EXT1, Regulated by MiR-665, Promotes Cell Apoptosis via ERK1/2 Signaling Pathway in Acute Lymphoblastic Leukemia

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: EXT1 is an endoplasmic reticulum-resident glycosyl transferase whose intracellular expression alters the biosynthesis and distribution of heparan sulfate. EXT1 is regarded as a classic tumor suppressor. MiR-665 can act as either an oncogene or tumor-suppressing gene in different tumors. The aim of the current study was to determine the function and molecular mechanisms of EXT1 and miR-665 in acute lymphoblastic leukemia (ALL).

Material/Methods: EXT1 expression in ALL was evaluated by real-time polymerase chain reaction (RT-PCR) and western blotting. The effects of EXT1 in ALL were explored by Cell Counting Kit-8 (CCK-8)/EdU assays, western blotting, flow cytometry, and in vivo tumorigenesis assays. Label-free quantification was used to detect differentially expressed proteins in EXT1-overexpressing Reh cells.

Results: EXT1 expression is downregulated in ALL and negatively correlated with miR-665 expression. Moreover, low EXT1 and high miR-665 expression levels in adult ALL bone marrow tissues are correlated with poor patient survival. Our study showed that EXT1 modulates the proliferation and apoptosis of ALL cells in vitro and in vivo and that miR-665 promotes cell growth and inhibits apoptosis by suppressing EXT1. EXT1 promotes cell apoptosis via deactivating the ERK1/2 pathway.

Conclusions: In conclusion, this study is the first to confirm the association between low EXT1 levels and several clinical features of ALL. Low bone marrow EXT1 levels independently predict poor prognoses in adult ALL patients. Thus, our study suggests that EXT1- or miR-665-targeted strategies can confer the therapeutic effect of promoting apoptosis by deactivating the ERK1/2 pathway.

MeSH Keywords: Apoptosis • Leukemia, Biphenotypic, Acute • MAP Kinase Signaling System • MicroRNAs

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/918295

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Background

Acute lymphoblastic leukemia (ALL) is a malignant neoplastic disease involving B or T lymphocytes in the bone marrow (BM). Abnormal hyperplasia can cause the original cells to accumulate in the BM and inhibit normal hematopoietic function. In addition, these cells can invade tissues outside the BM, such as the meninges, lymph nodes, gonads, and liver. The incidence of leukemia in China has been investigated, and the prevalence of ALL is approximately 0.67 per 100 000 persons. The peak age of disease onset occurs in childhood (0 to 9 years old), and approximately 80% of children are curable. The incidence of ALL in adults accounts for 20% of the total incidence; however, the therapeutic effect is significantly lower in adults than in children. Although multidrug combination chemotherapy can result in complete remission (CR) in more than 80% of adult ALL patients, more than 50% of patients eventually relapse, and the 5-year disease-free survival (DFS) rate is only 30% to 40% [1–3]. Currently, with the development of bioinformatics, improvements in gene sequencing technology [4], and in-depth studies on the molecular biology and pathogenesis of ALL [5,6], we are moving toward an era of individualized and precise treatment for ALL.

The EXT1 gene is located on chromosome 8q24.1 and contains 11 exons, spanning approximately 350 kb of genomic DNA [7]. EXT1 mRNA is expressed ubiquitously, especially in the liver. EXT1 is an endoplasmic reticulum-resident glycosyltransferase whose expression in cells alters the biosynthesis and distribution of cell surface heparan sulfate (HS) [8]. Germline mutations in EXT1 are responsible for hereditary multiple exostoses (HME), a disease characterized by the formation of cartilage-capped tumors (exostoses) that develop from the growth plates of endochondral bones [9]. Moreover, EXT1 is regarded as a classical tumor suppressor [10–12]. Daakour et al. [13] analyzed mutated genes found in ALL samples collected in the Sanger COSMIC database and found that EXT-1 might act as a tumor suppressor in ALL. However, the expression and mechanism of EXT1 in ALL are not clearly explained.

