

# The clinical significance of $\gamma$ -catenin in acute myeloid leukemia

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**Abstract:** Dysregulation of  $\gamma$ -catenin may function as an oncogenic factor in various malignancies. We investigated  $\gamma$ -catenin expression in acute myeloid leukemia (AML) and explored its role in the pathogenesis of AML.  $\gamma$ -Catenin was significantly overexpressed in AML patients compared to healthy donors. The  $\gamma$ -catenin expression in AML patients with lower white blood cells ( $<30 \times 10^9/L$ ) was significantly higher than those with higher white blood cells ( $\geq 30 \times 10^9/L$ ). The expression levels of  $\gamma$ -catenin in AML patients with mutated *CEBP $\alpha$*  were significantly higher than those with unmutated *CEBP $\alpha$* . AML patients with lower  $\gamma$ -catenin levels were more likely to achieve complete remission compared with patients who have higher  $\gamma$ -catenin levels. In K562 cells,  $\gamma$ -catenin knockdown suppressed cellular proliferation, while the cellular migration was greatly enhanced. Moreover, knocking down of  $\gamma$ -catenin enhanced the cytotoxicity of decitabine in K562 cells. Our investigation has indicated a potential role of  $\gamma$ -catenin in the pathogenesis of AML.

**Keywords:**  $\gamma$ -catenin, acute myeloid leukemia, AML, decitabine, prognosis, bone marrow, shRNA

## Introduction

Acute myeloid leukemia (AML) consists of a heterogeneous group of diseases. This disease results from abnormal self-renewal and suppressed differentiation of hematopoietic progenitor cells, which leads to replacement of normal marrow elements.<sup>1</sup> It has been well established that various clinical features, including old age, high white blood cells (WBC) count at diagnosis, and past history of chemotherapy, radiotherapy, or other hematological diseases, are associated with unfavorable treatment outcomes for AML patients. Furthermore, according to the current World Health Organization categorization of AML, cytogenetic and molecular analyses play an important role in prognostic stratification for AML patients. Prognosis of cytogenetically normal AML (CN-AML) that stratified into intermediated-risk group can be further potentiated by molecular abnormalities, ie, CN-AML with *NPM1* mutation or isolated *CEBP $\alpha$*  mutation in the absence of internal tandem duplications of *FLT3-ITD* implies low risk. Meanwhile, CN-AML with *FLT3-ITD* mutation in the absence of *NPM1* implies high risk.<sup>2</sup> However, numerous underlying genetic abnormalities as well as unknown prognostic markers of AML remain to be discovered for prospective novel therapeutic strategies for AML.

Both  $\beta$ -catenin and  $\gamma$ -catenin belong to the catenin family.  $\gamma$ -Catenin, also known as plakoglobin, which is a cytoskeletal protein and intracellular signal transduction protein, shares approximately 80% sequence homology with  $\beta$ -catenin.<sup>3</sup> Human  $\gamma$ -catenin gene is located on q12 of chromosome 17, with a protein molecular weight of 82 kDa.<sup>4</sup> Abnormal expression of  $\gamma$ -catenin gene reportedly occurs in a variety

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of hematologic malignancies and numerous solid tumors. However, reports on the roles of  $\gamma$ -catenin in different types of malignancies are varied or even contradictory. Our current study investigated the expression levels of  $\gamma$ -catenin gene in mononuclear cells in bone marrow (BM) from AML patients. The prognostic significance of  $\gamma$ -catenin level in de novo AML patients is also discussed. Finally, we explored the function of  $\gamma$ -catenin in the pathogenesis of AML, with the expectation that  $\gamma$ -catenin can serve as a potential therapeutic target for AML.

## Materials and methods

### Patients and samples

Between May 2008 and August 2012, BM samples were obtained from 71 patients with primary AML without treatment and 16 healthy donors at the First Affiliated Hospital of Nanjing Medical University. Informed consent were provided by all patients or their legally authorized representatives according to the Declaration of Helsinki for Cryopreservation and Medical Research, and all the methods of this study were approved of by the Institutional Review Board of The First Affiliated Hospital of Nanjing Medical University. A total of 16 healthy Chinese volunteers of Han nationality were recruited, who were unrelated residents in Jiangsu Province, People's Republic of China. All the recruited AML patients were aged between 13 and 85 years (median age: 47 years) and consisted of 40 males and 31 females. Diagnosis of AML was based on the morphologic and cytochemical criteria of the French American British classification. Mononuclear cells were isolated by the Ficoll Hypaque density gradient centrifugation method and stored at  $-80^{\circ}\text{C}$  for use. Follow-up data of the patients were provided by the hospital.

### Cytogenetic analyses

Regular cytogenetic analysis was conducted at diagnosis. In accordance with the criteria of the National Comprehensive Cancer Network guidelines of AML (version 1, 2012), all the patients were allocated to three groups according to their cytogenetic risks. Low-risk group: 16 or t (16; 16), t (8; 21), t (15; 17); high-risk group:  $-5/5q-$ ,  $-7/7q-$ , t (6; 9), t (9; 22), inv (3), t (3; 3), 11q23-non t (9; 11) or complex aberrations ( $\geq$  three independent clonal chromosomal abnormalities); and intermediate-risk group: +8, t (9; 11), normal or other nondefined cytogenetics.

### Molecular analyses

Analyses of FLT3/ITD, *NPM1*, *CEBP $\alpha$* , as well as *C-kit* mutations expression, were conducted.<sup>5,6</sup> Fusion genes,

*AML1/ETO* and *PML/RAR $\alpha$* , were assayed by quantitative reverse transcriptase-polymerase chain reaction.

### Quantitative real time-polymerase chain reaction

The expression of  $\gamma$ -catenin mRNA was determined by a quantitative RT-PCR.  $\beta$ -Actin was amplified to normalize the relative levels of  $\gamma$ -catenin. Total RNA was extracted from stored, frozen mononuclear AML cells using TRIZOL reagent. Moloney Murine Leukemia Virus reverse transcriptase was used to synthesize cDNA from total RNA. Each reaction mixture consisted of 2  $\mu\text{L}$  cDNA, 10  $\mu\text{L}$  SYBR Green PCR Master Mix, 1  $\mu\text{L}$   $\gamma$ -catenin primers (5 nmol/mL) or 1  $\mu\text{L}$   $\beta$ -actin primers (5 nmol/mL), and deionized water, making up to a total volume of 20  $\mu\text{L}$ . The sequences of primers were as follows: 5'-TCGCCATCTTCAAGTCGGG-3' (forward primer) and 5'-AGGGGCACCATCTTTTGACAG-3' (reverse primer) for  $\gamma$ -catenin; 5'-AAGCCACCCCACTTCTCTCTAA-3' (forward primer) and 5'-AATGCTATCACCTCCCCTGTGT-3' (reverse primer) for  $\beta$ -actin. The amplification cycling was performed with a denaturation step at  $95^{\circ}\text{C}$  for 10 minutes, then 40 cycles at  $95^{\circ}\text{C}$  for 30 seconds,  $62^{\circ}\text{C}$  for 40 seconds, and  $72^{\circ}\text{C}$  for 45 seconds. Experiments were performed in triplicate. The relative expression level was calculated using the  $2^{-\Delta\Delta\text{CT}}$  method.

