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MiR-212-5p regulates the proliferation and apoptosis of AML cells through targeting FZD5

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Abstract. – OBJECTIVE: To explore the effects of microRNA-212-5p (miR-212-5p) on biological functions of acute myeloid leukemia (AML) and to find the potential molecular mechanism.

PATIENTS AND METHODS: We measured the expression level of miR-212-5p in 35 AML patients and 20 patients with idiopathic thrombocytopenic purpura (ITP) as control cases. Besides, the miR-212-5p expression at cellular level was checked as well. In order to screen the functional targets of miR-212-5p, online prediction software was used and gene frizzled class receptor 5 (FZD5) attracted our attention. The effects of miR-212-5p on biological functions of AML cell line (Kasumi-1) were analyzed by subsequent experiments. The mRNA and protein expressions of FZD5 were detected by quantitative Real-time polymerase chain reaction (qRT-PCR) and Western blotting (WB) analysis, respectively. Cell proliferation was tested by cell counting kit-8 (CCK-8) assay. Cell cycle and apoptosis were measured by flow cytometry. Finally, protein expression of β-catenin was analyzed by WB assay.

RESULTS: In AML cases and cells, miR-212-5p was found to be lowly expressed. The potential target of miR-212-5p was predicated in three public databases. Through a series of experiments including qRT-PCR, WB and luciferase assay, we identified FZD5 as a functional target of miR-212-5p. In further cellular functional experiments on Kasumi-1, we found overexpression of miR-212-5p in Kasumi-1 cells greatly inhibited the cell viability and proliferation. The ratio of cells in G0/G1 phase and the proportion of apoptotic cells increased after miR-212-5p overexpression. Furthermore, Wnt/β-catenin signal pathway was the most apparent pathway that was regulated by miR-212-5p according to WB results. However, the effects of miR-212-5p were suppressed after restoring the expression of FZD5.

CONCLUSIONS: Expression of miR-212-5p was significantly lower in AML patients and cell lines, indicating that miR-212-5p served as a tumor-suppressor gene in AML. According to our *in vitro* experiments, miR-212-5p/FZD5 was likely to become a new therapeutic target for AML.

Key Words:
MiR-212-5p, Acute myeloid leukemia (AML), Frizzled class receptor 5 (FZD5), Wnt/β-catenin signal pathway.

Introduction

Acute myeloid leukemia (AML) is one of the most common hematological malignant tumors, directly threatening the life of affected patients. According to the statistics of the Surveillance and Epidemiology and End Results (SEER) database, there were 20,830 new cases of AML in the USA in 2015, accounting for 1.3% of the total in that year. Besides, there were 10,460 deaths of AML, accounting for 1.8% of total cancer deaths. AML accounts approximately 70% of acute leukemia cases, whose incidence rate increases year by year. More seriously, the mortality of AML sharply increases with the environmental pollution caused by industrial and agricultural production and development[1]. Currently, a great number of studies demonstrated that epigenetic changes, such as microRNA (miRNA) expression profile disorders, changes in DNA and RNA methylation levels, and histone modification disorders played important regulatory roles in the occurrence and development of AML. Therefore, it is particularly important to study the regulatory principle of epigenetics and pathogenesis of AML, so as to improve the therapeutic effect on AML. MiRNA is an important member in epigenetics, which is a non-coding RNA consisting of 19-25 nucleotides[2]. The first miRNA discovered was lin-4 reported in 1993[3]. After that, the miRNA library has been constantly improved, and more than 2,000 miR-

