

Research Article

TRIM31 promotes acute myeloid leukemia progression and sensitivity to daunorubicin through the Wnt/ β -catenin signaling

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Tripartite motif (TRIM) 31 is a member of TRIM family and exerts oncogenic role in the progression and drug resistance of several cancers. However, little is known about the relevance of TRIM31 in acute myeloid leukemia (AML). Herein, we investigated the role of TRIM31 in AML. We examined the expression levels of TRIM31 in the blood samples from 34 patients with AML and 34 healthy volunteers using qRT-PCR. The mRNA levels of TRIM31 in human bone marrow stromal cells (HS-5) and five AML cell lines were also detected. Loss/gain-of-function assays were performed to assess the role of TRIM31 in AML cells proliferation, apoptosis and sensitivity to daunorubicin. The expression levels of pro-caspase 3, cleaved caspase 3, Wnt3a, β -catenin, cyclin D1 and c-Myc were measured using Western blot. TRIM31 expression levels were significantly up-regulated in AML patients and cell lines. Knockdown of TRIM31 suppressed cell proliferation and promoted apoptosis in AML-5 and U937 cells. The IC_{50} of daunorubicin was significantly decreased in TRIM31 siRNA (si-TRIM31) transfected cells. Oppositely, induced cell proliferation and decreased cell apoptosis were observed in pcDNA-3.1-TRIM31 transfected cells. Furthermore, knockdown of TRIM31 suppressed the activation of Wnt/ β -catenin pathway in AML cells. Activation of Wnt/ β -catenin pathway by LiCl abolished the effects of si-TRIM31 on cell proliferation, apoptosis and sensitivity to daunorubicin in AML cells. In conclusion, the results indicated that TRIM31 promoted leukemogenesis and chemoresistance to daunorubicin in AML. The oncogenic role of TRIM31 in AML was mediated by the Wnt/ β -catenin pathway. Thus, TRIM31 might serve as a therapeutic target for the AML treatment.

Introduction

Acute myeloid leukemia (AML) is a kind of leukemia that starts in the myeloid cells, which have the capacities of self-renewing, sustaining malignant populations and producing subclones [1]. AML is the most common acute leukemia and can be very fatal in a short period without suitable intervention. Early and common symptoms of AML include fatigue, increased risk of infection, arthralgia and hemorrhage [2]. Available reports point to the risk factors implicated in the development of AML, such as chemical exposures, ionizing radiation, blood disorders and heredity [3]. In the past decades, the incidence of AML is gradually increasing.

Currently, the management of AML is divided into induction phase and consolidation phase [4,5]. Chemotherapy is a standard treatment option applied in both induction and consolidation phases to destroy leukemic cells [6]. However, this therapy usually presents high toxicity and high risk of recurrence due to the chemoresistance of AML stem cells [4]. Considering these alarming challenges, it is imperative to develop appropriate intervention for AML progression and chemoresistance.

Received: 15 December 2019
Revised: 27 February 2020
Accepted: 09 March 2020

Accepted Manuscript online:
31 March 2020
Version of Record published:
15 April 2020

Tripartite motif (TRIM) 31, a member of the TRIM protein family, was found to exert oncogenic role in the development and progression of several tumors. Overexpression of TRIM31 is observed in high-grade glioma tissues and associated with short survival time. Overexpressing TRIM31 promotes the proliferation, invasion and migration of glioma cells [7]. TRIM31 expression is significantly up-regulated in hepatocellular carcinoma (HCC) tissues and significantly correlated with advanced disease status. TRIM31 promotes the malignant behaviors of HCC cells through regulation of mammalian target of rapamycin complex1 (mTORC1) pathway [8]. In addition, TRIM31 has been proven to be associated with radiosensitivity and chemosensitivity in cancers. Knockdown of TRIM31 enhances radiosensitivity of colorectal cancer cells by inducing DNA damage and cell apoptosis [9]. High expression of TRIM31 in pancreatic cancer patients is associated with aggressive phenotype and poor prognosis. TRIM31 overexpression confers gemcitabine resistance in pancreatic cancer cells [10].

Considering the oncogenic roles of TRIM31 in these types of cancers, we speculated that TRIM31 might exhibit similar biological functions in AML. Thus, the aim of the present study was to uncover the role of TRIM31 in AML.

Materials and methods

Patient samples collection

The study was approved by the Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). A total of 34 patients diagnosed with AML and 34 healthy volunteers were enrolled. Prior informed consents were obtained from all participants. Up to 5 ml whole blood sample was obtained from each participant and used for the detection of TRIM31.

Cell culture and transfection

Human bone marrow stromal cells (HS-5) and four AML cell lines including AML-5, U937, K-562 and THP-1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). The cells were cultured in RPMI-1640 or DMEM (Life Technologies, Carlsbad, CA, USA). The medium was supplemented with 10% FBS (Life Technologies).

