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Inhibition of HOXB7 suppresses p27-mediated acute lymphoblastic leukemia by regulating basic fibroblast growth factor and ERK1/2

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ABSTRACT

Aims: Acute lymphoblastic leukemia (ALL) is characterized by abnormal proliferation of immature lymphocytes in the bone marrow, peripheral blood, and other tissues. HOXB7 is upregulated in tumors and is related to cell proliferation and cell cycle. However, the role of HOXB7 in ALL progression remains unclear. In this study, we explored the molecular mechanism of HOXB7 in cell viability and cell cycle in ALL cell lines.

Materials and methods: Peripheral blood lymphocytes was isolated by Isopycnic Ficoll-Hypaque solution; Relative mRNA expression of HOXB7 was measured by RT-qPCR; Relative protein expressions of HOXB7, p27, bFGF, pERK1/2 were tested by Western blot assay; Cell viability was tested by MTT; Cell proliferation was detected by BrdU assay; 2.8. Cell cycle was analyzed by flow cytometry.

Key findings: HOXB7 was significantly elevated in peripheral blood lymphocytes of patients with ALL. HOXB7 was inhibited by HOXB7 siRNA transfection; cell viability decreased; and cell cycle was arrested in ALL cell lines. Meanwhile, HOXB7 suppression significantly induced the protein expression of p27 (cyclin-dependent kinase inhibitor). We also demonstrated the molecular mechanism of HOXB7 regulation on p27. HOXB7 suppression obviously inhibited the protein expressions of b basic fibroblast growth factor (bFGF) and p-ERK1/2. Also, the inhibitory effects of HOXB7 suppression on p-ERK1/2, cell viability, and cell cycle in ALL cell lines were markedly reversed after culturing with bFGF (9 ng/mL) for 24 h. After incubating with bFGF, cells with HOXB7 inhibition were treated with a specific ERK1/2 inhibitor, PD98095, after which the effects of bFGF on protein expression of p27, cell viability, and cell cycle were obviously reversed.

Significance: Our study suggests that inhibiting HOXB7 suppresses p27-mediated ALL progression by regulating bFGF/ERK1/2.

1. Introduction

Acute lymphoblastic leukemia (ALL) is a malignant blood disease characterized by abnormal proliferation of immature lymphocytes in the bone marrow, peripheral blood, and other tissues [1,2]. Its biological characteristics and clinical heterogeneity are diverse [3]. Multidrug chemotherapy can induce complete remission in more than 80% of newly diagnosed adults with ALL, but the 5-year disease-free survival of patients with ALL is only 30% to 40% [4]. A better understanding of the molecular mechanisms of ALL is crucial for improving the treatment of acute leukemia.

The HOXB7 gene is a HOXB cluster in the homologous box gene HOX family [5,6]. It is believed that HOXB7 is a special type of transcriptional regulator that regulates the development and differentiation of embryonic cells, and its overexpression may promote certain tumors [7–9]. HOXB7 is highly expressed in breast, pancreatic, and lung cancers, and it is associated with tumor cell proliferation, differentiation, invasion, and angiogenesis [6,10–13]. It influences cell proliferation, cell cycle, and apoptosis in cancers such as lung cancer and gastric cancer [14–16]. HOXB7 also has been indicated to be overexpressed in leukemia [17]. However, the molecular mechanism and function of HOXB7 in ALL remain unclear. In our study, we aimed to investigate the molecular mechanism of HOXB7 in ALL, which could be used to improve treatment of the disease.

The p27 protein encoded by the tumor suppressor gene p27 binds to cyclin E/cyclin-dependent kinase2 in the nucleus and inhibits its activity [18]. P27 could prevent cells in the G1 phase from entering into the S phase by inhibiting cell growth and proliferation [19,20]. A

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decrease in p27 is closely related to tumorigenesis, tumor progression, and poor prognosis [21,22]. Liao et al. demonstrated that HOXB7 can downregulate p27, regulate cell cycle, and increase proliferation in colon cancer [23]. Its effect on p27 in ALL remains unclear. Thus, we explore whether and how HOXB7 regulates p27 in ALL.

Basic fibroblast growth factor (bFGF) is widely distributed in the heart, brain, liver, kidney, and other tissues [24]. Previous studies have shown that HOXB7 can regulate bFGF in breast cancer and that bFGF controls p27 by activating ERK1/2 in the Müller cell line [25,26]. As a strong mitogen that promotes the proliferation and differentiation of various cells, bFGF is highly expressed in certain dysplasia and tumor tissues [27,28] and upregulated in ALL [29]. We propose that HOXB7 can regulate cell proliferation and cycle in ALL by controlling bFGF, ERK1/2, and p27.

