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MiRNA-485-5p suppresses the proliferation of acute myeloid leukemia via targeting SALL4

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Abstract. – OBJECTIVE: To examine the expression level of microRNA-485-5p (miRNA-485-5p) in acute myeloid leukemia (AML) and its biological function in regulating the proliferative ability of AML through targeting SALL4.

PATIENTS AND METHODS: Serum level of miRNA-485-5p in AML patients and healthy controls was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). MiRNA-485-5p level in AML cell lines was detected by qRT-PCR as well. Proliferative and apoptotic changes in AML5 and U937 cells overexpressing miRNA-485-5p were assessed. Subsequently, the regulatory effect of miRNA-485-5p on SALL4 level was evaluated. Rescue experiments were conducted to uncover the role of miRNA-485-5p/SALL4 regulatory loop in regulating cellular behaviors of AML.

RESULTS: Compared with healthy controls, serum level of miRNA-485-5p was lower in AML patients. MiRNA-485-5p was similarly downregulated in AML cell lines. Overexpression of miRNA-485-5p stimulated proliferation and alleviated apoptosis in AML. SALL4 level was downregulated by transfection of miRNA-485-5p mimics in AML5 and U937 cells. Overexpression of SALL4 could reverse the regulatory effect of miRNA-485-5p on proliferative and apoptotic abilities of AML.

CONCLUSIONS: MiRNA-485-5p is downregulated in AML. Overexpression of miRNA-485-5p alleviates the malignant progression of AML through downregulating SALL4.

Key Words:
MiRNA-485-5p, SALL4, AML, Malignant progression.

Introduction

Acute myeloid leukemia (AML) is a malignant clonal disease of hematopoietic stem cells. Manifesting as the uncontrolled proliferation and maturation of bone marrow primordial cells, AML would lead to the excessive accumulation of naive cells in the blood and other organs[1,2].

Dual-changes in DNA sequences and non-DNA sequences (epigenetics) result in unrestricted self-renewal and non-differentiated state, thereby limiting cells to remain at a particular stage of development[3-5]. In addition to chromosome structural aberrations and abnormal numbers, leukemia subtypes are classified based on the discovery of mutant genes and abnormally expressed oncogenes (i.e., ERG and BAALC), which further deepens the knowledge on the prognosis of AM[6,7]. Gene researches on the abnormally expressed molecules discovered in AML contribute to the understanding of AML pathogenesis[8,9].

MicroRNAs (miRNAs) are a type of non-coding RNAs with 21-25 nucleotides long. They suppress the transcription of target genes or impair mRNA stability by binding to the 3′-untranslated region (3′-UTR) of target genes[10,11]. Recent researches[12-14] have demonstrated that some miRNAs are stable and re-generated in serum and plasma. Plenty of miRNAs are closely related to tumor diseases. These tumor-related miRNAs may be utilized as diagnostic and therapeutic targets for tumors[14,15]. It is demonstrated that miRNAs are crucial regulators in a diverse biological progression. Abnormalities in miRNA expressions and functions could be involved in the pathogenesis of AML[16,17]. In the past years, several differentially expressed miRNAs have been found in AML. MiRNA-485-5p is found to be upregulated in many solid tumors as a proto-oncogene. Overexpression of miRNA-485-5p stimulates the tumor growth[18,19]. In this paper, we first detected the expression pat-

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tern of miRNA-485-5p in the serum of AML patients and AML cell lines. Its potential influences on proliferative and apoptotic abilities of AML were subsequently explored. Our study may provide a theoretical basis for clinical treatment of AML.

Patients and Methods

Patients and AML Samples

A total of 35 AML patients and 35 healthy volunteers were enrolled. Serum samples were taken from them and preserved for the following experiments. Each subject has been fully informed. This investigation was approved by the Ethics Committee of Luoyang Central Hospital. Signed written informed consents were obtained from all participants before the study.

Cell Culture

Human bone marrow stromal cells (HS-5) and AML cells (AML2, AML193, Kasumi-1, HL-60, AML5, and U937) were provided by American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD, USA) and maintained in a 37°C, 5% CO_2 incubator.