MicroRNAs (miRNAs) are a class of noncoding single-stranded RNAs containing 19 to 25 nucleotides; miRNAs are encoded by endogenous genes [14] and participate in different types of physiological and pathological processes [15]. Notably, miRNAs play an important role in the pathogenesis and progression of tumors [16]. Evidence indicates that miRNAs can be either oncogenes or tumor suppressors [17–19]. The role of miRNA in ALL has been extensively reported and studied. For example, MIR335 targeting MAPK1 is reduced, and MIR335 could thus be a biomarker closely related to ALL prognosis [20]. MiR-100, miR-196b, and let-7e were shown to be downregulated in childhood ALL BM samples compared with normal samples, but miR-128a and miR-181b were overexpressed [21].

MiRNAs that regulate EXT1 expression have been found in astrocytoma [22] and osteosarcoma [23]. To the best of our knowledge, this study is the first to demonstrated that EXT1 acts as a tumor suppressor regulated by miR-665 in ALL.

Material and Methods

Patients and clinical samples

This study was approved by the Institutional Review Board and the Ethics Committees of Sun Yat-sen University Cancer Center. All 70 patients were newly diagnosed with adult ALL. Before treatment, written informed consent to obtain BM samples and medical information was obtained from the normal control group (20 normal BM samples from patients with non-hematological malignancies) and the ALL patients. Our research was based on the guidelines of the Declaration of Helsinki.

Cell culture and reagents

Reh, BALL-1, Jurkat, MOLT-4, and CCRF cell lines were purchased from ATCC (Manassas, VA, USA) and cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) at 37°C with 5%CO_2. We changed the medium every 2 to 3 days. Antibodies were obtained from the following sources: EXT1 (sc-515144) (Santa Cruz Biotechnology, USA), GAPDH, ERK1/2, and pERK1/2, Bax, Bcl-2 (Proteintech, Wuhan, China). PD98059 (Selleck Chemicals, Houston, USA) was used as the ERK1/2 inhibitor. Hsa-miR-665 inhibitor was chemically synthesized by GeneCopoeia (Guangzhou, China) and its sequence was as follows: UGGUCCUCCGACUCCGGGGA.

Real-time polymerase chain reaction (RT-PCR)

TRIzol reagent (Thermo Fisher Scientific, USA) was used to extract total RNA. RNA was purified with a total RNA isolation kit (Thermo Fisher Scientific, USA). The synthesis of complementary DNA (cDNA) from total RNA (100 ng of mRNA) was performed using SYBR® qPCR RT Master Mix (TOYOBO, Japan) and qPCR was then performed using a SYBR® qPCR Mix (TOYOBO, Japan). The following primer sequences were used:
EXT1 sense: 5'-GGCAAAAGCACAAGGATTCTCGC-3',
antisense: 5'-CTGCAAAGCCTCCAGGAATCTG-3';
GAPDH sense: 5'-GTCTCCTCTGACTTCAACAGCG -3',
antisense: 5'-ACCACCCTGTTGCTGTAGCCAA-3';
hsa-miR-665 (HmiRQP0778, GeneCopoeia, USA).

Western blotting

Cells were lysed in RIPA buffer (KGP2100, keyGEN, China). The extracted proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then

transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with primary antibodies in 5% BSA (bovine serum albumin), followed by incubation with secondary antibodies (Proteintech, Wuhan, China). Immunoreactive bands were detected by using electrochemiluminescence (ECL) (Pierce, Hercules, CA, USA).

Lentiviral production and infection

The EXT1-overexpressed, the control CON238, the EXT1-sh1, the EXT1-sh2, and the control CON313 lentiviruses were constructed by GENECHEM (Shanghai, China), and then transferred into the Reh or BALL-1 cell lines. After expansion and maintenance, the infected cells were positively selected with puromycin. The efficiency of transfection was tested by western blotting analysis. The EXT1-sh1 sequence: GCACTTAGACAGCAGACACAA the EXT1-sh2 sequence: CAGCGACAGAGCTGCATGAAT.