We aimed to explore the molecular mechanisms of HOXB7 in ALL by detecting expression of HOXB7 in leukomonocytes from blood samples of patients with ALL. We tested cell proliferation and cell cycle in ALL cell lines after suppressing HOXB7. We also investigated the molecular mechanism underlying HOXB7's suppression of cell cycle and proliferation in ALL.

2. Materials and methods

2.1. Peripheral blood samples and cell lines

The study comprised 20 (male, n = 12; female, n = 8) cases of untreated ALL. Patient ages ranged from 5 to 23 years, with an average age of 9 years. Pathological diagnoses were in line with the FAB diagnostic criteria. Control peripheral blood samples (n = 20) were collected from patients without ALL. The patients were along with written informed consent. This research was approved by the ethics committee at Zhumadian Central Hospital.

The human leukemia cell lines CCRF-CEM (T-cell, ALL) and Nalm-6 (B cell precursor, ALL) were purchased from ATCC (Rockville, MD, USA) and DSMZ (Braunschweig, Germany), respectively. The two cell lines were cultured in RPMI 1640 (Gibco, Rockville, MD, USA) with 10% fetal bovine serum at 37 °C in a 5% CO_2 atmosphere.

2.2. Isolation of peripheral blood lymphocytes

Isopycnic Ficoll-Hypaque solution (Amersham Pharmacia Biotech, Uppsala, Sweden) was mixed with the venous blood (15 mL). Next, the mixture was centrifuged at 1500 rpm for 30 min at room temperature. The resulting middle layer contained the lymphocytes, which were isolated for subsequent peripheral blood lymphocytes testing.

2.3. Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from the cells and reverse transcribed into cDNA, which was used as the template in the real-time quantitative polymerase chain reaction. GAPDH was the reference gene. Then, 10 μL of SYBR Green I Premix (Takara Biotechnology, Dalian, China) was added to the 25-μL reaction system. The reaction program was as follows: 94 °C for 30 s, 40 cycles at 94 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s. The primers were HOBX7, forward 5′-ACC GAC ACT AAA ACG TCC CTG-3′ and reverse 5′-TTT GTT CTG GGA AGG CTC CG-3′ and GAPDH, forward 5′-GTC TGG CGT TTT TGG ATG TT -3′ and reverse 5′-CGT CTT CAC CAC CAT GGA GA-3′.

2.4. Western blot

Cells were lysed in cell lysis buffer (Beyotime, Haimen, China), and the protein was extracted. The protein was quantified using a BCA kit (Beyotime). Next, the protein (25 μg) was loaded and separated in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to the polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). The membranes were blocked in 2% non-fat dry milk at room temperature for 2 h. After washing in tris-buffered saline, the membranes were incubated in the following primary antibodies: anti-HOXB7 (1:500), anti-bFGF (1:700), anti-ERK1/2 (1:500), anti-p27 (1:800), and anti-GAPDH (1:1000) (Abcam Inc., Cambridge, MA, USA), followed by incubation in horseradish peroxidase–conjugated secondary antibody (1:1000) (Abcam) for 1 h at room temperature. Finally, protein bands were visualized using an enhanced chemiluminescence kit (Amersham, Little Chalfont, UK). GAPDH was the reference protein.

2.5. Cell transfection

The ALL cell lines were separately plated in 12-well plates and cultured in the incubator (Thermo Fisher Scientific, Waltham, MA, USA) for 24 h. The cells were transfected with HOXB7 siRNA (5′-GCU AUUGUAAGGUCUUUGUTT-3′, 2 μg) or non-specific siRNA (2 μg) using Turbofect (Thermo Fisher Scientific) and incubated at 37 °C in 5% CO_2 for 24 h. Transfection efficiency was measured by RT-qPCR and Western blot assays.

2.6. Cell growth and vitality assay

Cell growth and viability were measured by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the standard instructions. Cells were plated in 96-well plates and cultured at 37 °C for 24 h. The culture medium was then changed to 20 μL of MTT solution (5 mg/mL of phosphate-buffered solution) and incubated for 5 h. Then, 150 μL of dimethyl sulfoxide per well was added to dissolve the formazan. Results were read using a microplate reader (Thermo Fisher Scientific) at 490 nm.

2.7. Bromodeoxyuridine assay

Cell proliferation was tested by using a bromodeoxyuridine (BrdU) cell proliferation assay kit (Cell Signaling Technology, Danvers, MA, USA). Briefly, the cells were seeded in 96-well plates and cultured with BrdU solution (12 μL) at 37 °C for 1 h. After discarding the culture medium, a denaturing solution (180 μL) was added and incubated for 30 min. Next, cells were incubated with anti-BrdU conjugated with peroxidase for 30 min. The optical density at 450 nm was measured using a SpectroFluor Plus multi-well plate reader (Tecan, Research Triangle Park, NC, USA).

