

## Original Article

# The anti-apoptosis effect of MLAA-34 in leukemia and the $\beta$ -catenin/T cell factor 4 protein pathway

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**Abstract:** Objective This study aims to observe the effects of MLAA-34 gene in leukemia and explore its mechanism. Methods MLAA-34 RNAi vector was constructed. NOD/SCID mice and 293T cells, K562 cells, HL60 cells and U937 cells were used in this study. They were divided into MLAA-34-siRNA group and control group. The proliferation ability and apoptosis of cells were detected. The expression levels of  $\beta$ -Catenin and TCF 4 were determined using western blotting and immunohistochemical methods. Results There was significant correlation between MLAA-34 gene and U937 cell proliferation, the apoptosis rates of U937 cells with siRNA infection were higher than that of control group. There were significant differences in the expression levels of  $\beta$ -Catenin and TCF 4 between U937 cells and U937 with MLAA-34-siRNA cells group. Conclusions MLAA-34 gene had anti-apoptotic effect in leukemia, which maybe through the  $\beta$ -Catenin/TCF 4 pathway.

**Keywords:** MLAA-34, leukemia,  $\beta$ -Catenin/TCF 4 pathway, apoptosis

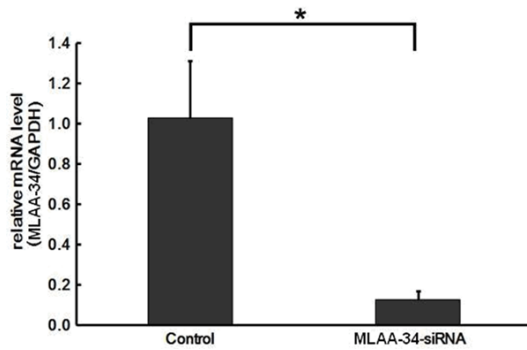
## Introduction

Acute monocytic leukemia (AML) is a cancer of the myeloid line of blood cells which is the most common acute leukemia affecting adults. It is characterized by the rapid growth of abnormal white blood cells that accumulate in the bone marrow and interfere with the production of normal blood cells. The treatment of AML has considerably improved recently, the complete remission (CR) rates in younger patients reached 65-75% with intensive induction therapy. However, they often relapsed and 5 years survival rate was only 30-40%, it was worse in patients older than 60 years [1-4]. AML-M5 is one of the most common subtypes of AML defined by the French-American-British (FAB). It was hard to cure and its relapse rate was high. M5 has been reported to have a worse prognosis than other subtypes of AML [5-7]. The patients usually developed resistance to standard anti-leukaemic drugs and therefore need to be treated with new drugs that act through other cellular pathways to reduce the risk of cross-resistance and to improve response to treatment. Allogeneic hematopoietic stem cell

transplantation has proved to be the most effective therapeutic method for acute leukemia, but its application so far has been much limited [8, 9]. These situations clearly call for novel and more effective therapy.

Immunotherapeutic methods taking leukemia associated antigens (LAAs) as target may represent a promising novel treatment option to prevent relapse and improve the outcomes of AML patients. LAAs induced specific T-cell immune responses in leukemia patients and could be potential targets for specific immunotherapies. Some peptides derived from leukemia-associated antigens were under clinical investigation for AML [10, 11]. MLAA-34 is one of the novel leukemia associated antigens that has recently been identified. It exclusively reacts with sera from allogeneic leukemia patients but not with normal donor sera [12]. In our previous study we found that the down-regulation of MLAA-34 expression significantly suppresses the proliferation and increases the spontaneous apoptosis of U937 cells in vitro, there was cross-link between MLAA-34 and the Ras, Wnt, calcium and chemokine signaling pathways in AML and

## MLAA-34 gene and $\beta$ -catenin/TCF4 pathway



**Figure 1.** RT-PCR results of MLAA-34 siRNA in U937 cells. The levels of MLAA-34 in MLAA-34-siRNA group were significantly lower than that of control group, \* $P < 0.05$ .

thirteen of the annotated proteins may interact with MLAA-34 and participate in carcinogenesis directly, the Ras, Wnt, calcium and chemokine signaling pathways may be involved in anti-apoptosis with MLAA-34 in U937 cells [7-9]. Therefore, we explored the anti-apoptotic effect of MLAA-34 gene in leukemia and its mechanism in this study.

### Materials and methods

#### Experimental animals and cell culture

This study was approved by the Ethics Committee of The Second Affiliated Hospital of Xi'an Jiao Tong University. Experimental procedures were carried out under the supervision of the Ethics Committee to minimize the suffering of animals. NOD/SCID mice (female, aged 6~8 weeks) were purchased from Vital River Laboratories (VRL, Beijing, China). The mice were kept in clean and quiet environment with room temperature and had free access to food and drinking water in SPF laboratory. They and were pre feeding for one week to adapt to the environment.