Cell proliferation assays

Cell Counting Kit-8 (CCK-8, Tong Ren, Japan) assay can be used to detect cell viability. Cells were seeded in a density of 1×10^4 cells/well in 96-well multi-plates and different treatments were performed prior to CCK-8 solution. The absorbance values were detected at 450 nm using a microplate reader. An iClick™ EdU Andy Fluor™ 488 Flow Cytometry Assay Kit (GeneCopoeia, Guangzhou, China) was used to characterize the proliferating cells by flow cytometry.

Cell apoptosis assay

The apoptotic cells were measured by fluorescein isothiocyanate (FITC)-Annexin V and PI Apoptosis Kit (GeneCopoeia, Guangzhou, China). According to the manufacturer's instruction, each 100 μL of cell suspension washed by chilled phosphate-buffered saline (PBS) was added in 5 μL of FITC-annexin V and 2 μL 100 μg/mL of propidium iodide (PI), then incubated at room temperature for 15 minutes in the dark and finally analyzed by flow cytometry. The apoptosis cells in flow cytometry were defined as annexin V positivity cells.

Luciferase assay

Luciferase reporter constructs containing the wild-type (WT) or mutant-type (MT) in the 3'untranslated regions (3' UTR) of EXT1 gene were generated using the miRNA 3'UTR target clones (Promega, Madison, WI, USA). A total of 30 000 cells were seeded into 24-well culture plates in phenol red-free medium. After transfection with the plasmids for 48 hours using a Lipofectamine 3000 kit (Thermo Fisher Scientific, USA), the luciferase activity was determined by a Luc-Pair™ Duo-Luciferase HS Assay Kit (GeneCopoeia, Guangzhou, China) according to the manufacturer's instructions. The pre-miR-665

sequence: TCTCCTCGAGGGGTCTCTGCCTCTACCCAGGAC TCTTTCATGACCAGGAGGCTGAGGCCCCTCACAGGCGGC. The WT sequences in the 3' UTR of EXT1 gene was: GCTAGCGGAATCCGGCTGAGTGGGGGAGGGGAAGCAAGAAGGG ATGGGGGTCAAGCTGCTCTCTCTTCCCAGTGCAGATCCACTCATCAGCA GAGCCAGATTGTGCCAACTATCCAAAAACTTAGATGAGCA GAATGACAAAAAAAAAAAAGGCCAATGAGAACTCAACTCCTGG CTCCTG GGACTGCACCAGACTGCTCCAAACTCACCTCACTGGCTTCTGT GTCCCAAGACTAGGTTGTGTACAGTTTAATTATG GAACATTAAATAATT ATTTTTGAAATGATTGCTATGCAGGTTTAAACTTTTTTAATGA TCAAAACTATTAAAAACCAGAGTTCTTTGTTTAATCAGTCGAC. The MT sequences in the 3' UTR of EXT1 gene was: GCTAGCGGAATCC GGCTGAGTGGGGGAGGGGAAGCAAGAAGGGATGGGGGTCA AGCTGCTCTCTCTTCCCAGTGCAGATCCACTCATCAGCAGAGCCAGA TTGTGCCAACTATCCAAAAACTTAGATGAGCAGAATGACA AAAAAAAAAAAGGCCAAACTCTTGAGTTGAGGACCGAGGACC CACTGCACCAGACTGCTCCAAACTCACCTCACTGGCTTCTGTGTCC CAAGACTAGGTTGTGTACAGTTTAATTATGGAACATTAAATAATT ATTTTTGAAATGATTGCTATGCAGGTTTAAACTTTTTTAATGATC AAAACTATTAAAAACCAGAGTTCTTTGTTTAATCAGTCGAC.