The TRIM31 siRNA (si-TRIM31), non-targeting scrambled siRNA (si-NC) and TRIM31-overexpressing plasmid (pcDNA-3.1-TRIM31) were procured from GenePharma (Shanghai, China). Cell transfections were carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, U.S.A.) based on the manufacturer's instructions.

qRT-PCR

Total RNA was isolated from blood samples using RNAPrep Pure Blood Kit (Tiangen Biotech, Beijing, China). Total RNA was extracted from the cell lines using a Total RNA Purification kit (Invitrogen) in accordance with the manufacturer's protocols. After the assessment of quantity and concentration of RNA, total RNA was used for the reverse transcription reaction with the Prime-Script RT reagent kit (TaKaRa, Dalian, China). Quantitative RT-PCR was performed using an miScript SYBR green PCR kit (Qiagen, Hilden, Germany). The relative mRNA level of TRIM31 was calculated using the comparative $2^{-\Delta\Delta C_t}$ method.

Western blot

Total proteins were extracted from AML-5 and U937 cells using radioimmunoprecipitation assay (RIPA) lysis buffer (Sangon Biotech, Shanghai, China). The protein samples were loaded for electrophoresis and then subjected to Western blot analysis for the measurement of pro-caspase 3, cleaved caspase 3, Wnt3a, β -catenin, cyclin D1 and c-Myc expression levels. The specific primary and corresponding secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The protein bands were detected by enhanced chemiluminescence (ECL) reagent (Thermo Fisher Scientific, Waltham, MA, U.S.A.) and analyzed by ImageJ Software (NIH, Bethesda, MD, U.S.A.).

MTT assay

MTT assay was performed to assess cell proliferation and sensitivity to daunorubicin. For the cell proliferation assay, AML-5 and U937 cells (1×10^4 cells/well) were plated in a 96-well plate and cultured for adhesion. After incubating for 0, 24, 48, 72, 96 h, each well was added with 20 μ l MTT (5 mg/ml, Sigma-Aldrich, St. Louis, MO, U.S.A.) and incubated for additional 4 h. Then, 150 μ l DMSO was added the cells to dissolve formazan. Finally, the optical density (OD) at 490 nm was measured using a Microplate Reader (Molecular Devices, Sunnyvale, CA, U.S.A.).

For the chemosensitivity assay, AML-5 and U937 cells (1×10^4 cells/well) were incubated with a series of concentrations of daunorubicin (0, 2, 4, 8, 16 μM) for 24 h. Then the cell viability was measured using MTT assay as described above.

Cell apoptosis assay

Cell apoptosis was detected by flow cytometry using AnnexinV-FITC/Propidium Iodide (PI) staining kit (Invitrogen). In brief, after transfection, cells were harvested and resuspended in binding buffer. Subsequently, AnnexinV-FITC and PI were added and stained for 15 min at room temperature in dark. Finally, cells were subjected to flow cytometry (BD Biosciences, San Jose, CA, U.S.A.).

Caspase-3 activity

Caspase-3 activity of AML-5 and U937 cells was measured using a caspase-3 activity assay kit (Nanjing Jiancheng Bioengineering Institute). According to the instructions, cell lysates were incubated with 50 μl Ac-DEVD-pNA for 4 h at 37°C. The absorbance at 450 nm was measured using a microplate Reader (Bio-Rad).

Statistical analysis

All statistical analyses were performed using Student GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, U.S.A.). The Student's *t* test or one-way ANOVA was respectively used to assess the significant differences between two groups or among multiple groups. Differences were considered statistically significant when *P*-value <0.05.

Results

TRIM31 was highly expressed in AML patients and cell lines

In the present study, we first validated the relative expression of TRIM31 in AML patients. Results in Figure 1A showed that up-regulation of TRIM31 expression was observed in blood samples from AML patients compared with samples from healthy volunteers. Next, the TRIM31 expression levels in human bone marrow stromal cells (HS-5) and four AML cell lines (AML-5, U937, K-562 and THP-1 cells) were also detected using qRT-PCR and Western blot. The results showed that high expression levels of TRIM31 were observed in AML cell lines, especially in AML-5 and U937 cells (Figure 1B,C).

Knockdown of TRIM31 inhibited the proliferation and induced the apoptosis of AML cells

Loss-of-function assays were then performed in AML-5 and U937 cells through transfection with si-TRIM31 or si-NC (Figure 2A,B). MTT assay demonstrated that TRIM31 knockdown significantly decreased AML-5 and U937 cells proliferation, compared with si-NC transfected cells (Figure 2C,D). Furthermore, we found that knockdown of TRIM31 significantly promoted caspase-3 activity in AML-5 and U937 cells, respectively, as compared with the si-con group (Figure 2E,F). Similarly, we also found that knockdown of TRIM31 greatly promoted cell apoptosis, increased cleaved caspase 3 expression and reduced pro-caspase 3 expression in AML-5 and U937 cells, respectively (Supplementary Figures S1A,B and S2A,B).

Overexpression of TRIM31 promoted the proliferation and inhibited the apoptosis of AML cells

Subsequently, gain-of-function assays were performed through transfection with TRIM31-overexpressing plasmid (pcDNA-3.1-TRIM31) or pcDNA-3.1 empty plasmid (Figure 3A,B). As illustrated in Figure 3C,D, after transfection with pcDNA-3.1-TRIM31, AML-5 and U937 cells proliferation were obviously promoted. Meanwhile, pcDNA-3.1-TRIM31 transfection led to significant reduction in caspase-3 activity in both AML-5 and U937 cells (Figure 3E,F). Furthermore, TRIM31 overexpression significantly inhibited cell apoptosis, down-regulated cleaved caspase 3 expression and up-regulated pro-caspase 3 expression in AML-5 and U937 cells, respectively (Supplementary Figures S1C,D and S2C,D).