### Cell culture

To investigate whether  $\gamma$ -catenin was implicated in AML or not, two cell lines were involved: the acute monocytic leukemia line THP-1 and the human erythroleukemia line K562. THP-1 and K562 were cultured in Dulbecco's Modified Eagle's Medium and Roswell Park Memorial Institute-1640 medium, respectively. All culture media (Thermo Fisher Scientific, Waltham, MA, USA) were supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific). All cells (KeyGEN BioTECH, Nanjing, People's Republic of China) were maintained at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$ /95%  $\text{O}_2$  environment.

### Western blotting

Western blotting was performed according to widely established protocols. The antibodies were as follows: anti- $\gamma$ -catenin (Becton, Dickinson and Company, BD, Oxford, UK), anti-glyceraldehyde-3-phosphate dehydrogenase antibody (Cell Signaling Technology, Danvers, MA, USA), and the secondary horseradish-peroxidase-conjugated antibodies (Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, People's Republic of China).

## Establishment of K562/KD

On the basis of Western blot analysis,  $\gamma$ -catenin protein was found to be highly expressed in K562 cells in comparison to THP-1 cells. Thereby, K562 was selected for further investigation. The parent cell line K562 was transfected with short hairpin RNA (shRNA) to produce a K562/KD cell model with diminished  $\gamma$ -catenin expression. To minimize off-target effects, three types of shRNA plasmids were used for transient transfection into K562 cells (Table 1).

## Cell proliferation and migration analysis

Cell proliferation analysis was conducted with a Cell Counting Kit-8 (CCK-8) kit (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. After establishing the K562-shRNA model, cells were divided into three groups for further study, including shRNA- $\gamma$ -catenin cells (knock-down, K562/KD), shRNA-NC cells (transfection negative control, K562/NC), and normal cells (K562 without transfection, K562/WT). In every group, the cell viability was determined at 0, 12, 24, 36, 48, and 60 hours posttransfection using the CCK-8 reagent. Each assay was run in triplicate and repeated three times.

$$\text{Relative cell viability (\%)} = \frac{\text{OD}_{450} \text{ of K562/KD or K562/NC}}{\text{OD}_{450} \text{ of K562/WT}} \quad (1)$$

$$\text{Inhibitor rate} = \left( 1 - \text{OD}_{450} - \frac{\text{Treated}}{\text{OD}_{450}} - \text{Untreated} \right) \times 100\% \quad (2)$$

Cell migration experiment was conducted with the Transwell kit (Becton, Dickinson and Company) according to the instructions of manufacturer.

## Statistical analysis

The definition of complete remission (CR) followed the recommended criteria,<sup>7</sup> and we defined relapse-free survival (RFS) as the interval from the date of documented CR until relapse or death in CR (failure), or alive in CR at final follow-up (censored). Overall survival (OS) was defined as the interval from the date of initial diagnosis

until death (failure) or alive at final follow-up (censored). Mann–Whitney *U*-test was employed to evaluate quantitative parameters, and  $\chi^2$  test was used for qualitative parameters. Statistical analyses were performed using Student's *t*-test. The cumulative survival rate was calculated by the Kaplan–Meier method, and statistical significance was analyzed by the log-rank test. Multivariate Cox proportional hazard model was adopted to explore the prognostic effect of the various clinical variables. A *P*-value less than 0.05 was considered statistically significant, and all reported *P*-values were two-sided. All the statistical analyses were performed using the Statistical Program for Social Sciences (SPSS Inc., Chicago, IL, USA; 16.0). Graphs were plotted using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

## Results

### The expression of $\gamma$ -catenin in all samples

An overview of the clinical characteristics of 71 patients is summarized in Table 2.  $\gamma$ -Catenin gene transcript levels were examined in BM mononuclear cells from 71 AML patients and 16 healthy individuals by RT-PCR. A statistically significant difference was found in  $\gamma$ -catenin expression levels between these two groups ( $P=0.020$ , Figure 1A). Compared with the healthy donors, AML patients exhibited significantly higher expression levels of  $\gamma$ -catenin.

### Correlation between $\gamma$ -catenin expression and clinical characteristics in AML

We further explored the relationship between  $\gamma$ -catenin expression and the clinical features as well as known prognostic factors in AML patients. We compared the  $\gamma$ -catenin expression level among different subtypes (M0–M6) of AML and found significant overexpression in patients with AML-M2 in comparison to the other subtypes ( $P=0.053$ , Figure 1B). The expression levels of  $\gamma$ -catenin gene in patients with lower WBC ( $<30 \times 10^9/\text{L}$ ) were significantly higher than those with higher WBC ( $\geq 30 \times 10^9/\text{L}$ ;  $P=0.032$ , Figure 1C). In addition, patients with *CEBP $\alpha$*  mutation had higher  $\gamma$ -catenin levels compared to those in the unmutated group ( $P=0.047$ , Figure 1D). In terms of age, sex, cytogenetics, extramedullary presentation (lymphadenectasis and/or hepatosplenomegaly), mutation status of *NPM1* and *FLT3-ITD*, and fusion genes AML1-ETO, no significant correlations with  $\gamma$ -catenin expression levels (all  $P>0.05$ , Figure 2A–H) were established. Because of the limited number of samples tested for PML-RAR $\alpha$ , we were unable

**Table 1** Three types of shRNA plasmids

Target site	Target sequence of shRNA
JUP-home-1307	GCTGAAGATTCTGGTGAATCA
JUP-home-855	GTCCTGTCTATGCCATCACC
JUP-homo-1553	CGCTGTGCGTCTCAACTATGG