Label-free quantification proteomics

Proteins were isolated by nano-HPLC reverse-phase chromatography and detected by mass spectrometry Q Exactive (Thermo Fisher Scientific, USA), EASY-nLC1200 (Thermo Fisher Scientific, USA). For label-free quantification, MaxQuant database search software was implemented. Protein-protein comparisons for all results were exported in MPP format from MaxQuant. The resulting feature files for each sample were processed by quantile normalization and principal component analysis (PCA) to assess data quality. Before statistical analysis, the compounds (entities) present in all sample groups were determined by the first filter (frequency analysis). This experiment was conducted on 3 groups of biological replicates.

Animal experiment

Animal experiment was performed following a protocol approved by the Animal Care and Ethics Committee of Sun Yat-sen University. Twenty NOD/SCID mice (4–5 weeks old, 16–22 g) were raised at the animal facility of the Laboratory Animal Center of Sun Yat-sen University. The 4 randomly assigned groups: vector-EXT1, oe-EXT1, sh-control, and shEXT1. Vector-EXT1 Reh, oe-EXT1 Reh, sh-control BALL-1, and shEXT1 BALL-1 were injected subcutaneously into the NOD/SCID mice. The tumor volumes were measured in 3-dimensions (length, width, and height) and calculated as follows: volume=($\pi \times$length$\times$width$\times$height)/6. The tumor xenograft weights were also measured. All mice were sacrificed humanely by CO_2 inhalation prior to death. In all animal studies, all efforts were made to minimize the suffering of the mice.

Statistical analysis

The data are presented as the means±standard deviations. Statistical analysis was performed by one-way ANOVA with Dunnett's post hoc test and a 2-tailed unpaired *t*-test using SPSS 16.0 software. Progression-free survival (PFS) and overall survival (OS) were estimated by the Kaplan-Meier method and were compared between groups with a log-rank test. A Cox regression model was used for multivariate analyses.

Bioinformatics analysis

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were conducted on the DAVID website (*https://david.ncifcrf.gov/*). Protein interaction analysis and presentation was conducted with the STRING database (R-package: STRINGdb).

Results

EXT1 is downregulated in ALL and is negatively correlated with miR-665

By mining the Oncomine database (*https://www.oncomine.org/*), we found that EXT1 expression was significantly downregulated in childhood ALL (87 ALL BM samples compared with 6 normal BM samples, *P*<0.001, Figure 1A), and a similar result was observed for our clinical specimens (70 adult ALL BM samples compared with 20 normal BM samples, Figure 1G, *P*<0.001). RT-PCR analysis verified that EXT1 mRNA expression was downregulated in MOLT-4 and Reh ALL cells compared with that in normal peripheral blood mononuclear cells (PBMC) (Figure 1E). Furthermore, wester n blotting showed that EXT1 protein expression was lower in MOLT-4 an d Reh cells than in normal PBMC (Figure 1C). Western blotting revealed that the protein expression of EXT1 was lower in the 3 adult ALL BM tissues than in the 1 normal tissue (Figure 1D). In summary, these results strongly suggest that EXT1 is downregulated in ALL. We used the online tool DIANA to select 8 miRNAs based on a predicted score of greater than 0.7. MiR-665 was the most significantly upregulated miRNA in both adult ALL BM tissues and ALL cells, as verified by RT-PCR (Figure 1F, 1H). Moreover, we found a strong negative correlation between miR-665 and EXT1 levels (Figure 1B, *P*<0.05, r=−0.4131), suggesting the existence of a regulatory mechanism between miR-665 and EXT1 in ALL.

