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Original Article

miR-149 regulates the proliferation and apoptosis of human colonic carcinoma cells by targeting FZD5

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Abstract: Objective: To explore the effects of miR-149 on the cell proliferation and apoptosis of colorectal cancer (CRC) and its potential molecular mechanism. Methods: miR-149 expression patterns were detected in human CRC cell lines by quantitative real-time RT-PCR (Q-PCR). Online prediction software and luciferase reporter assay were performed to screen the functional targets of miR-149. CRC cells were transfected with miR-149 mimics or siRNAs of FZD5 and then divided into NC group (negative control), miR-149 mimics group (cells transfected with miR-149 mimics) and miR-149 mimics + SiFZD5 group (cells transfected by miR-149 mimics and SiFZD5). Moreover, the effects of miR-149 on the proliferation and apoptosis of CRC cells were also analyzed by MTT and flow cytometry assay. In addition, the expression of Wnt/β-catenin signal pathways related factors were shown by western blot analysis. Results: Q-PCR results demonstrated that the expression of miR-149 was significantly lower in SW480 than that in the FHC cell line. Frizzled class receptor 5 (FZD5) was identified as a functional target of miR-149 through a series of experiments including Q-PCR, western blot analysis, and luciferase assay. Cellular functional experiments demonstrated that the cell viability and proliferation were greatly inhibited after miR-149 overexpression in SW480 cells. Furthermore, the proportion of apoptotic cells increased significantly after introducing miR-149 into SW480 cells. Furthermore, Wnt/β-catenin signal pathway was activated because of the lower expression of β-catenin and cyclinD1 in miR-149 mimics group. However, reducing FZD5 expression restored the expression of β-catenin and cyclin D. Conclusions: Our data suggested that miR-149 may function as a tumor suppressor in CRC cells lines by targeting FZD5. miR-149/FZD5 may become a new therapeutic target for CRC.

Keywords: miR-149, colorectal cancer (CRC), Frizzled class receptor 5 (FZD5), Wnt/β-catenin signal pathway

Introduction

Colorectal cancer (CRC) is one of the most common cancer diagnoses and causes of mortality in the world [1]. The morbidity and mortality rates remained elevated in many developing countries including China in the past decades [2, 3]. Recent research found that the expressions of small noncoding RNA species expression were orchestrated at the posttranscriptional level [4].

microRNAs (miRNAs) are a class of endogenous, small, single-stranded, non-coding RNAs that have shown strong associations with human cancers [5]. miRNAs can be classified as oncogenes or as tumor suppressors by binding the sites in the 3'untranslated regions (UTRs) of various targeting genes [6]. Evidence has established miRNAs as pivotal players in the initiation, promotion, and progression of various human cancers [7]. For example, loss of miR-149 leads to some oncogenes' gain of function in prostate carcinoma [8], and elevated expression of miR-149 has been reported to be important in the progression of nasopharyngeal carcinoma [9]. Therefore, miRNAs may represent a novel and attractive therapeutic strategy for human cancers.

Wnt signaling pathway is highly conserved and plays principal regulatory roles in many developmental processes [10]. Aberrant Wnt signaling has been associated with many types of cancer and other diseases [11-13]. However, the expression pattern and role of miR-149 in the development of CRC still need to be exploited. In this study, the role of miR-149 during the occurrence and development of CRC and its related molecular mechanism were investigated.

Table 1. Sequences of miR-149 mimics and SiFZD5 used for transfection

Gene	Name	Sequences (5' to 3')
miR-149	miR-149 mimics	UCUGGCUCCGUGUCUUCACUCCC
mimics-NC	miR-149 mimics	GGGAGUGAAGACACGGAGCCAGA
SiEphB3	SiEphB3-sense	UCCUAAGGUUGGCGUUGUA
	SiEphB3-antisense	UACAACGCCAACCUUAGGA
SiRNA-NC	SiRNA control-sense	GGAAAUCGAGUUCGCCGUU
	iRNA control-antisense	AACGGCGAACUCGAUUUCC