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NAs have been successively discovered in human body so far[4]. MiRNAs promote the degradation of messenger RNAs (mRNAs) or inhibit their translation through the complementary base pairing with the 3' UTR of the target gene, thereby regulating expressions of the target genes at the transcriptional or post-transcriptional level[2, 5, 6]. According to the computer prediction, each miRNA can regulate about 200 mRNAs. Hence, about 1/3 to 1/2 of human protein-encoding genes can be regulated. Moreover, one protein-encoding gene can be regulated by multiple miRNAs, indicating that a miRNA is an important physiological regulator[7]. Functionally, miRNAs are involved in the regulation of cell proliferation, apoptosis, cycle, metabolism, migration, angiogenesis and other physiological processes. We believed that their abnormal expressions would inevitably lead to physiological function disorders in the body, resulting in the occurrence and development of diseases[8]. Up to now, expression features of miRNAs in multiple tumors have been identified through exploring the miRNA expression microarrays. In addition, further functional studies have confirmed that some differentially-expressed miRNAs played important roles in the occurrence and development of the tumor[9,10]. In recent years, the regulating effects of miRNA in AML have also been reported in many studies[11-14]. Expression features and biological functions of these miRNAs in AML have been well concerned. iRMiR-212-5p, as a member of the highly regarded microRNA family, was found to be abnormally expressed in many diseases including breast Cancer[15] and colorectal cancer[16]. However, few reports mentioned the effects of miR-212-5p on AML. In our study, the role of miR-212-5p during the occurrence and development of AML and its related molecular mechanism were clarified.

Materials and Methods

Clinical Cases and Cells

The bone marrow specimens were taken from 35 AML patients altogether with 20 patients with Idiopathic Thrombocytopenic Purpura (ITP) treated in the Department of Hematology and Oncology, the First Affiliated Hospital of the Xi'an Medical University, from October 2016 to October 2017. Before collection, this study was approved by the Medical Ethics Committee of First Affiliated Hospital of the Xi'an Medical University and the informed consent was obtained from patients. French - American - British (FAB) and World Health Organization (WHO) typing criteria were used for references. Enrolled patients were definitely diagnosed with AML initially without a history of other malignant tumors and any anti-tumor drug therapy. The AML Kasumi-1 cell line was purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The human embryonic kidney 293T cell lines were purchased from the Institute of Hematology, Chinese Academy of Medical Sciences (Tianjin, China). Cells were cultured with the complete culture solution containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), and those in the logarithmic growth phase were taken for the experiment.

Luciferase Reporter Assay

The primers were designed and synthesized according to the 3'UTR sequences of the target gene FZD5. PCR amplification was performed with the DNA extracted from HEK293T cells as a template. Product was recycled and the recombinant plasmid was constructed named Psi-Check2-FZD5-wt. The mutant recombinant plasmid with the loss of binding site of FZD5 and miR-212-5p was also constructed named Psi-Check2-FZD5-Mut in the same way. Psi-Check2-FZD5-wt or Psi-Check2-FZD5-Mut were respectively transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to instructions. The luciferase activity was detected using the dual-luciferase reporter gene assay kit. The experiment was repeated for 3 times.

Transfection

MiR-212-5p mimics and si-FZD5 were synthesized and transfected to AML cell lines (Kasumi-1) to analyze biological function of miR-212-5p. Transfection efficiency was subsequently detected by quantitative Real-time polymerase chain reaction (qRT-PCR). d:Cells were divided into three group based on their specific treatment: NC group (negative control), miR-212-5p mimics (cells transfected with miR-212-5p mimics) and miR-212-5p mimics + si-FZD5 (cells transfected by miR-212-5p mimics and si-FZD5). All the transfection plasmids were purchased form RiboBio (Guangzhou, China). Cell transfection was performed using Lipofectamine RNAiMAX (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions.

