

HES1 activation suppresses proliferation of leukemia cells in acute myeloid leukemia

Chen Tian¹ · Yong Yu¹ · Yongsheng Jia¹ · Lei Zhu¹ · Yizhuo Zhang¹

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Abstract Although aberrant Notch activation contributes to leukemogenesis in T cells, the role of Notch pathway in acute myeloid leukemia (AML) remains controversial. To address this issue, we compared the expression levels of its downstream effector HES1 and p21 in bone marrow mononuclear cells (BMNCs) from 30 newly diagnosed AML patients and three AML cell lines to normal BMNCs. The results showed that both of them were downregulated in AML cells. In vitro, induced activation of HES1 by retrovirus in AML cell lines consistently led to AML cell growth arrest and apoptosis induction, which was associated with enhanced p21 expression. Furthermore, overexpression of HES1 in primary AML cells inhibited growth of AML in a xenograft mice model. In conclusion, we demonstrated the tumor suppressor role of HES1 in AML.

Keywords HES1 · p21 · AML · Apoptosis · Proliferation

Introduction

In hematopoietic cells, Notch pathway plays either oncogenic or tumor suppressor roles, depending on the cell types [1–3]. In T cell acute lymphoblastic leukemia (T-ALL), Notch mutations are found in 50–70 % of patients [4]. In contrast to

T-ALL, where the role of Notch pathway is well defined, there are conflicting opinions on the role of Notch pathway in AML. Li et al. reported that Notch pathway was activated in HL60 [5], while Lobry et al. demonstrated that it was silenced in human AML samples [6]. Recently, Kannan et al. reported that although Notch receptors were expressed, Notch pathway was not activated in human AML samples and cell lines [7].

HES1 is the downstream effector of Notch pathway, which is reported to affect cell differentiation and maintain the cells in an immature state in various tissues, including the hematopoietic tissue [8]. Our previous studies found that overexpression of HES1 inhibited cycling of hematopoietic stem and progenitor cells (HSPCs) in vitro and cell expansion in vivo, associated with upregulation of p21 [9]. Recently, it was reported that HES1 acted as a tumor suppressor in MLL-AF9 AML mice model [10]. But the role of HES1 in primary AML cells with other gene mutations and AML cell lines has not been well demonstrated. So we focused to clarify the function of HES1 in AML.

Method

AML cell lines and AML samples

AML cell lines HL60, U937, and KG1a were purchased from the Cell Bank of Beijing Union Medical College, and cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS) at 37° centigrade, in 5 % CO₂ incubator with standard humidity. Bone marrow (BM) samples were obtained from newly diagnosed AML patients before treatment and healthy donors after informed consent and with the approval of the ethics committee of Tianjin Cancer Hospital. Baseline morphology, karyotype, and genetic mutations such as Flt3

✉ Yizhuo Zhang
yizhuozhang111@163.com

¹ National Clinical Research Center for Cancer, Key laboratory of Cancer Prevention and Therapy, Tianjin Medical University Cancer Institute and Hospital, Tianjin 300060, China

and CEBPA, were performed as part of the routine clinical evaluation of the patients. The characteristics of patients are shown in Table 1. BMNCs were isolated with Ficoll (Sigma).

Real-time RT-PCR analyses

Total RNA of AML cells was extracted with Trizol (Invitrogen) from 30 patients. Reverse transcription was achieved using QuantiTect Reverse Transcription Kit (Qiagen). Real-time PCR was performed using an ABI-Prism 7500 Sequence Detector (Applied Biosystems). The parameters for the thermal cycling of PCR were as follows: 15 s at 95 °C and 60 s at 60 °C, 45 cycles. The sequences of HES1 primers were upper: 5-GCAGATGACGGCTGCGCTGA-3, lower: 5-AAGCGGTCACCTCGTTCATGC-3. The sequences of p21 primers were upper: 5-AATCCTGGTGATGTCCGACC-3, lower: 5-TTGCAGAAGACCAATCTG-3.

GAPDH was used as housekeeper. The primer sequences of GAPDH were upper: 5-CGGAGTCAACGGATTTGGTCGTAT-3, lower: 5-AGCCTTCTCCATGGTGGTGAAGAC-3.