Table 2. Primers used for Q-PCR analysis. Primers were designed using Primer Express version 2.0 software. Primer specificity was confirmed using Primer-BLAST web software (National Centre for Biotechnology Information)

gene	name	Primers (5' to 3')
hsa-miR-149	miR-149-RT	GTCGTATCCAGTGCAGGGTCCGAG GTATTCGCACTGGATACGACGGGAGT
	miR-149-F	GGCTCTGGCTCCGTGTCTT
	miR-149-R	CAGTGCAGGGTCCGAGGTATT
U6	U6-RT	GTCGTATCCAGTGCAGGGTCCGAG GTATTCGCACTGGATACGACAAAAAT
	U6-F	CAAATTCGTGAAGCGTTCCATA
	U6-R	AGTGCAGGGTCCGAGGTATTC
β-actin	β-actin-F	CCTGTACGCCAACACAGTGC
	β-actin-R	ATACTCCTGCTTGCTGATCC
FZD5	FZD5-2-F	ATCGGCTACAACCTGACGCAC
	FZD5-R	GCAGACAGATGGGCGTGTAC

Materials and methods

Cell culture

The human CRC cell line SW480 and normal colon epithelial FHC cell were was purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and used in this study. Cells were cultured in DMEM containing 10% FBS and 2 mM L-glutamine and maintained at 37°C in a 5% CO_2 atmosphere.

Cell transfection

The human miR-149 duplex mimic and negative control oligonucleotide duplex mimic (miR-NC), small interfering RNA (siRNA) for Frizzled class receptor 5 (FZD5) and the negative control RNA (siRNA-NC) were designed and synthesized by Genepharma (Suzhou, China). The sequences of miRNAs and siRNAs were shown in **Table 1**. Cells were divided into NC group (negative control), miR-149 mimics group (cells transfected with miR-149 mimics) and miR-149 mimics + SiFZD5 group (cells transfected by miR-149 mimics and SiFZD5). Cultured SW480 cells were transfected with miRNAs or siRNAs with the Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's protocol to analyze biological function of miR-149. Transfection efficiency was subsequently detected by quantitative real-time RT-PCR (Q-PCR).

Q-PCR analysis

Total RNA was isolated and purified from CRC cells using TRIZOL Reagent (Invitrogen). The cDNA was reversed according to the manufacturer's instructions of cDNA Synthesis Kit (Takara). Quantitative PCR was accomplished to detect the expression levels of miRNA and mRNA using the BioRad CFX96 detection system (Bio-Rad) and interaction dye SYBR Green. U6 snRNA was used for normalization. Primer sequences were listed in **Table 2**. Three repeats were set for each specimen. The relative expressions of miRNAs and mRNAs were calculated using $2^{-\Delta\Delta CT}$.

Western blot analysis

Total proteins were prepared from the CRC cells by radioimmunoprecipitation assay (RIPA) (Beyotime). The protein concentration was checked using Bio-Rad protein assay system according to instructions of the kit. Proteins were analyzed with SDS polyacrylamide gel electrophoresis. After electrophoresis, they were electro-transferred to the PVDF membrane. The blots were probed with appropriate primary antibodies (Abcam) at 4°C overnight, and incubated with HRP-conjugated appropriate secondary antibody. The protein bands were scanned and quantified. Signals were visualized using ECL Substrates (Millipore). The protein expression was normalized to endogenous β-actin.

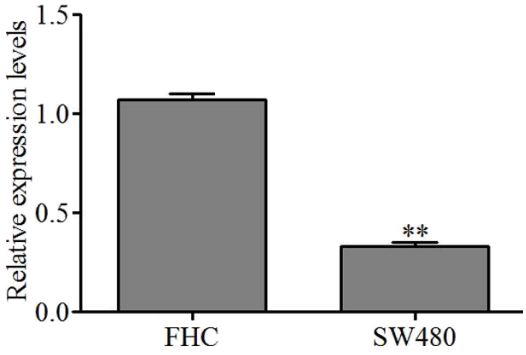

Figure 1. Expression pattern of miR-149 in human CRC cell lines and the normal human colonic epithelial FHC cell lines. Data are the mean ± SD; each bar represents the mean of three independent experiments carried out in triplicate. **P<0.01, compared with FHC group.

