CLINICAL RESEARCH ARTICLE

CD82 supports survival of childhood acute myeloid leukemia cells via activation of Wnt/ β -catenin signaling pathway

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BACKGROUND: Stem cell marker CD82 plays a vital role in the oncogenesis and progression of acute myelogenous leukemia (AML), especially in sharing properties of leukemia stem cells (LSCs). The Wnt/ β -catenin pathway is required for the development of LSCs in AML. The present study aimed to validate whether CD82 supports the survival of LSCs in pediatric AML via activation of Wnt/ β -catenin signaling pathway.

METHODS: CD82 expression and its correlation with molecules downstream of Wnt/ β -catenin pathway in samples from pediatric AML patients were analyzed. Forced or downregulated expression of CD82 in AML cells was evaluated for the effects of CD82 on cell proliferation, cycle regulation, apoptosis, and adriamycin chemoresistance and to validate the underlying mechanism.

RESULT: Aberrant expression of CD82 in pediatric AML patients was found. CD82 messenger RNA expression correlated positively with downstream molecules of Wnt/ β -catenin pathway in AML children. Knockdown of CD82 induced apoptosis, suppressed growth, and decreased adriamycin chemoresistance in AML cells. CD82 accelerated β -catenin nuclear location and then stimulated the expression of downstream molecules of Wnt/ β -catenin pathway.

CONCLUSION: CD82 regulates the proliferation and chemotherapy resistance of AML cells via activation of the Wnt/ β -catenin pathway, which suggest that the CD82 may be a potential therapeutic target in AML children.

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INTRODUCTION

Acute myelogenous leukemia (AML), originating from hematopoietic stem/progenitor cells, is characterized by increased immature myeloid blasts within the bone marrow (BM) that account for approximately 20% acute leukemia in children and adolescents.¹ With an increasing number of chemotherapy drugs being made available, AML still remains a highly fatal disease due to its significant relapse rate after standard treatment.² The high relapse rate in AML is thought to be due to the lack of eradication of the leukemia stem cells (LSCs)—a subset of cells that are capable of self-renewable and extensive proliferation. LSCs have been studied as potential therapeutic targets,^{3,4} and of particular interest to researchers are the signaling pathways that control the development and survival of LSCs.

CD82/KAI1, initially identified as a metastasis suppressor of some solid malignant tumors,^{5,6} could only suppress the formation of metastatic lesions without affecting the growth of primary tumors. In hematopoietic malignancies, it was reported that CD82 is highly expressed in leukemia cells from patients with human leukemias, including AML.⁷ In the previous study,⁸ it has been found that CD82 was highly expressed in pediatric patients with ALL. In a methylcellulose medium, CD82 levels are correlated with the colony-forming abilities in CD34+/CD38– AML cells⁹ whose cells fulfilled the criteria for LSCs in vivo.^{10,11} Recently, CD82 upregulation has been identified in chemotherapy-resistant CD34+/CD38– AML cells,¹² which are

often responsible for disease relapse. A previous study found that CD82 regulated the hematopoietic stem and progenitor cells' BM maintenance, homing, and engraftment.¹³ The above evidences conformed that CD82 is important to maintain the character of LSCs as a biomarker for LSC in AML.

The Wnt/ β -catenin is active in chronic myeloid leukemia (CML)¹¹ and AML.^{14,15} Deregulation of canonical or noncanonical Wnt signaling pathway plays critical roles in the pathogenesis of various cancers including AML.¹⁶ While β -catenin is not required for self-renewal of adult HSC,^{17,18} Wang et al.¹⁹ show that the Wnt/ β -catenin signaling pathway is required for self-renewal of LSCs, which are derived from either hematopoietic stem cells (HSCs) induced by co-expression of the Hoxa9 and Meis1a oncogenes or more differentiated granulocyte macrophage progenitors induced by the fusion oncoprotein MLL-AF9. All the evidences above confirmed that the Wnt/ β -catenin pathway is required for the development of LSCs in AML.

CD82 has been found to promote the adhesion and colony-forming ability of AML cells by several signaling pathways, including STAT5/IL-10²⁰ and P38-MAPK.²¹ Since the Wnt/ β -catenin signaling pathway plays an important role in the self-renewal and proliferation of LSC,^{22–24} it is hypothesized that it might also be involved in the CD82 regulating survival of AML LSC cells. The present study demonstrated the essential role of Wnt/ β -catenin signaling pathway CD82 in regulating the proliferation and chemotherapy resistance of AML cells.