2.8. Cell cycle detection

Cells were dissociated using trypsin and centrifuged for harvesting. Cells were resuspended in 75% ethanol and fixed overnight at −20 °C. Phosphate-buffered solution was used to wash cells twice, and each sample was resuspended in 450 μL of phosphate-buffered solution with 50 μL propidium iodide (1 μg/mL). After incubating at 37 °C for 30 min, cell cycle was measured and analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA).

2.9. Statistical analysis

Differences were determined by *t*-test for two groups or one-way ANOVA followed by Bonferroni's test for multiple groups. For each analysis, $P < .05$ was recognized as statistically significant. Data are expressed as mean ± standard deviation (SD).

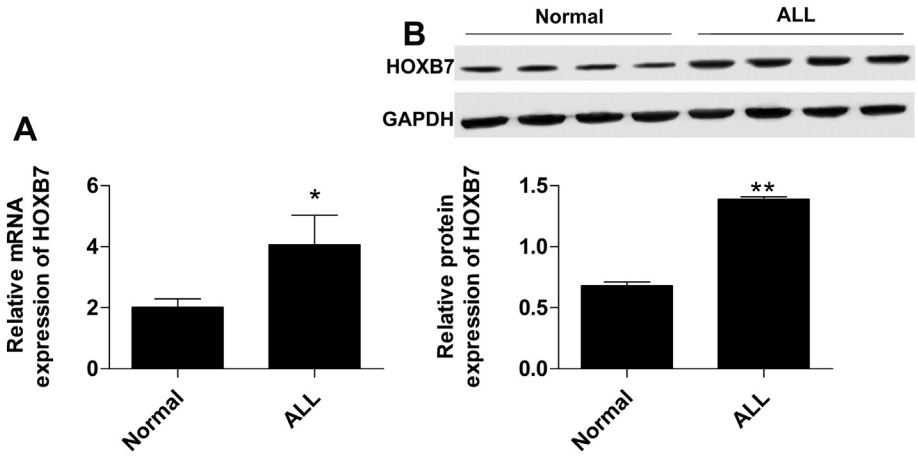

Fig. 1. HOXB7 expression in peripheral blood lymphocytes of patients with acute lymphoblastic leukemia, compared to normal group. (A) The mRNA expression of HOXB7 was measured by real-time quantitative polymerase chain reaction. (B) The protein expression of HOXB7 was measured by Western blot. Normal, lymphocytes collected from blood samples of patients without ALL; ALL, lymphocytes collected from blood samples of patients with untreated acute lymphoblastic leukemia; $^{*}P < .05$ versus the normal group.

3. Results

3.1. HOXB7 is increased in peripheral blood lymphocytes of patients with acute lymphoblastic leukemia

To explore the expression of HOXB7 in ALL, we tested the mRNA (Fig. 1A) and protein (Fig. 1B) expressions of HOXB7 in peripheral blood lymphocytes of patients with ALL and found that it was twice that of the normal group.

3.2. HOXB7 inhibition constrains cell viability and proliferation

To investigate the effect of HOXB7 on ALL, we measured the cell viability and proliferation of the ALL cell lines CCRF-CEM and Nalm-6 with HOXB7 inhibition, using MTT and BrdU assays, respectively. First, the mRNA (Fig. 2A) and protein (Fig. 2B) expressions of HOXB7 were markedly decreased in the loss-of-function experiment of HOXB7, indicating that HOXB7 inhibition was successful. Moreover, the results demonstrated that cell viability (Fig. 2C) and proliferation (Fig. 2D) in the HOXB7 siRNA group was significantly decreased, compared to the non-spe siRNA group.

3.3. HOXB7 inhibition induces cell cycle arrest and p27 expression

To determine whether the growth inhibitory effect of HOXB7 was caused by cell cycle arrest, we assessed cell cycle in ALL cell lines with HOXB7 inhibition using flow cytometry. Data indicated that the HOXB7 suppression obviously accumulated G1 phase of the cell cycle in ALL