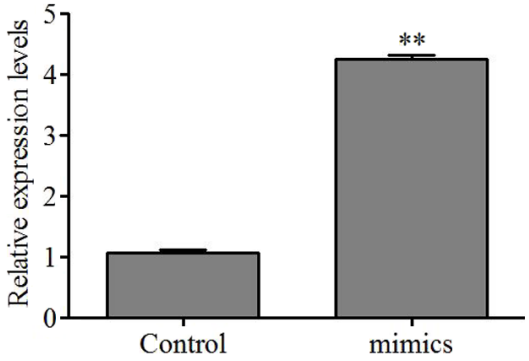

Figure 2. Transfection efficiency of miR-149 mimics by Q-PCR method. Data are the mean ± SD; each bar represents the mean of three independent experiments carried out in triplicate. **P<0.01, compared with control group.

Cell proliferation assay

MTT method was performed to determine the cell viability. The 2×10^3 transfected cells in different groups were inoculated into a 24-well plate. The viability of SW480 cells were assessed on day 1, 2, 3 and 4. Then, MTT [3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich] (20 μL, 5 mg/mL) was added to each well and incubated for 4 h. The reaction was terminated through removal of the supernatant and addition of DMSO to dissolve the formazan product. After 0.5 h, absorbance was read at 570 nm on a microplate reader (Multiskan Spectrum).

Luciferase reporter assay

The full-length 3'UTR of human FZD5 mRNA containing the putative miR-149 binding sequence was amplified and cloned into the pGL3 promotor vector (Promega) to construct FZD5 3'UTR recombinant plasmid. The mutant recombinant plasmid with the loss of binding site of FZD5 and miR-149 was also constructed in the same way. For the dual luciferase assay, CRC cells were transfected with miR-149 mimics or miR-NC using Lipofectamine 2000. The luciferase activity was examined using the dual-luciferase reporter gene assay kit according to instructions. Three repeats were conducted.

Cell apotosis assay

Apoptotic cells can be distinguished from normal cells by flow cytometry using an Annexin V-FITC/PI apoptosis kit (Invitrogen). CRC cells of each group were collected and washed with phosphate-buffered saline (PBS). After digestion, the 1×10^6/mL cell suspension was prepared via centrifugation and discarding the supernatant. Then, CRC cells were incubated with 5 μl FITC-Annexin V and 10 μl propidium iodide (PI) for 10 min in a dark condition. The apoptotic rate was measured by flow cytometry analysis.

Statistical analyses

The results are presented as mean ± SD. One-way analysis of variance and Student's t-test was performed using SPSS 17.0 statistical software (SPSS). Outcomes were considered significant if two-tailed $P<0.05$.

Results

miR-149 expression is reduced in CRC cells

Q-PCR was used to investigate the expression pattern of miR-149 in CRC cells. Q-PCR results demonstrated that the expression of miR-149 was significantly lower in SW480 than that in FHC cell line (**Figure 1**). Therefore, miR-149 was expected to be involved in CRC development.

Transfection efficiency of miR-149 mimics

To analyze the role of miR-149 in CRC, miR-149 mimics was transferred into SW480 cells, and the transfection efficiency was also detected by Q-PCR assay. As displayed in **Figure 2**, the expression of miR-149 in SW480 cells