Knockdown of TRIM31 enhanced cell sensitivity to daunorubicin

As shown in Figure 4A,B, daunorubicin significantly reduced cell viability in both AML-5 and U937 cells in dose-dependent manner. The growth rate in si-TRIM31 transfected AML-5 cells was less than that in normal AML-5

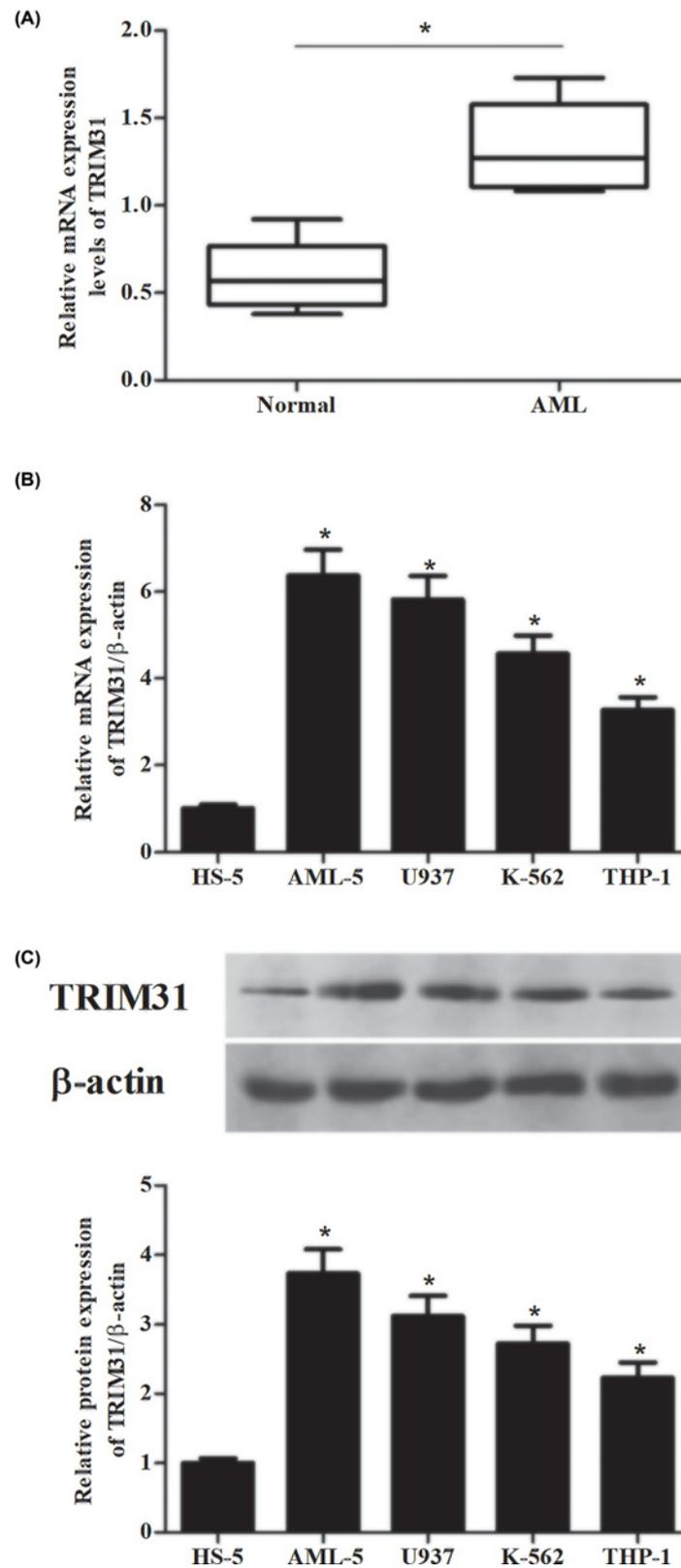


Figure 1. Relative expression levels of TRIM31 in blood samples and cell lines

(A) Expression levels of TRIM31 in blood samples from AML patients ($n=34$) and healthy volunteers ($n=34$). $*P<0.05$ compared with control group (healthy volunteers). (B,C) The mRNA and protein levels of TRIM31 in human bone marrow stromal cells (HS-5) and four AML cell lines (AML-5, U937, K-562 and THP-1 cells). $*P<0.05$ compared with HS-5 cells.