Transduction of AML cells

The plasmid MSCV-HES1-IRES-GFP, pCMV-VSV-G, and pKAT, kindly gifted from Professor Tao Cheng (State Key Laboratory of Experimental Hematology, Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College) were cotransfected into package cell line 293T using lipofectamine 2000 (Invitrogen). Virus supernatant was harvested 48 and 72 h after transfection. HL60, U937, KG1a, and primary AML BMNCs were then transduced with virus supernatant. Cells were sorted by FACS (BD Aria II) 48 h after

Table 1 Characteristics of AML patients included in the study

Number	Sex	Age (year)	FAB	Karyotype	Genetic mutation	Disease status
1	M	17	M1	Normal	Flt3 mutation	Newly diagnosed
2	M	12	M4	Normal	Normal	Newly diagnosed
3	M	39	M4	Normal	Normal	Newly diagnosed
4	F	33	M4	Normal	Normal	Newly diagnosed
5	M	31	M4	t (16; 16)	Normal	Newly diagnosed
6	F	25	M2	t (8; 21)	Normal	Newly diagnosed
7	F	30	M4	Normal	Normal	Newly diagnosed
8	F	33	M4	Normal	Normal	Newly diagnosed
9	F	25	M1	Normal	CEBPA	Newly diagnosed
10	M	56	M5	Normal	Normal	Newly diagnosed
11	M	47	M2	Normal	Normal	Newly diagnosed
12	M	37	M4	Normal	Flt3 mutation	Newly diagnosed
13	F	59	M4	Normal	Normal	Newly diagnosed
14	M	65	M5	Normal	Normal	Newly diagnosed
15	F	16	M5	Normal	Normal	Newly diagnosed
16	M	16	M5	Normal	CEBPA	Newly diagnosed
17	M	24	M1	Normal	Normal	Newly diagnosed
18	F	36	M2	t (8; 21)	Normal	Newly diagnosed
19	M	45	M4	Normal	Normal	Newly diagnosed
20	M	28	M4	Normal	Flt3 mutation	Newly diagnosed
21	F	32	M6	Normal	Normal	Newly diagnosed
22	F	64	M5	Normal	Flt3 mutation	Newly diagnosed
23	F	59	M2	t (8; 21)	Normal	Newly diagnosed
24	F	38	M6	Normal	Normal	Newly diagnosed
25	M	56	M5	Normal	Normal	Newly diagnosed
26	F	40	M5	Normal	CEBPA	Newly diagnosed
27	F	31	M2	Normal	Normal	Newly diagnosed
28	M	23	M4	Normal	Normal	Newly diagnosed
29	F	29	M4	inv (16)	Normal	Newly diagnosed
30	F	35	M5	Normal	CEBPA	Newly diagnosed

FAB French-American-British classification, M male, F female

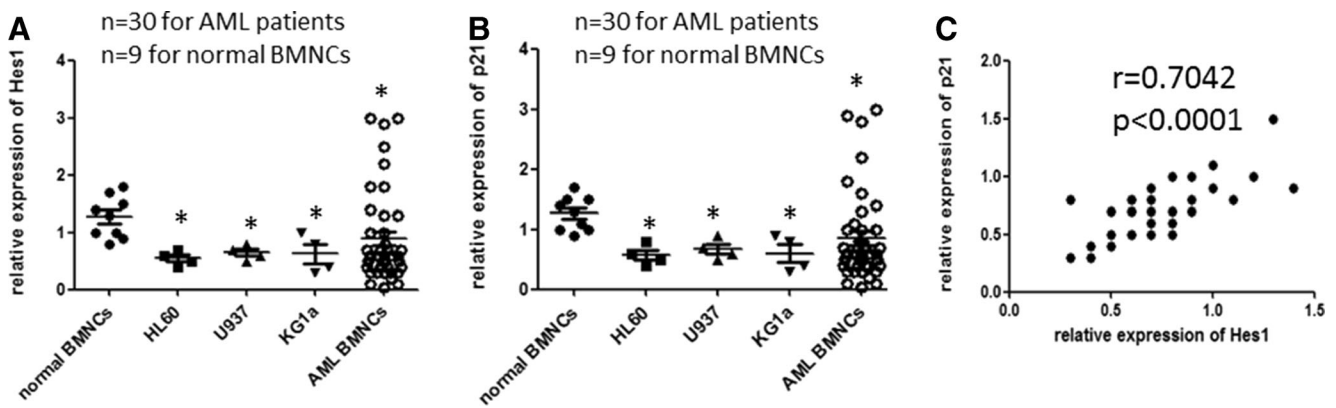


Fig. 1 Expression of HES1 and p21 in AML cells and normal BMNCs. BM samples from 30 AML patients were obtained. The expression of HES1 (a) and p21 (b) were determined by real-time PCR ($*p<0.05$). c The correlation between the expression of HES1 and p21 were analyzed ($p<0.0001$)

transduction, based on GFP fluorescence. MSCV-IRES-GFP was used as blank vector.

