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ORIGINAL ARTICLE

MicroRNA-31 inhibits the growth and metastasis and enhances drug sensitivity of the human colon cancer cells by targeting PAX6

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Summary

Purpose: *Every year more than 2 million cases of colon cancer are detected across the world. There is a pressing need for identification of efficient therapeutic targets for the management of this disease. Herein, we explored the role of miR-31 in colon cancer via regulation of paired box 6 (PAX6).*

Methods: *The expression profile of miR-31 was determined by qRT-PCR. Cell viability was determined by qRT-PCR. Colony formation potential was assessed by clonogenic assay. Transwell assay was used for the assessment of the cell migration and invasion. Protein expression was determined by western blot analysis.*

Results: *The findings showed that miR-31 is significantly suppressed in colon cancer. Restoration of the miR-31 in RKO colon cancer cells resulted in significant decline in their viability and colony formation. Conversely, inhibition of miR-31 resulted in the promotion of proliferation and colony formation of the RKO cells. The miR-31 overexpression also caused a remarkable decrease in the migration and invasion potential of the RKO cells. Bioinformatic approaches showed that PAX6 acts as the target of miR-31 in colon cancer and the interaction between these two also confirmed by dual-luciferase assay. The expression of PAX6 was found to be significantly upregulated in colon cancer cells and miR-31 overexpression suppressed its expression. Additionally, PAX6 silencing resulted in decline in the RKO cell viability. However, PAX6 overexpression promoted the proliferation of RKO cells by avoiding the tumor suppressive effects of miR-31.*

Conclusion: *Taken all together, miR-31 may prove essential therapeutic target for the treatment of colon cancer.*

Key words: *colon cancer, microRNA-31, proliferation, apoptosis, invasion*

Introduction

MicroRNAs (miRs) have great functional diversity by regulating a vast number of cellular and physiological processes [1]. Regulating around 30% of the human genes, they have been shown to be involved in processes such as cell cycle, differentiation, proliferation and apoptosis to name a few [2]. MiRs have also been implicated in cancer-related processes such as tumorigenesis and metastasis. MiRs have been categorized as oncomiRs which promote the growth of cancer or as tumor-suppressors which suppress the growth of tumors [3]. MiR-31 has been revealed to be involved in the development of bladder cancer [4]. In gastric cancer low expression of miR-31 is linked with poor prognosis [5]. Involvement of miR-31 in the regulation of colon cancer invasion has been reported [6]. However, the role of miR-31 in colon cancer growth and metastasis via regulation of Paired

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box 6 (PAX6) expression has not been studied. This study was undertaken to unveil the role of miR-31 in colon cancer via modulation of PAX6 expression. Colon cancer is responsible for around a million deaths annually across the world. Currently, it is considered as the 3rd prevalent cancer and 4th main reason for cancer associated deaths [7]. Annually, around two million new colon cancer patients are added to the list worldwide. Although a decline has been observed in colon cancer over the last few decades, owing to changing life style and environmental factors, colon cancer incidence is expected to increase significantly in the coming years [8]. According to reports, it is believed that the colon cancer incidence will increase by more than 60% [9]. Different treatment regimens are being used for the treatment of this disease but late diagnosis and frequent relapses add to the hurdles faced in colon cancer treatment. Herein, we showed that miR-31 is significantly suppressed in colon cancer and its upregulation suppresses its growth and metastasis via post-transcriptional suppression of PAX6.

Methods

Tissue samples, cell lines and culture conditions

Specimens of colon cancer tissues and normal surrounding tissues came from the patients undergoing treatment at the Tianjin Medical University General Hospital, Tianjin, China. Written informed consent from the patients was taken prior to the collection of tissues. The institutional ethical guidelines were strictly followed for the collection and laboratory usage of clinical specimens.. The specimens were snap-frozen and stored in liquid nitrogen. The normal colon CCD-18Co and the colon cancer cell lines HT-29, SW-948, RKO and SW480 were procured from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. The cell lines were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 0.2% penicillin and streptomycin (Invitrogen, Carlsbad, California, United States). All cells were cultured in a 5% CO_2 incubation chamber at 37°C.