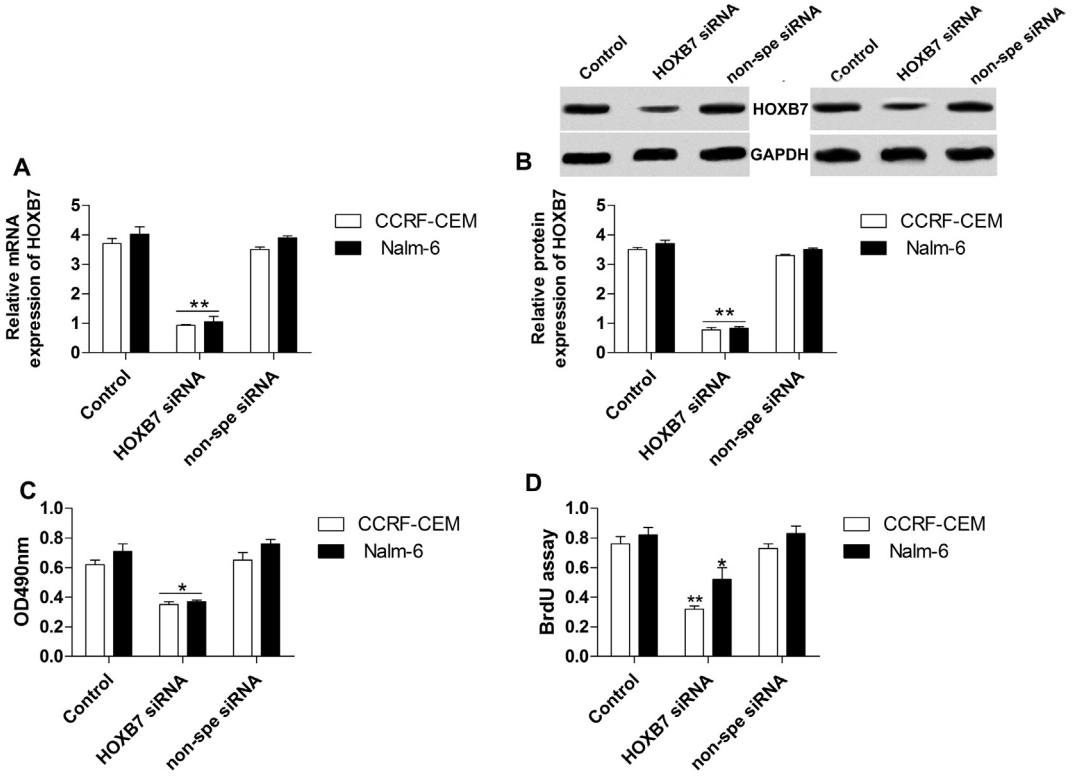

Fig. 2. Effect of HOXB7 inhibition on cell viability and proliferation. The relative HOXB7 mRNA (A) and protein (B. left: CCRF-CEM, right: Nalm-6) levels after cell transfection were tested using real-time quantitative polymerase chain reaction and Western blot, respectively. Cell viability (C) and proliferation (D) were measured by MTT and BrdU assays, respectively. Control, untreated acute lymphoblastic leukemia (ALL) cells; HOXB7 siRNA, ALL cells transfected with HOXB7 siRNA; non-spe siRNA, ALL cells transfected with non-specific siRNA. N = 3 for each group. $^{*}P < .05$ and $^{**}P < .01$ for the HOXB7 siRNA group versus the non-spe siRNA group.