MTT assay

Cell proliferation was quantified using MTT assay. Transduced cell lines were seeded in 96-well plates (8000 cells/well) after sorting. MTT reagent (Sigma) was then added, followed by 3 h incubation at 37 °C. Finally, the solution was decanted, and 100 μ L DMSO was added to dissolve the purple formazan crystals. The absorbance of the resulting solution was measured at 570 nm with a microplate reader (Synergy 2, BioTek, VT, USA).

Cell cycle and apoptosis analysis

Transduced cell lines were permeabilized and stained with Hoechst 33342 (Sigma) followed by 1 μ g/ml PY (Sigma). The proportion of cells in G0 phase was determined by flow cytometry with quantitation of DNA and RNA. Transduced cell lines were stained with annexin V and PI (BD Pharmingen) for 20 min at room temperature. The ratio of apoptosis was assessed by FACS.

In vivo xenograft model

NOD/SCID mice were bought from the Animal Center of the Institute of Hematology and Blood Diseases Hospital. The NOD/SCID mice were inoculated subcutaneously with 1×10^6 HES1+ HL60, U937, and KG1a cells suspended in 100 μ L PBS, respectively. Mice were injected with blank vector-transduced AML cells as vehicle control. Each treatment group and control group consisted of eight mice. Tumor size was assessed using a digital caliper.

NOD/SCID mice of 6 to 9 weeks old were sub-lethally irradiated, and then injected by tail vein with 5×10^5 GFP+ primary AML cells transduced with HES1 or control plasmid. On day 14, 18, and 21, proportion of CD45+ (APC-H7 mouse

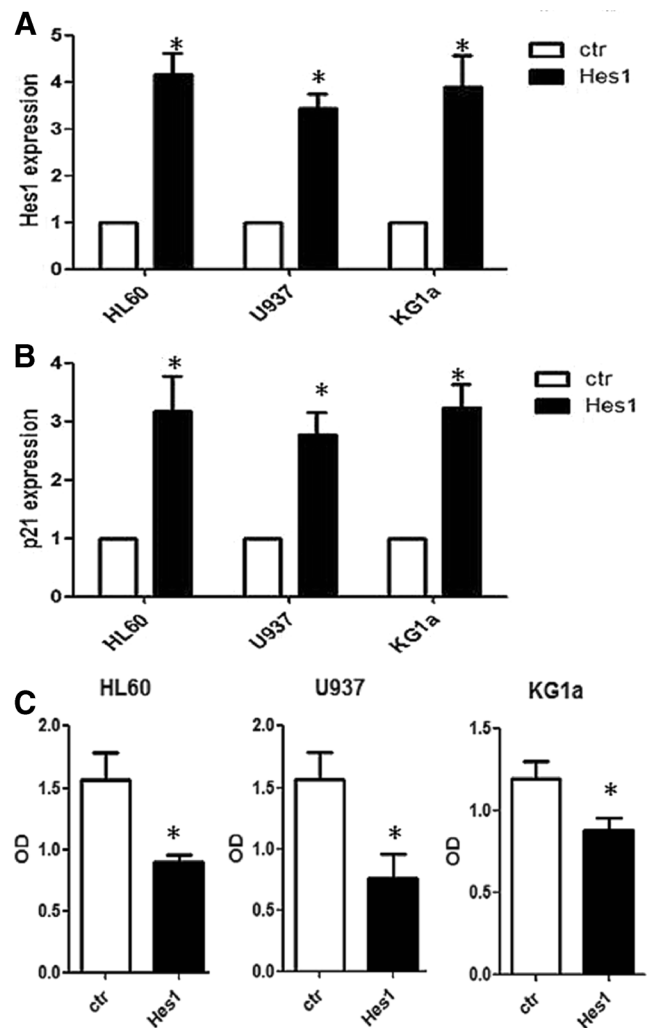


Fig. 2 HES1 expression was upregulated in AML cell lines after transfection and overexpression of HES1 suppressed the proliferation of AML cells. AML cell lines were transduced with retroviral vectors expressing HES1. a, b RT-PCR was done to test the expression of HES1 and p21 after transfection ($*p<0.05$). c MTT assay showed that proliferation of AML cell lines after transfection was reduced

anti-human CD45, BD Pharmigen, #560274) AML cells in peripheral blood (PB) was analyzed. On day 21 after transplantation, mice were sacrificed; the spleen weight and BM cells were analyzed.