RNA isolation and qRT-PCR analysis

Total RNA was isolated from the cells with RNAiso reagent (Takara, Japan). The RNA extracted was subjected to DNAse I treatment (Thermo Fisher Scientific, Waltham, Massachusetts, USA). cDNA synthesis was performed with the help of Primescript™ reverse transcription reagent (Takara, Japan). Quantitative real time-PCR (qRT-PCR) was performed on QuantStudio 3 Real Time-PCR system (Thermo Fisher Scientific) following the manufacturer's guidelines. The relative expression was normalized with human GADPH gene and $2^{-\Delta\Delta Ct}$ method was used to quantify the relative expression values. RT primers were synthesized through Primer3 v. 0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/) online software.

Transfection

The transfection of miR-negative control (NC), miR-31 mimics and miR-31 inhibitor were performed with Lipofectamine 2000 (Invitrogen) following the manufacturer's guidelines. Since RKO cell line exhibited the lowest expression of miR-31, only this cell line was used for further experimentation.

Cell viability assay

The assessment of the RKO cell viability was done by MTT assay. The transfected RKO cells were incubated at 37°C for 24 h and then treated with MTT for 4 h. Subsequently dimethyl sulfoxide (DMSO) (10%) was added to dissolve the formazan crystals and finally absorbance was taken at 570 nm by spectrophotometer to determine the RKO cell viability.

Colony formation assay

For evaluation of the colony formation potential of the miR-31 overexpressing RKO cells, around 500 log-phase RKO cells were plated in 6-well plates and then subjected to incubation at 37°C for 2 weeks. The cells were then fixed and stained with Giemsa. The colonies were finally counted and also photographed.

Migration and invasion assay

Transwell chamber with Matrigel coating was used to assess the invasion of transfected cancer cells. Briefly, 100µl cell culture containing 6000 cells was added to the upper chamber of the transwell and lower chamber was given 750 µl of DMEM medium supplemented with 10% FBS. After 48-h incubation at 37°C/5%CO_2, cells from the surface of membrane's upper side were removed carefully with cotton swabs, while those sticked to lower side of the membrane were fixed with 70% ethyl alcohol and stained with 0.1% crystal violet. Light microscope (×100) was used for visualization of cells and photographs were taken. At least 7 random fields were used for counting the invasive cells.

Western blotting

The RKO cells were lysed in ice cold hypotonic buffer containing protease inhibitors. The protein content of the RKO cell lysates were evaluated by bicinchoninic acid (BCA) assay. Similar quantities of the proteins from each sample were loaded and then subsequently separated on SDS-PAGE. After transferring the gels to nitrocellulose membranes, the membranes were treated with primary antibodies for 55 min for at 23°C. This was followed by incubation with secondary antibody. The visualisation of the bands was carried out by chemiluminescence reagent.

Statistics

The experiments were performed in triplicate. The values are shown as mean ± SD. Student's t-test (for comparison between two samples) and one-way ANOVA followed by Tukey's test (for comparison between more than two samples) were used for statistical analysis using GraphPad Prism software v.7 (GraphPad Prism Inc, La Jolla, CA, USA)S. $P<0.05$ was considered to indicate statistically significant difference.

Results

miR-31 is significantly suppressed in colon cancer

The expression analysis of miR-31 in the colon cancer tissue and cell lines by qRT-PCR was first carried out to know about the expression prolife of the miR-31 in human colon cancer. The outcomes of the qRT-PCR as determined by ΔΔCT methodology revealed a remarkable downregulation of miR-31 in colon cancer cell lines relative to the normal cells. The expression of miR-31 in colon cancer tissues was upto 7 folds downregulated (Figure 1A). Additionally, the determination of the expression in miR-31 in colon cancer tissues revealed upto 6 folds downregulation of miR-31 (Figure 1B). Subsequently, the RKO colon cancer cells which exhibited the lowest transcript levels of miR-31 were selected for further experimentation.