**Abbreviation:** shRNA, short hairpin RNA.

**Table 2** Patient characteristics

Characteristic	Value	P-value <sup>a</sup>
Age, years (median, range)	47 (13–85)	0.945
<60, n (%)	50 (70.4)	
≥60, n (%)	21 (29.6)	
Sex, n (%)		0.066
Male	40 (56.3)	
Female	31 (43.7)	
WBC at diagnosis, ×10 <sup>9</sup> /L (median, range)	20.8 (0.7–299.7)	0.032
<30, n (%)	39 (56.5)	
≥30, n (%)	30 (43.5)	
Missing data, n	2	
Hemoglobin at diagnosis, g/L (median, range)	81.0 (11.0–159.0)	0.111
Normal (male 120–160, female 110–150), n (%)	11 (17.7)	
Anemia (male <120, female <110), n (%)	51 (82.3)	
Missing data, n	9	
Platelet at diagnosis, ×10 <sup>9</sup> /L (median, range)	40.0 (2.0–279.0)	0.284
Normal (100–300), n (%)	9 (14.3)	
Thrombocytopenia (<100), n (%)	54 (85.7)	
Missing data, n	8	
BM blasts at diagnosis, % (median, range)	73.6 (20.4–99.0)	
French American British subtype, n (%)		0.292
M0	2 (2.8)	
M1	17 (23.9)	
M2	26 (36.6)	
M3	9 (12.7)	
M4	3 (4.2)	
M5	8 (11.3)	
M6	5 (7.0)	
Unclassified	1 (1.4)	
FLT3-ITD mutation status, mutated +/-total (%)	5/56 (8.9)	0.605
NPM1 mutation status, mutated +/-total (%)	11/52 (21.2)	0.831
CEBPα mutation status, mutated +/-total (%)	13/46 (28.3)	0.047
Cytogenetics, n (%)		0.114
Favorable	13 (21.0)	
Intermediate	44 (70.9)	
Unfavorable	5 (8.1)	
Missing data, n	9	
Extramedullary presentation, n (%)		0.286
Yes	19 (26.8)	
No	52 (73.2)	

**Notes:** <sup>a</sup> $\gamma$ -Catenin mRNA levels were compared among different groups. Mann-Whitney U-test was employed to evaluate quantitative parameters, and  $\chi^2$  test was used for qualitative parameters. Statistical analyses were performed using Student's t-test. All reported P-values were two-sided.

**Abbreviations:** WBC, white blood cells; BM, bone marrow.

to further explore the relationship between  $\gamma$ -catenin expression and PML-RAR $\alpha$ .

## $\gamma$ -Catenin expression level in patients achieving CR

Apart from six patients who received alleviative treatment and nine patients diagnosed as M3, the remaining 56 patients, according to their conditions, received various standard induction regimens, including idarubicin and cytarabine (Ara-C), daunorubicin and cytarabine, mitoxantrone and cytarabine,

aclacimomycin and cytarabine, homoharringtonine plus cytarabine and granulocyte-colony stimulating factor (G-CSF), and decitabine combined with half-dose CAG (cytarabine, aclarubicin, G-CSF). Among the 56 patients, 38 achieved CR after one to two therapy cycles. The rate of CR in the group of low  $\gamma$ -catenin expression was 71.4%, compared with 64.3% in the high-expression group. Fifty-six patients were divided into CR group (n=38) and not CR (n=18) group; the  $\gamma$ -catenin level in CR group was markedly lower than that in the not-CR group ( $P=0.047$ , Figure 3).

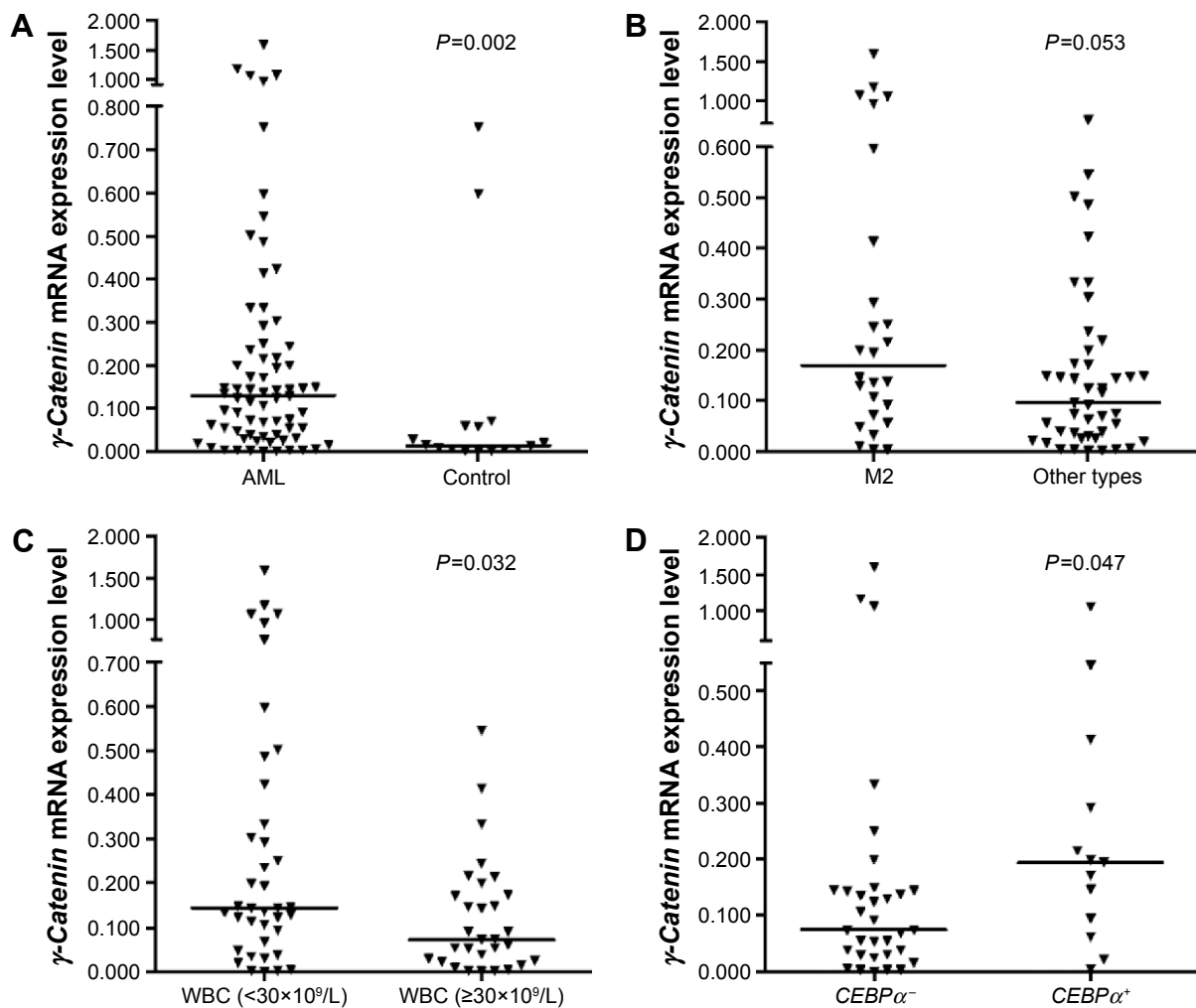
## Correlations between $\gamma$ -catenin expression levels and OS and RFS in AML patients with intermediate-risk cytogenetics

To identify the clinical prognostic effect of  $\gamma$ -catenin, we analyzed the prognosis of 56 patients with non-M3 AML. The median follow-up duration for OS was 12.22 months (ranging from 0.07 to 51.17 months). Between patients with high and low  $\gamma$ -catenin expressions, there was no statistically significant difference in OS and RFS (Figure 4A and B). We further analyzed the prognostic significance of  $\gamma$ -catenin expression level in subgroups with intermediate-risk cytogenetics, and no difference was revealed in OS and RFS (Figure 4C and D). Moreover, the effects of  $\gamma$ -catenin on OS and RFS in patients under 60 years of age and with intermediate-risk cytogenetics still remained unknown (Figure 4E and F).