Statistical analysis

Data were presented as means±SD. The significant difference was examined using the Student's *t* test. *p* values of less than 0.05 were considered to be significant. Statistical analyses were performed using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA, <http://www.graphpad.com/>).

Results

Expression of HES1 and p21 was low in human AML samples and cell lines

We first investigated the expression of HES1 and p21 in primary AML patient samples and AML cell lines. Using real-

time PCR, we compared HES1 expression in 30 AML patient BMNCs and three AML cell lines to that in normal donor BMNCs. We found that expression of HES1 (Fig. 1a) and its downstream effector p21 (Fig. 1b) was lower in primary AML BMNCs, HL60, U937, and KG1a compared to that in normal BMNCs. Furthermore, a positive correlation between the expression of HES1 and p21 was observed, which suggested that HES1-p21 pathway were differentially activated between AML cells and normal BMNCs ($r=0.7042$, $p<0.0001$) (Fig. 1c).

Overexpression of HES1 after transfection

Because HES1 appeared to be inactive in AML BMNCs and cell lines, we used a construct that encodes HES1, as well as GFP (MSCV-HES1-IRES-GFP) to transfect AML cell lines. After transfection, we used fluorescent microscope and FACS to test the transfection efficiency. Real-time PCR results showed that the expression of HES1 and p21 were both upregulated after transfection (Fig. 2a, b).