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MATERIALS AND METHODS

Patients and samples

A total of 31 untreated pediatric patients with de novo AML were enrolled in the present study. All patients were diagnosed according to the World Health Organization Classification. BM samples have been aspirated before treatment. A total of 25 hematologically normal age-matched BM samples were obtained from patients by a BM aspiration as a part of routine investigation for non-malignant hematological disease (such as idiopathic thrombocytopenia) or lymphoma for staging (proven to be uninvolved on BM biopsy). All of the samples were collected at the Department of Pediatrics, Shandong Provincial Hospital Affiliated to Shandong University (Jinan, China). Written informed consent was obtained from parents on behalf of the children enrolled. The present study was approved by the Medical Ethical Committee of Shandong Provincial Hospital Affiliated to Shandong University.

Cell culture and reagents

Human AML cell lines THP1 and kasumi cell line purchased from Chinese Academy of Medical Sciences (Tianjin, China) were cultured in RPMI-1640 medium (Gibco, CA, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin in an incubator maintained at 37 °C in an atmosphere containing 5% CO₂.

Lentiviral particles production and stable transfection

CD82 overexpression (CD82OE) lentiviral particles and CD82-shRNA (short hairpin RNA) lentiviral vector and control plasmid were purchased from Genechem (Shanghai, China). A total of 1×10^4 /well cells were seeded in 24-well culture plates and transfected with lentivirus. After 48 h incubation, 2 µg/mL puromycin (Sigma-Aldrich, St. Louis, MO, USA) was added for 3–4 days to select the cells that were transfected stably by the lentivirus. Reverse transcription-polymerase chain reaction (RT-PCR) and western blot were used to detect the efficiency of transfection.

Cell viability assay

Cells transfected with virus or control cells (5000/well) were seeded into 96-well culture plates and were cultured in 37 °C for 0, 24, 48, and 72 h. At the time of measurement, cells were incubated with 10 µL of CCK8 (Dojindo, Tokyo, Japan) at 37 °C for 4 h. The absorbance was determined at a test wavelength of 450 nm, and 650 nm was used as the reference wavelength. Cell viability was expressed by the relative optical density (OD) value in treated samples as compared with control cells after correction for background absorbance.

Apoptosis assay

The apoptosis analysis was performed according to the manufacturer's protocol using the Annexin V/propidium iodide (PI) Apoptosis Detection Kit (BestBio, Shanghai, China). In brief, 3×10^5 cells were resuspended in 500 µL of binding buffer, stained with 5 µL Annexin V in the dark for 15 min at 4 °C, and were incubated for another 5 min after 10 µL PI being added. The percentage of apoptotic cells was determined by using FACS flow cytometer equipped with the Cell Quest software (Beckman Coulter, CA, USA).

Cell cycle assay

Cells were collected for cell cycle analysis. Cells were fixed in 75% alcohol overnight at 4 °C, resuspended with phosphate-buffered saline (PBS) containing 0.2 mg/mL RNase A, and incubated at 37 °C for 30 min. Cells were incubated in the dark for another 30 min at 4 °C after adding PI solution (BestBio, Shanghai, China). DNA content was then determined by using a FACS Calibur flow cytometer (Beckman Coulter, CA, USA).

Table 1. Primers used for qRT-PCR in human samples

Gene primer sequence		
CD82	Forward	5'-TGT CCT GCA AAC CTC CTC CA-3'
	Reverse	5'-CCA TGA GCA TAG TGA CTG CCC-3'
β-Catenin	Forward	5'-CTC TGG TGG ATA TGG CCA GGA-3'
	Reverse	5'-CAG ATC TGG CAG CCC ATG AA-3'
c-myc	Forward	5'-TAC AAC ACC CGA GCA AGG AC-3'
	Reverse	5'-GAG GCT GCT GGT TTT CCA CT-3'
CyclinD1	Forward	5'-TGA GGG ACG CTT TGT CTG TC-3'
	Reverse	5'-GCC TTT GGC CTC TCG ATA CA-3'
Survivin	Forward	5'-TCCACTGCCCACTGAGAAC-3'
	Reverse	5'-TGGCTCCAGCCTTCCA-3'
β-Actin	Forward	5'-CCT TCC TGG GCA TGG AGT CCT G-3'
	Reverse	5'-GGA GCA ATG ATC TTG ATC TTC-3'
qRT-PCR quantitative reverse transcription-PCR		

Reverse transcription-polymerase chain reaction

The total RNA was obtained from cells and isolated by Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For the reverse transcription reaction, Prime Script RT Reagent Kit (Takara Bio Inc., Dalian, China) was used according to the manufacturer's instruction. Reverse transcription reaction was done at 37 °C for 15 min, followed by 85 °C for 5 s. Real-time quantitative PCR was conducted by using an ABI Prism 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA) by following the manufacturer's instructions. In the final 10 µL reaction volume, the real-time PCR contained 5 µL of 2 × SYBR Green Real-Time PCR Master Mix, 3.2 µL of ddH₂O, 1 µL of complementary DNA sample, and 0.4 µL of the forward and reverse primers. All quantitative reverse transcription-PCR (qRT-PCR) were conducted on the Roche LightCycler[®] 480 PCR system (Roche) and performed in triplicates. The primers used are shown in Table 1. The results were expressed relatively according to the number of β-actin transcripts used as an internal control. Relative gene expression level (the amount of target, normalized to endogenous control gene) was calculated by using the comparative Ct method formula $2^{-\Delta Ct}$.