## Knockdown of $\gamma$ -catenin expression suppressed K562 viability

On the basis of Western blot analysis,  $\gamma$ -catenin protein was highly expressed in K562 cells in comparison to THP-1 cells (Figure 5A). So, K562 was selected for further investigation. The transfection efficiency was determined at 24, 48, and 72 hours posttransfection by fluorescent microscopy (Figure 5B). Consequently, the peak time in transfection efficiency was at 72 hours. The protein expression was further analyzed by Western blotting at 72 hours, showing up to 80%  $\gamma$ -catenin knockdown in K562/KD cells in comparison to K562/NC cells (Figure 5C). K562/KD cells exhibited significantly lower viability than K562/NC cells, indicating that the suppression of  $\gamma$ -catenin expression inhibited cellular viability of K562 cells. Thus,  $\gamma$ -catenin may be involved in cell proliferation of K562 cells ( $P<0.05$ , Figure 6A). Moreover, the cell migration experiment was performed in Transwell to compare the migration abilities among K562/KD, K562/NC, and K562/WT cell groups. K562/KD cells exhibited enhanced migration ability over the other two





**Figure 1**  $\gamma$ -Catenin expression and clinical characteristics.

**Notes:** (A) The level of  $\gamma$ -catenin gene compared between the 71 AML patients and the 16 healthy controls. (B) The level of  $\gamma$ -catenin gene compared between patients with AML-M2 and the others. (C) The expression level of  $\gamma$ -catenin gene in patients with lower WBC ( $<30 \times 10^9/L$ ) and patients with higher WBC ( $\geq 30 \times 10^9/L$ ). (D) The expression level of  $\gamma$ -catenin gene in patients with mutated  $CEBP\alpha$  gene in contrast with patients with nonmutated  $CEBP\alpha$  gene.

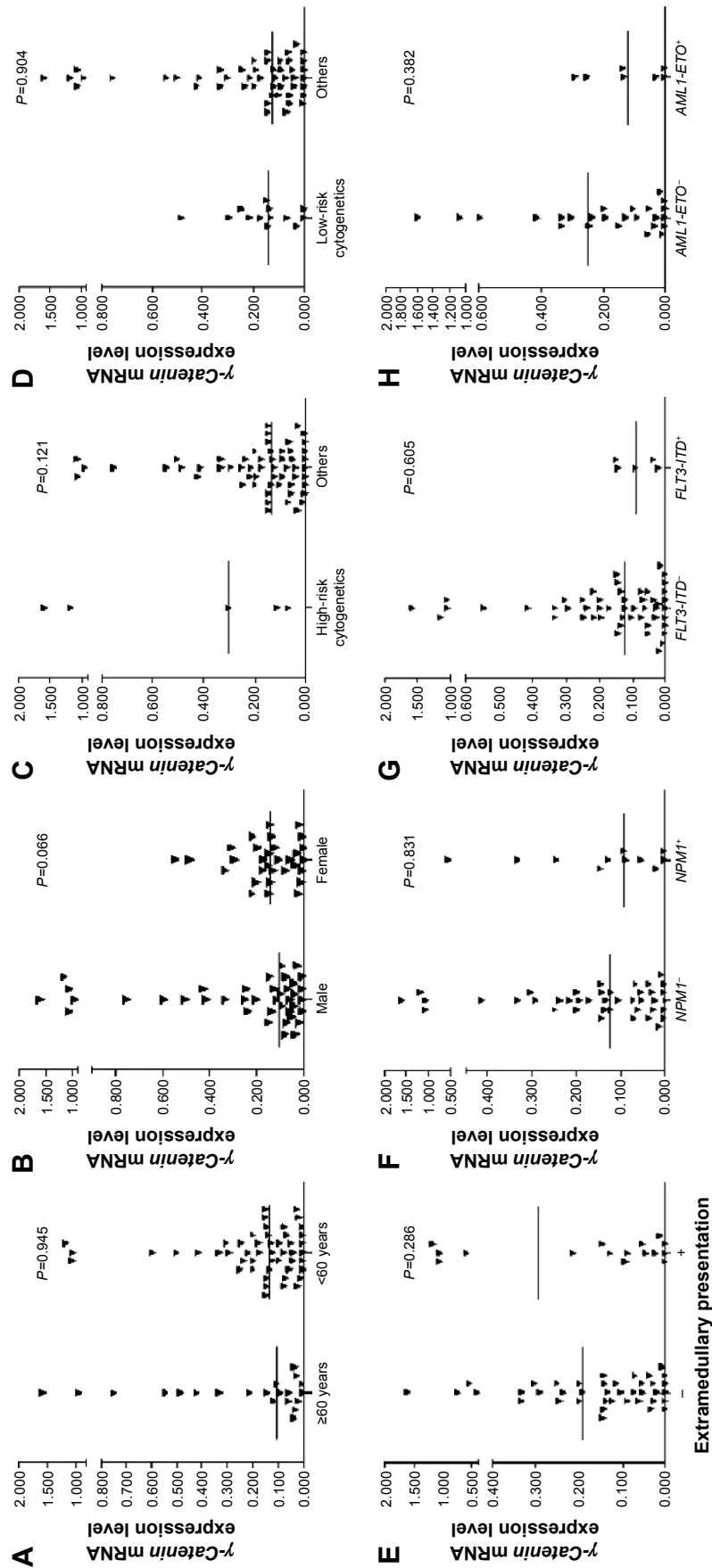
**Abbreviations:** AML, acute myeloid leukemia; mRNA, messenger RNA; WBC, white blood cells.

groups ( $P < 0.05$ , Figure 6B), indicating that  $\gamma$ -catenin is a potential inhibitor for K562 cell migration.

