wnt/β-catenin signaling.

Taken together, we presented evidences for a role of miR-96 in colon cells for the first time and showed that FOXF2 is the direct target of miR-96. Moreover, we confirmed that miR-96 target FOXF2 to modulate the Wnt/β-catenin signaling in colon cells. These findings fulfill our knowledge on the mechanism of colon cancer and may provide a new perspective for the clinical therapy.

References

1. Gellad Z.F and Provenzale D: Colorectal cancer: national and international perspective on the burden of disease and public health impact. Gastroenterology 138: 2177-2190, 2010.
2. Mccain J: Carlos fernández-del castillo confronts the challenge of pancreatic cancer: although progress has been slow, a renowned expert on the disease sees reasons for hope. P & T 39: 281, 2014.

3. Xu L.H, Deng C.S, Zhu Y.Q, et al: Synergistic effect of tumor necrosis factor-related apoptosis inducing ligand (TRAIL) and doxorubicin on human colon cancer SW480 cells. Chin J Cancer 2: 816, 2003.
4. Pandey G.K: Regulatory Roles of Noncoding RNA in Development and Disease. 2013.
5. Bolton E.M, Tuzova A.V, Walsh A.L, et al: Noncoding RNAs in prostate cancer: the long and the short of it. Clin Cancer Res 20: 35-43, 2014.
6. Seligmann B.A: Methods of co-detecting mRNA and small non-coding RNA. 2016.
7. Yin Z, Li Y, Han X, and Shen F: Genome-wide profiling of miRNAs and other small non-coding RNAs in the Verticillium dahliae-inoculated cotton roots. Plos One 7: e35765, 2012.
8. Pal M and Pal P: BRCA1 and miRNAs: An Emerging Therapeutic Target and Intervention Tool in Breast Cancer. diabetes 22: 23, 2013.
9. Zhou X, Jin W, Jia H, et al: MiR-223 promotes the cisplatin resistance of human gastric cancer cells via regulating cell cycle by targeting FBXW7. J Exp Clin Cancer Res 34: 28, 2015.
10. Yu X.F, Zou J, Bao Z.J, et al: miR-93 suppresses proliferation and colony formation of human colon cancer stem cells. World J Gastroenterol 19: 3770, 2013.
11. Deyao W.U, Ding J, Wang L, et al: microRNA-125b inhibits cell migration and invasion by targeting matrix metallopeptidase 13 in bladder cancer. Oncol Lett 5: 829-834, 2013.
12. Ohshima K, Inoue K, Fujiwara A, et al: Let-7 microRNA family is selectively secreted into the extracellular environment via exosomes in a metastatic gastric cancer cell line. Plos One 5: e13247, 2010.
13. Bagnyukova T.V, Pogribny I.P, and Chekhun V.F. MicroRNAs in normal and cancer cells: a new class of gene expression regulators. Exp Oncol 28: 263-269, 2006.
14. Guttilla I.K and White B.A: Coordinate regulation of FOXO1 by miR-27a, miR-96, and miR-182 in breast cancer cells. J Biol Chem 284: 23204-23216, 2009.
15. Sacheli R, Nguyen L, Borgs L, et al: Expression patterns of miR-96, miR-182 and miR-183 in the development inner ear. Gene Expr Patterns 9: 364, 2009.