miR-31 suppresses the proliferation of colon cancer cells

To understand the function of miR-31 in colon cancer, we overexpressed miR-31 in RKO colon cancer cells (Figure 1C) and then subjected them to MTT assay. The outcomes of the assay revealed that miR-31 overexpression caused a significant reduction of the viability of RKO cells in a time-dependent manner. Nonetheless, the proliferation of the RKO cells was promoted upon miR-31 inhibition (Figure 1D). The colony formation assay was also performed to assess the colon formation

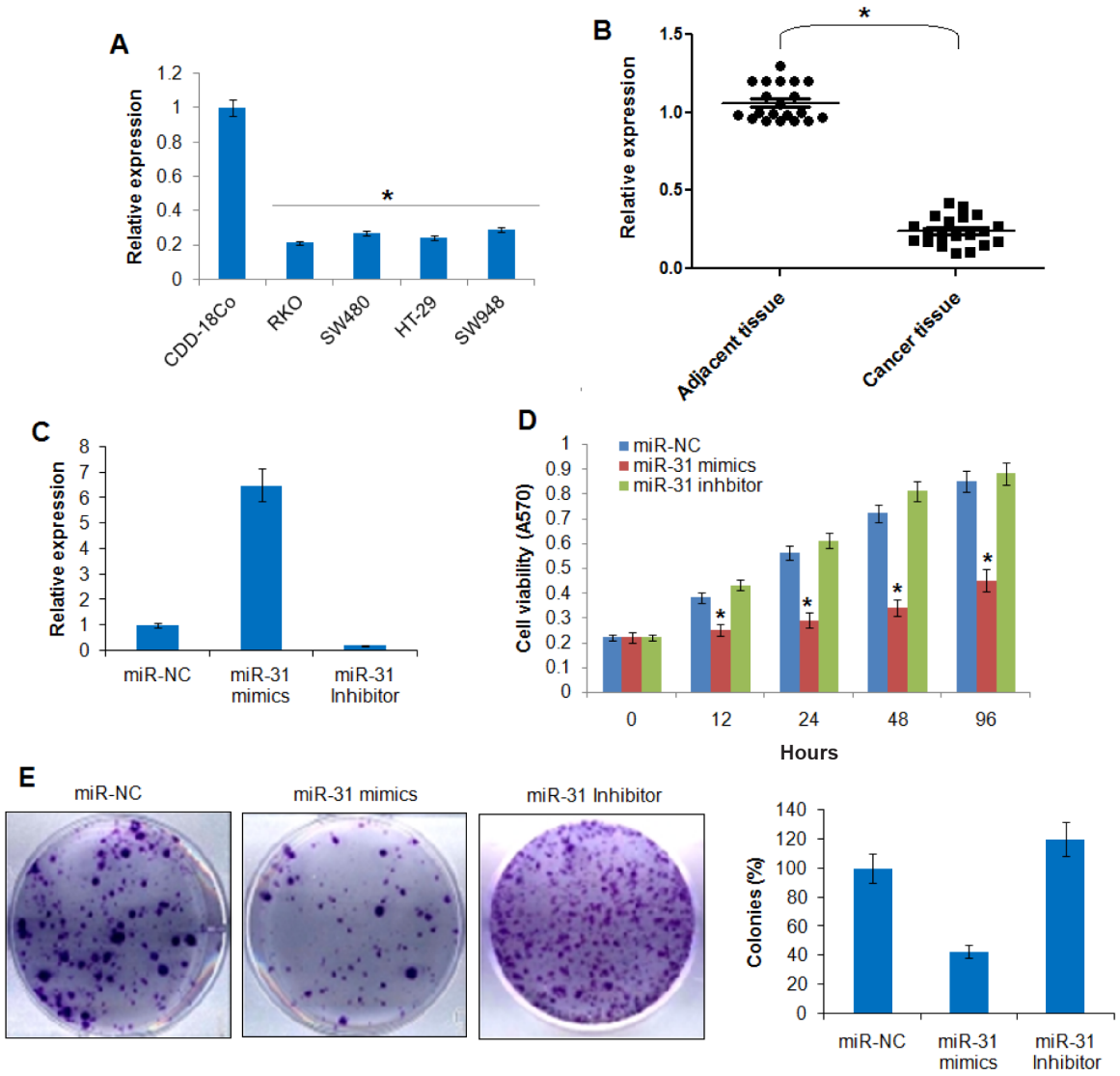

Figure 1. miR-31 suppresses the proliferation of human colon cancer. **A:** Expression of miR-31 in normal CDD-18Co and colon cancer cells. **B:** Expression of miR-31 in cancer and normal adjacent tissues. **C:** Expression of miR-31 in miR-NC, miR-31 mimics and miR-31 inhibitor transfected RKO cells. **D:** Cell viability of miR-NC, miR-31 mimics and miR-31 inhibitor transfected RKO cells. **E:** Colony formation potential of the miR-NC, miR-31 mimics and miR-31 inhibitor transfected RKO cells. The experiments were performed in triplicate and expressed as mean ± SD (*p< 0.05).

potential of the miR-31 overexpressing cells. The findings showed that miR-31 overexpression remarkably inhibited the colony formation potential of the RKO cells (Figure 1E). However, the suppression of miR-31 expression promoted the colony formation of the RKO cells. Taken together, miR-31 suppresses the viability and colony formation potential of the colon cancer cells.

miR-31 enhances drug sensitivity of human colon cancer cells