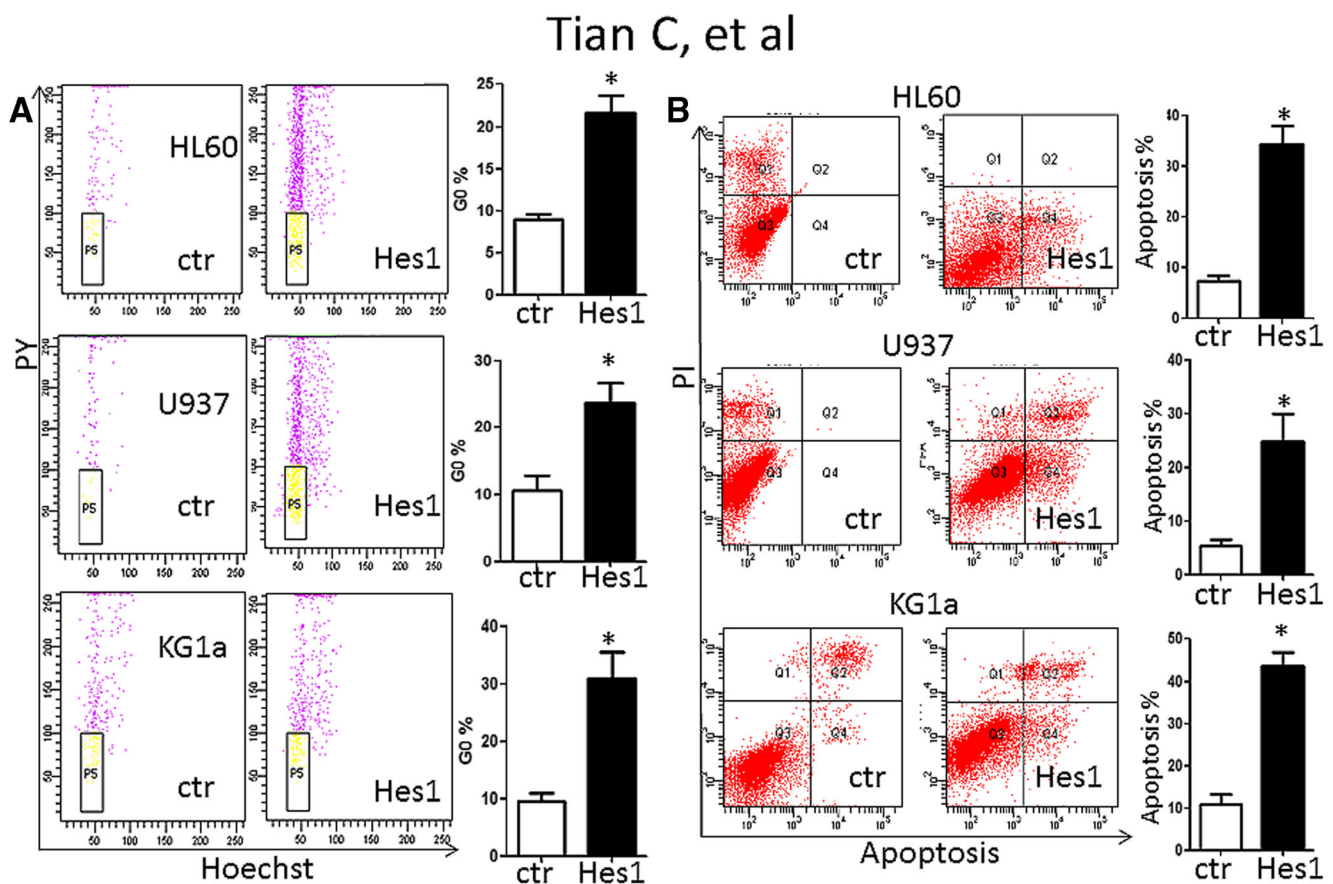


Fig. 3 HES1 activation induced growth arrest and apoptosis. **a** Flow cytometry-based cell cycle analysis of GFP⁺ cells on day 2 after transfection with Hoechst/PY. **b** Annexin V staining of GFP⁺ cells on

day 2 after transfection. The data in the bar graphs were means with standard deviations. * $p<0.05$ (*t* test)

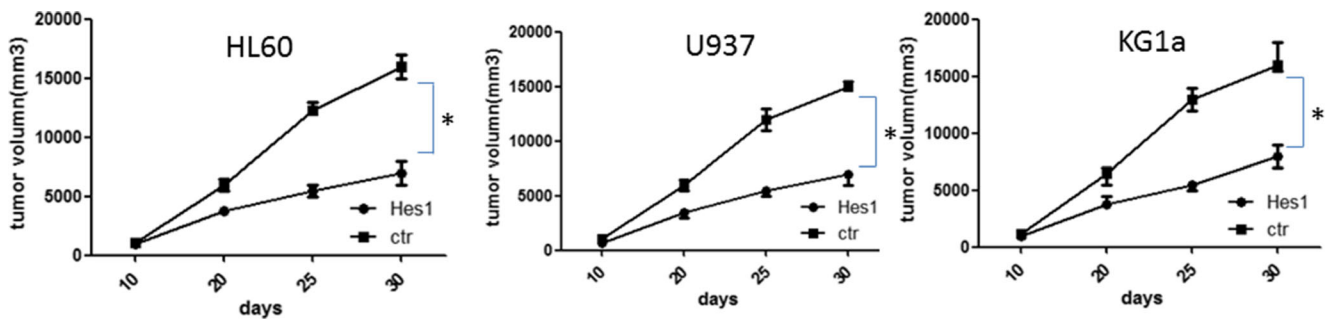


Fig. 4 HES1 overexpression inhibited AML cell growth in vivo. HES1+ AML cell lines (10^6) were subcutaneously injected into NOD/SCID mice. The graph showed tumor size measured at the indicated days after cell injection ($*p < 0.05$)

HES1 activation inhibited AML cell growth and induced apoptosis

In MTT assay, we compared the effect of HES1 on the growth of AML cells. We found that overexpression of HES1 inhibited the proliferation of all AML cell lines (Fig. 2c). Next,

we evaluated the effect of HES1 on cell cycle and apoptosis. After transfection, HES1+ cells had a significant decrease in S phase of the cell cycle and accumulation in G0 phase (Fig. 3a) compared to control, which was consistent with growth arrest. Apoptosis analysis showed that cells with HES1 overexpression had a significant increase in apoptosis (Fig. 3b).