QRT-PCR Analysis

The total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and then reversely transcribed into complementary DNA (cDNA) according to instructions of the M-MLV reverse transcriptase kit. The mRNA expressions of miR-212-5p and FZD5 were detected using the SYBR PrimeScriptTM RT-PCR kit (TaKaRa, Otsu, Shiga, Japan) on the Roche Light Cycler 480 fluorescence quantitative PCR system. Three repeated wells were set for each specimen. The relative expressions of miRNAs were calculated using $2^{-\Delta\Delta CT}$. U6 was used as the internal reference in the quantitative analysis of miR-212-5p expression, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal reference in the quantitative analysis of the FZD5 expression. The experiment was repeated for 3 times. Primer sequences used in this study were as follows: FZD5, F: 5'-GGAGATGGCACAGGAGGAA-3', R: 5'-GCCCGTAGTGCTTCAGTTT-3'; miR-212-5p, F: 5'-CCTCGACTGGGGGTGTAAACAT-3', R: 5'-GTGGAGTCGATTGCGTGTC-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot Analysis

The Kasumi-1 cell protein was extracted via radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) after transfection. Protein concentration was subsequently detected using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA) according to instructions of the kit. After denaturation, 10 μg total protein samples were taken and separated using 100 g/L polyacrylamide gel. Next, the protein was transferred onto the membrane for 1 h, blocked at room temperature for 2 h, and incubated with the primary antibodies (FZD5, β-catenin and GAPDH, diluted at 1:1000, Cell Signaling Technology (CST) Inc., Danvers, MA, USA) at 4°C overnight. After rinsing with Tris buffered saline-Tween (TBST), the diluted secondary antibody was added for incubation at room temperature for 1 h. The protein band was detected using the enhanced chemiluminescence (ECL) reagent (Thermo Fisher Scientific, Waltham, MA, USA), followed by Alpha gel imaging, photography using chemiluminescence system and analyzed using ImageJ analysis software. GAPDH was used as the internal reference protein. All experiments were repeated for 3 times.

Cell Proliferation

The cell proliferation was detected using the cell counting kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan) as follows. The cells in different groups after transfection were inoculated into a 24-well plate. At 24, 48, 72 and 96 h after transfection, an equal amount of Kasumi-1 cells were inoculated into a 96-well plate. 10 μL CCK-8 were then added, followed by incubation with 5% CO_2 at 37°C for 2 h. The absorbance (A) value at 450 nm was detected using the multi-functional microplate reader, and 630 nm was used as the reference wavelength. Cells were counted for 3 times in each group, and the above experiment was repeated for 3 times.

Cell Cycle Assay

Kasumi-1 cells in the logarithmic growth phase were inoculated into the 6-well plate, and the cell concentration was adjusted to 1×10^4 cells/mL (1 mL/well). After transfection for 48 h, cells were detected according to the cell cycle assay kit. Cells were digested using the EDTA-free trypsin (ethylene diamine tetraacetic acid), centrifuged and resuspended. Then, 5 μL RNase adjusted at a final concentration of 10 mg/mL were added for incubation at 37°C for 1 h. The propidium iodide (PI) was added for staining at room temperature in a dark place for 30 min, followed by cell cycle detection using the flow cytometer. Three parallel repeated wells were set in each experimental group.

Cell Apoptosis Detection

According to instructions of the flow cytometry kit, Kasumi-1 cells in each group were collected and washed with phosphate-buffered saline (PBS) (Beyotime, Shanghai, China). After digestion, the cell suspension with concentration of 1×10^6 /mL was prepared, followed by centrifugation and the supernatant was discarded. 5 μL Annexin V-FITC and 10 μL Propidium Iodide (PI) were added into cells and gently mixed, followed by incubation for 5-15 min. The apoptotic rate was detected using Influx Flow Cytometer & Cell Sorter System (BD, Franklin Lakes, NJ, USA).

Statistical Analysis

Statistical analysis was performed with a Student's t-test or F-test. $p<0.05$ (two-sided) was considered significant. Data were analyzed by Prism 6.02 software (La Jolla, CA, USA).

Results

MiR-212-5p Expression Reduced in AML Cases and Cells

QRT-PCR results showed that the expression level of miR-212-5p in AML cases was significantly lower than that in ITP cases (Figure 1A). The same results were got in the *in vitro* determination; the expression level of miR-212-5p was much lower in AML cells (Kasumi-1) by comparing with that in HEK293T cells (Figure 1B). Taken together, miR-212-5p was expected to be involved in AML development.