Low EXT1 and high miR-665 expression levels in adult ALL BM tissues are correlated with poor patient survival

The median follow-up time was 36 months (range 9–84 months). The baseline clinical characteristics (including age, sex, immunophenotype, and risk stratification) for patients

are shown in Table 1. The Kaplan-Meier survival curves suggested that the PFS and OS times were significantly longer for patients with high EXT1 expression than for patients with low EXT1 expression levels (Figure 2A, 2B, *P*<0.001). In contrast, patients with high expression levels of miR-665 had worse prognosis than those with lower miR-665 expression levels (Figure 2C, 2D, *P*<0.001). We analyzed the association between EXT1/miR-665 and the clinicopathological features of adult ALL patients. As shown in Table 2, strong associations were observed between EXT1 expression and risk stratification (*P*=0.029). Furthermore, miR-665 expression was closely related to risk stratification (*P*=0.006). Cox regression analysis revealed that the independent prognostic factors for PFS and OS were the BM EXT1 level and risk stratification (Table 3). Taken together, these results suggest that low EXT1 and high miR-665 expression levels in adult ALL BM tissues are correlated with poor survival.

EXT1 modulates the proliferation and apoptosis of ALL cells

To verify the function of EXT1, we ectopically overexpressed EXT1 in Reh and BALL-1 cells (Figure 3A, 3E). Both EdU and CCK-8 (Figure 3B, 3F) assays revealed that the overexpression of EXT1 markedly inhibited cell proliferation. Meanwhile, we generated EXT1 knockdown BALL-1 ALL cells using EXT1 shRNAs (Figure 3I). EXT1 knockdown markedly increased the proliferation of ALL cells (Figure 3J). Moreover, the overexpression of EXT1 significantly increased cell apoptosis detected by flow cytometry (Figure 3C, 3G) and western blotting (Figure 3D, 3H). Apoptosis in BALL-1 EXT1-shRNA cells was inhibited compared with that in BALL-1 or NC cells (Figure 3K, 3L). To further confirm the role of EXT1 in the growth and survival of ALL cells, we used an ALL xenograft mouse model to assess the effect of EXT1 overexpression or inhibition on tumor growth *in vivo*. EXT1-overexpressing Reh cells or EXT1-knockdown BALL-1 