293T cells, K562 cells, HL60 cells and U937 cells were obtained from ATCC (American Type Culture Collection). 293T cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and 0.02 mg/ml kanamycin (GIBCO) at 37°C in 5% CO<sub>2</sub>. U937 and K562 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. They were divided into MLAA-34-siRNA group and control group.

#### Construction of lentiviral vector for MLAA-34 RNA interference

According to the MLAA-34 gene sequences in GenBank, we designed shRNA oligonucleotide sequences using the software (Ambion) in accordance with the design principles of RNA interference sequences. PSCSI2891-1: CCGG-AGCAGTGGGAAGCTATTCAGAATTCAAGAGATTC-TGAATAGCTTCCACTGCTTTTTT; PSCSI2891-2: AATTCAAAAAAGCAGTGGGAAGCTATTCAGAATCTC-TTGAATTCTGAATAGCTTCCACTGCT. Then it was inserted into pGCSIL -GFP vector to construct the recombinant plasmid, PCR analysis and DNA sequencing were performed to confirm the accuracy.

#### Package the virus

Briefly, 293T cells at confluence of 70%~80% were used for transfection. The lentiviral packaging plasmid mixture was co-transfected into cells and incubated at room temperature for 25 min, then added into 6-well plate and cultured at 37°C with 5% CO<sub>2</sub>. The medium containing transfection mixture residues was discarded and fresh medium was added after 12 hours, the cell supernatant was collected after 48 hours and removal of cell debris was carried out by centrifuge, then the 0.45  $\mu$ m PVDF film was used to filter and harvest the packaged virus particles.

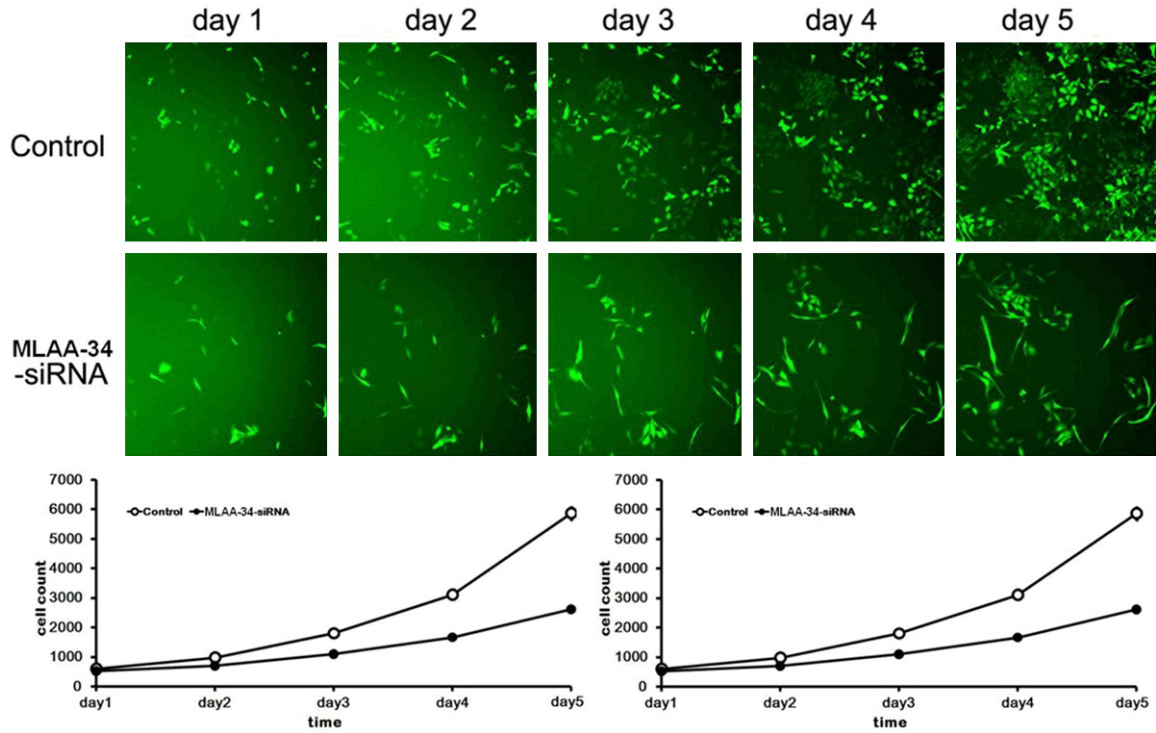
#### RNA interference and plasmid transfection

SiRNA oligonucleotides corresponding to human MLAA-34 gene (5'-AGCAGTGGGAAGCTATTC-AGAA-3') were used for infection of U937 cells using lipofectamine 2000 (Life Technologies) according to the manual.