Western blotting

Cells were lysed according to the manufacturer's instruction. To be specific, cells were washed twice with cold PBS, and lysed in cell lysis buffer for Western blotting on ice for 30 min. The protein concentration was qualified by the Bicinchoninic Acid Assay Protein Reagent Kit (Novoprotein, Shanghai, China) according to a standardized curve. Equal amounts of cell extract (50 µg) were subjected to electrophoresis in sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% nonfat milk in PBS/0.1% Tween-20 and then incubated with appropriate primary antibodies overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G (IgG) at room temperature for 1 h. After washing, immunoreactive bands were detected by ECL (Millipore, Billerica, MA, USA). Western blot were visualizes by FluorChem E Chemiluminescent Western Blot Imaging System (Cell Biosciences, Santa Clara, CA, USA). Primary antibodies against CD82 and CD44 were purchased from Abcam (Cambridge, UK). Primary antibodies against c-myc, cyclinD1, and survivin were obtained from Cell Signaling Technology (Beverly, MA, USA). Primary antibody against β-actin was from Sigma-Aldrich (St. Louis, MO, USA). Primary antibody against β-catenin was from Santa Cruz Biotechnology

(Santa Cruz, TX, USA). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG were purchased from ZSGB-BIO (Beijing, China).

Immunofluorescence

Immunofluorescence was performed to identify subcellular localization of β -catenin. Cells were harvested by centrifugation. Drops of cells were plated on polysine-coated slides and incubated at room temperature for 25 min, and then fixed with 4% polyoxymethylene. Cells were permeabilized with 0.5% Triton X-100 in PBS for 15 min, and then blocked with goat serum for 30 min to minimize nonspecific binding of the primary antibody. The β -catenin antibody (Abcam, Cambridge, UK) was applied at 1:250 dilution overnight in 4°C. Images were captured by Zeiss Microscopy LSM780 fluorescent microscope and analyzed with the Image J software.

Statistical analysis

Experiments were performed three times and quantitative data are expressed as mean $\pm$ SD or median (range). The Student's *t* test, χ^2 test, and Wilcoxon's test were carried out to assess the differences between two groups. Analysis of variance was used to assess the differences between three or more groups. The Pearson's or Spearman's correlation test was used for correlation analysis depending on data distribution. SPSS version 19.0 software (SPSS, Chicago, IL, USA) was utilized for statistical analysis. *P* value <0.05 was considered statistically significant.

RESULTS

CD82 was aberrantly expressed in AML cells of BM from childhood AML

To explore the biological functions of CD82 in childhood AML, we first examine its expression patterns in newly diagnosed AML children. Messenger RNA (mRNA) of AML cells from BM was extracted according to the manufacturer's instructions, and the expressions were directed by RT-qPCR. It is found that CD82 mRNA expression in AML patients (median, 0.007785; range,

0.0008193–0.02225, *n* = 31) was obviously elevated compared with the controls (median, 0.001994; range, 0.0001789–0.01566, *n* = 25) (Fig. 1).

Clinical significance of CD82 expression was discovered in childhood AML

We have also collected the clinical data of the same patients and conducted statistical analysis to evaluate the clinical significance of CD82. The results demonstrated that CD82 mRNA expression in AML-M2 pediatric patients was significantly higher than other types (*p* = 0.0486). It was found that AML patients with AML/ETO gene mutation has the higher CD82 mRNA expression than patients with other genes mutation (*p* = 0.0586). No association has been found between CD82 expression and other clinical factors, including gender, age, and white blood cells in patients with AML (Table 1).