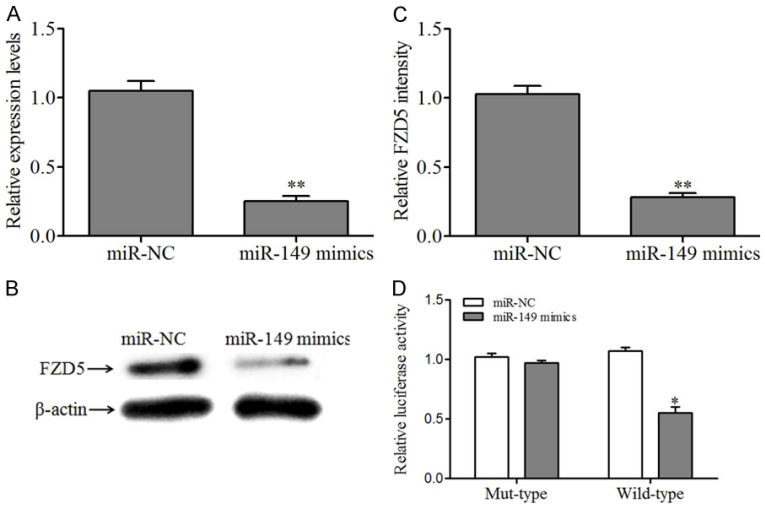

Figure 3. FZD5 is a direct target of miR-149. SW480 cells were transfected with miR-149 mimics or miR-NC. A. Expression level of FZD5 detected by Q-PCR method. B, C. Expression level of FZD5 checked by western blot. D. Relative activities of luciferase reporters. Expression level of FZD5 detected by qRT-PCR analysis. Data are the mean ± SD; each bar represents the mean of three independent experiments carried out in triplicate. *P<0.05, compared with miR-NC group. **P<0.01, compared with miR-NC group.

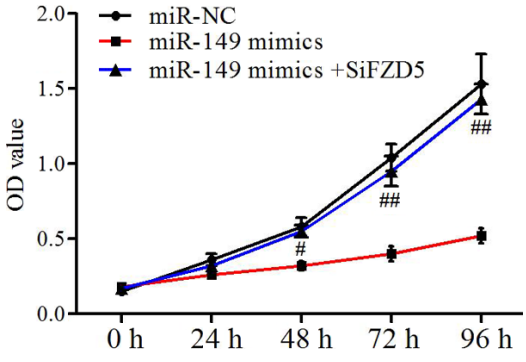

Figure 4. miR-149 regulates the cell proliferation by targeting FZD5. Data are the mean ± SD; each bar represents the mean of three independent experiments carried out in triplicate. #P<0.05, compared with miR-mimics group. ##P<0.01, compared with miR-mimics group.

was significantly increased after transfection with miR-149 mimics.

Screening of the candidate target genes of miR-149

Commonly, miRNAs exert their functions through imperfect binding with the 3'UTR of theirs target mRNAs. The miRecords resource from three independent prediction tools on the website were used to obtain potential targets of miR-149, and to further assess the potential role and the underlying molecular mechanism of miR-149 in CRC. Among these putative targets of miR-149, FZD5 was chosen for its vital roles in human cancers as the previous reports. Then, the mRNA and protein expression of FZD5 were checked by Q-PCR and western blot analysis after transferred with miR-149 mimics or mimic-NC sequences. The observation indicated that FZD5 mRNA and protein levels were significantly suppressed compared with negative control (**Figure 3A-C**).

To obtain further direct evidence that FZD5 is a target of miR-149, luciferase assay was performed. As shown in **Figure 3D**, relative luciferase activity was decreased dramatically in the wild type group after being introduced into miR-149 mimics. Taken together, these data suggest that FZD5 is a direct target gene of miR-149 in CRC cells.

miR-149 regulates the cell proliferation by targeting FZD5

MTT assay was conducted to detect the cell proliferative rate in each group. There was a significant reduction in SW480 cells cultured at 48 h, 72 h and 96 h after miR-149 mimics transfection (**Figure 4**). Conversely, the cell growth was partially recovered after FZD5 knockdown by RNA interference (**Figure 4**).

miR-149 regulates the cell apoptosis by targeting FZD5

Flow cytometry analysis was taken to examine apoptosis rate in each group. As shown in **Figure 5**, the apoptotic rate of SW480 cells was significantly higher in the miR-149 mimic group than that of the miR-NC group. However, apoptotic rate decreased significantly after reduced FZD5 expression (**Figure 5**).

miR-149 inhibits the activation of Wnt/β-catenin signaling pathway

Western blot was employed to further determine the effects of miR-149 on the expre-