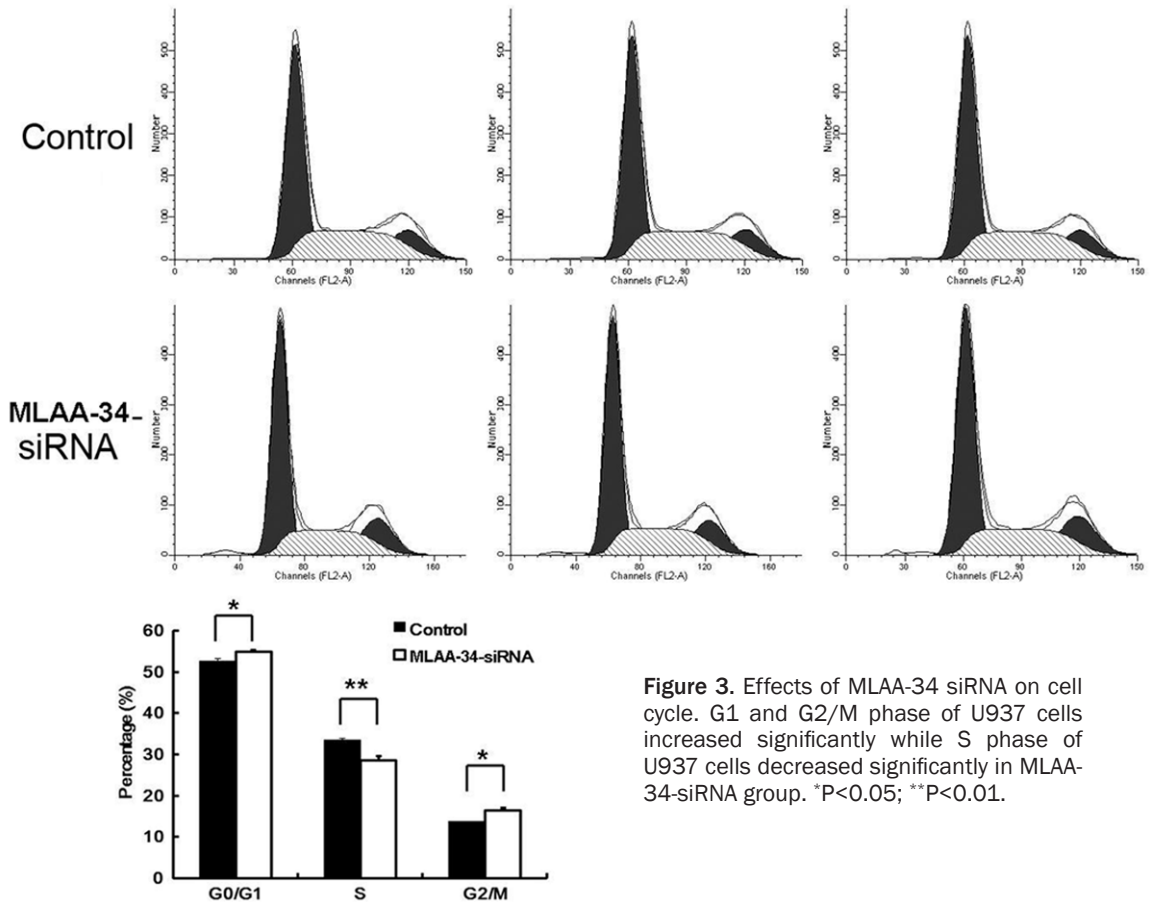
#### RNA extraction and real-time PCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manual protocol. 1  $\mu$ g total RNA was subjected to reverse transcription using reverse transcription system (Promega). Real-time PCR were performed using SYBR Green PCR Master Mix (Qigen). At the end of each reaction, a melting curve analysis was performed to confirm the absence of primer dimers. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as an internal control for normalization of RNA quantity