The effects of miR-31 were also investigated on the sensitivity of the RKO colon cancer cells to 5-FU. The miR-31 overexpressing, 5-FU treated and miR-31 overexpressing plus 5-FU treated were subjected to MTT assay. The results revealed that miR-31 overexpression made the RKO colon cancer cells more sensitive to the cytotoxic effects of 5-FU (Figure 2).

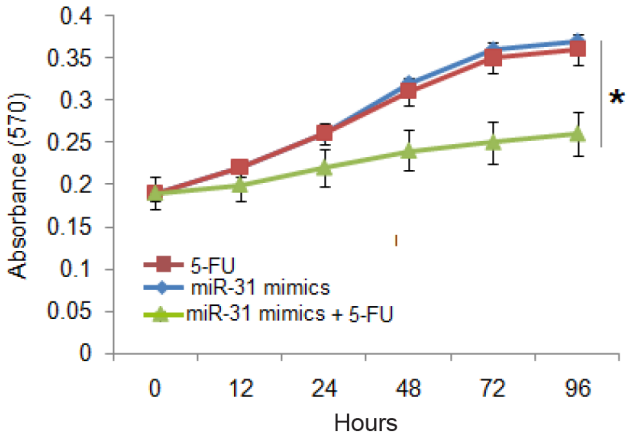

Figure 2. The miR-31 enhances the chemosensitivity of the RKO cells to 5-FU. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

miR-31 suppresses the metastasis of human colon cancer cells

To gain insights about the anti-metastatic potential of miR-31 overexpression, we performed transwell migration and invasion assays. It was observed that the migration of the RKO colon cancer cells was decreased by 58% (Figure 3A) and the invasion was inhibited by 61% (Figure 3B). Nonetheless, the suppression of miR-31 expression promoted both migration and invasion of the RKO colon cancer cells.

miR-31 exerts its effects by targeting PAX6

The bioinformatic analysis showed that miR-31 targets the PAX6 (Figure 4A). The dual luciferase assay proved the interaction between the miR-31 and PAX6 (Figure 4B). Next, we performed the qRT-PCR and the outcomes revealed that PAX6 is remarkably upregulated in human colon cancer cells (Figure 4C). Nonetheless, the western blot analysis showed that the overexpression of miR-31 caused remarkable decrease in the expression of PAX6 (Figure 4D). To further confirm that miR-31 exerts its effects via post-transcriptional suppression of PAX6, we performed the MTT assay of the si-PAX6 mimics and si-NC transfected RKO cells. The outcomes showed that silencing the PAX6 caused significant decline of the RKO cell proliferation (Figure 5A). However, PAX6 overexpression promoted the proliferation of the RKO cells by avoiding the tumor-suppressive effects of miR-31 (Figure 5B).