Transfection

Transfection plasmids were provided by GenePharma (Shanghai, China). Cells were pre-seeded in the 6-well plates and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) at 70% confluence. At 48 h, cells were harvested for subsequent experiments.

Cell Counting Kit-8 (CCK-8)

Cells were seeded in the 96-well plate with 2×10^3 cells per well. At the appointed time points, absorbance (A) at 450 nm of each sample was recorded using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for depicting the viability curves.

Colony Formation Assay

Cells were seeded in a 6-well plate with 200 cells per well and incubated for 10-14 days. Colonies were washed with phosphate-buffered saline (PBS) twice, fixed in 4% paraformaldehyde and dyed with Giemsa solution for 30 min. After PBS wash, colonies containing over 50 cells were counted.

Flow Cytometry

Cells were washed with PBS twice and suspended in binding buffer. Subsequently, cells were incubated with 5 μL of AnnexinV-FITC (fluorescein isothiocyanate) and 5 μL of Propidium Iodide (PI) at room temperature in dark. The apoptotic rate was subjected to flow cytometry determination (Partec AG, Arlesheim, Switzerland).

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

Total RNA was extracted from cells or serum samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), purified by DNase I treatment, and reversely transcribed into cDNA using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNA was subjected to qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as internal references. Each sample was detected in triplicate, and relative level calculated by the $2^{-\Delta\Delta Ct}$ method was analyzed by iQ5 2.0. Primer sequences used in this study were as follows: SALL4, F: 5'-CGGATGAATGGCACTCATACTA-3', R: 5'-GCTAGTTGATGTGAACGACAGGA-3'; microRNA-485-5p, F: 5'-GCCTGTGCACATCATCGCGTG-3', R: 5'-AGGTTGAATGTATGAAGTCAG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot

Total protein was extracted from cells using radioimmunoprecipitation assay (RIPA) and quantified by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). The protein sample was loaded for electrophoresis and transferred on a polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking in 5% skim milk for 2 hours, membranes were subjected to incubation with primary and secondary antibodies. Bands were exposed by enhanced chemiluminescence (ECL) and analyzed by Image Software (NIH, Bethesda, MD, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) was used for data analyses. Data were expressed as mean

± standard deviation. Intergroup differences were analyzed by the *t*-test. Chi-square test was performed to evaluate the relationship between two genes. $p<0.05$ was considered as statistically significant.

Results

MiRNA-485-5p was Lowly Expressed in AML

The serum level of miRNA-485-5p in AML patients and healthy controls was determined. As qRT-PCR data revealed, the serum level of miRNA-485-5p remained lower in AML patients than those of healthy volunteers (Figure 1A). Compared with human bone marrow stromal cells HS-5, miRNA-485-5p was identically downregulated in AML cell lines (Figure 1B). Particularly, AML5 and U937 cells expressed the lowest abundance of miRNA-485-5p among the six selected AML cell lines, which were chosen for the following experiments.

Overexpression of MiRNA-485-5p Inhibited Proliferation and Promoted Apoptosis in AML

Transfection of miRNA-485-5p mimics markedly upregulated miRNA-485-5p level in AML5 and U937 cells, indicating a great transfection efficacy (Figure 2A). As CCK-8 assay revealed, transfection of miRNA-485-5p mimics reduced the viability in AML5 and U937 cells at 72 and 96 h (Figure 2B). Besides, colony number was reduced by transfection of miRNA-485-5p mimics in AML cells, suggesting the attenuated proliferative ability (Figure 2C). As flow cytometry results demonstrated, the apoptotic rate was remarkably elevated in AML cells overexpressing miRNA-485-5p (Figure 2D).

Overexpression of MiRNA-485-5p Downregulated SALL4

To uncover the potential involvement of SALL4 in AML progression regulated by miRNA-485-5p, SALL4 level in AML cells was examined. Both mRNA and protein levels of SALL4 were downregulated in AML cells transfected with miRNA-485-5p mimics (Figures 3A, 3B). In addition, SALL4 was upregulated in serum samples of AML patients and AML cell lines (Figures 3C, 3D). By detecting serum samples of AML patients, a negative correlation was identified between expression levels of miRNA-485-5p and SALL4 (Figure 3E).