MLAA-34 gene and  $\beta$ -catenin/TCF4 pathway

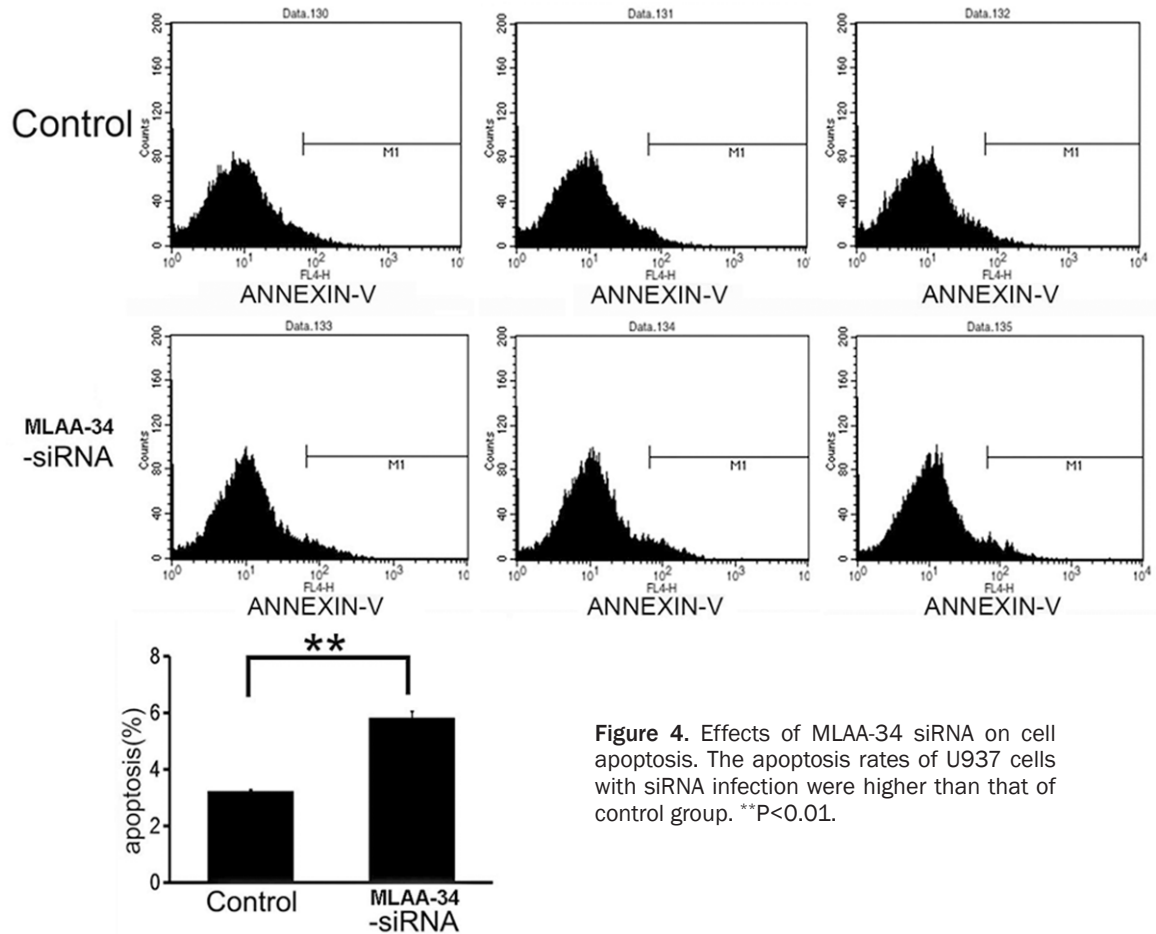


**Figure 2.** Effects of MLAA-34 siRNA on cell proliferation in different group. The proliferation rate of U937 cells in MLAA-34-siRNA group was inhibited after siRNA transfection.



**Figure 3.** Effects of MLAA-34 siRNA on cell cycle. G1 and G2/M phase of U937 cells increased significantly while S phase of U937 cells decreased significantly in MLAA-34-siRNA group. \* $P < 0.05$ ; \*\* $P < 0.01$ .

## MLAA-34 gene and $\beta$ -catenin/TCF4 pathway



**Figure 4.** Effects of MLAA-34 siRNA on cell apoptosis. The apoptosis rates of U937 cells with siRNA infection were higher than that of control group. \*\* $P < 0.01$ .

and quality differences in all samples. Quantifications of target genes mRNA was performed using the  $2^{-\Delta\Delta Ct}$  method. Primers sequences were as follows: GAPDH-F: 5'-TGACTTCAACAGCGACACCCA-3', GAPDH-R: 5'-CACCTGTGCTGTAGCCAAA-3'; MLAA-34-F: 5'-GGGAAAGGAA-TGGTGAA-3', MLAA-34-R: 5'-TGCATAGAGCCG-GTTAG-3'.

### *The effects of MLAA-34 on cells*

The proliferation ability of cells was detected by cell counting methods. The cell density was adjusted to  $2 \times 10^4$  cells/ml and inoculated in 96 well plates with 100  $\mu$ l/well and incubated at 37°C with 5%  $CO_2$  for 5 days. The cells were counted after culture for 1, 2, 3, 4 and 5 days. The cell cycle was detected by PI-FACS methods. The cell density was inoculated in 6cm dish and used for detection when they reached to 85% fusion.