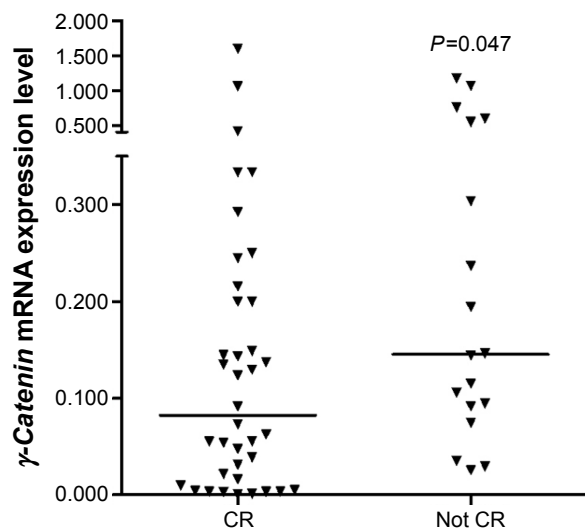
### Suppression of $\gamma$ -catenin expression sensitizes K562 cells to decitabine

To understand how the knockdown of  $\gamma$ -catenin affects K562 cells, we treated K562/KD, K562/NC, and K562/WT cells with decitabine (4.4, 8.8, and 17.6  $\mu M$ ) and examined the inhibitory effects of this agent using CCK-8 assay kit, and then the inhibitory rate and half maximal inhibitory concentration ( $IC_{50}$ ) were calculated. The results showed that the inhibitory rate of decitabine in K562/KD cells ( $IC_{50} = 6.24 \pm 0.77 \mu M$ ) was significantly higher than that in K562/NC cells ( $IC_{50} = 12.14 \pm 1.38 \mu M$ ) or K562/WT cells ( $IC_{50} = 22.51 \pm 3.83 \mu M$ ), indicating that the downregulation of  $\gamma$ -catenin could sensitize K562 cells to decitabine ( $P < 0.05$ , Figure 7A).

Benzyloxycarbonyl-Val-Ala-Asp (zVAD) as a caspase inhibitor can block the apoptotic pathway. Necrostatin-1 (Nec-1) is an inhibitor of programmed necrotic pathway. To investigate the pathway of decitabine-induced cell death, cells were treated separately with 1) growth media, 2) decitabine, 3) zVAD (20  $\mu M$ ) + decitabine, and 4) Nec-1 (30  $\mu M$ ) + decitabine. From this, we observed that the cytotoxicity of decitabine was inhibited by either zVAD or Nec-1 in any cell group, indicating that decitabine could cause cell death by both apoptotic and programmed necrotic pathways ( $*P=0.001$ ,  $**P=0.000$ ,  $***P=0.001$ ,  $^{\#}P=0.000$ ,  $^{\#\#}P=0.002$ ,  $^{\#\#\#}P=0.000$ , Figure 7B). Meanwhile, the cell viability of K562/KD was significantly lower than that of K562/NC in the same processing method ( $P < 0.05$ ), indicating once again that the downregulation of  $\gamma$ -catenin could sensitize K562 cells to decitabine.



**Figure 2**  $\gamma$ -Catenin expression and clinical characteristics.  
**Notes:** Correlation between  $\gamma$ -catenin expression and other clinical characteristics including age (A), sex (B), cytogenetics (C and D), extramedullary presentation (lymphadenectasis and/or hepatosplenomegaly) (E), mutation status of *NPM1* (F) and *FLT3-ITD* (G), and fusion genes *AML1-ETO* (H) in AML.  
**Abbreviations:** AML, acute myeloid leukemia; mRNA, messenger RNA.

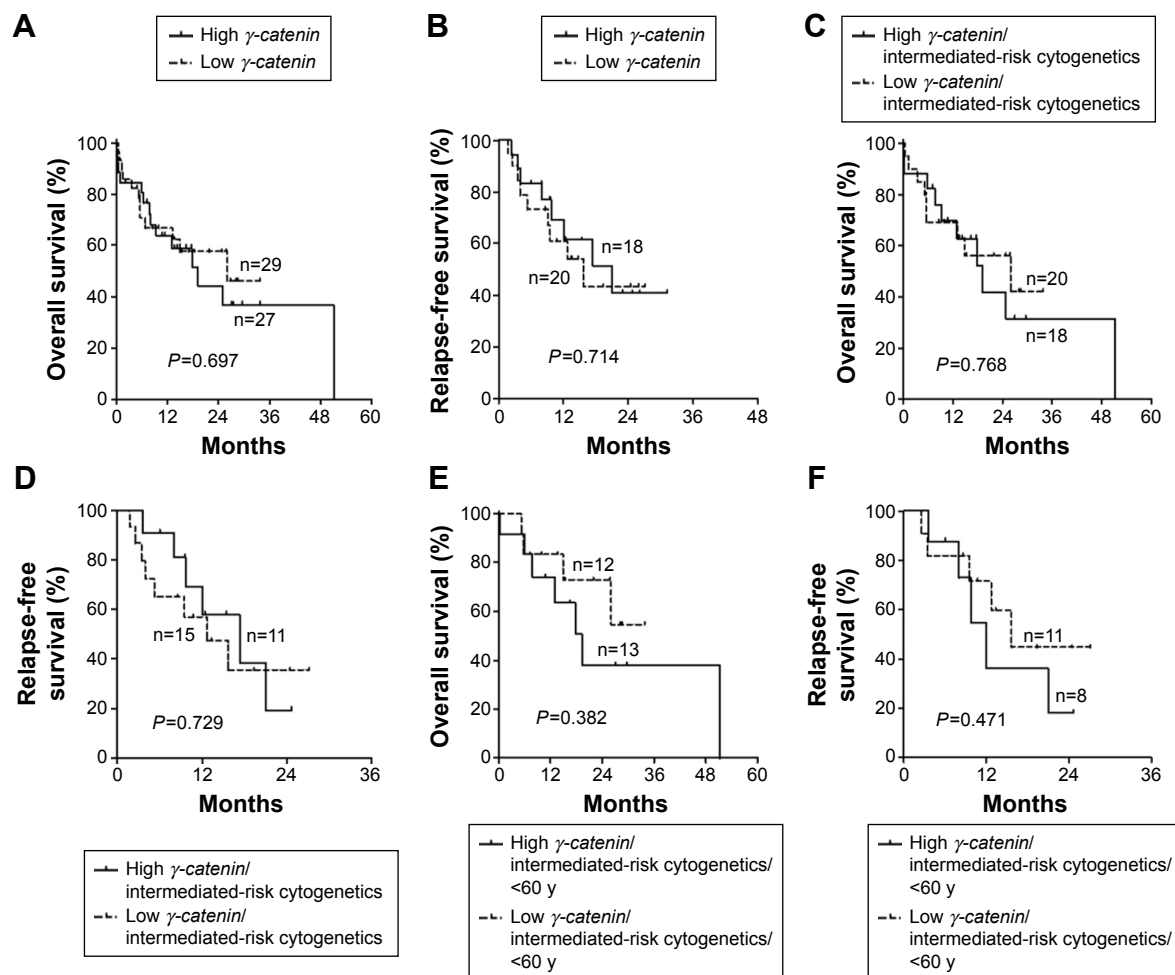


**Figure 3** The expression level of  $\gamma$ -catenin gene compared between patients (n=38) who achieved CR following one to two cycles of standard induction chemotherapy and those who could not achieve CR after two cycles of induction chemotherapy (n=18). **Abbreviation:** CR, complete remission.