MiRNA-485-5p Regulated Cellular Behaviors of AML Through SALL4

The interaction between miRNA-485-5p and SALL4 in influencing the proliferative and apoptotic abilities of AML was explored. First of all, downregulated mRNA and protein levels of SALL4 in AML cells overexpressing miRNA-485-5p were partially reversed by co-transfection of pcDNA-SALL4 (Figures 4A, 4B). Of note, reduced viability in AML5 and U947 cells overexpressing miRNA-485-5p was reversed by overexpression of SALL4 (Figure 4C). Colony formation assay also obtained a similar result. The decreased number of colonies owing to overexpression of miRNA-485-5p was partially elevated by overexpression of SALL4 (Figure 4D). Furthermore, overexpression of miRNA-485-5p increased the apoptotic rate in AML cells, which was reversed by SALL4 overexpression (Figure 4E). Hence, it is believed that miRNA-485-5p mediated AML proliferation and apoptosis through negatively regulating SALL4 level.

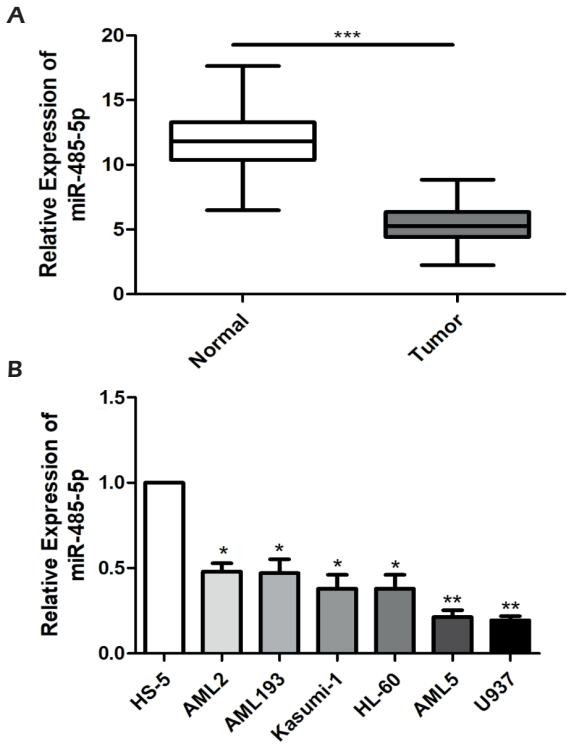

Figure 1. MiR-485-5p was lowly expressed in AML. ***A,*** Serum level of miR-485-5p in AML patients and healthy controls. ***B,*** MiR-485-5p level in human bone marrow stromal cells (HS-5) and AML cells (AML2, AML193, Kasumi-1, HL-60, AML5, and U937).