CD82 was correlated with β -catenin and molecules downstream of Wnt/ β -catenin

We also detected the mRNA expression level of β -catenin and its downstream molecules such as survivin, cyclinD1, and c-myc in

Fig. 1 Messenger RNA (mRNA) expression of CD82 in the bone marrow of pediatric acute myelogenous leukemia (AML) patients and health controls. ****p* <0.001

Fig. 2 **a** Positive correlations were found between CD82 and β -catenin. **b** Positive correlations were found between CD82 and c-myc. **c** Positive correlations were found between CD82 and cyclinD1. **d** Correlation of CD82 with survivin. **e** Upregulating CD82 expression, and mRNA level of survivin, c-myc, and cyclinD1 were all upregulated. **f** Downregulating CD82 mRNA expression, and the mRNA expression of survivin, c-myc, and cyclinD1 were all downregulated. **p* < 0.05

Table 2. CD82 mRNA expression and clinic pathological characteristics of pediatric patients with newly diagnosed AML

Characteristic	Patients, <i>n</i>	CD82 mRNA relative expression ^a	<i>P</i> value
Sex			
Female	6	0.005826 ± 0.001632	0.1259
Male	9	0.01973 ± 0.01031	
Age			
1–10 years	12	0.01357 ± 0.005359	0.0710
<1 year or >10 years	3	0.002651 ± 0.002651	
WBCs (× 10 ⁹)			
<10	4	0.005026 ± 0.001081	0.6885
10–50	5	0.02254 ± 0.01223	
>50	6	0.006344 ± 0.002164	
FAB of AML			
M2	6	0.02182 ± 0.0099	0.0486
Other	9	0.00443 ± 0.0008	
M3	2		
M4	3		
M5	3		
M7	1		
Fusion gene			
AML/ETO	6	0.02301 ± 0.01203	0.0586
Other	9	0.005577 ± 0.0014	
MLL/AF9	2		
PML/RARα	2		
MLL/AF6	1		
CBFB/MYH11	1		
EV11	1		
Negative	2		

AML acute myelogenous leukemia, mRNA messenger RNA, FAB French-American-British, MLL mixed-lineage leukemia, PML promyelocytic leukemia, RARα retinoic acid receptor-α, CD cluster of differentiation, WBC white blood cell

^aData are presented as mean plus or minus standard deviation

newly diagnosed AML children. The present study has found positive correlations between CD82 mRNA expression with β -catenin ($r = 0.5151$, $p = 0.0018$), c -myc ($r = 0.6305$, $p < 0.001$), cyclinD1 ($r = 0.4032$, $p = 0.0411$) (Fig. 2a–c). However, the correlation between CD82 mRNA expression with survivin was not obviously significant (Fig. 2d). These observations led to the hypothesis that CD82 regulates both the expression of β -catenin and its downstream molecules in AML cells. To verify the above hypothesis, additional experiments were performed in AML cell line THP1, and cells that were transfected with CD82-expressing lentiviral particles (CD82OE) expressed increased β -catenin, c -myc, cyclinD1, and survivin by a factor, compared with the control cells (Fig. 2e). Meanwhile, the expression of all these genes decreased following shRNA-mediated downregulation of CD82 (CD82DR) (Fig. 2f). Further observations support the hypothesis that CD82 functions in regulating both the β -catenin and its downstream molecules in AML cells (Table 2).

CD82 accelerated the proliferation of AML cells
Then, we focused on the roles of CD82 on the biological function in AML cells. It is supposed that CD82 was crucial in preventing the apoptosis of AML cells or promoting the proliferation of AML cells, thus the expression level of CD82 in AML cells might influence the

growth of AML cells in all probabilities. To verify this hypothesis, AML cells with CD82-expressing lentiviral particles were transduced to make CD82 overexpression (CD82OE) or shRNA-mediated CD82DR in AML cells. Having been evaluated by fluorescence microscope, both transfection efficiencies were more than 90%. Both Real-time RT-PCR and western blot showed that CD82 mRNA and protein level in CD82OE THP1 cells were obviously elevated, while cells in CD82DR THP1 decreased (Fig. 5a, b). CD82OE has accelerated cell growth of THP1 cells compared with negative control by CCK8 analyses (Fig. 3a). Inversely, CD82DR suppressed cell growth of THP1 cells compared with the negative control (Fig. 3b). Accordingly, the underlying mechanism of CD82 that accelerates the proliferation of AML cells was explored by detecting the cell cycle and apoptosis with FACS. As shown in Fig. 3e, upregulation of CD82 significantly increased the ratio of cells undergoing the S phase. These results provided evidence that CD82 promoted proliferation of THP1 by mediating G0/G1 arrest of AML cells. We also found that CD82 inhibited apoptosis of THP1 induced by adriamycin. CD82OE and control cells were treated with 100 μ g/L adriamycin for 48 h. The cell apoptosis was detected by Annexin-V-APC/7AAD (7-aminoactinomycin D) double staining. According to the flow cytometry results, the percentage of apoptotic cells of CD82OE THP1 was 7.83%, which was markedly less than the control THP1 cell (20.41%) (Fig. 4). Furthermore, when the concentration of adriamycin was doubled, the difference of percentage of apoptotic cells between CD82OE THP1 cells with control THP1 cells was even more pronounced (Fig. 4).