16. Laine S.K, Alm J.J, Virtanen S.P, et al: MicroRNAs miR-96, miR-124, and miR-199a regulate gene expression in human bone marrow-derived mesenchymal stem cells. J Cell Biochem 113: 2687, 2012.
17. Cai T, Long J, Wang H, et al: Identification and characterization of miR-96, a potential biomarker of NSCLC, through bioinformatic analysis. Oncol Rep 38: 1213, 2017.
18. Budzinska M, Owczarz M, Pawlikpachucka E, et al: miR-96, miR-145 and miR-9 expression increases, and IGF-1R and FOXO1expression decreases in peripheral blood mononuclear cells of aging humans. BMC Geriatr 16: 200, 2016.
19. Yu S, Lu ZC, Meng Y, et al: miRNA-96 suppresses KRAS and functions as a tumor suppressor gene in pancreatic cancer. Cancer Res 70: 6015, 2010.
20. Kong PZ, Yang F, Li L, et al: Decreased FOXF2 mRNA expression indicates early-onset metastasis and poor prognosis for breast cancer patients with histological grade II tumor. Plos One 8: e61591, 2013.
21. Xie YK, Huo SF, Zhang G, et al: CDA-2 induces cell differentiation through suppressing Twist/SLUG signaling via miR-124 in glioma. J Neurooncol 110: 179, 2012.
22. Menc á A, Modamioh øybj ør S, Redshaw N ,et al: Mutations in the seed region of human miR-96 are responsible for nonsyndromic progressive hearing loss. Nat Genet 41: 609, 2009.
23. Yamada Y, Enokida H, Kojima S, et al: MiR-96 and miR-183 detection in urine serve as potential tumor markers of urothelial carcinoma: correlation with stage and grade, and comparison with urinary cytology. Cancer Sci 102: 522–529, 2011.
24. Kuhn S, Johnson S.L, Furness D.N, et al: miR-96 regulates the progression of differentiation in mammalian cochlear inner and outer hair cells. Proc Natl Acad Sci U S A 108: 2355-2360, 2011.
25. Ito YA: Contribution of FOXC1 to the development of axenfeld-rieger syndrome and glaucoma. 2013.
26. Adell T and M üller W.E: Isolation and characterization of five Fox (Forkhead) genes from the sponge Suberites domuncula. Gene 334: 35-46, 2004.
27. Lo PK, Ji SL, Liang X, et al: The forkhead box-F (FOXF) proteins, important regulators in development, function as tumor suppressors and are epigenetically silenced in breast cancer. Mol Cancer Ther 3615S, 2007.

28. Lo PK: The controversial role of forkhead box F2 (FOXF2) transcription factor in breast cancer. Pras Open 1, 2017.
29. Wang QS, Kong PZ, Li XQ, et al: FOXF2 deficiency promotes epithelial-mesenchymal transition and metastasis of basal-like breast cancer. Breast Cancer Res 17: 30, 2015.
30. Van dHL, Dits N, Van IW et al: The FOXF2 pathway in the human prostate stroma. Prostate 69: 1538-1547, 2010.
31. Lin Q, Carmon K, Gong X: Regulation of Wnt/beta-Catenin signaling. U.S. Patent No. 9,057,096. 2013.
32. Zhou G. Wnt/β-Catenin Signaling and Oral Cancer Metastasis. Oral Cancer Metastasis. Springer, New York, pp231-264, 2010.
33. Minli MO, Mengru LI, Chen Z, et al: Inhibition of the Wnt palmitoyltransferase porcupine suppresses cell growth and downregulates the Wnt/β-catenin pathway in gastric cancer. Oncol Lett 5: 1719-1723, 2013.
34. Akiyama T, Sekiya T, Ohwada S: Anti-BAMBI antibody and diagnostic or remedy for colon cancer and liver cancer containing the same. U.S. Patent 7,491,802, 2009.
35. Okuda Y, Nakano K, Suzuki K, et al: Wnt signaling as a possible promoting factor of cell differentiation in pleomorphic adenomas. Int J Med Sci 11: 971-978, 2013.
36. Garzon R, Fabbri M, Cimmino A, et al: MicroRNA expression and function in cancer. Trends Mol Med 12: 580, 2006.
37. Hiroshi H, Koji U, Varahram S, et al: MicroRNA-182-5p promotes cell invasion and proliferation by down regulatingFOXF2,RECK and MTSS1 genes in human prostate cancer. Plos One 8: e55502, 2013.
38. Clevers H: Wnt/β-catenin signaling in development and disease. Cell 127: 469-480, 2006.
39. Akiyama T: Wnt/β-catenin signaling. Cytokine Growth Factor Rev 11: 273-282, 2000.