Transfection Efficiency of miR-212-5p Mimics

Transfection efficiency was detected by qRT-PCR assay. As shown in Figure 2, the expression of miR-212-5p in Kasumi-1 cells was significantly up-regulated after transfection with miR-212-5p mimics.

FZD5 Was a Direct Target of miR-212-5p

MiRNAs exert their biological functions through regulating their target genes. As a result, it is necessary to verify its target genes to further clarify the regulatory mechanism of miR-212-5p in AML. The potential target site FZD5 was selected through bioinformatics prediction in this study (Figure 3A). To further verify the target gene predicted by bioinformatics, 293T cells were co-transfected with the recombinant plasmids Psi-Check2-FZD5-wt, Psi-Check2-FZD5-Mut and miR-212-5p. Luciferase reporter assay manifested that after the wild-type plasmid Psi-Check2-FZD5-wt and miR-212-5p were co-transfected into 293T cells, the relative activity of Renilla luciferase was significantly inhibited. However, co-transfection of the mutant-type plasmid PsiCheck2-FZD5-Mut and miR-212-5p into 293T cells did not change the activity of Renilla luciferase (Figure 3B). The above results suggested that miR-212-5p might target the FZD5 gene.

MiR-212-5p Decreased The Expression Level of FZD5

Both qRT-PCR (Figure 3C) and WB (Figure 3D) results showed that the expression level of FZD5 was regulated by miR-212-5p. Specifically, FZD5 expression in AML cells was downregulated after transfection of miR-212-5p mimics.

MiR-212-5p Promoted the Cell Proliferation

CCK-8 assay was taken to detect the cell proliferative rate in each group. There was a significant reduction in Kasumi-1 cells after miR-212-5p mimics transfection. Interestingly, the cell growth was partially recovered after FZD5 knockdown (Figure 4A).

Influence of miR-212-5p on the Cell Cycle of AML

It was validated from flow cytometry analyses that overexpression of miR-212-5p leads to G0/G1 phase arrest in AML cells, However, the arrested G0/G1 phase in AML cells was accelerated by FZD5 knockdown (Figure 4B).

MiR-212-5p Accelerated the Cell Apoptosis

Flow cytometry analysis showed that in the miR-212-5p mimics group, the apoptotic rate of Kasumi-1 cells was significantly higher than that of the control group. However, apoptotic rate was not statistically significant between the co-transfection group and the control group (Figure 4C).

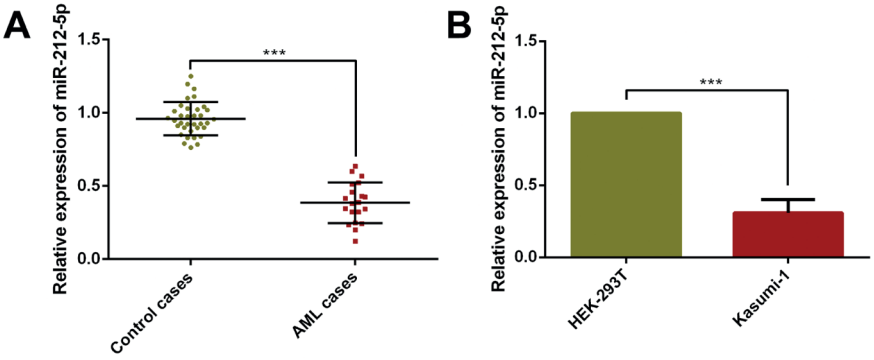

Figure 1. The expressions of miR-212-5p in AML bone marrow specimens and cells. ***A,*** Difference in the expression of miR-212-5p between AML and control bone marrow specimens. ***B,*** Difference in the expression of miR-212-5p between human AML cell line Kasumi-1 and human embryonic kidney cell line HEK-293T. ($^{***}p<0.001$).