CD82 promoted the AML cells resistant to adriamycin

To verify whether CD82 was associated with resistance to chemotherapeutic drugs, the expression of CD82 in K562 and its imatinib-resistant cells line KA have been detected. CD82 mRNA expression was obviously elevated in KA cells (Fig. 3f). Thus, CD82 was hypothesized to be associated with AML cells' resistance to chemotherapeutic drugs. To verify this hypothesis, we detected the IC₅₀ (drug concentration leads to 50% decrease of cell viability) values of adriamycin of forced expression of CD82, downregulation expression of CD82, and the control THP1 cells. Forced expression of CD82 was found to be able to weaken the inhibitory effect of adriamycin against THP1 cells, with the IC₅₀ values of overexpression of CD82 and control as 98.67 and 113.2 μ g/L, respectively (Fig. 3c). While the downregulated expression of CD82 promoted the inhibitory effect of adriamycin against THP1, the IC₅₀ values of CD82DR and control were 137.8 and 75.34 μ g/L, respectively (Fig. 3d).

CD82 was involved in Wnt/ β -catenin signaling pathway and accelerated its activation

The molecular mechanism of CD82 in AML progression has been investigated as well. Because of the correlation of CD82 with β -catenin and its downstream molecules, it was supposed that CD82 influences the proliferation of AML cells through Wnt/ β -catenin signaling pathway. First, the activity of Wnt signaling following up- and down-regulation of CD82 in AML cells has been detected. It was found that total β -catenin protein level increased significantly in CD82OE THP1 cells and decreased in CD82 DR THP1 cells. Western blot analysis of subcellular fractions confirmed that β -catenin increased in the nucleus of CD82OE THP1 cells (Fig. 5d). To make the localization of β -catenin clear, the subcellular localization of β -catenin has been investigated by immunofluorescence.

β -Catenin was found primarily in the nucleus and slightly in the cytoplasm of CD82OE THP1 cells (Fig. 6). β -Catenins translocating and accumulating in the nucleus results in the transcription of its downstream genes. We then assessed whether expression of the Wnt/ β -catenin signaling pathway target genes could be impaired by CD82 up- or down-regulation. RT-PCR revealed that the mRNA

Fig. 3 **a** Proliferation of THP1-CD82OE and control cells were assessed by CCK8 assays, and proliferation rates at 24, 48, and 72 h were calculated compared to the absorbance at 0 h. **b** Proliferation of THP1-CD82DR and control cells. **c** Half-maximal inhibitory concentration (IC_{50}) values of THP1-CD82OE and control cells were calculated according to cell growth inhibition after adriamycin (ADM) treatment (100 μg/L, 48 h). * $p < 0.05$, ** $p < 0.01$. **d** IC_{50} values of THP1-CD82DR and control cells. **f** Upregulation of CD82 significantly increased the ratio of cells undergoing the S phase

Fig. 4 Apoptotic cells were detected by Annexin V and 7-aminoactinomycin D (7AAD) staining using flow cytometry after adriamycin (ADM) treatment. Representative dot plots and graphs are shown * $p < 0.05$

expression of c-myc, cyclinD1, and survivin increased significantly when CD82 was overexpressed in THP1 and downregulated; the mRNA level of c-myc, cyclinD1, and survivin decreased obviously. We further detected the protein level of the Wnt/β-catenin signaling pathway target genes by western blot. Results revealed that the protein level of β-catenin and cyclinD1 decreased when CD82 was downregulated in THP1 cells. However, the protein level of c-myc and cyclinD1 were changed diametrically when CD82 was up- or down-regulated compared with negative controls (Fig. 4e, f).

DISCUSSION

Approximately 20% of childhood leukemias are of myeloid origin and they represent a spectrum of hematopoietic malignancies. It is initiated and maintained by a subset of self-renewing LSCs, which are quantifiably associated with worse clinical outcomes, and often persist after conventional chemotherapy resulting in relapse^{25,26}

In the present study, we provide new insights into how tetraspanin CD82 support the survival of LSCs that control signal

transduction in childhood AML. As Wnt/β-catenin is a critical signaling hub for controlling AML cell proliferation and survival, we focused on identifying the properties of tetraspanin CD82 that contributes to aberrant Wnt/β-catenin signaling.