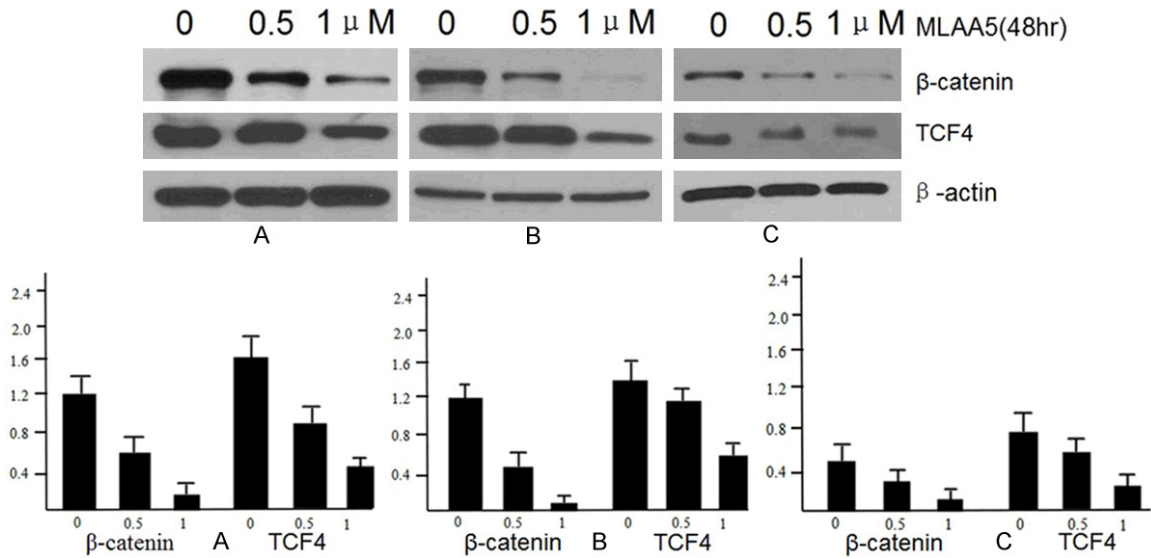
Fluorescein Annexin V-FITC/PI double labeling was performed with the Annexin V-FITC Apo-

ptosis Detection Kit (Beckman) to detect the apoptotic effects of MLAA-34 gene on U937 cells. The cells were stained with Annexin V-FITC and PI according to the instructions of the kit manual. The apoptotic cells were determined with a FACS Calibur flow cytometer (BD Biosciences) and analyzed with CELLQUEST software (BD Biosciences). Clone forming test was also performed in this study. The cells were seeded at density 400 cells/well in 6 well plates and incubated at 37°C with 5%  $CO_2$  for 14 days. The numbers of cell colony were measured after that.

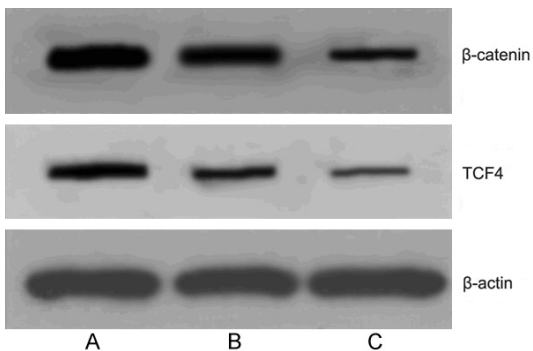
### *The effects of MLAA5 on $\beta$ -catenin and T cell factor 4*

The K562, HL60 and U937 cells were treated with different concentration of MLAA5 (0.5  $\mu$ M and 1  $\mu$ M) respectively and cultured at 37°C with 5%  $CO_2$  for 48 h. The expression levels of  $\beta$ -catenin and T Cell Factor 4 (TCF4) were determined by western blotting method. Briefly, the cells were lysed with protein lysis buffer and

## MLAA-34 gene and $\beta$ -catenin/TCF4 pathway



**Figure 5.** Western blotting results of the effects of MLAA5 on  $\beta$ -catenin and TCF4 in different cells. The levels of  $\beta$ -catenin and TCF4 was significant lower in 1  $\mu$ M of MLAA5 group than that of control group, there was significant difference in the levels of  $\beta$ -catenin and TCF4 among different concentration of MLAA5 group. A: U937; B: K562; C: HL60.



**Figure 6.** Western blotting results of the effects of MLAA-34 siRNA on  $\beta$ -catenin and TCF4. There were significant differences in the expression levels of  $\beta$ -catenin and TCF4 between mononuclear cells of M5 patients and U937 with MLAA-34-siRNA cells group. A: Control group; B: Mononuclear cells of M5 patients; C: U937 with MLAA-34-siRNA cells group.

the lysates were harvested by centrifugation (12,000 rpm) at 4°C for 5 min. Protein samples were then separated by SDS polyacrylamide gel electrophoresis and were transferred onto a polyvinylidene fluoride (PVDF) membrane. After blocking the nonspecific binding sites for 60 min with 5% nonfat milk, the membranes were incubated overnight at 4°C with required primary antibodies. The membranes were then washed three times with Tris-buffered saline with Tween-20 (TBST) for 10 min and were probed with the horseradish peroxidase (HRP)-

conjugated goat anti-mouse IgG antibody at room temperature for 1 h. The membranes were developed using an enhanced chemiluminescence system after washes. The protein levels were normalized to  $\beta$ -actin control.