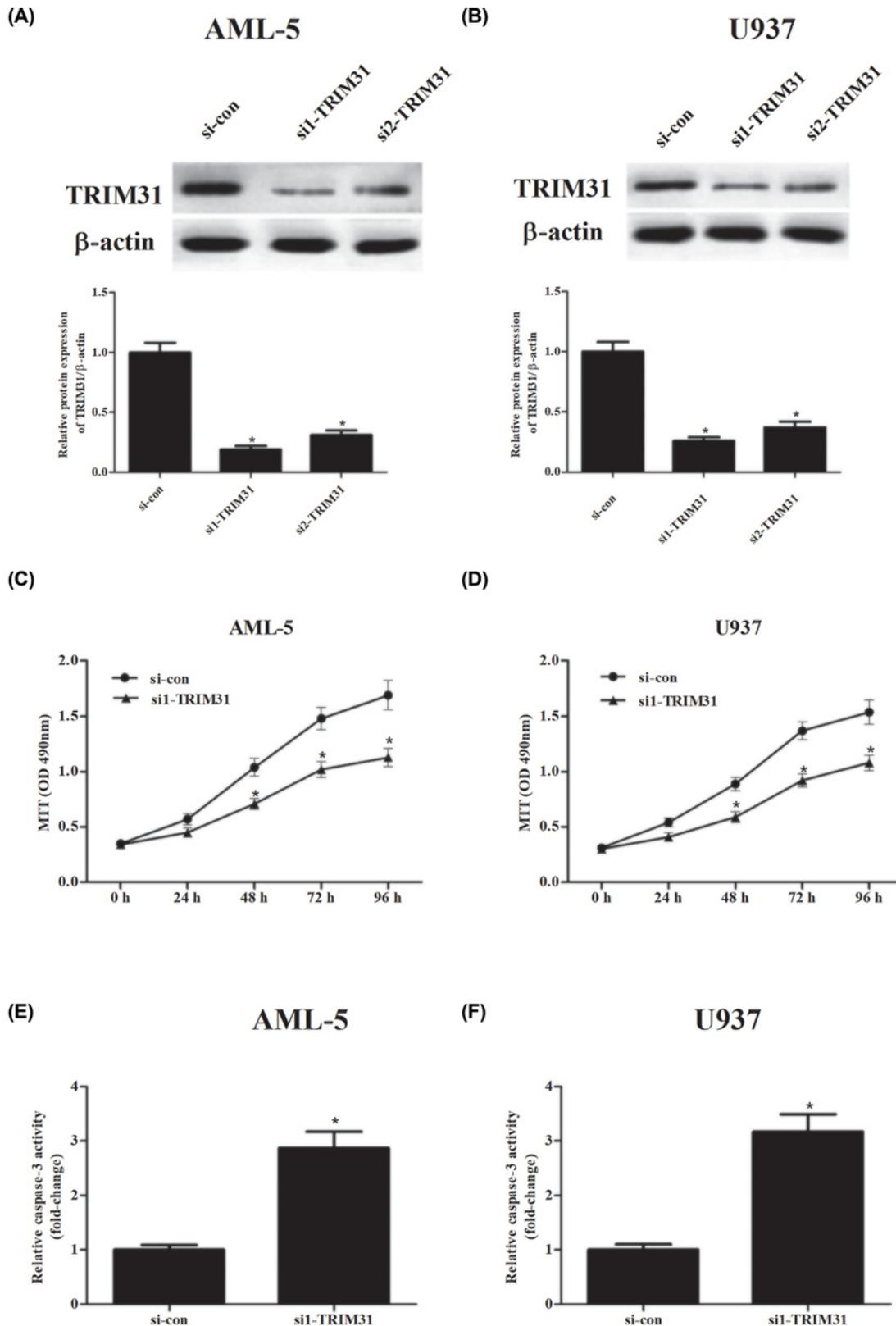


Figure 2. Loss-of-function assays were performed after transfection with si-TRIM31

(A,B) AML-5 and U937 cells were transfected with si1/2-TRIM31 or si-con, and transfection efficiency was confirmed by Western blot. (C,D) MTT assay was performed to detect AML-5 and U937 cells proliferation. (E,F) Caspase-3 activity was carried out to analyze apoptosis of AML-5 and U937 cells. * $P < 0.05$.