CD82 is highly expressed in leukemia cells from patients with AML, CML, and chronic lymphocytic leukemia.⁷ In our previous study, frequencies of CD34+CD82+ cells in childhood ALL were higher than those in controls.⁸ Furthermore, aberrant expression of CD82 in CD34+CD38- LSCs was detected by comparing the protein expression profile of CD34+/CD38- LSCs freshly isolated from individuals with AML to that of their CD34+/CD38+ counterparts.⁹ Importantly, it was revealed that CD82DR in CD34+CD38- AML cells could inhibit the colony-forming ability of these cells. Aberrant constitutive activation of β-catenin is linked to the initiation and progression of AML and other cancers.²⁷ β-Catenin is required for LSC development and maintenance in AML, but not necessary for the development of adult HSCs. We focused on CD82 because it functions as an activated molecule of β-catenin that is important to maintain the character of LSCs. Our experiments utilizing RT-qPCR confirmed aberrant mRNA expression of CD82, β-catenin, and its downstream molecules in freshly

Fig. 5 **a** THP1 cells were infected with lentiviruses harboring CD82 (CD82OE), shRNA-CD82 (CD82DR), or negative control (control), and CD82 expression was determined by quantitative reverse transcription-PCR (qRT-PCR) and western blot. **b** THP1 cells were infected with lentiviruses harboring shRNA-CD82 (CD82DR) or negative control (control), and CD82 expression was determined by qRT-PCR and western blot. CD82 levels were normalized to the expression of β-actin. ** $p < 0.01$. **c–f** Expression of protein level were measured by western blot

Fig. 6 Subcellular localization of β-catenin measured by immunofluorescence

isolated AML cells of BM from newly diagnosed AML children. The results indicated that CD82 was overexpressed in samples isolated from AML children. A novel function of CD82 in AML cells of BM from patients has also been examined, and CD82 was found positively correlated with the expression of β-catenin, probably via activation of Wnt/β-catenin signaling, which results in the activation of β-catenin's downstream molecule genes, such as *c-myc*, *cyclinD1*, and *survivin*. It suggests the possibility that CD82 might regulate the survival of AML cells by Wnt/β-catenin signaling pathway. The clinical relevance of CD82 was also analyzed. Interestingly, CD82 expression correlated with AML-M2 and AML/ETO mutation. However, the number of samples in the present study was limited. Additional larger prospective studies are required to verify the present observations.

Inhibiting Wnt signaling pathway by CD82 was originally reported to be involved in cancer metastasis in solid tumors. This pathway has been extensively studied in tumorigenesis and has been shown to be involved in the development of several types of leukemia.^{22,28} In a recent study, Wnt signaling pathway has been implicated in the pathogenesis of leukemia through the maintenance of LSC.²⁹ The other studies reported that CD82 modulated adhesion of leukemia cells to the BM microenvironment and survival of AML cells via the STAT5/IL-10 signaling pathway and JAK2/STAT5 signaling.^{9,20,30} However, few studies have focused on CD82 promoting proliferation in childhood AML via Wnt/β-catenin signaling pathway. Our present study explored the regulation of Wnt/β-catenin by CD82 and its impact on the survival of AML cells, and verified the hypotheses in the AML cell

line THP1. It is found that forced expression of CD82 significantly elevated levels of β -catenin mRNA and β -catenin's downstream molecule c-myc, cyclinD1, and survivin mRNA level in AML cells. Meanwhile, CD82DR by shRNA decreased levels of β -catenin in AML cells, and expression of the target genes in downstream of Wnt/ β -catenin involved. The positive correlation was noted between mRNA levels of β -catenin and CD82 in AML cells. Further western blot analysis detected transformation in protein levels of these genes. The protein level of cyclinD1 was overexpressed, while the protein level of c-myc and survivin was underexpressed when CD82 gene was upregulated.

β -Catenin is the central molecule in the canonical Wnt signaling pathway and accumulation of β -catenin in the nucleus has been demonstrated in both AML cell lines and primary blasts, indicating the activation of Wnt/ β -catenin signaling pathway.³¹ In the present study, immunofluorescence and western blot analysis demonstrated that forced expression of CD82 accelerated β -catenin locating more in the nucleus of THP1 cells, and next stimulated the expression of its downstream molecules compared with the control cells. This result suggests that CD82 promoted the activity of Wnt/ β -catenin by accelerating β -catenin locating more in the nucleus and then stimulating the expression of cyclinD1, which was responsible for the cell cycle arrest and promoting cells get into the proliferative phase. CD82 may regulate c-myc and survivin expression on the transcriptional level.