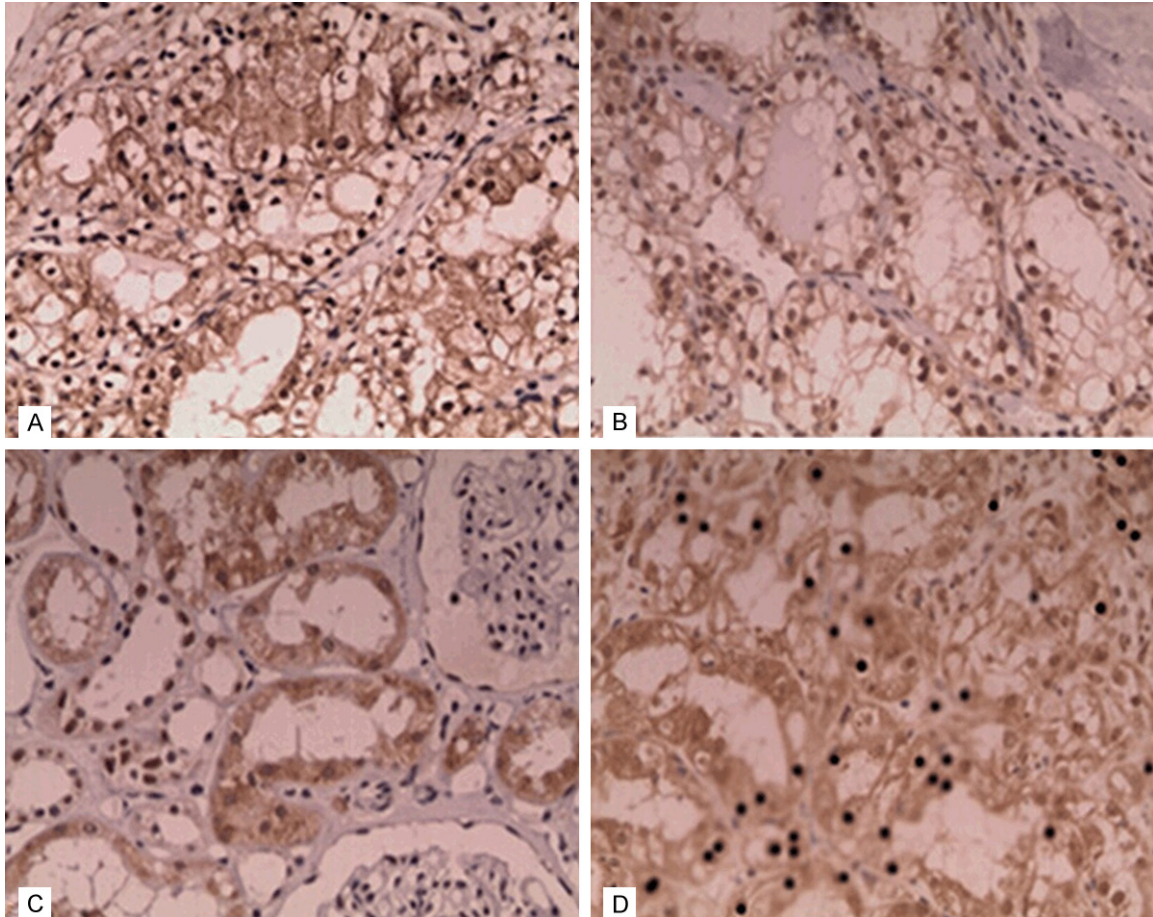
### Establishment of NOD/SCID mouse model

The bone marrow hematopoiesis in NOD/SCID mice was inhibited by intraperitoneal injection of cyclophosphamide (CTX). The peripheral blood mononuclear cells of M5 patients and U937 with MLAA-34-siRNA cells were transplanted by the intraperitoneal injection respectively after 4 consecutive days. The content of IgG was detected by ELISA.

### $\beta$ -catenin and TCF4 pathway

The expression levels of  $\beta$ -catenin and TCF4 were determined by western blotting method. The process was similar with that of the above.

The levels of  $\beta$ -catenin and TCF4 in kidney and spleen tissues were determined by immunohistochemical methods. Briefly, the specimens fixed in 10% formaldehyde were taken out and washed with PBS, they were paraffin-embedded and were sliced at thickness of 4  $\mu$ m. Following deparaffinization, dehydration, and antigen retrieval, the sections were blocked with



**Figure 7.** The immunohistochemical results of the levels of  $\beta$ -catenin and TCF4 in kidney tissues. The levels of  $\beta$ -catenin and TCF4 in kidney and spleen tissues in MLAA-34-siRNA group were lower than that of control group. A:  $\beta$ -catenin in MLAA-34-siRNA group; B:  $\beta$ -catenin in control group; C: TCF4 in MLAA-34-siRNA group; D: TCF4 in control group.

5% BSA and incubated at 37°C for 20 min, and then they were incubated with required antibodies at 4°C overnight. After that, they were washed with PBS and incubated at 37°C for 2 h after drop-adding the 2nd antibody and washed with PBS. After treated with the DAB solution, they were flushed completely, counterstained with hematoxylin and washed with water, treated with dehydration and transparency, then mounted on slides and observed under microscope.