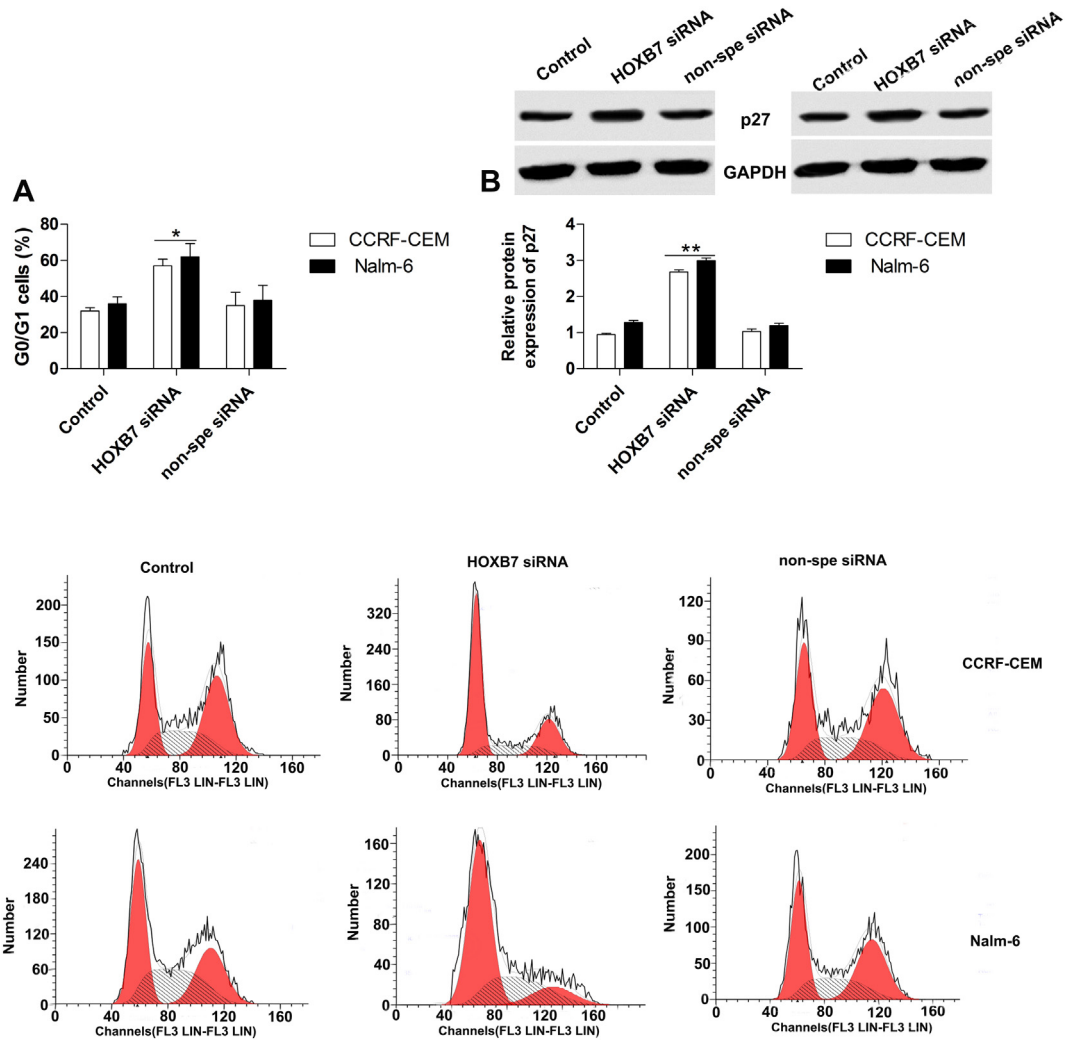

Fig. 3. Effects of HOXB7 inhibition on cell cycle and p27 expression in ALL cell lines. Cell cycle (A) was assessed using flow cytometry after HOXB7 inhibition in ALL cells lines. Relative protein expression of p27 was detected by Western blot in ALL cell lines (B. (left: CCRF-CEM, right: Nalm)). N = 3 for each group. $^{*}P < .05$ and $^{**}P < .01$ for the HOXB7 siRNA group versus the non-spe siRNA group.

cell lines (Fig. 3A). Also, p27 was elevated under HOXB7 suppression. The protein expression of p27 was markedly improved by HOXB7 downregulation (Fig. 3B).

3.4. HOXB7 regulates cell viability and cell cycle via bFGF and ERK1/2 signaling

It was reported that bFGF can control p27 by regulating phosphorylated ERK1/2 and that HOXB7 can regulate bFGF [25,26]. Thus, we hypothesized that HOXB7 could regulate bFGF/ERK1/2 signaling in ALL cell lines. We found that HOXB7 downregulation significantly restrained the protein expression of bFGF and p-ERK1/2 in ALL cell lines (Fig. 4A). Next, the cell lines with HOXB7 inhibition were treated with bFGF (9 ng/mL) for 24 h, and the results showed that ERK1/2 phosphorylation levels were increased by one time (Fig. 4B). Meanwhile, cell viability (Fig. 4C) increased and cell cycle arrest (Fig. 4D) arrested following treatment of bFGF in ALL cell lines with HOXB7 suppression. Thus, HOXB7 could regulate bFGF/ERK1/2 signaling and then affect cell viability and cell cycle in ALL cell lines.

3.5. HOXB7 controls p27 by regulating bFGF and ERK1/2 signaling

We found that HOXB7 could regulate bFGF and ERK1/2 signaling and affect p27. Other studies have indicated that p-ERK1/2 controls p27. Therefore, we supposed that HOXB7 could affect p27 by regulating bFGF and ERK1/2 signaling. The ALL cell lines with HOXB7 inhibition were incubated with bFGF at 9 ng/mL for 10 h and then 60 μmoL of PD98095 (specific ERK1/2 inhibitor) or DMSO (control) for 7 h. The results indicated that protein expression of p27 in the HOXB7 si-bFGF-PD group was more than twice that of the HOXB7 si-bFGF-DM group (Fig. 5A). Moreover, cell viability (Fig. 5B) decreased and cell cycle arrest improved (Fig. 5C) in the HOXB7 si-bFGF-PD group, compared to HOXB7 si-bFGF-DM group. These findings indicate that HOXB7 controls p27 by modulating bFGF and ERK1/2 signaling, thus regulating cell viability and cell cycle in ALL cell lines.