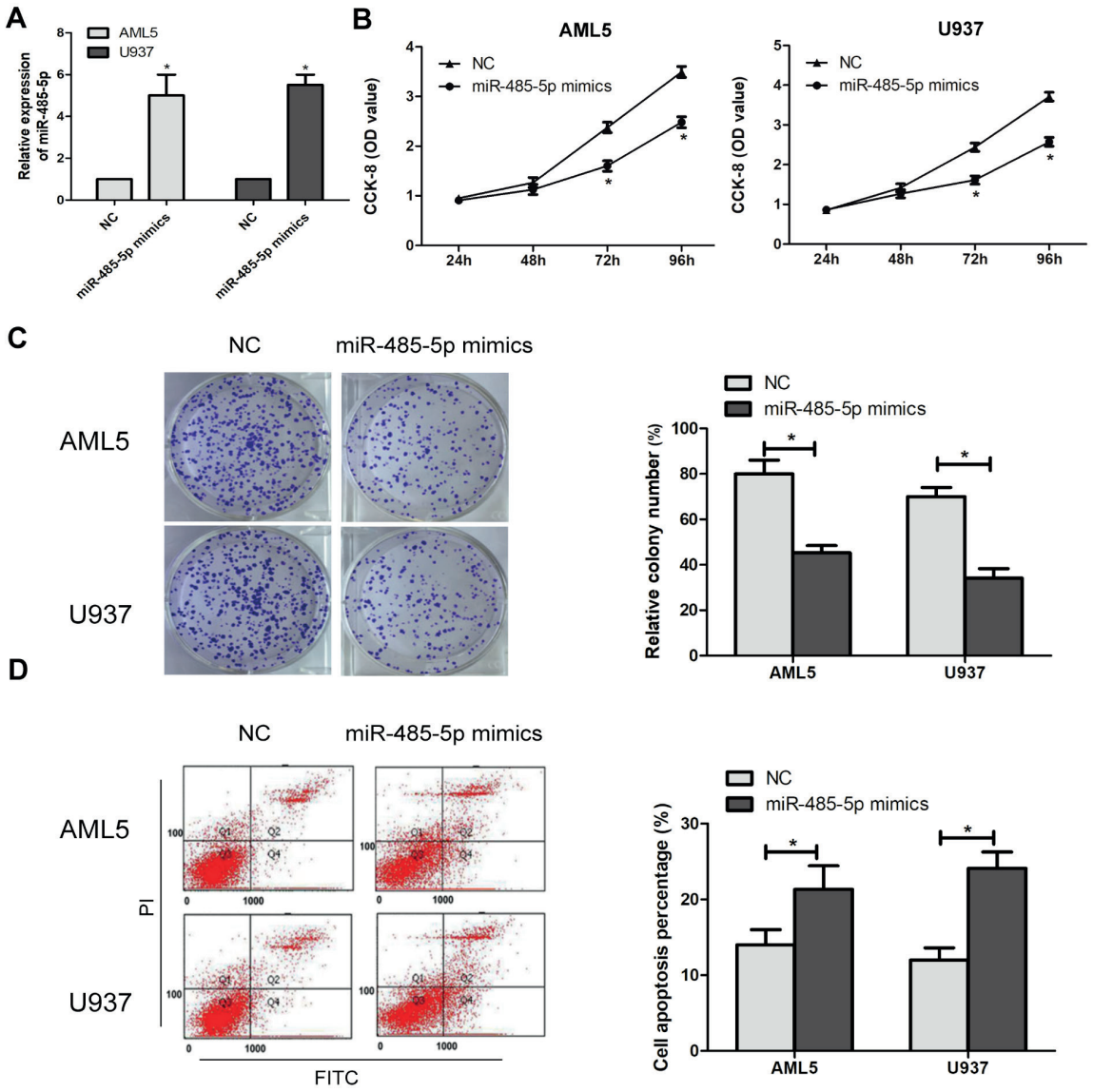

Figure 2. Overexpression of miR-485-5p inhibited proliferation and promoted apoptosis in AML. ***A,*** Transfection efficacy of miR-485-5p mimics in AML5 and U937 cells. ***B,*** Viability in AML5 and U937 cells transfected with NC or miR-485-5p mimics. ***C,*** Colony number in AML5 and U937 cells transfected with NC or miR-485-5p mimics (magnification ×'40). ***D,*** Apoptotic rate in AML5 and U937 cells transfected with NC or miR-485-5p mimics.

Discussion

The occurrence of leukemia is a complex process involving multiple genetic and epigenetic events in an independent way[1-3]. Many factors can lead to hematopoietic dysfunction, including abnormalities in chromosome behaviors, certain oncogenes, and intracellular signaling pathways[6,7]. Some authors[16,17] have found that abnormally expressed miRNAs may be involved in the development of leukemia. According to the specific regulatory functions in the progression of leukemia, miRNAs are classified into carcinogenic ones (i.e. miR-155) and tumor-suppressor ones (i.e., miR-29b)[20, 21]. Several differentially expressed miRNAs have been identified in AML, which may be utilized as diagnostic, therapeutic and prognostic markers in AML[13-17]. As a result, searching for abnormally expressed miRNAs in AML and analyzing their functions may contribute to improving the prognosis of AML patients[16].