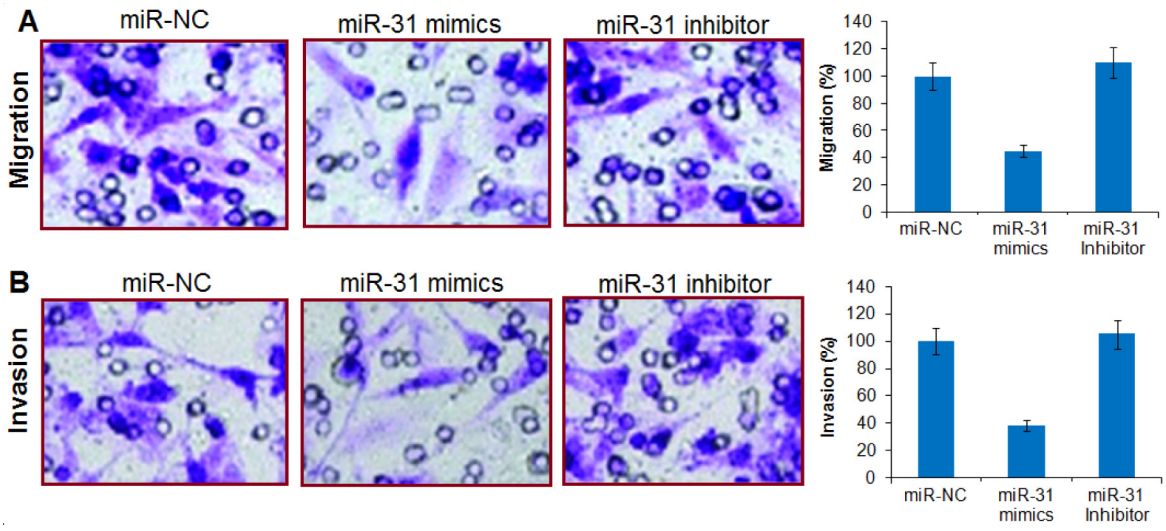

Figure 3. miR-31 suppresses the metastasis of colon cancer cells. **A:** Cell migration and **B:** Cell invasion of the miR-NC, miR-31 mimics and miR-31 inhibitor transfected RKO cells as determined by transwell assays. The experiments were performed in triplicate and expressed as mean ± SD (p< 0.05).