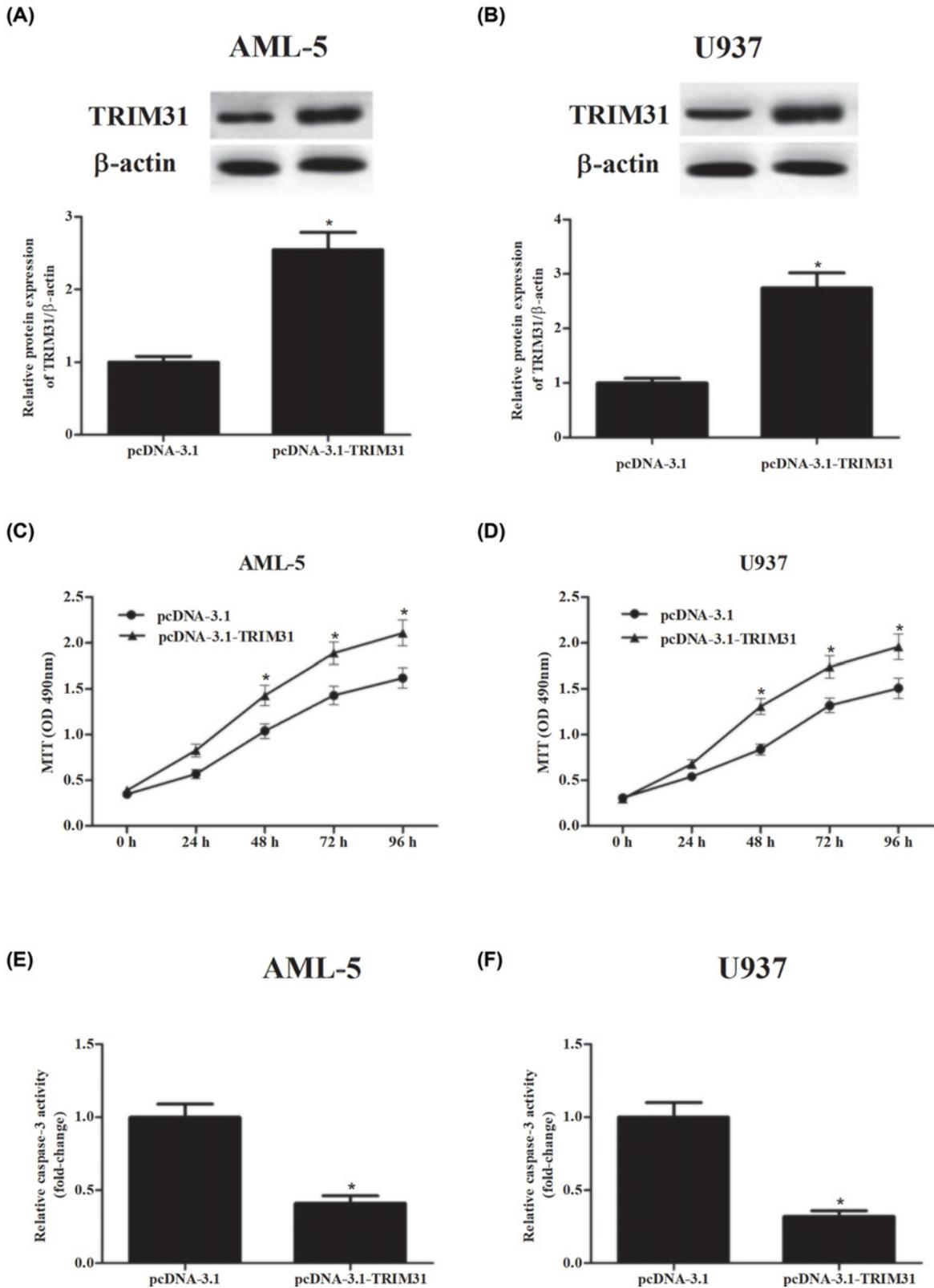
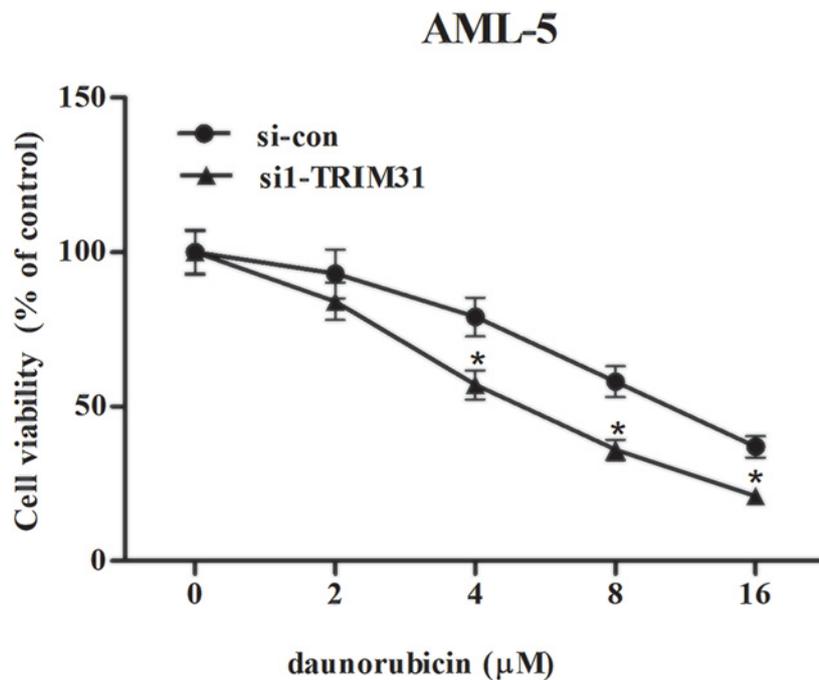


Figure 3. Gain-of-function assays were performed after transfection with pcDNA-3.1-TRIM31

(A,B) Western blot was applied to assess the protein levels of TRIM31 after transfection with pcDNA-3.1-TRIM31 or pcDNA-3.1 empty plasmid in AML-5 and U937 cells. (C,D) MTT assay was performed to detect AML-5 and U937 cells proliferation. (E,F) Caspase-3 activity was carried out to analyze apoptosis of AML-5 and U937 cells. * $P < 0.05$.