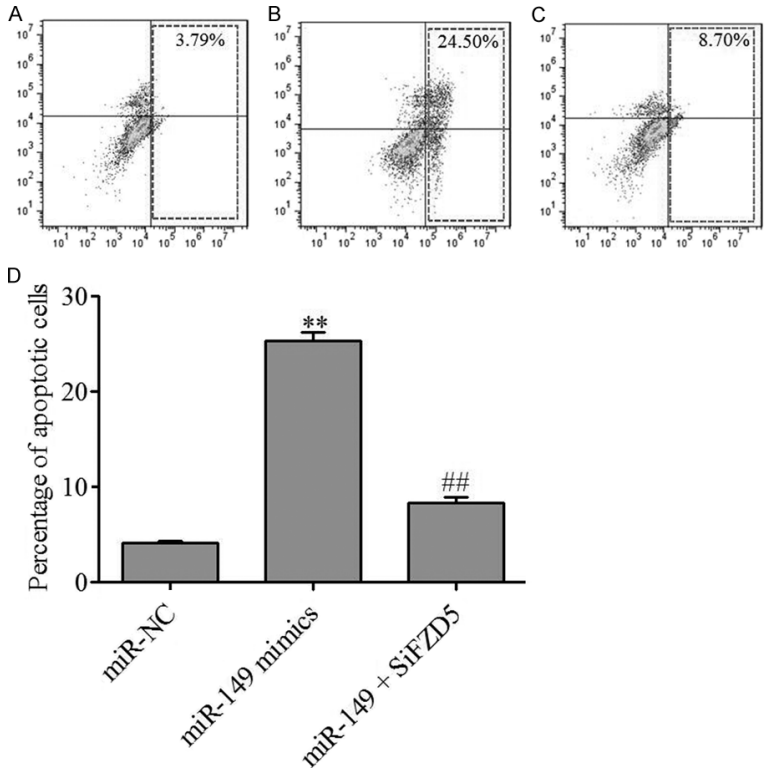

Figure 5. miR-149 regulates cell apoptosis by targeting FZD5. A. miR-NC group. B. miR-149 mimics group. C. miR-149 mimics + SiFZD5 group. D. Quantitative analysis of cell apoptosis in CRC cells of each group. Data are the mean ± SD; each bar represents the mean of three independent experiments carried out in triplicate. **P<0.01, compared with miR-NC group. ##P<0.01, compared with the miR-mimics group.

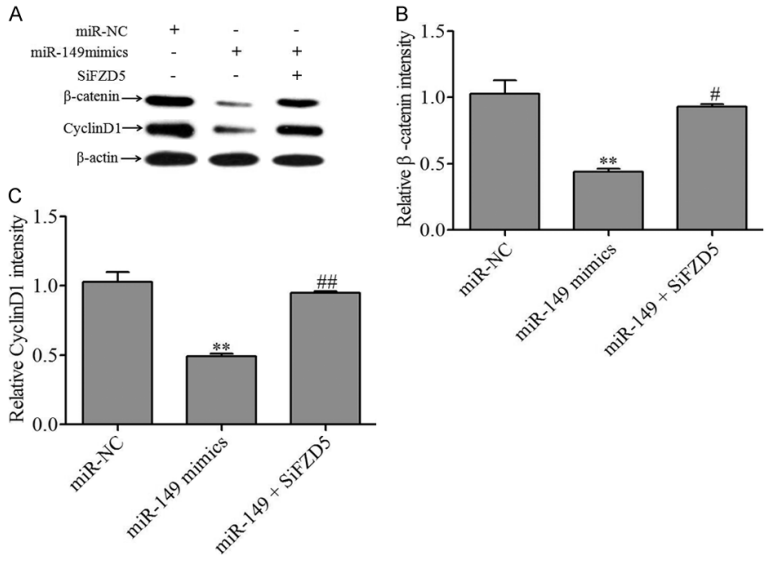

Figure 6. miR-149 inhibits activation of the Wnt/β-catenin signaling pathway. The expression of β-catenin and cyclinD1 were detected by western blot. Data are the mean ± SD; each bar represents the mean of three independent experiments carried out in triplicate. **P<0.01, compared with miR-NC group. ##P<0.01, compared with miR-mimics group.

ssions of relative factors in Wnt/β-catenin signal pathways. The observation manifested that the expression of β-catenin and cyclin D1 in the miR-149 mimics group were notably lower than miR-NC group (**Figure 6**). Interestingly, the inhibitory effects of miR-149 on β-catenin and cyclinD1 expression were reversed after reduced FZD5 expression by siRNA.