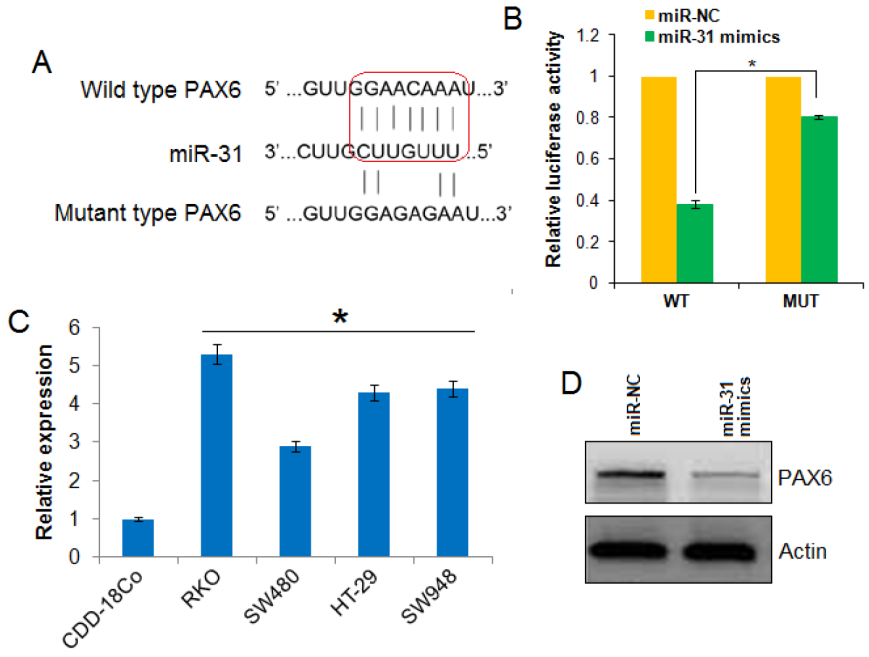

Figure 4. miR-31 targets PAX6 to exert its effects. **A:** TargetScan analysis showing PAX6 as the target of miR-31. **B:** Dual luciferase assay. **C:** Expression of PAX6 in normal and colon cancer cells. **D:** Western blot analysis showing the expression of PAX6 in miR-NC and miR-31 mimics transfected RKO cells. The experiments were performed in triplicate and expressed as mean ± SD (*p< 0.05).

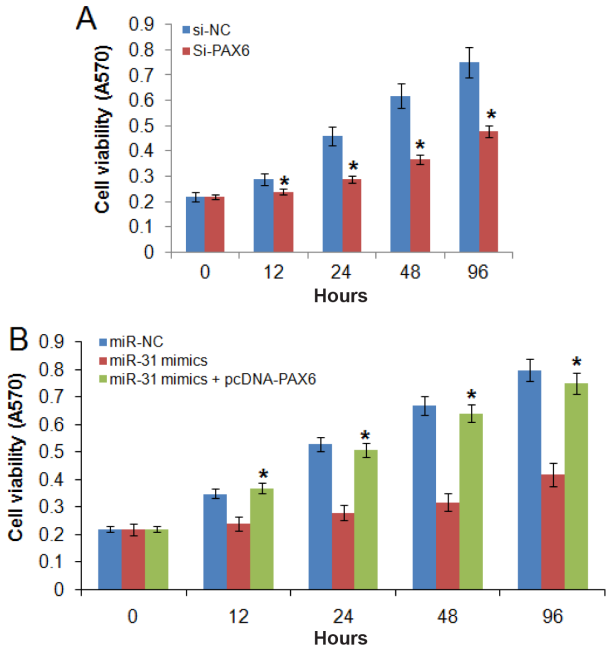

Figure 5. PAX6 overexpression abolishes the effects of miR-31. **A:** Cell viability of si-NC and si-PAX6 transfected RKO cells. **B:** miR-NC, miR-31 mimics, miR-31 mimics + pcDNA-PAX6 transfected RKO cells. The experiments were performed in triplicate and expressed as mean ± SD (*p< 0.05).

Discussion

Colon cancer is one of the devastating malignancies and is currently considered to be the 3rd prevalent type of cancer across the globe [10]. The therapeutic targets for an efficient treatment of colon cancer are limited. In this article we studied the function of miR-31 in colon cancer via regulation of PAX6 and the findings showed that miR-31 is remarkably downregulated in colon cancer and its overexpression suppresses the viability and colony formation of the colon cancer cells. However, the inhibition of miR-31 promoted the proliferation and colony formation potential of the colon cancer cells. These findings suggest that miR-31 acts as a tumor-suppressor in colon cancer. These results are also supported by a previous study wherein miR-31 has been reported to be suppressed in bladder cancer [4]. The miR-31 also enhanced the drug sensitivity of the colon cancer to 5-FU. This is in agreement with a previous observation wherein miR-31 has been reported to increase the resistance of the ovarian cancer cells to cisplatin [11]. Additionally, the mitomycin-C sensitivity of the urothelial bladder cancer cells is also increased by miR-31 [12]. The migration and invasion of cancer cells are among the essential processes required for the metastasis of cancer cells [13]. The findings of our study revealed that miR-31 suppressed the migration and invasion of the human colon cancer cells. Different miRs exert their effects via post-transcriptional suppression of different genes and each miR may have multiple molecular targets [14]. Herein, we found that miR-31 targets PAX6. The interaction between the miR-31 and PAX6 were confirmed

by the dual luciferase assay. PAX6 has previously been shown to be aberrantly expressed in several types of cancers such as lung cancer, glioma and retinoblastoma to name a few [15-17]. As such it may be involved in the development and progression of different types of cancers including colon cancer. Silencing of PAX6 resulted in suppression of the proliferation of colon cancer cells and overexpression of PAX6 could avoid the tumor suppressive effects of miR-31. Taken all together, miR-31 may prove essential in the management of colon cancer.