(A)



(B)

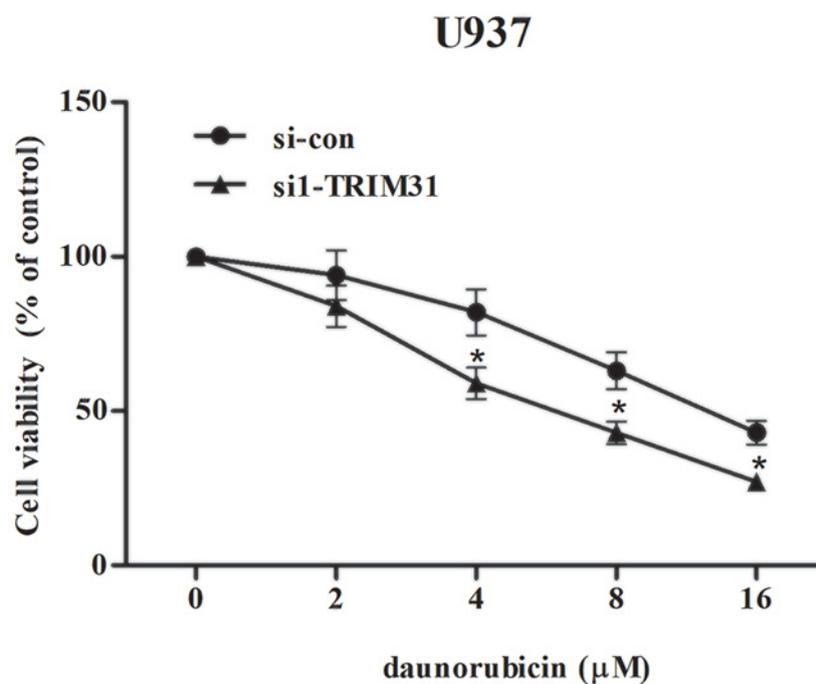


Figure 4. Evaluation of cell sensitivity to daunorubicin after transfection with si-TRIM31

AML-5 and U937 cells transfected with si-con or si1-TRIM31 were treated with various concentrations of ADR (0, 2, 4, 8, 16 μM) for 24 h. Then, cell viability was measured in AML-5 (A) and U937 cells (B) using MTT assay. * $P < 0.05$.

cells. Similarly, the growth rate was lower in si-TRIM31 transfected U937 cells than normal U937 cells. The results indicated that daunorubicin sensitivity was significantly increased in si-TRIM31 transfected AML cells.

Knockdown of TRIM31 suppressed the activation of Wnt/ β -catenin signaling pathway in AML cells

To further investigate the mechanism of TRIM31, the expression levels of Wnt3a and β -catenin in U937 cells were measured using Western blot. As shown in Figure 5, knockdown of TRIM31 in U937 cells resulted in obvious decrease in Wnt3a and β -catenin expressions. Furthermore, TRIM31 knockdown also significantly down-regulated the protein expression levels of cyclin D1 and c-Myc (Supplementary Figure S3). These data indicating that TRIM31 knockdown inhibited the activation of Wnt/ β -catenin signaling pathway in U937 cells.

LiCl, the Wnt/ β -catenin pathway activator, reversed the effects of TRIM31 knockdown on AML cells

To further confirm the role of Wnt/ β -catenin signaling pathway, U937 cells were treated with LiCl, which is an activator of Wnt/ β -catenin signaling pathway. We found that LiCl increased the protein expression levels of Wnt3a and β -catenin in si-TRIM31-transfected U937 cells (Figure 6A). Treatment with LiCl abolished the effects of si-TRIM31 on cell proliferation and caspase-3 activity in U937 cells (Figure 6B,C). As shown in Figure 6D, U937 cells treated with LiCl exhibited higher cell viability to daunorubicin, as compared with the U937 cells transfected with si-TRIM31 to daunorubicin. These findings suggested that activation of Wnt/ β -catenin signaling pathway reversed the effects of si-TRIM31 on cell proliferation, apoptosis and daunorubicin sensitivity in U937 cells.

Discussion

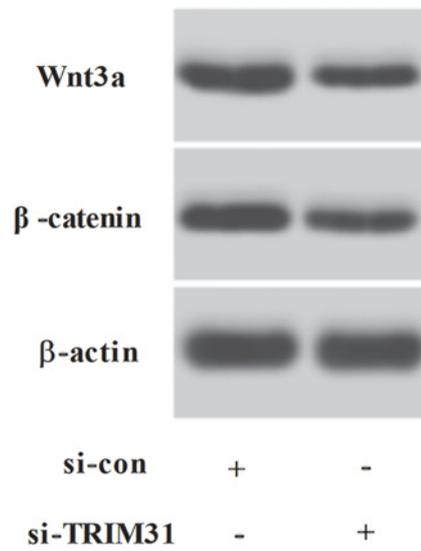
As described in the present study, TRIM31 protein could promote cell proliferation and induce cell apoptosis, as well regulate chemoresistance of AML cells. In addition, the roles of TRIM31 were mediated by the Wnt/ β -catenin signaling pathway.