## Discussion

Wnt signaling pathway plays a critical role in the regulation of early embryogenesis and is progressively shut off in differentiated mature cells.<sup>8</sup> The aberrant activation of Wnt pathway has been found to play a role in the pathological progresses in various malignant diseases, including AML.<sup>9</sup> Wnt signaling pathways are generally termed as “canonical” pathways, which are  $\beta$ -catenin dependent, and “noncanonical” pathways, which are  $\beta$ -catenin independent. The mechanism of the canonical pathway has been well defined, while the noncanonical one still remains unclear.

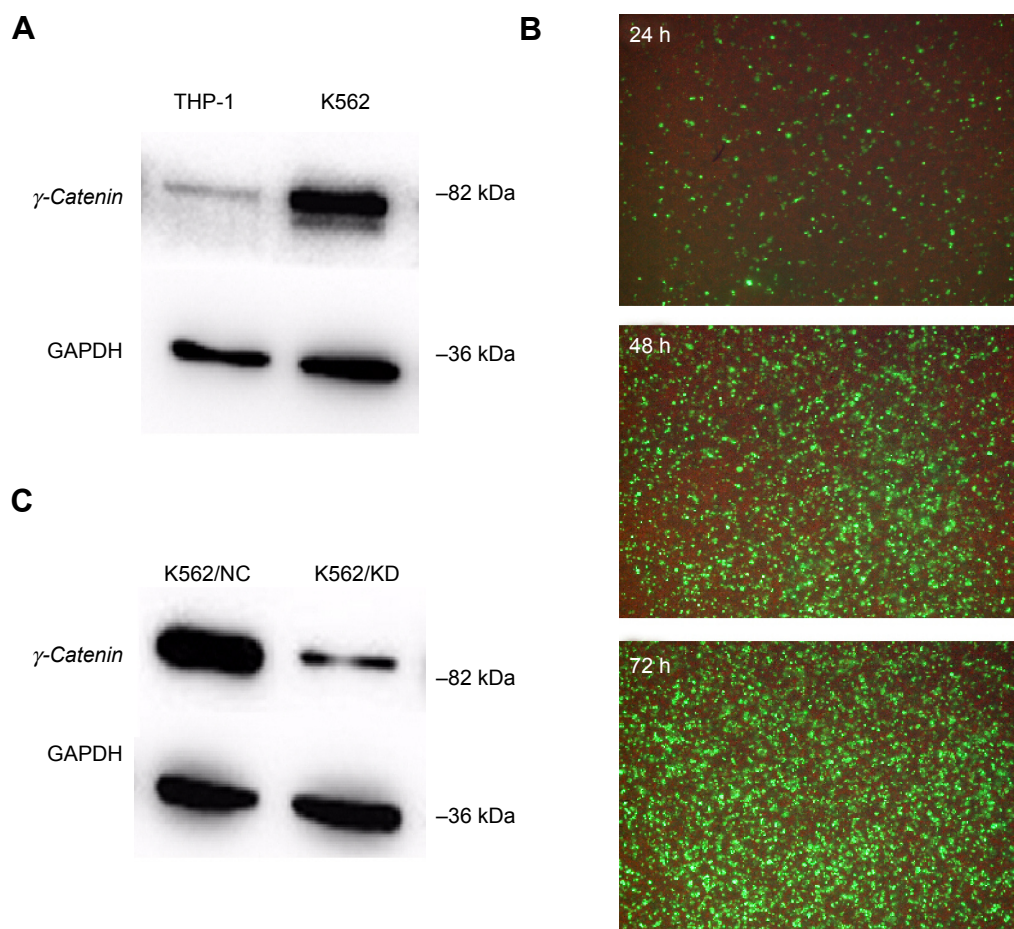
Cytoplasmic  $\beta$ -catenin, the central mediator, is degraded by the APC/Axin-GSK-3 $\beta$  complex. In this complex, APC and GSK-3 $\beta$ 's function is to facilitate the phosphorylation of  $\beta$ -catenin.<sup>10</sup> In the presence of Wnt signaling, the pathway is initiated via binding of Wnt proteins and the dimeric cell surface receptors composed of frizzled protein (Fzd) and



**Figure 4**  $\gamma$ -Catenin expression and OS and RFS in AML.

**Notes:** (A and B) Correlations between  $\gamma$ -catenin expression levels and OS and RFS in AML patients. (C and D) Correlations between  $\gamma$ -catenin expression levels and OS and RFS in subgroups with intermediated-risk cytogenetics. (E and F) Correlations between  $\gamma$ -catenin expression levels and OS and RFS in subgroups who were younger than 60 years and with intermediated-risk cytogenetics.

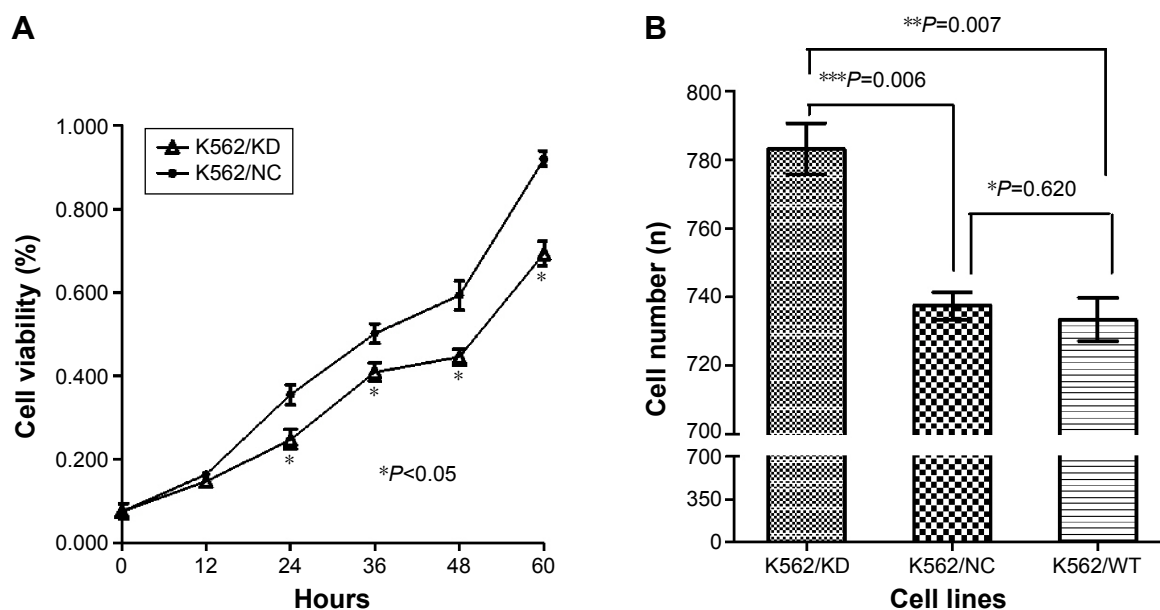
**Abbreviations:** OS, overall survival; RFS, relapse-free survival; AML, acute myeloid leukemia.