MiRNAs are non-coding RNAs with 22 nt long. First, the miRNA-encoding gene in the nucleus transcribes a 400-nucleotide primary

miRNA (pri-miRNA) with a stem-loop structure under the assistance of RNA polymerase II. Subsequently, the pri-miRNA is translocated into the nucleus, where it is recognized and cleaved by the Drosha-DGCR8 protein complex to form a 70-nucleotide miRNA precursor (pre-miRNA) with a stem-loop structure. Subsequently, the Exportin-5 transporter transports the pre-miRNA to the cytoplasm[10,11]. Finally, the RNaselIIDicer enzyme processes the pre-miRNA into a double-stranded RNA of about 22 nucleotides, and the duplex is broken into a mature miRNA[12,13]. Mature miRNAs, alongside with RNA-induced silencing complexes (RISC) containing Argonaute protein, contribute to form asymmetric RISC-miRNA complexes (miRISC) to negatively regulate target gene expressions at the post-transcriptional level[14,15]. The miRNA recognizes the target mRNA mainly by interacting with a miRNA regulatory element (MRE) located on the 3' UTR of the target mRNA[22-24]. Base pairing degree between the miRNA and the target mRNA determines the way of miRISC to inhibit the translation of target mRNAs or directly degrade them. Highly matched miRNAs cause cleavage and degradation of target RNA through a mechanism similar to siRNA; otherwise, miRNAs inhibit the translation of target RNAs[24, 25].

In this study, we explored the clinical characteristics of miRNA-485-5p in AML. Serum level of miRNA-485-5p was found to be lower in AML patients relative to healthy controls. It is suggested that miRNA-485-5p may be a tumor-suppressor gene in AML. To further uncover the biological function of miRNA-485-5p in AML, we constructed the overexpression model in AML5 and U937 cells. Overexpression of miRNA-485-5p stimulated proliferation and alleviated apoptosis in AML.

With the in-depth researches in recent years, a growing number of oncogenes and tumor-suppressor genes have been discovered[26]. Oncogenes activated by mutation, translocation, and other mechanisms stimulate the progression of tumorigenesis.