4. Discussion

The clinical cure rate for ALL has been significantly improved with the application of bone marrow transplantation and multi-drug combination therapy. Unfortunately, some patients respond poorly to treatment [30]. The HOX gene family is mainly responsible for regulating spine and human embryonic organ development [31]. As the main gene that regulates cell proliferation and differentiation, HOXB7 is closely related to tumor progression and metastasis [32]. Kovochich et al. find that HOXB7 is overexpressed and induces cell survival and invasion in pancreatic adenocarcinoma [13]. HOXB7 also is upregulated in melanoma and colon cancer [33].

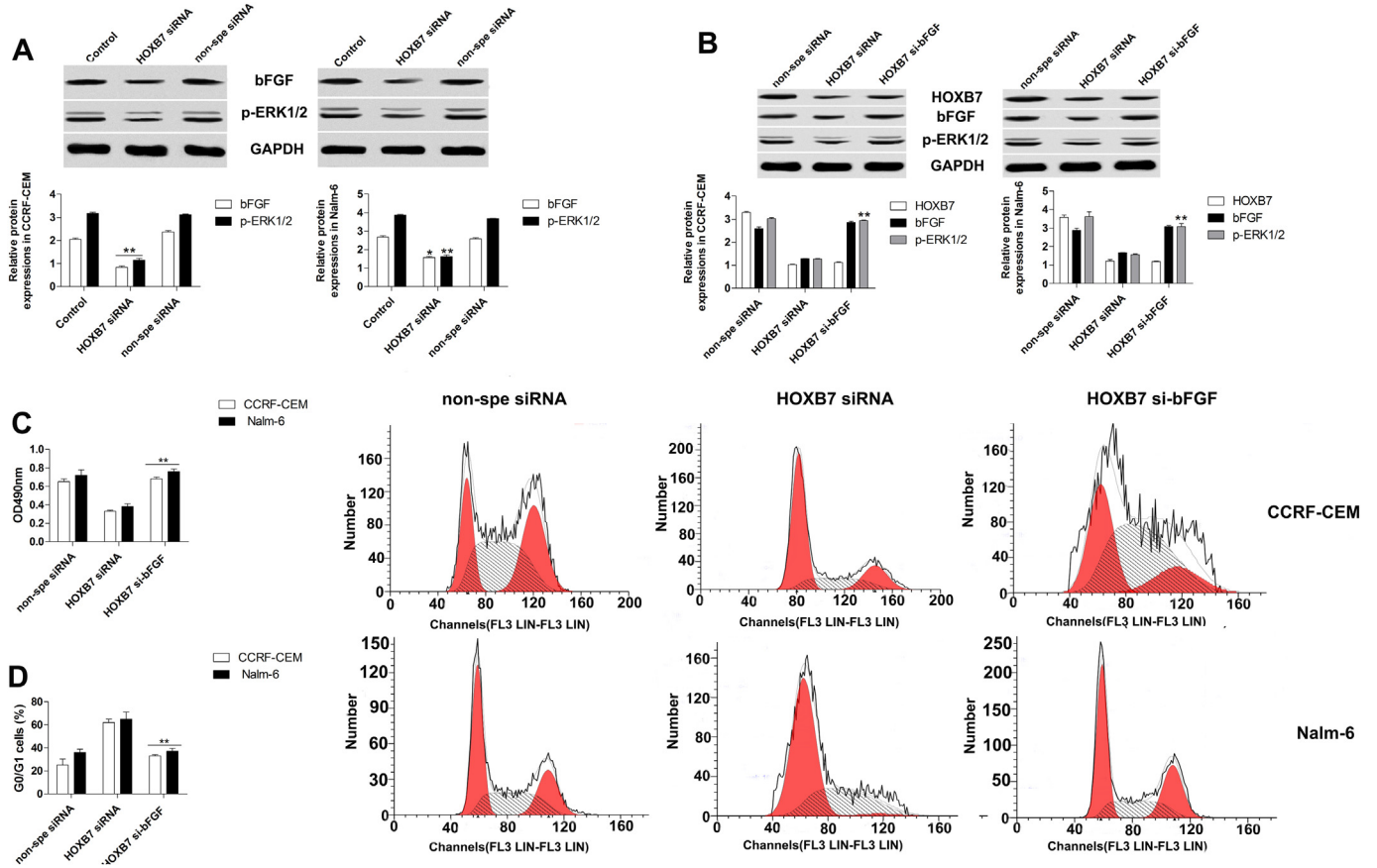

Fig. 4. Effects of HOXB7 on bFGF and ERK1/2 signaling, cell viability, and cell cycle in ALL cell lines. The relative protein levels (A) of bFGF and p-ERK1/2 after HOXB7 inhibition were tested using Western blot analysis in ALL cell lines. N = 3 for each group. $^{*}P < .05$ and $^{**}P < .01$ for the HOXB7 siRNA group versus the non-specific siRNA group. The relative protein levels (B) after incubation with bFGF were tested using Western blot analysis in ALL cell lines with HOXB7 inhibition. Cell viability (C) and cell cycle (D) were assessed by MTT and by flow cytometry, respectively. N = 3 for each group. $^{**}P < .01$ for the HOXB7 si-bFGF group versus the HOXB7 siRNA group. HOXB7 si-bFGF, ALL cell lines with bFGF incubation after HOXB7 inhibition.