Discussion

Anecdotal evidence suggested that miRNAs could be used as diagnostic, or prognostic markers, owing to their remarkably stable forms and abnormal expression in a variety of cancer patients compared to that in healthy individuals [14]. miR-149 was one of the miRNAs that had received extensive attention in recent years [15]. It was reported that miR-149 was a tumor suppressor and ectopic expression of miR-149 in gastric cancer cells inhibits proliferation and cell cycle progression by down-regulating target gene ZBTB2 [16]. In prostate cancer, miR-149 expression was reduced and as a diagnostic marker, could discriminate between malignant and normal tissues in prostate cancer patients [17]. In addition, miR-149 enhanced the sensitivity of EC cell lines to cisplatin by targeting polβ [18]. Profiling and functional research indicated that a large number of miRNAs' had potential prognostic values in CRC [19]. However, the effect and mechanisms underlying the miR-149 effect in CRC still needed more description. Here, we found that miR-149 expression was

significantly decreased in SW480 cells lines compared with normal colon epithelial FHC cells. Furthermore, FZD5 was identified as a direct target gene of miR-149 in CRC cells. It was also observed that miR-149 regulated the cell proliferation and apoptosis by targeting FZD5. Taken together, our data suggested that miR-149 may function as a tumor suppressor in CRC cells lines by targeting FZD5.

Wnt/β-catenin is a key molecular signaling regulator in a variety of human cancers including CRC. Evidence had demonstrated that miRNAs dysregulation and the aberrant activation of the Wnt pathway was highly associated with carcinogenesis and cancer metastasis [20]. It was commonly known that Wnt signaling cascades were usually triggered through the secreted Wnt ligands binding to Frizzled (FZD) receptor proteins [21]. In this study, FZD5 was verified as a direct target gene of miR-149. Therefore, the expression of β-catenin and cyclin D1, factors involved in the Wnt/β-catenin signaling pathway, were checked using western blot. Results showed that β-catenin and cyclin D1 expression were reduced significantly. However, the inhibitory effects of miR-149 on β-catenin and cyclin D1 expression were reversed after reduced FZD5 expression by siRNA. Based on these observations, miR-149 may inhibit the activation of Wnt/β-catenin signaling pathway in CRC.

Further evidence also revealed that each miRNA may control many target genes which can affect carcinogenesis in different ways. Wang et al reported that miR-149 was epigenetically silenced in CRC and overexpression of miR-149 inhibited cell growth and invasion of into CRC cells *in vitro* [22]. They also identified *Sp1* as a target of miR-149. Liu's results indicated that miR-149 increased the sensitivity of CRC cells to 5-fluorouracil by targeting *FOXM1* [23]. In our study, we revealed that *FZD5*, one regulator of Wnt/β-catenin signaling, could be also targeted by miR-149. Whether there was crosstalk between these targets of miR-149 needs investigation. Besides, more cautious studies are indispensable to further probe the effect of FZD5 on tumor growth *in vivo*.

Conclusions

Our data suggested that miR-149 may function as a tumor suppressor in CRC cells lines by targeting FZD5. miR-149/FZD5 is likely to become a new therapeutic target for CRC.

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Disclosure of conflict of interest

None.

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References

[1] Siegel RL, Miller KD, Fedewa SA, Ahnen DJ, Meester RGS, Barzi A and Jemal A. Colorectal cancer statistics, 2017. CA Cancer J Clin 2017; 67: 177-193.

[2] Arnold M, Sierra MS, Laversanne M, Soerjomataram I, Jemal A and Bray F. Global patterns and trends in colorectal cancer incidence and mortality. Gut 2017; 66: 683-691.

[3] Favoriti P, Carbone G, Greco M, Pirozzi F, Pirozzi RE and Corcione F. Worldwide burden of colorectal cancer: a review. Updates Surg 2016; 68: 7-11.

[4] Mohammadi A, Mansoori B and Baradaran B. The role of microRNAs in colorectal cancer. Biomed Pharmacother 2016; 84: 705-713.

[5] Moridikia A, Mirzaei H, Sahebkar A and Salimian J. MicroRNAs: potential candidates for diagnosis and treatment of colorectal cancer. J Cell Physiol 2018; 233: 901-913.