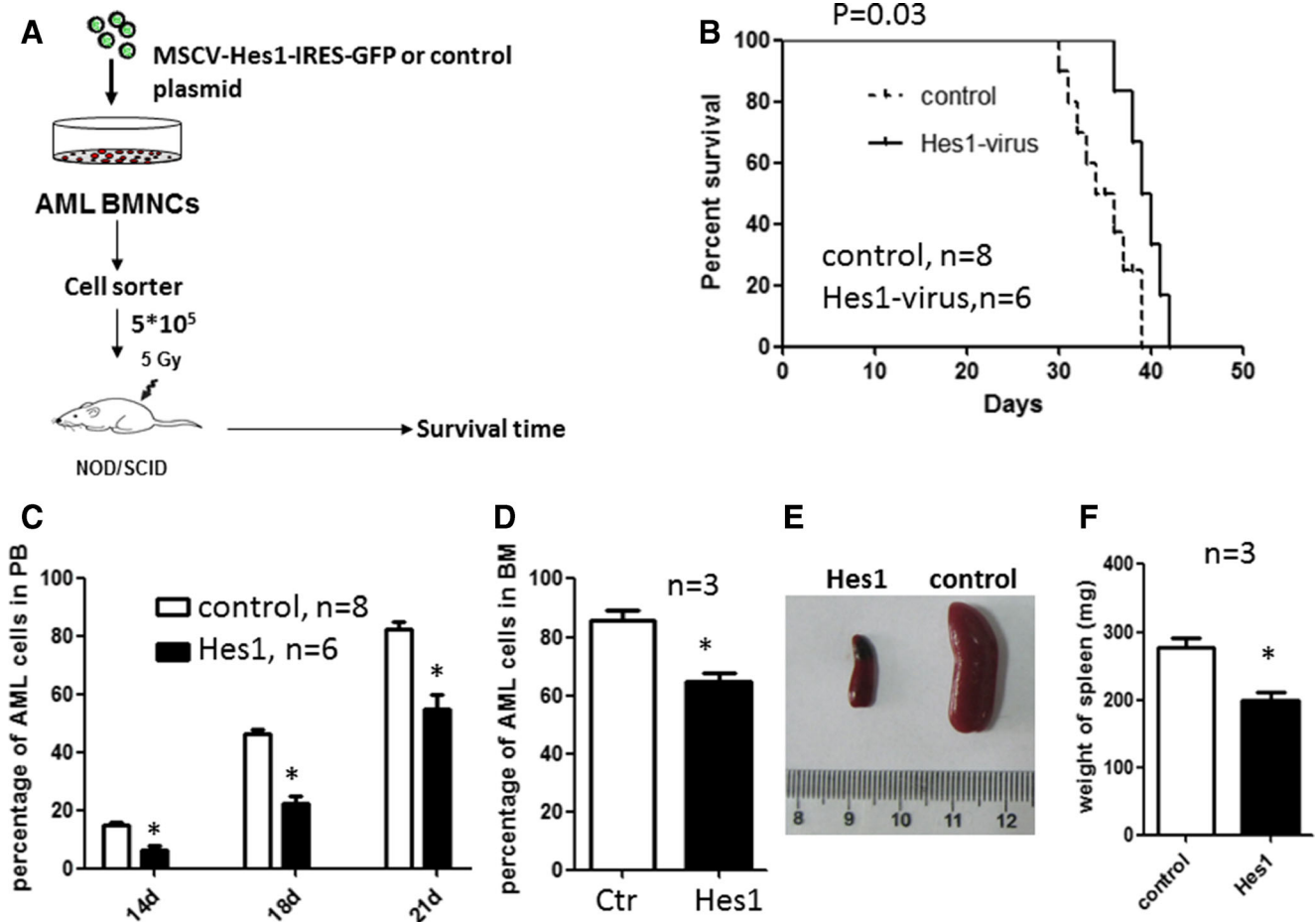


Fig. 5 Impact of HES1 activation on the survival of xenograft mice. **a** Establishment of HES1 overexpressed AML mice model. **b** Survival curves of the different mouse groups ($n=6$ for test group and $n=8$ for control group). **c** The percentage of leukemia cells in PB of control and HES1+ mice on day 14, 18, and 21 after transplantation ($n=6$ for test group and $n=8$ for control group). **d** Detection of leukemia cells in BM of

control and HES1+ mice on day 21 after transplantation ($n=3$). **e** The typical size of spleen in two groups on day 21 after transplantation was shown. **f** The weight of spleen from control and HES1+ AML mice on day 21 after transplantation. The data in the bar graphs were means with standard deviations. $*p < 0.05$ (t test)

Overexpression of HES1 suppressed AML formation in vivo

We next tested whether activation of HES1 could suppress AML in vivo. GFP⁺ AML cells were sorted and injected (1×10^6 cells) subcutaneously into NOD/SCID mice. As shown in Fig. 4, a more robust growth of tumors was observed in control group than in HES1⁺ group.