In our study, HOXB7 expression was significantly increased in the peripheral blood lymphocytes of ALL patients, which is consistent with studies by Kovochich et al. [13] and others in different cancers [10,34]. For example, HOXB7 promotes cell proliferation and cell cycle and is related to poor prognosis in gastric cancer [35]. HOXB7 suppression by miR-376c-3p could control proliferation, migration, cell cycle, and apoptosis in human oral squamous cancer cells [36]. Similarly, we found that HOXB7 inhibition effectively suppressed cell viability, proliferation, and cell cycle in ALL cell lines.

The cyclin-dependent protein kinase inhibitor p27 is an important negative regulator of cell cycle [37]. P27 inhibits cell proliferation and tumor formation and is recognized as a tumor suppressor gene [38]. Zhao et al. elucidated that a decrease in p27 confers resistance to anti-HER2-targeted therapy in breast cancer [39]. Lu et al. proposed that nuclear retention of p27 caused by Nrdp1 suppresses growth of colorectal cancer cells [40]. Agarwal et al. have indicated that reduced p27 promotes leukemia [41]. Furthermore, p27 has been reported to be modulated by HOXB7 and to regulate cell cycle and proliferation in colon cancer [23]. In our research, p27 expression was markedly constrained in ALL cell lines by HOXB7 inhibition, leading to suppressed cells viability, proliferation, and cell cycle.

The growth factor bFGF has several functions to promote wound healing, tissue repair, vascular proliferation, and embryonic development and differentiation [42]. It is an important regulator of cell proliferation, cell differentiation, apoptosis, and the synthesis and degradation of the extracellular matrix [43]. bFGF may induce human pluripotent stem cell survival and proliferation [44]. Kitamura et al. found that bFGF may stimulate endothelial cell proliferation [45]. Additionally, bFGF can accelerate fibroblast differentiation of bone mesenchymal stem cells to promote tumor growth [46]. It is indicated to be a transcriptional target of HOXB7 in epithelial cells [47] and to control p27 by activating ERK1/2 in the Müller cell line [25].

To explore whether HOXB7 can modulate p27 by regulating bFGF and ERK1/2 in ALL, we performed a loss-of-function experiment of HOXB7 in ALL cell lines. Results demonstrated that HOXB7 inhibition significantly reduced the protein levels of bFGF and p-ERK1/2 and constrained cell viability, proliferation, and cell cycle. Incubation with bFGF of ALL cell lines with HOXB7 inhibition abolished the inhibitory effect of HOXB7 on p-ERK1/2, cell viability, proliferation, and cell cycle. These findings suggest that HOXB7 regulates cell viability, proliferation, and cell cycle via bFGF/ERK1/2 signaling. Furthermore, p27 was upregulated and cell viability, proliferation, and cell cycle were inhibited when PD98095 was used to inhibit ERK1/2 in ALL cell lines with HOXB7 suppression and bFGF incubation. Therefore, inhibiting HOXB7 may suppress cell viability, proliferation, and cell cycle by regulating bFGF, ERK1/2, and p27 in ALL cell lines.

In brief, this study revealed that HOXB7 is induced in the peripheral blood lymphocytes of ALL patients and that HOXB7 inhibition upregulated p27 and constrained cell viability, proliferation, and cell cycle in ALL lines. Additionally, HOXB7 inhibition suppressed p27-mediated ALL progression by regulating bFGF and ERK1/2. Our study reveals the unique role of the HOXB7 in regulating p27-mediated ALL progression and thus provides novel insight into developing a potential therapeutic strategy for the treatment of ALL.