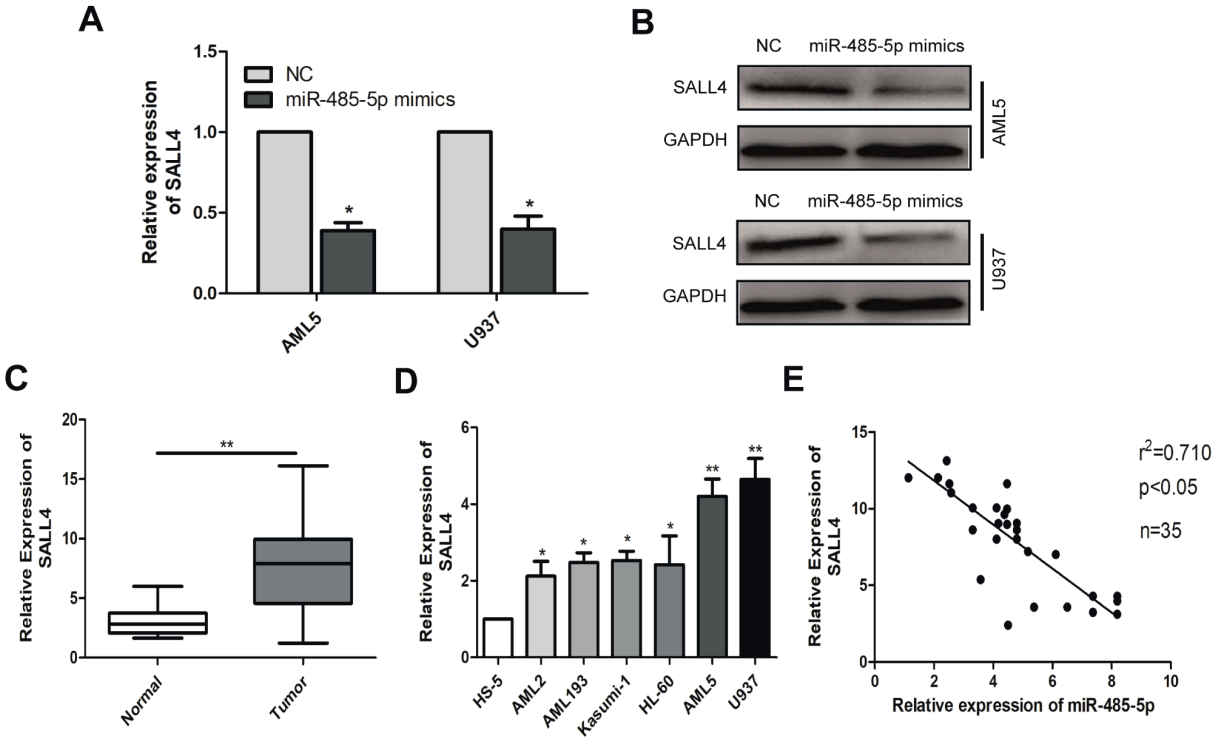

Figure 3. Overexpression of miR-485-5p downregulated SALL4. ***A,*** Relative level of SALL4 in AML5 and U937 cells transfected with NC or miR-485-5p mimics. ***B,*** Protein level of SALL4 in AML5 and U937 cells transfected with NC or miR-485-5p mimics. ***C,*** Serum level of SALL4 in AML patients and healthy controls. ***D,*** SALL4 level in human bone marrow stromal cells (HS-5) and AML cells (AML2, AML193, Kasumi-1, HL-60, AML5, and U937). ***E,*** A negative correlation between serum levels of miR-485-5p and SALL4 in AML patients.