**Figure 5**  $\gamma$ -Catenin protein level in K562 and THP-I cell line and the process of transfection in K562.

**Notes:** (A) The expression level of  $\gamma$ -catenin protein was determined by Western blot in K562 cell line and THP-I cell line. (B) The transfection efficiency was determined posttransfection at 24, 48, and 72 hours by fluorescent microscopy ( $\times 10$ ). (C) shRNA was transfected into K562 cell line to suppress the expression of  $\gamma$ -catenin protein.

**Abbreviations:** GAPDH, glyceraldehyde 3-phosphate dehydrogenase; KD, knockdown; h, hours; NC, negative control; shRNA, short hairpin RNA.

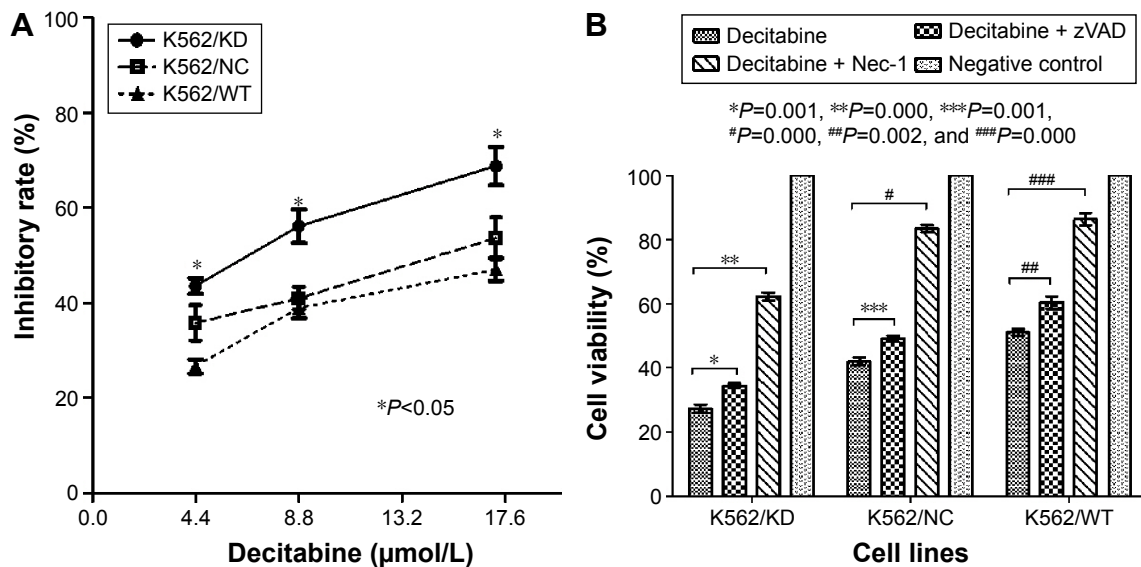


**Figure 6** The cell viability and ability of migration.

**Notes:** (A) The cell viability of K562 cell compared between K562/KD and K562/NC after the suppression of  $\gamma$ -catenin protein expression. (B) The ability of migration of K562 cell line after the suppression of  $\gamma$ -catenin protein expression.

**Abbreviations:** KD, knockdown; NC, negative control; WT, wild type.





**Figure 7** The effect of decitabine.

**Notes:** (A) The sensitivity of K562 cell line to decitabine therapy after the suppression of  $\gamma$ -catenin protein expression. (B) The cell viability of K562 cell line after treatment separately with 1) growth media, 2) decitabine, 3) zVAD (20  $\mu$ M) + decitabine, and 4) Nec-1 (30  $\mu$ M) + decitabine.

**Abbreviations:** KD, knockdown; Nec-1, Necrostatin-1; NC, negative control; WT, wild type; zVAD, Benzyloxycarbonyl-Val-Ala-Asp.

low-density lipoprotein receptor-related protein 5 or 6, leading to the recruitment and activation of the dishevelled protein. This process turns the low-density lipoprotein receptor-related protein 5 or 6 into phosphorylated form and thereby leads to the dissociation of APC/Axin-GSK-3 $\beta$  complex. As a result, the phosphorylation and degradation of  $\beta$ -catenin are inhibited. Subsequently,  $\beta$ -catenin in the dephosphorylated form translocates into the nucleus, where it accumulates and acts as the ultimate effector by binding to TCF/LEF-1 to form a complex and activates the downstream target genes, such as cyclin D<sub>1</sub> and C-myc,<sup>11–13</sup> which are well-established oncogenes, and regulates the cellular proliferation and differentiation.<sup>14</sup>

Several previous studies have reported that in various hematological malignancies, including chronic myelogenous leukemia,<sup>15</sup> chronic lymphocytic leukemia,<sup>16</sup> acute lymphoblastic leukemia,<sup>17</sup> as well as AML,<sup>9</sup> dysregulation of Wnt signaling has been identified as one of the key roles in leukemogenesis.