Relapse and drug resistance are still difficult to avoid in AML children, and LSCs are considered one of the most important contributors. CD82 upregulation was identified in chemotherapy-resistant CD34⁺/CD38[−] AML cells, which are often the cells responsible for disease relapse. The use of blocking antibody against CD82 in combination with cytarabine significantly prolonged survival of immune-deficient mice-bearing human AML cells than did treatment with cytarabine alone.³² Blockade of CD82 could mobilize leukemic blasts into the peripheral circulation and potentiate the cytotoxic effects of anti-leukemic agents. This provided evidence to prove that CD82 correlates with the resistant of chemotherapy of AML cells. In this study, we transduced AML cells with CD82-expressing lentiviral particles to make CD82OE or shRNA-mediated CD82DR in AML cells. We found that forced CD82 expression accelerated cell growth of THP1 cells by the cell viability assay. Inversely, downregulated expression of CD82 suppressed cell growth of THP1 cells. Further cell cycle and apoptosis experiments revealed the mechanism of CD82 accelerating the proliferation of AML cells. Upregulation of CD82 significantly increased the ratio of cells undergoing proliferative phase. In CD82OE THP1 cells, both the mRNA and protein level of cyclinD1, which was responsible for the cell cycle arrest and promoting cells get into the proliferative phase, were elevated. These results provided evidences that CD82 promoted proliferation of AML cells.

We also found that CD82 inhibited apoptosis of AML cells. THP1 cells transfected with CD82OE lentiviral particles were treated with adriamycin in different concentrations. The cells' apoptosis were detected. Moreover, the percentage of apoptotic cells of CD82OE THP1 was markedly less than the controls. Following the increased concentration of adriamycin, the percentage of apoptotic cells of CD82OE THP1 cells were significantly less than the controls. This experiment demonstrates that forced CD82 inhibited apoptosis of AML cells. This result suggests that CD82, as a marker of LSC, is involved in chemotherapy resistance of AML. The specific mechanism is not very clear now, and works are going on at present.

Knowledge uncovered by studying pediatric AML may provide insights to improve treatment and outcome of childhood malignancies in general. We hope that our success in treating AML will someday rival that achieved for acute lymphoblastic leukemia.

CONCLUSIONS

In conclusion, this study demonstrated that CD82 regulates the proliferation and chemotherapy resistance of AML cells, and the underlying mechanism is related to activation of the Wnt/ β -catenin pathway. These observations suggest that the CD82 may be a potential therapeutic target for attenuating aberrant signal transduction in AML children.

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AUTHOR CONTRIBUTIONS

Contributions to conception and design, P.Z. and H.J. Acquisition and analysis of data: H.J., L.C., and Y.X. Drafting and revising the article, H.J., S.L., and J.D. Final approval of the version to be published, P.Z. and Y.W.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

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REFERENCES

- Rubnitz, J. E. How I treat pediatric acute myeloid leukemia. *Blood* **119**, 5980–5988 (2012).
- Hasle, H. & Kaspers, G. J. Strategies for reducing the treatment-related physical burden of childhood acute myeloid leukaemia—a review. *Br. J. Haematol.* **176**, 168–178 (2017).
- Dick, J. E. Stem cell concepts renew cancer research. *Blood* **112**, 4793–4807 (2008).
- Yassin M. et al. A novel method for detecting the cellular stemness state in normal and leukemic human hematopoietic cells can predict disease outcome and drug sensitivity. *Leukemia*, 31 (2019). <https://doi.org/10.1038/s41375-019-0386-z>. [Epub ahead of print].
- Zeng, T. D., Zheng, B., Zheng, W. & Chen, C. CD82/KAI1 inhibits invasion and metastasis of esophageal squamous cell carcinoma via TGF- β 1. *Eur. Rev. Med. Pharmacol. Sci.* **22**, 5928–5937 (2018).
- Luan, M., He, X., Huang, X., Zhang, Q. & Ma, K. The peptide mimicking small extracellular loop domain of CD82 inhibits tumor cell migration, adhesion and induces apoptosis by inhibiting integrin mediated signaling. *Biochem. Biophys. Res. Commun.* **503**, 2206–2211 (2018).
- Burchert, A. et al. CD82 (KAI1), a member of the tetraspan family, is expressed on early haemopoietic progenitor cells and up-regulated in distinct human leukaemias. *Br. J. Haematol.* **107**, 494–504 (1999).
- Ji, H. et al. Aberrant expression of CD133 and CD82 in patients with pediatric acute lymphoblastic leukemia and the clinical significance. *Oncol. Lett.* **14**, 5811–5818 (2017).
- Nishioka, C. et al. CD34(+) / CD38(−) acute myelogenous leukemia cells aberrantly express CD82 which regulates adhesion and survival of leukemia stem cells. *Int. J. Cancer* **132**, 2006–2019 (2013).
- Clarke, M. F. et al. Cancer stem cells—perspectives on current status and future directions: AACR Workshop on cancer stem cells. *Cancer Res.* **66**, 9339–9344 (2006).
- Ishikawa, F. et al. Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. *Nat. Biotechnol.* **25**, 1315–1321 (2007).
- Mine, M. et al. miR-203 inhibits frizzled-2 expression via CD82/KAI1 expression in human lung carcinoma cells. *PLoS ONE* **10**, e0131350 (2015).
- Saito-Reis, C. A., Marjon, K. D., Pascetti, E. M., Floren, M. & Gillette, J. M. The tetraspanin CD82 regulates bone marrow homing and engraftment of hematopoietic stem and progenitor cells. *Mol. Biol. Cell* **29**, 2946–2958 (2018).
- Majeti, R. et al. Dysregulated gene expression networks in human acute myelogenous leukemia stem cells. *Proc. Natl. Acad. Sci. USA* **106**, 3396–3401 (2009).
- Moon, J. S. et al. Inhibition of human mesenchymal stem cell proliferation via Wnt signaling activation. *J. Cell Biochem.* **119**, 1670–1678 (2018).
- Tsukimoto, I. et al. Risk-stratified therapy and the intensive use of cytarabine improves the outcome in childhood acute myeloid leukemia: the AML99 trial