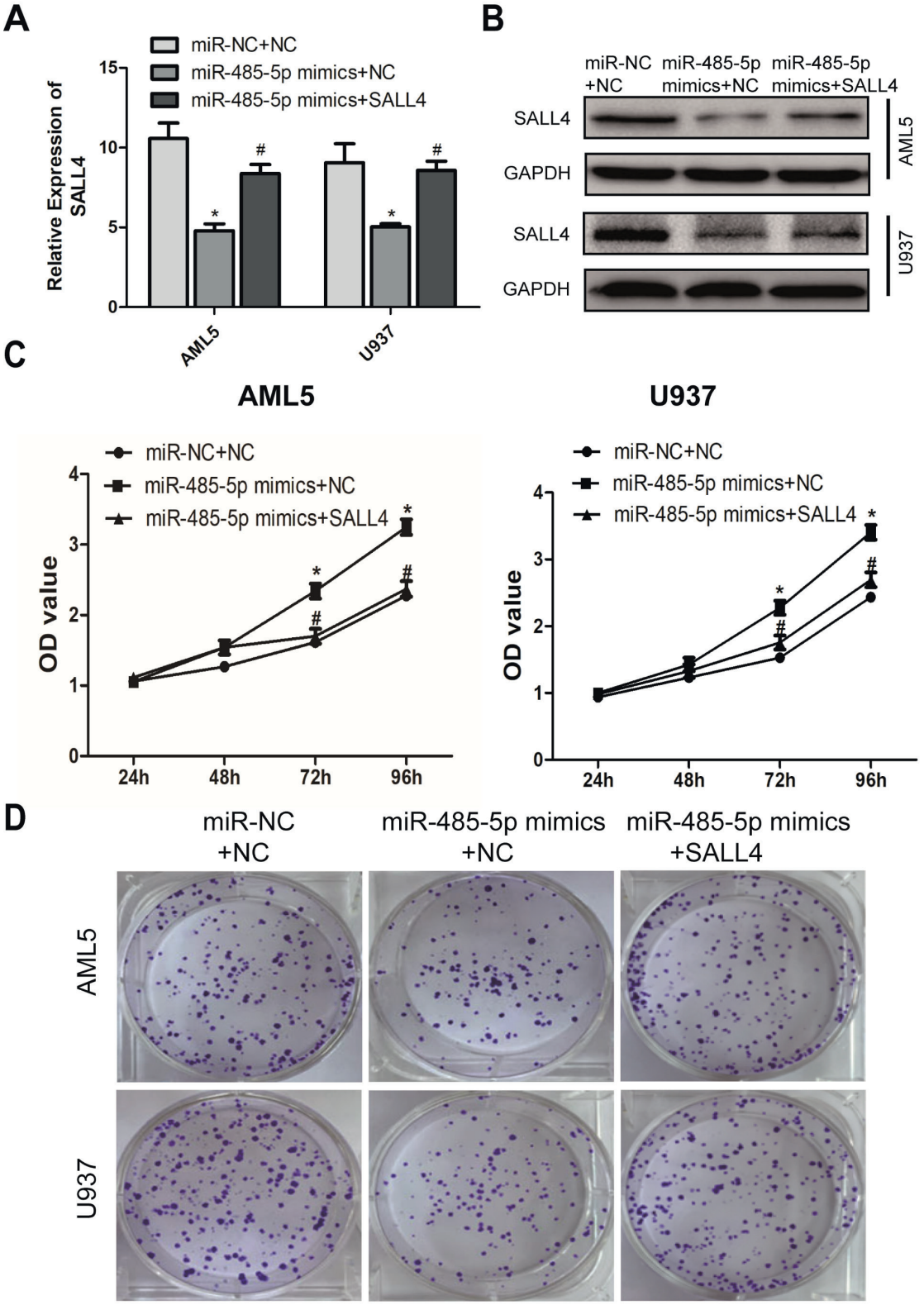

Figure 4. MiR-485-5p regulated cellular behaviors of AML through SALL4. AML5 and U937 cells were transfected with miR-NC + NC, miR-485-5p mimics + NC or miR-485-5p mimics + pcDNA-SALL4. ***A,*** Relative level of SALL4; ***B,*** Protein level of SALL4; ***C,*** Viability at 24, 48, 72, and 96 h; ***D,*** Relative colony number (magnification × 40);

Figure Continued

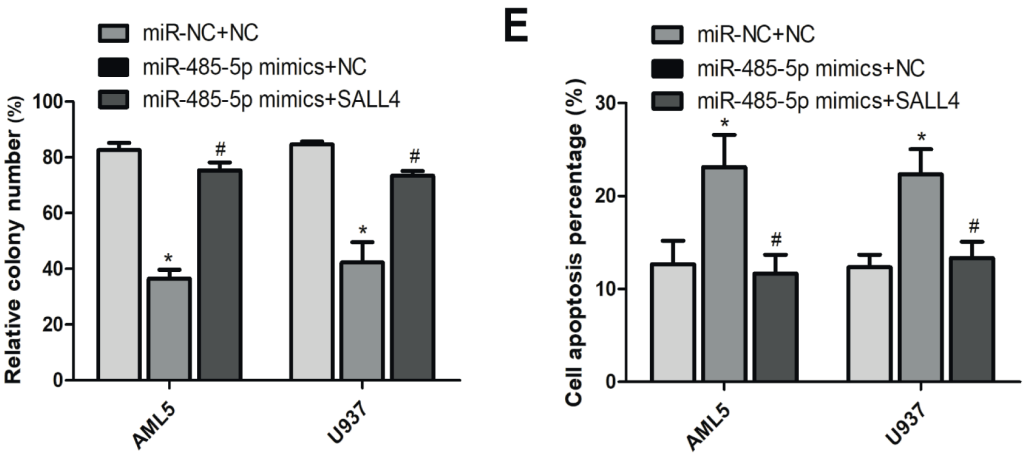

Figure 4. *(Continued)*. ***E,*** Apoptotic rate.

Meanwhile, the loss of function of tumor-suppressor genes fails to inhibit the malignant progression of cells[27,28]. Some investigations have shown the presence of SALL4 in hematopoietic stem cells and progenitor cells. With the differentiation and maturation of the blood cells, SALL4 expression gradually decreases until disappears[29]. Our work showed that SALL4 was highly expressed in the serum of AML patients and cell lines, suggesting that SALL4 exerted a carcinogenic role in AML. SALL4 level was downregulated by miRNA-485-5p overexpression in AML5 and U937 cells, indicating a regulatory interaction with miRNA-485-5p. Notably, overexpression of SALL4 could reverse the regulatory effect of miRNA-485-5p on proliferative and apoptotic abilities of AML. It is suggested that miRNA-485-5p alleviated the malignant progression of AML through targeting SALL4.

Conclusions

We found that miRNA-485-5p is downregulated in AML. Overexpression of miRNA-485-5p alleviates the malignant progression of AML through downregulating SALL4.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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