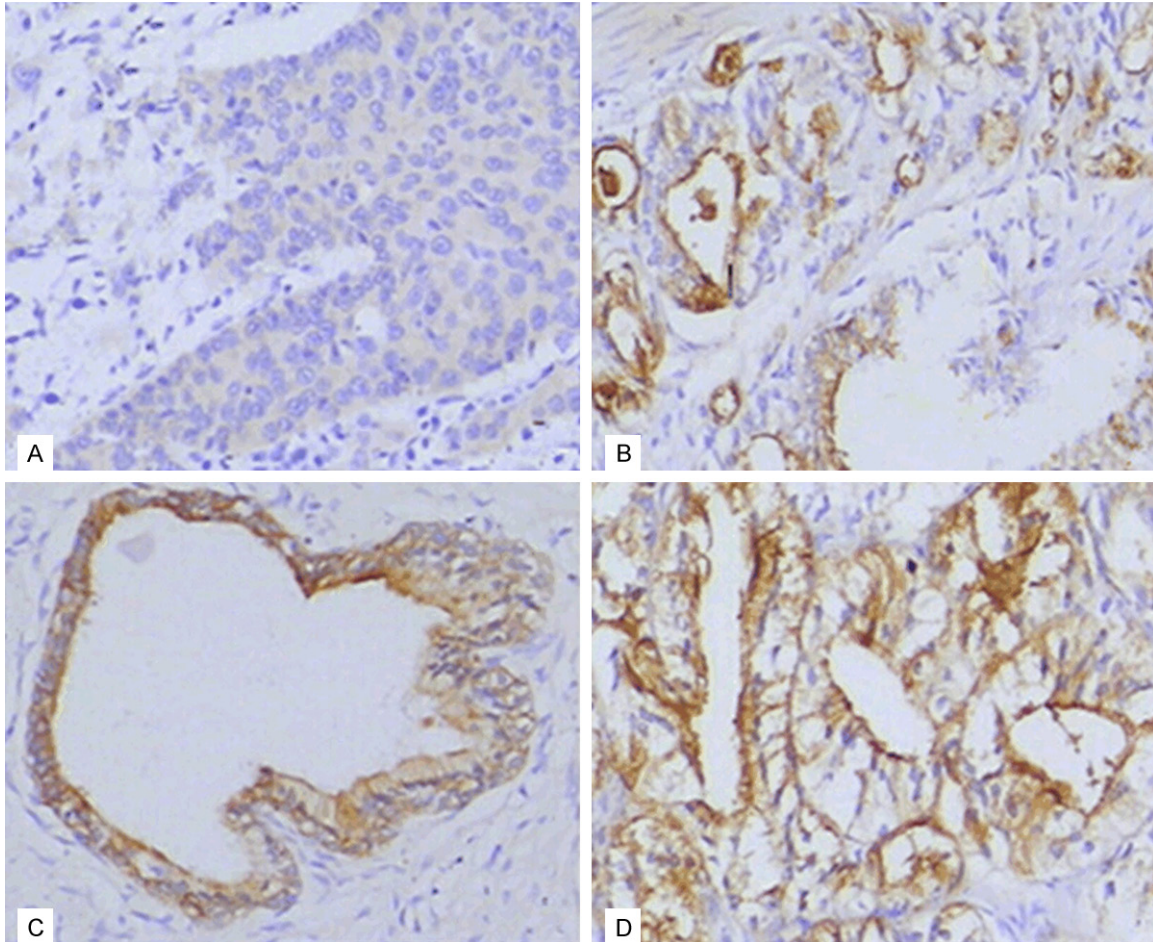
#### Statistical analysis

The results are expressed as mean  $\pm$  SD, and the Mann-Whitney U test was used to evaluate the differences between groups. A value of  $P < 0.05$  and  $P < 0.01$  was taken to denote statistical significance.

## Results

### Effects of MLAA-34 siRNA on U937 cells

The RT-PCR results of MLAA-34 siRNA in U937 cells were shown in **Figure 1**. The expression of MLAA-34 gene in U937 cells was inhibited after siRNA transfection. The levels in MLAA-34-siRNA group were lower than that of control group ( $P < 0.05$ ). These suggested that MLAA-34 siRNA was established successfully. The effects of MLAA-34 siRNA on U937 cell proliferation were shown in **Figure 2**. We found that the proliferation rate of U937 cells in MLAA-34-siRNA group was inhibited after siRNA transfection ( $P < 0.05$ ). It suggested that there was significant correlation between MLAA-34 gene and U937 cell proliferation. The **Figure 3** showed the effects of MLAA-34 siRNA on U937 cells cycle. It showed that G1 and G2/M phase



**Figure 8.** The immunohistochemical results of the levels of  $\beta$ -catenin and TCF4 in spleen tissues. The levels of  $\beta$ -catenin and TCF4 in kidney and spleen tissues in MLAA-34-siRNA group were lower than that of control group. A:  $\beta$ -catenin in MLAA-34-siRNA group; B:  $\beta$ -catenin in control group; C: TCF4 in MLAA-34-siRNA group; D: TCF4 in control group.

of U937 cells increased significantly while S phase of U937 cells decreased significantly in MLAA-34-siRNA group. It suggested that MLAA-34 gene was significantly related with U937 cell cycle distribution. The results of effects of MLAA-34 siRNA on cell apoptosis were shown in **Figure 4**. Apoptotic analysis showed a pronounced induction increase of apoptosis cell death by MLAA-34 silencing in U937 cells. The apoptosis rates of U937 cells with siRNA infection were higher than that of control group. The differences were statistically significant ( $P < 0.05$ ). It suggested that MLAA-34 gene was significantly related with cell apoptosis. After siRNA virus infection, the cell colony numbers of U937 cells in MLAA-34-siRNA group significantly decreased, which suggested that MLAA-34 gene was significantly related to the clone formation ability of U937 cells.