[6] Lin S and Gregory RI. MicroRNA biogenesis pathways in cancer. Nat Rev Cancer 2015; 15: 321-33.

[7] Peng Y and Croce CM. The role of microRNAs in human cancer. Signal Transduct Target Ther 2016; 1: 15004.

[8] Schaefer A, Jung M, Mollenkopf HJ, Wagner I, Stephan C, Jentzmik F, Miller K, Lein M, Kristiansen G and Jung K. Diagnostic and prognostic implications of microRNA profiling in prostate carcinoma. Int J Cancer 2010; 126: 1166-76.

[9] Luo Z, Zhang L, Li Z, Li X, Li G, Yu H, Jiang C, Dai Y, Guo X, Xiang J and Li G. An in silico analysis of dynamic changes in microRNA expression profiles in stepwise development of nasopharyngeal carcinoma. BMC Med Genomics 2012; 5: 3.

[10] Duchartre Y, Kim YM and Kahn M. The Wnt signaling pathway in cancer. Crit Rev Oncol Hematol 2016; 99: 141-9.

[11] Tai D, Wells K, Arcaroli J, Vanderbilt C, Aisner DL, Messersmith WA and Lieu CH. Targeting the Wnt signaling pathway in cancer therapeutics. Oncologist 2015; 20: 1189-98.
[12] Zhan T, Rindtorff N and Boutros M. Wnt signaling in cancer. Oncogene 2017; 36: 1461-1473.
[13] Peng Y, Zhang X, Feng X, Fan X and Jin Z. The crosstalk between microRNAs and the Wnt/β-catenin signaling pathway in cancer. Oncotarget 2017; 8: 14089-14106.
[14] Svoronos AA, Engelman DM and Slack FJ. OncomiR or tumor suppressor? The duplicity of microRNAs in cancer. Cancer Res 2016; 76: 3666-70.
[15] He Y, Yu D, Zhu L, Zhong S, Zhao J and Tang J. miR-149 in human cancer: a systemic review. J Cancer 2018; 9: 375-388.
[16] Wang Y, Zheng X, Zhang Z, Zhou J, Zhao G, Yang J, Xia L, Wang R, Cai X, Hu H, Zhu C, Nie Y, Wu K, Zhang D and Fan D. MicroRNA-149 inhibits proliferation and cell cycle progression through the targeting of ZBTB2 in human gastric cancer. PLoS One 2012; 7: e41693.
[17] Hashemi M, Moradi N, Ziaee SA, Narouie B, Soltani MH, Rezaei M, Shahkar G and Taheri M. Association between single nucleotide polymorphism in miR-499, miR-196a2, miR-146a and miR-149 and prostate cancer risk in a sample of Iranian population. J Adv Res 2016; 7: 491-8.
[18] Wang Y, Chen J, Zhang M, Zhang W, Li M, Zang W, Dong Z and Zhao G. MiR-149 sensitizes esophageal cancer cell lines to cisplatin by targeting DNA polymerase β. J Cell Mol Med 2018; [Epub ahead of print].
[19] Ow SH, Chua PJ and Bay BH. miR-149 as a potential molecular target for cancer. Curr Med Chem 2018; 25: 1046-1054.
[20] Ghahhari NM and Babashah S. Interplay between microRNAs and Wnt/β-catenin signalling pathway regulates epithelial-mesenchymal transition in cancer. Eur J Cancer 2015; 51: 1638-49.
[21] Yang Y and Mlodzik M. Wnt-Frizzled/planar cell polarity signaling: cellular orientation by facing the wind (Wnt). Annu Rev Cell Dev Biol 2015; 31: 623-46.
[22] Wang F, Ma YL, Zhang P, Shen TY, Shi CZ, Yang YZ, Moyer MP, Zhang HZ, Chen HQ, Liang Y and Qin HL. SP1 mediates the link between methylation of the tumour suppressor miR-149 and outcome in colorectal cancer. J Pathol 2013; 229: 12-24.
[23] Liu X, Xie T, Mao X, Xue L, Chu X and Chen L. MicroRNA-149 increases the sensitivity of colorectal cancer cells to 5-fluorouracil by targeting forkhead box transcription factor FOXM1. Cell Physiol Biochem 2016; 39: 617-29.