$\gamma$ -Catenin belongs to the catenin family, which includes  $\alpha$ -,  $\beta$ -, and  $\gamma$ -catenin. Sharing approximately 80% sequence homology,  $\gamma$ -catenin is closely related to  $\beta$ -catenin.  $\beta$ -Catenin and  $\gamma$ -catenin not only play directly an important role in cell–cell adhesion along with transmembranous E-cadherin<sup>18</sup> but also influence the signal transduction through the Wnt canonical cascade. Unlike the clearly defined oncogenic effect of  $\beta$ -catenin, the role of  $\gamma$ -catenin in the aspect of signal transduction remains confusing. There are reports suggesting that  $\gamma$ -catenin may possess indirect oncogenic activity by blocking the degradation of  $\beta$ -catenin and in

turn increasing the level of  $\beta$ -catenin in the cytoplasm.<sup>19,20</sup> Recently, in 2013, Morgan et al<sup>21</sup> concluded that  $\gamma$ -catenin was overexpressed in AML and could promote the stabilization and nuclear localization of  $\beta$ -catenin. Moreover, it has been reported that in the absence of endogenous  $\beta$ -catenin expression,  $\gamma$ -catenin had transcriptional regulatory activity, which is TCF/LEF-family dependent.<sup>22</sup> Moreover, in 2004, Zheng et al<sup>23</sup> reported that the AML-associated translocation products, including PML/RAR, promyelocytic leukemia zinc finger/RAR(X-RAR), and AML-1/ETO, activated the Wnt pathway via upregulating  $\gamma$ -catenin and in turn encouraged the self-renewal of hematopoietic stem cells and contributed to the leukemogenesis in AML. However, from 2000 to 2006, several groups demonstrated that  $\gamma$ -catenin acted as a suppressor of tumors and metastasis. Decreased level of  $\gamma$ -catenin might result in poorer prognosis in several solid tumors, such as bladder, pituitary, oral, skin, and lung cancers.<sup>24–28</sup>

In this study, we compared  $\gamma$ -catenin mRNA levels in marrow mononuclear cells between naive AML patients and healthy donors, investigated the potential correlation between  $\gamma$ -catenin and various clinical parameters, and analyzed the clinical significance of  $\gamma$ -catenin in AML.  $\gamma$ -Catenin mRNA expression level was significantly higher in AML patients than in healthy donors, which is consistent with previous studies.<sup>21,23,29</sup> Therefore, elucidating the role of  $\gamma$ -catenin in AML would help to understand the pathological mechanism of the disease and help develop a potential biomarker for clinical diagnosis and prognosis. Our data further showed that patients who had achieved CR in one to two cycles

had significantly lower  $\gamma$ -catenin mRNA expression level in comparison to refractory patients with slow response. So,  $\gamma$ -catenin mRNA level in naive AML patients might act as an indicator for therapeutic efficacy. Lower level of  $\gamma$ -catenin suggested better response to chemotherapies. However, in all the 71 AML patients, we did not find any correlation between  $\gamma$ -catenin expression and OS or RFS. Surprisingly,  $\gamma$ -catenin mRNA was highly expressed in AML patients with *CEBP $\alpha$*  mutation or low WBC count at first visit. It is well known that *CEBP $\alpha$*  mutation indicates better prognosis, while high WBC number at first-visit acts as an adverse prognostic indicator for AML. Thus, further investigation is necessary to better clarify the specific relationship between  $\gamma$ -catenin and AML development.

Furthermore, we initiated in vitro study with two cell lines: the acute monocytic leukemia line THP-1 and the chronic myeloid leukemia-derived erythroleukemia line K562. On the basis of Western blot analysis,  $\gamma$ -catenin protein was highly expressed in K562 in comparison to THP-1, and so K562 was chosen for further investigation. At present, K562 is widely used as one of the model cell lines for hematopoietic study. It has the potential to differentiate into granulocyte, erythrocyte, and megakaryocyte lineages.<sup>30</sup>

Our results showed that downregulation of  $\gamma$ -catenin reduced the viability and growth rate of K562 cell, which is consistent with the study led by Niu et al.<sup>31</sup> It has been demonstrated that suppression of  $\gamma$ -catenin can inhibit the expression of two  $\beta$ -catenin downstream oncogenes, c-Myc and cyclin D<sub>1</sub>. As suggested by Morgan et al,<sup>21</sup> the overexpression of  $\gamma$ -catenin could stabilize  $\beta$ -catenin. So we speculate that  $\gamma$ -catenin could stimulate leukemia cellular growth by affecting  $\beta$ -catenin in Wnt signal pathway.

We found that downregulation of  $\gamma$ -catenin enhanced the toxicity of decitabine to K562 cells. Decitabine (5-aza-2'-deoxycytidine) is a cytosine analog and a DNA demethylation agent. It can induce leukemia cell differentiation and apoptosis and is the first-line therapeutic agent for AML showing good tolerance. The detailed mechanism of action of decitabine is as yet unclear. However, it was reported in 2007 and 2008 that the cytotoxicity of decitabine may rely on the demethylation of target genes, thus accelerating the expression of tumor suppressor genes.<sup>32,33</sup> To our knowledge, this is the first study which established that downregulation of  $\gamma$ -catenin can sensitize K562 cells to decitabine and enhance the drug's activity. The results suggested that  $\gamma$ -catenin may play a role in the cytotoxicity of decitabine and may serve as a potential therapeutic target for AML. We also found that the cytotoxicity of decitabine was inhibited by either zVAD or Nec-1. zVAD is a caspase inhibitor, which can block the

apoptotic pathway. Nec-1 is an inhibitor of programmed necrotic pathway. This finding indicates that decitabine could cause both apoptosis and programmed necrosis, in accordance to a study by Steinhart et al.<sup>34</sup>

In several solid tumors,  $\gamma$ -catenin was found to regulate cellular invasion and migration. Mukhina et al<sup>35</sup> have found that the downregulation of  $\gamma$ -catenin increased cell migration and invasion in MCF-7 cells, which express membrane-localized E-cadherin and  $\gamma$ -catenin. To discern the specific role of  $\gamma$ -catenin in these processes, the authors reexpressed  $\gamma$ -catenin in MCF-7 cells, which resulted in decreased migration and invasiveness of MCF-7 cells. The metastasis suppression activity of  $\gamma$ -catenin has also been described in bladder carcinomas.<sup>36</sup> In the study by Franke,<sup>37</sup> different types of classic and new junction proteins/structures were detected in K562 cells, including  $\gamma$ -catenin in the desmosome junction. These cell-cell junctions are maintained with passages. In our investigation, knockdown of  $\gamma$ -catenin enhanced K562 cell migration, presumably by weakening intracellular junctions.

## Conclusion

$\gamma$ -Catenin gene was significantly overexpressed in AML patients compared with healthy donors. The patients who had lower level of  $\gamma$ -catenin expression were more likely to achieve CR in comparison to those with higher  $\gamma$ -catenin level.  $\gamma$ -Catenin protein might accelerate the growth of AML cells. Knocking down the  $\gamma$ -catenin gene could enhance the cytotoxicity of decitabine in K562 cells. However,  $\gamma$ -catenin seemed to be capable of suppressing the migration of K562 cell line, and this needs further investigation.

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## Disclosure

The authors report no conflicts of interest in this work.

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