- from the Japanese Childhood AML Cooperative Study Group. *J. Clin. Oncol.* **27**, 4007–4013 (2009).
17. Jeannot, G. et al. Long-term, multilineage hematopoiesis occurs in the combined absence of beta-catenin and gamma-catenin. *Blood* **111**, 142–149 (2008).
18. Cobas, M. et al. Beta-catenin is dispensable for hematopoiesis and lymphopoiesis. *J. Exp. Med.* **199**, 221–229 (2004).
19. Wang, Y. et al. The Wnt/beta-catenin pathway is required for the development of leukemia stem cells in AML. *Science* **327**, 1650–1653 (2010).
20. Nishioka, C. et al. CD82 regulates STAT5/IL-10 and supports survival of acute myelogenous leukemia cells. *Int. J. Cancer* **134**, 55–64 (2014).
21. Nishioka, C., Ikezoe, T., Yang, J. & Yokoyama, A. Tetraspanin family member, CD82, regulates expression of EZH2 via Inactivation of p38 MAPK signaling in leukemia cells. *PLoS ONE* **10**, e0125017 (2015).
22. Luis, T. C., Ichii, M., Brugman, M. H., Kincade, P. & Staal, F. J. Wnt signaling strength regulates normal hematopoiesis and its deregulation is involved in leukemia development. *Leukemia* **26**, 414–421 (2012).
23. Seke Etet, P. F. et al. Normal hematopoiesis and hematologic malignancies: role of canonical Wnt signaling pathway and stromal microenvironment. *Biochim. Biophys. Acta* **1835**, 1–10 (2013).
24. Ueno, K., Hirata, H., Hinoda, Y. & Dahiya, R. Frizzled homolog proteins, microRNAs and Wnt signaling in cancer. *Int. J. Cancer* **132**, 1731–1740 (2013).
25. Shlush, L. I. et al. Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. *Nature* **506**, 328–333 (2014).
26. Thomas, D. & Majeti, R. Biology and relevance of human acute myeloid leukemia stem cells. *Blood* **129**, 1577–1585 (2017).
27. Zoller, M. Tetraspanins: push and pull in suppressing and promoting metastasis. *Nat. Rev. Cancer* **9**, 40–55 (2009).
28. Lu, D. et al. Salinomycin inhibits Wnt signaling and selectively induces apoptosis in chronic lymphocytic leukemia cells. *Proc. Natl. Acad. Sci. USA* **108**, 13253–13257 (2011).
29. Ysebaert, L. et al. Expression of beta-catenin by acute myeloid leukemia cells predicts enhanced clonogenic capacities and poor prognosis. *Leukemia* **20**, 1211–1216 (2006).
30. Nishioka, C. et al. The novel function of CD82 and its impact on BCL2L12 via AKT/STAT5 signal pathway in acute myelogenous leukemia cells. *Leukemia* **29**, 2296–2306 (2015).
31. Morgan, R. G. et al. Gamma-catenin is overexpressed in acute myeloid leukemia and promotes the stabilization and nuclear localization of beta-catenin. *Leukemia* **27**, 336–343 (2013).
32. Nishioka, C., Ikezoe, T. & Yokoyama, A. Blockade of CD82 by a monoclonal antibody potentiates anti-leukemia effects of AraC in vivo. *Cancer Med.* **4**, 1426–1431 (2015).