#### *MLAA5 inhibited $\beta$ -catenin and TCF4*

We used K562, HL60 and U937 cells to test the inhibition effect of MLAA5 on  $\beta$ -catenin and TCF4. The results were shown in **Figure 5**. The levels of  $\beta$ -catenin and TCF4 was significant lower in 1  $\mu$ M of MLAA5 group than that of control group, there was significant difference in the levels of  $\beta$ -catenin and TCF4 among different concentration of MLAA5 group. It suggested that MLAA5 could inhibit the expression of  $\beta$ -catenin and TCF4 in different cells with dose-dependent.

#### *Detection of IgG in NOD/SCID mouse*

The content of IgG in U937 with MLAA-34-siRNA cells group after injection for 4, 8 and 12 weeks was 390, 1100 and 1040  $\mu$ g/ml respec-

tively, while it was less than 31.8  $\mu\text{g}/\text{ml}$  in mononuclear cells of M5 patients group.

### *MLAA-34-siRNA enhanced the $\beta$ -catenin and TCF4*

Western blotting results the  $\beta$ -catenin and TCF4 were shown in **Figure 6**. We found that there were significant differences in the expression levels of  $\beta$ -catenin and TCF4 between mononuclear cells of M5 patients and U937 with MLAA-34-siRNA cells group. It suggested that MLAA-34-siRNA increased the  $\beta$ -catenin and TCF4. The immunohistochemical results of the levels of  $\beta$ -catenin and TCF4 in kidney and spleen tissues were shown in **Figures 7 and 8** respectively. They showed that the levels of  $\beta$ -catenin and TCF4 in kidney and spleen tissues in MLAA-34-siRNA group were higher than that of control group, MLAA-34-siRNA could increase  $\beta$ -catenin and TCF4.

### Discussion

MLAA-34 is a novel M5-associated antigen that plays a role in the apoptosis of U937 cells [7]. In our previous studies, we found that MLAA-34 may be a novel anti-apoptotic factor in vitro [7, 9]. In this study we verified the anti-apoptotic effects in vitro and in vivo. AML was divided into 8 subtypes (M0 to M7) including subtypes from granulocytic- or myeloid-derived progenitors (M0 to M3), from monocytic myeloid-derived progenitors (M4 and M5), and the relatively rare leukemias deriving from megakaryocytic and erythroid progenitors (M6 and M7) [13, 14]. M5 was largely incurable because of its high relapse rates, infiltration and the median remission duration of approximately only six months [7].

Apoptosis is the process of programmed cell death (PCD) that may occur in multicellular organisms. It is the regulated destruction of a cell PCD and characterized by many biological and morphological changes and involved in the cascade of integrating pathways and factors [15]. Previous studies suggested that many types of cancer cells are very prone to undergo apoptosis [16]. Wnt signaling pathway was required for normal embryological development especially in the intestinal epithelium, its signal transduction target genes were implicated in various cancer types and developmental disorders, abnormal Wnt signalling contributed to the patho-

genesis of many cancers [17-19]. TCF factors play a pivotal role in the Wnt signalling pathway in which they may function as transcriptional activators or repressors depending on the availability of  $\beta$ -catenin in the nucleus [20]. The human TCF-4 gene located on the long arm of chromosome 10 (10q25.3) [21]. Over-activation of the canonical  $\beta$ -catenin/TCF4 pathway was one of the most frequent signaling abnormalities in many types of cancer. The stabilization and nuclear translocation of  $\beta$ -catenin played an important in this pathway, it binded to the TCF family and subsequently activated a series of genes that ultimately establish the oncogenic phenotype [22, 23]. In this study we found that MLAA-34-siRNA could inhibit the apoptosis of K562, HL60 and U937 cells, it also significantly increase the levels of  $\beta$ -catenin and TCF4, so MLAA-34 played the anti-apoptotic effect maybe through  $\beta$ -catenin and TCF4 pathway.

### Conclusions

In a word, MLAA-34 played the anti-apoptotic effect in AML in vitro and in vivo, the mechanism was partly associated with  $\beta$ -catenin and TCF4 pathway. The findings will lead to a better understanding of the pathogenic mechanism in M5, MLAA-34 could serve as a potential biomarker for the early diagnosis and gene therapy of M5.

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### Disclosure of conflict of interest

None.

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