Furthermore, we established an AML xenotransplant mouse model by injecting 5×10^5 GFP⁺ AML cells to the sub-lethally irradiated mice (Fig. 5a, $n=15$ for test and control group, respectively). Nine mice in the test group developed leukemia while eleven mice in the control group developed leukemia. The results showed that the survival of mice with HES1⁺ cells was significantly longer than that with control cells (Fig. 5b, $n=6$ in test group and $n=8$ in control group). Leukemia cells in PB of mice injected with HES1⁺ cells appeared later and proliferated slower than that in control mice (Fig. 5c, $n=6$ in test group and $n=8$ in control group). On day 21, mice were killed. Leukemia cells in BM were detected by FACS, and weight of spleen was recorded. The percentage of leukemia cells in BM of HES1⁺ mice was lower than that in control mice (Fig. 5d, $n=3$). Furthermore, low degree of splenomegaly was detected in HES1⁺ mice when compared with control counterparts (Fig. 5e, f; $n=3$).

Discussion

Abnormal activation of Notch pathway can lead to hematopoietic malignancies [11, 12]. In contrast, a definite role for Notch pathway in the development of AML is less clear. Chiamonte reported that Notch pathway was not activated in AML [13]. Similarly, Kannan and Lobry reported that Notch was silenced in AML [6, 7]. However, there were contradictory results as well. G. H. Li, Tohda, and X. Xu reported that Notch1 receptors were upregulated in AML BMNCs [2, 5, 14, 15]. Yin demonstrated that activated Notch1 inhibited proliferation of K562 chronic myelogenous leukemia (CML) cells [16].

HES1, the downstream effector of Notch pathway, is a member of basic helix-loop-helix transcription factors which belongs to the HES family [17]. Its roles in embryogenesis, chronic myelogenous leukemia and normal hematopoiesis were reported [18–20]. HES proteins generally act as repressors of transcription [21]. It has been reported that HES1 was involved in cell cycle, and maintained multipotent precursor cells in an undifferentiated state in several tissues during development and adulthood [22]. Recently, it was reported that HES1 acted as a tumor suppressor in MLL-AF9 AML mice model [10].

Given these contrasting findings of Notch pathway in AML, we sought to determine whether there was evidence

of HES1 activation in human AML cell lines and patient samples. In order to investigate the relationship between HES1 and AML, we first tested the expression level of HES1 in HL60, U937, KG1a, and AML patients. We found that expression of HES1 was low in AML cell lines and patient samples compared to normal BMNCs. We also found that p21, target gene of HES1, was low in AML cells which suggested that HES1 may affect AML cells through HES1-p21 pathway.

Then, we enhanced the expression of HES1 in AML cells and studied whether cell cycle of these cells were affected. MTT assay showed that the proliferation of AML cells with HES1 overexpression was inhibited in vitro. Cell cycle analysis revealed that more HES1⁺ AML cells were maintained in G0 phase and apoptosis of these cells was induced. In vivo, tumor formation analysis showed a more robust growth of tumors in mice receiving control cells than in mice injected with HES1⁺ AML cells. To show the therapeutic potential of targeting HES1 in vivo, we established an AML xenotransplant mouse model by injecting 5×10^5 HES1⁺ AML cells to the sub-lethally irradiated mice. Results showed that the survival of mice with HES1⁺ AML cells was significantly longer than that with control cells, which suggested that overexpression of HES1 in AML cells resulted in reduction of tumor growth in an AML xenograft mouse model.

In summary, we provided the antitumor activity of HES1 activation by inhibiting proliferation and inducing apoptosis of AML cells, and suggested that HES1 could be a potential approach for AML therapy.

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Authors' contribution Chen Tian did all the experiments and wrote the paper; Lei Zhu, Yongsheng Jia, and Yong Yu provided clinical samples and information; Yizhuo Zhang designed the experiments, interpreted the data, and wrote the manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

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