TRIM family is a group of proteins characterized by three novel functional domains: a ‘really interesting new gene’ (RING) domain in the N-terminus, 1 or 2 zinc-finger domains called B boxes (B1 box and B2 box) and a coiled-coil motif [11,12]. Due to the specific nature of TRIM proteins, they have been found to be implicated in a variety of biological processes, such as cell proliferation, cell division and developmental processes and cell metabolism [13]. A growing body of evidence shows that several TRIM members are involved in the tumorigenesis and serve as therapeutic targets for the treatment of cancers [14]. Additionally, several TRIM proteins have been found to be involved in blood cancers, including acute promyelocytic leukemia (APL), B-cell acute lymphocytic leukemia (B-ALL), chronic lymphocytic leukemia (CLL), chronic myeloid leukemia (CML), chronic myelomonocytic leukemia (CMML) and AML [15–18].

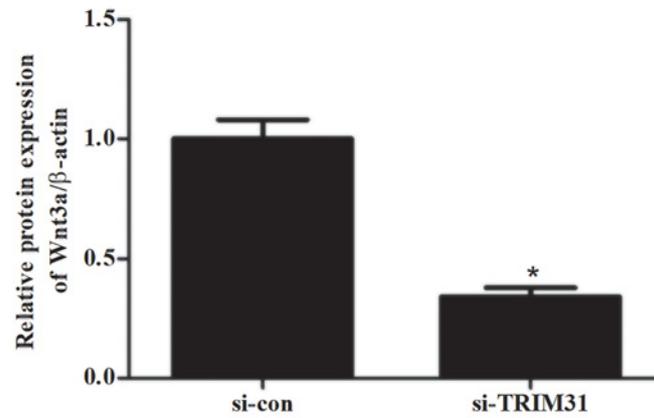
Overexpression of TRIM32 in the human promyelogenous leukemic cell line HL60 suppresses cellular proliferation and induces granulocytic differentiation, implying that TRIM32 may be a potentially therapeutic target for APL [16]. TRIM62 levels are significantly lower in AML cells than those in normal CD34-positive cells. Lower TRIM62 level is associated with shorter complete remission duration, shorter event-free and overall survival rates in AML patients, indicating that TRIM62 is an independent adverse prognostic factor in AML [19]. TRIM31 is a cancer-related TRIM protein that has been observed to exert oncogenic activity in several types of cancers [20–22]. Given its involvement in these cancers, we speculated that TRIM31 might represent therapeutic target in AML. To that end, we examined the expression levels of TRIM31 in the blood samples from 34 patients with AML and 34 healthy volunteers. The results showed that TRIM31 was highly expressed in AML patients. Moreover, loss/gain-of-function assays proved that TRIM31 promoted cell proliferation and induced caspase-3 activity AML cells. Knockdown of TRIM31 in AML cells elevated the drug sensitivity to daunorubicin. These findings were in consonance with the notion of TRIM62 as an oncogene in AML.

Transduced Wnt signals are highly conserved and regulate various developmental processes [23,24]. β -catenin is a core downstream protein in the the canonical Wnt pathway. Activation of Wnt leads to β -catenin accumulation and translocation to the nucleus. Here it interacts with T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors and thereby regulate the expression of target genes, which are important for embryonic development and proliferation [25,26]. Recently, Wnt/ β -catenin pathway has been found to play an important role in the development of AML [27–29]. The β -catenin protein is readily detected in primary AML samples, and is required for the development of leukemia stem cells (LSCs) in AML [30]. Targeting the Wnt/ β -catenin pathway may represent a new

(A)



(B)



(C)

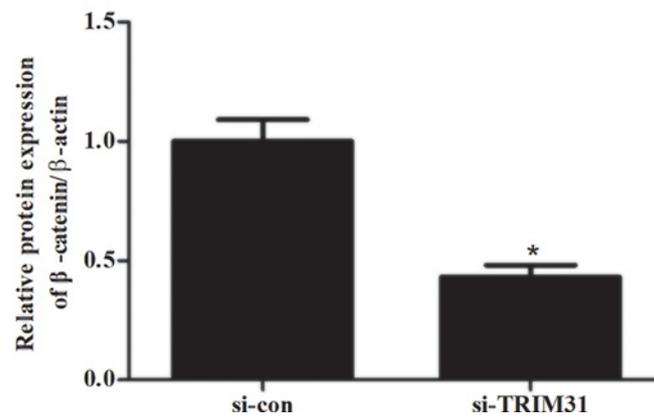


Figure 5. Effects of TRIM31 knockdown on the Wnt/ β -catenin signaling pathway in U937 cells

(A) After transfection with si-TRIM31 or si-con, the expression levels of Wnt3a and β -catenin in U937 cells were measured using Western blot. (B,C) Quantification analysis of Wnt3a and β -catenin. * P <0.05 compared with si-con transfected U937 cells.