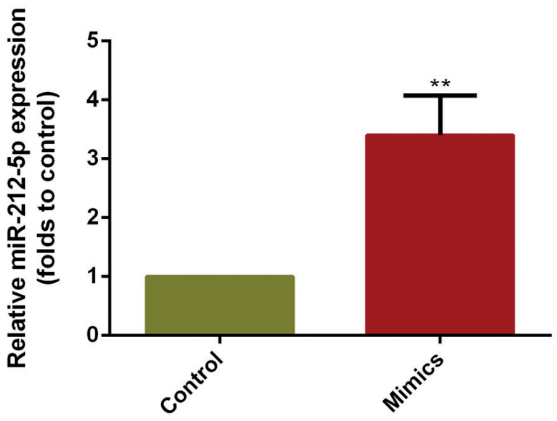

Figure 2. Transfection efficiency of miR-212-5p mimics by qRT-PCR. (**p<0.01).

MiR-212-5p Inhibited the Activation of Wnt/β-Catenin Signaling Pathway

WB assay was employed to detect the effects of miR-212-5p on the expressions of relative genes in Wnt/β-catenin signal pathways. The results manifested that after overexpression of miR-212-5p in Kasumi-1 cells, the expression of β-catenin notably decreased. However, FZD5 could reverse the inhibitory effects of miR-212-5p on β-catenin expression (Figure 4D).

Discussion

AML is a kind of malignant hematologic diseases with highly heterogeneous, and its complex and diverse pathogenesis has not been fully clarified yet. MiRNAs possess very powerful regulatory function in human body involving a wide regulatory range, and its important correlation with tumor has been confirmed in a large number of researches. The first study on AML and miRNAs was reported in 2007. Debernardi et al[17] detected 5 specific miRNAs that could regulate the HOX family genes through the literature review based on preliminary studies on the HOX family genes. In subsequent studies, Bryant et al[18] found that miR-10a expression was abnormally high in AML patients accompanied with nucleophosmin 1 (NPM 1) mutation.