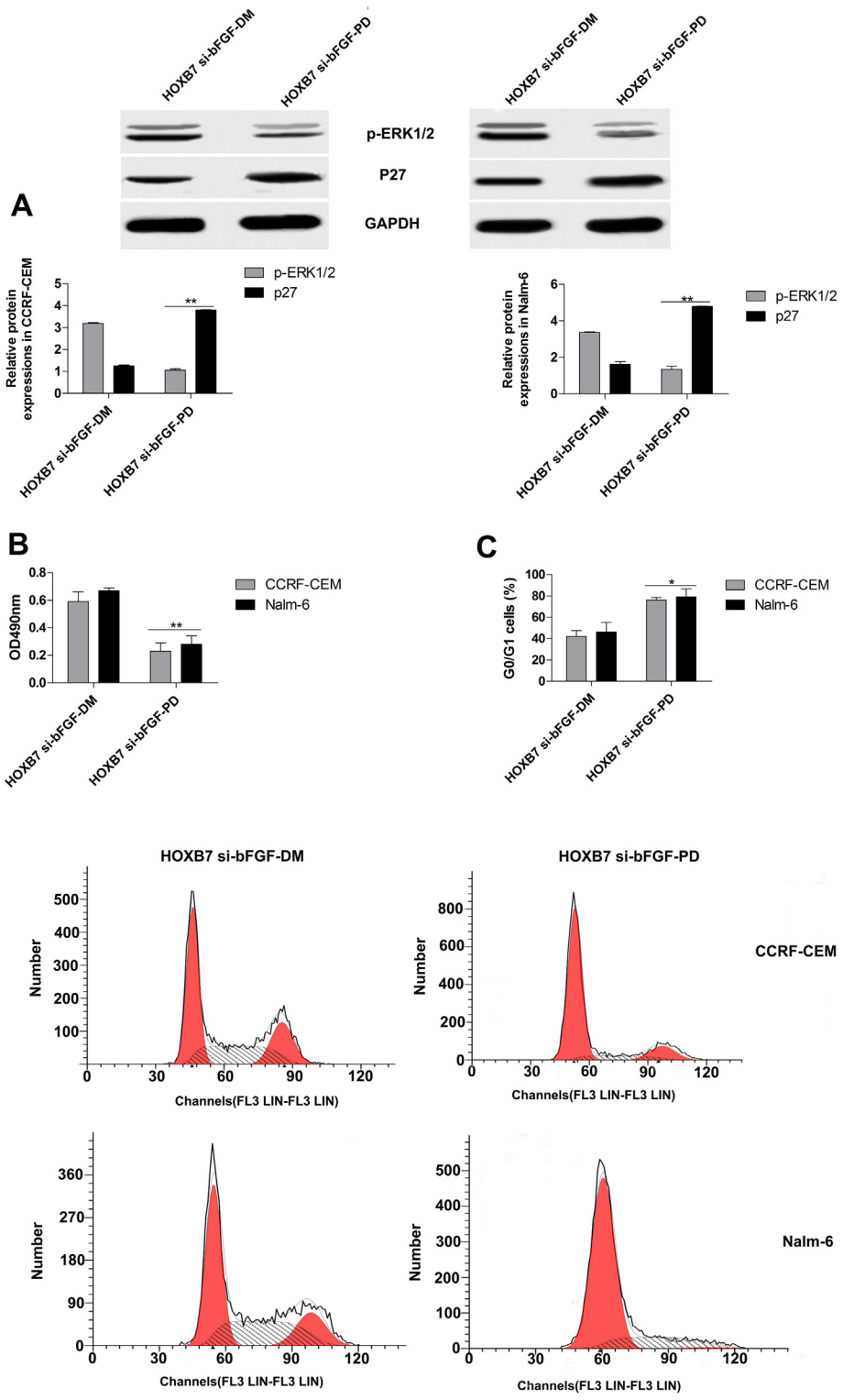

Fig. 5. HOXB7 controls p27 by regulating bFGF and ERK1/2 signaling. The relative protein levels (A) of p-ERK1/2 and p27 after bFGF and PD98095 incubations were tested using Western blot analysis in ALL cell lines with HOXB7 inhibition. Cell viability (B) and cell cycle (C) were assessed by MTT and by flow cytometry, respectively. HOXB7 si-bFGF-DM, ALL cell lines with bFGF and DMSO incubations after HOXB7 inhibition. HOXB7 si-bFGF-PD, ALL cell lines with bFGF and PD98095 incubations after HOXB7 inhibition. N = 3 for each group. $^{*}P < .05$ and $^{**}P < .01$ for the HOXB7 si-bFGF-PD group versus the HOXB7 si-bFGF-DM group.

5. Conclusion

HOXB7 may be an important regulator of p27-mediated ALL progression. Our data indicated that HOXB7 inhibition suppressed p27-mediated ALL progression by regulating bFGF and ERK1/2. We define a novel molecular mechanism underlying ALL progression.

Abbreviations

ALL	acute lymphoblastic leukemia
bFGF	basic fibroblast growth factor
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

BrdU	bromodeoxyuridine
RT-qPCR	real-time quantitative polymerase chain reaction
PVDF	polyvinylidene fluoride
SD	standard deviation

Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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None.

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