Conclusion

Taken all together, it is concluded that miR-31 is significantly suppressed in human colon cancer. The miR-31 overexpression suppresses the growth, drug sensitivity and metastasis of the human colon cancer cells via post-transcriptional suppression of PAX6. As such miR-31 may be utilised as a therapeutic target for the colon cancer treatment.

Conflict of interests

The authors declare no conflict of interests.

References

1. Bushati N, Cohen SM. microRNA functions. Annu Rev Cell Dev Biol 2007;23:175-205.
2. Nana-Sinkam SP, Croce CM. MicroRNAs as therapeutic targets in cancer. Translat Res 2011;157:216-25.
3. Carthew RW, Sontheimer EJ. Origins and mechanisms of miRNAs and siRNAs. Cell 2009;136:642-55.
4. Wang S, Li Q, Wang K et al. Decreased expression of microRNA-31 associates with aggressive tumor progression and poor prognosis in patients with bladder cancer. Clin Transl Oncol 2013;15:849-54.
5. Wang H, Zhang X, Liu Y et al. Downregulated miR-31 level associates with poor prognosis of gastric cancer and its restoration suppresses tumor cell malignant phenotypes by inhibiting E2F2. Oncotarget 2016;7:36577.
6. Wang CJ, Stratmann J, Zhou ZG, Sun XF. Suppression of microRNA-31 increases sensitivity to 5-FU at an early stage, and affects cell migration and invasion in HCT-116 colon cancer cells. BMC Cancer 2010;10:616.
7. Siegel R, DeSantis C, Jemal A. Colorectal cancer statistics, 2014. CA: Cancer J Clin 2014;64:104-17.
8. Arnold M, Sierra MS, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global patterns and trends in colorectal cancer incidence and mortality. Gut 2017;66:683-91 .
9. Potter JD, Slattery ML, Bostick RM, Gapstur SM. Colon cancer: a review of the epidemiology. Epidemiol Rev 1993;15:499-545.
10. Van Cutsem E, Cervantes A, Adam R et al. ESMO consensus guidelines for the management of patients with metastatic colorectal cancer. Ann Oncol 2016;27:1386-422.
11. Dong Z, Zhong Z, Yang L, Wang S, Gong Z. MicroRNA-31 inhibits cisplatin-induced apoptosis in non-small cell lung cancer cells by regulating the drug transporter ABCB9. Cancer Lett 2014;343:249-57.
12. Xu T, Qin L, Zhu Z et al. MicroRNA-31 functions as a tumor suppressor and increases sensitivity to mitomycin-C in urothelial bladder cancer by targeting integrin α5. Oncotarget 2016;7:27445.
13. Choi JH, Song YS, Yoon JS, Song KW, Lee YY. Enhancer of zeste homolog 2 expression is associated with tumor cell proliferation and metastasis in gastric cancer. Apmis 2010;118:196-202.
14. Wu W, Sun M, Zou GM, Chen J. MicroRNA and cancer: Current status and prospective. Int J Cancer 2007;120:953-60.
15. Luo J, Li H, Zhang C. MicroRNA 7 inhibits the malignant phenotypes of non small cell lung cancer in vitro by targeting Pax6. Mol Med Rep 2015;12:5443 8.
16. Cheng Q, Cao H, Chen Z et al. PAX6, a novel target of miR 335, inhibits cell proliferation and invasion in glioma cells. Mol Med Rep 2014;10:399 404.
17. Meng B, Wang Y, Li B. Suppression of PAX6 promotes cell proliferation and inhibits apoptosis in human retinoblastoma cells. Int J Mol Med 2014;34:399 408.