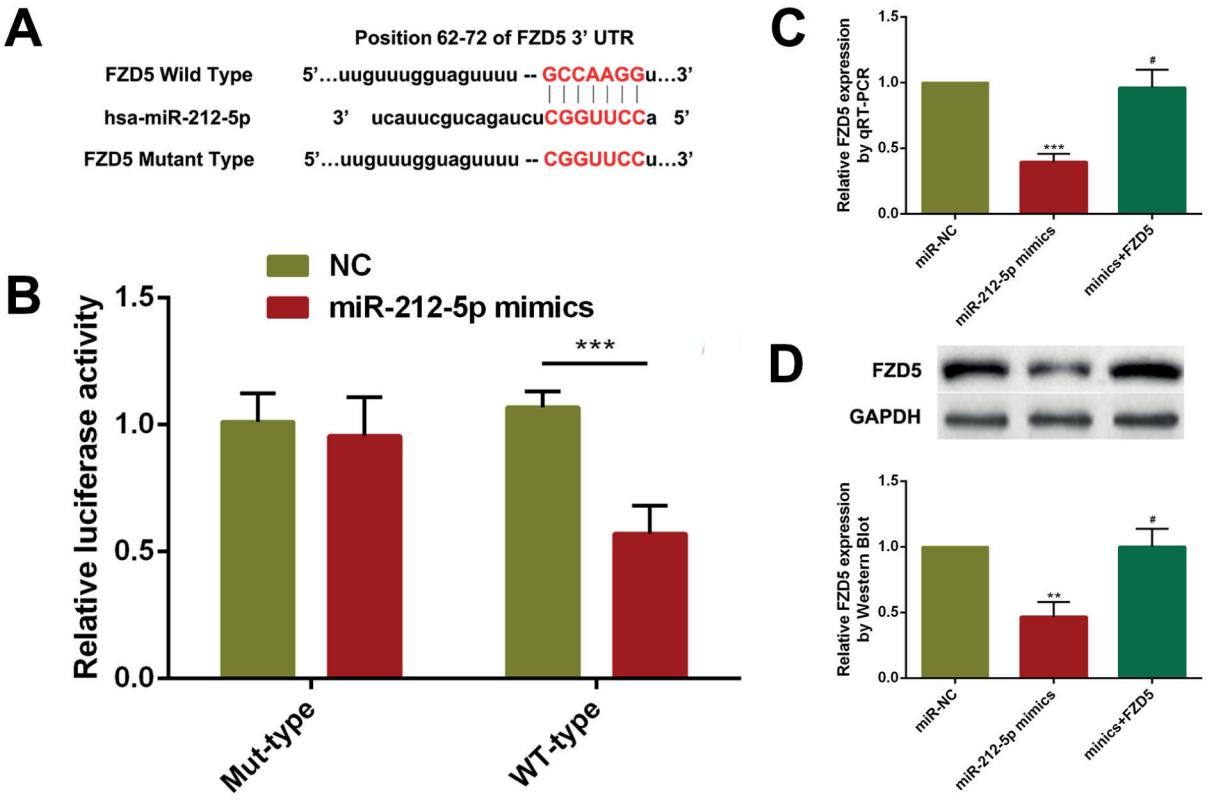

Figure 3. FZD5 is a direct and functional target of miR-212-5p. Kasumi-1 cells were transfected with miR-212-5p mimics or inhibitor. ***A,*** Diagram of putative miR-212-5p binding sites of FZD5. ***B,*** Relative activities of luciferase reporters (***p<0.001). ***C,*** Expression level of FZD5 detected by Western blot. ***D,*** Expression level of FZD5 detected by qRT-PCR analysis. All data were presented as means ± standard deviations. (**p<0.01, ***p<0.001 vs. NC group; #p<0.05 vs. Mimics group).

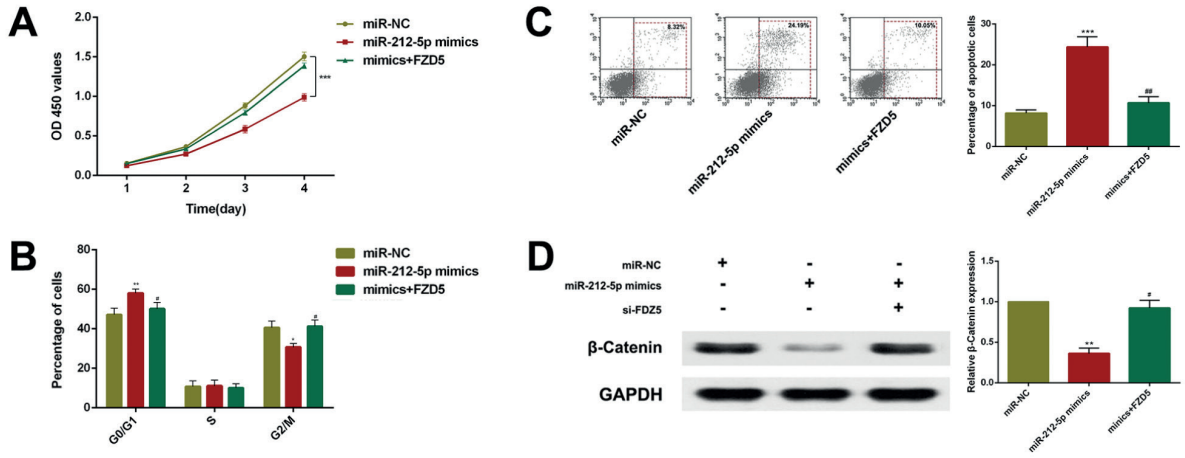

Figure 4. MiR-212-5p inhibited the biological function of AML cells. ***A,*** and ***B,*** MiR-212-5p decreased the cell proliferation (***$p<0.001$) and caused the G0/G1 arrest (*$p<0.05$, **$p<0.01$ vs. NC group; #$p<0.05$ *vs*. Mimics group). ***C,*** Apoptosis level of AML cells after transfection was detected by flow cytometer. ***D,*** The β-catenin expression was detected by WB assay. All data were presented as means ± standard deviations (**$p<0.01$, ***$p<0.001$ vs. NC group; #$p<0.05$, ##$p<0.01$ *vs*. Mimics group).