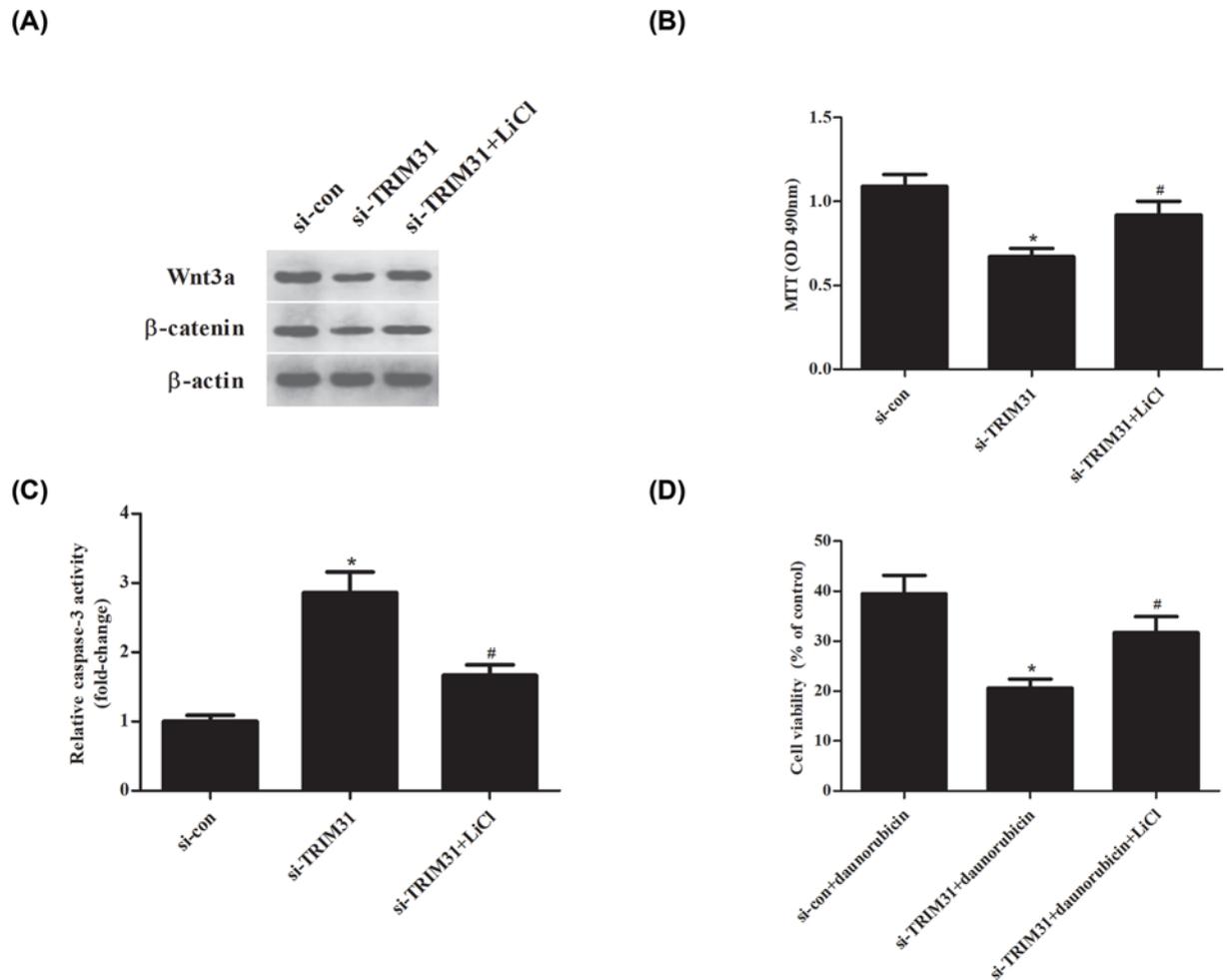


Figure 6. Effects of LiCl on si-TRIM31 transfected U937 cells

U937 cells were treated with LiCl to activate Wnt/β-catenin signaling pathway. **(A)** The expression levels of Wnt3a and β-catenin in U937 cells were measured using Western blot. **(B)** MTT assay was performed to detect U937 cells proliferation. **(C)** Flow cytometry was carried out to analyze apoptotic rates of U937 cells. **(D)** Cell viability in U937 cells with or without LiCl treatment. **P* < 0.05 compared with si-con transfected U937 cells; #*P* < 0.05 compared with si-TRIM31 transfected U937 cells.

therapeutic opportunity for the treatment of AML [31]. We found that knockdown of TRIM31 suppressed the activation of Wnt/β-catenin pathway in AML cells. Activation of Wnt/β-catenin signaling pathway by LiCl reversed the effects of si-TRIM31 on AML cells. The results indicated that the oncogenic role of TRIM31 in AML was mediated by the Wnt/β-catenin signaling pathway.

In conclusion, our findings demonstrated that TRIM31 was involved in leukemogenesis and chemoresistance. Wnt/β-catenin signaling pathway played a critical role in the oncogenic potential of TRIM31 in AML. Collectively, TRIM31 might serve as a therapeutic target for the AML treatment.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Funding

This work supported by the Applied Basic Research Project of Wuhan City [grant number 2017060201010156]; the Natural Science Foundation of China [grant number 81873444]; the Health Commission of Hubei Province Scientific Research Project [grant number WJ2019M126]; and the Research Clinician Funding Program in China [grant number 2017014].

Author Contribution

Yi Xiao designed the experiments and wrote the manuscript. Taoran Deng and Xi Ming performed the experiments. Jinhuang Xu analyzed the data.

Abbreviations

AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; HCC, hepatocellular carcinoma; PI, Propidium Iodide; si-TRIM31, TRIM31 siRNA; TRIM, tripartite motif.

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