MiR-10a knockdown would promote apoptosis of AML cells. According to the dual-luciferase reporter assay, researchers found that miR-10a resisted apoptosis via inhibiting two downstream target genes, KLF4 and RB1CC1. With the advanced detection technique, miRNA expression profile of AML would be constantly improved. MiRNAs would play a new role in the diagnosis of the occurrence and development of AML, providing new ideas for the diagnosis and targeted therapy of AML. miRNAs exert their biological functions by regulating their target genes, so exploring downstream target genes of miRNAs is a core link in the study on miRNAs. To further clarify the biological function of miR-212-5p, the downstream potential target genes of miR-212-5p were predicted using the TargetScan miRNA database and other bioinformatics websites. Finally, an important target gene, FZD5, also known as HFZS or C2orf31, was screened out to be the target gene of miR-212-5p. FZD5 is located on chromosome 2q33.3, which is a member of the frizzled gene family encoding the seven-span transmembrane protein, as well as one of the important receptors in the Wnt signaling pathway. It was generally believed that Wnt5A is a ligand of its interaction. The Wnt/β-catenin signaling pathway is a key factor regulating the proliferation, differentiation and apoptosis of hematopoietic stem cells[19-21]. Dysfunctional Wnt/β-catenin signaling pathway may be involved in the occurrence of AML[22]. Griffths et al[23] pointed out the abnormally activated Wnt/β-catenin signaling pathway in AML. The β-catenin overexpression was an important prognostic hallmark for AML. The mRNA and protein levels of FZD5 in Kasumi-1 cells with miR-212-5p overexpression were detected via qRT-PCR and WB assay, respectively. Results were well consistent with the previous bioinformatics prediction data. Both mRNA and protein levels of FZD5 were significantly down-regulated, indirectly indicating that FZD5 was a target gene of miR-212-5p in AML. Dual-luciferase reporter assay further verified that miR-212-5p could indeed inhibit the luciferase activity via direct complementary pairing with the wild-type 3'-UTR of FZD5. However, miR-212-5p was unable to reduce the relative expression level of luciferase in mutant-type 3'UTR of FZD5. The above results directly proved the regulatory relationship between miR-212-5p and FZD5. Blocked apoptosis, cycle disorder and malignant hyperplasia were important pathological mechanisms of AML. Most of drugs used in the clinical treatment of AML currently are based on the abovementioned mechanisms[24]. Although the chemotherapy could alleviate the disease in the initial stage in some AML patients, the 5-year long-term survival rate is still less than 30%, especially in elderly patients[25]. Therefore, searching new regulatory factors in AML and improving existing pathological mechanism would make new breakthroughs in targeted therapy undoubtedly. In functional experiments of this study, overexpressed miR-212-5p could reduce prolifera-

tion and promote apoptosis of Kasumi-1 cells. However, the G0/G1 cell cycle arrest, advanced apoptosis and suppressed proliferation caused by miR-212-5p could be partially reversed after restore of FZD5. It is suggested that miR-212-5p indeed regulated the phenotype of AML cells through inhibiting the downstream target gene FZ5D. Finally, it was confirmed via WB results that miR-212-5p could inhibit the activity of the Wnt/β-catenin signaling pathway. The abnormal activation of Wnt/β-catenin signaling pathway was closely related to malignant behavior of cells in AML. We demonstrated that miR-212-5p inhibited the activity of Wnt/β-catenin signaling pathway through regulating FZD5.

Conclusions

We demonstrated taht miR-212-5p expression was significantly down-regulated in AML. MiR-212-5p acted as an anti-oncogene, and affected the biological functions by regulation of the expression of FZD5 of AML cells. We provide experimental evidence for searching for new molecular therapeutic targets for AML.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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