cells were injected subcutaneously into the NOD/SCID mice in the experimental group, and oe-Control Reh cells or sh-Control BALL-1 cells were injected subcutaneously into the mice in the control group. The tumors became visible at the injection site 7 days later. As shown in the growth curves, EXT1 overexpression significantly inhibited tumor growth (Figure 4A), while EXT1 knockdown significantly promoted tumor growth (Figure 4B). At day 35, the tumors were removed and weighed. EXT1 overexpression effectively inhibited the tumor volumes and tumor weights compared with those observed in the control group (Figure 4C, *P*<0.05). We also observed that tumor volume and weight were higher in the EXT1-silenced group than in the control group (Figure 4D, *P*<0.05). These results indicate that EXT1 may play an important role in inhibiting proliferation and promoting apoptosis in ALL cells.
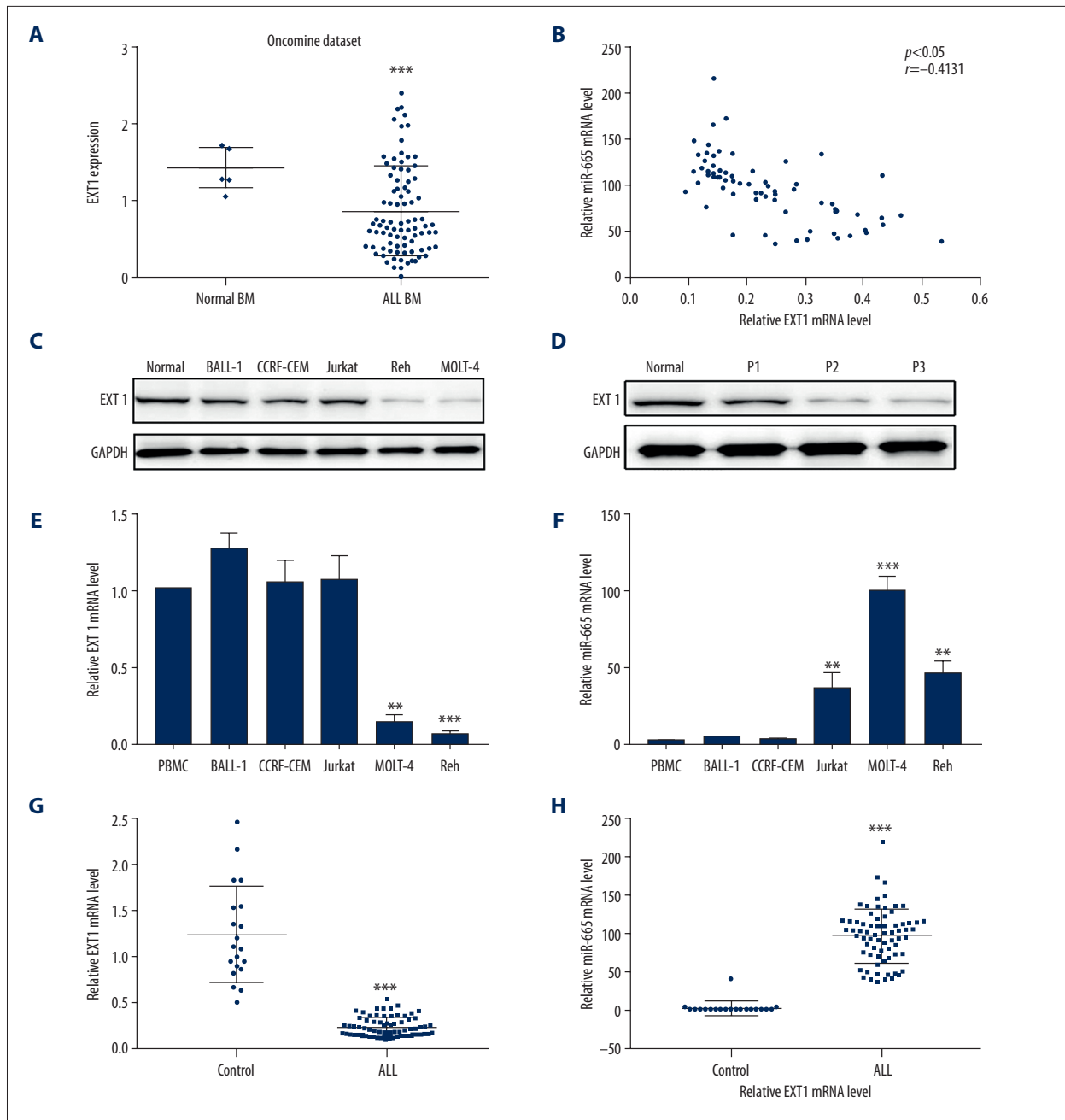
Figure 1. EXT1 is downregulated in ALL and negatively correlated with miR-665. (**A**) EXT1 mRNA expression was frequently downregulated in childhood ALL samples in the Andersson Leukemia Statistics (121 patients with childhood acute leukemia were analyzed on Swegene 27K microarrays and 6 normal bone marrow samples were used as controls) of Oncomine database. We compared 87 childhood ALL with 6 normal bone marrow samples. (**B**) The relationship between the expression of miR-665 and EXT1 in adult ALL. Log-rank (Mantel-Cox), $P<0.05$, r=–0.4131. (**C**) Western blotting of EXT1 expression in normal PBMC and ALL cell lines. (**D**) EXT1 protein expression levels in one healthy person and 3 ALL patients. (**E**) RT-PCR analysis of EXT1 expression in normal PBMC and ALL cell lines. Data represent the mean±SD of 3 independent experiments. (**F**) Real-time PCR analysis of miR-665 expression in normal PBMC and ALL cell lines. Data represent the mean±SD of 3 independent experiments. (**G**) Real-time PCR analysis of EXT1 expression in 70 ALL BM samples and 20 normal BM samples. (**H**) Real-time PCR analysis of miR-665 expression in 70 ALL BM samples and 20 normal BM samples. (The RNA levels were normalized to GAPDH. RNA levels are shown as the mean CT values±SDs. ** $P<0.01$, *** $P<0.001$, compared with the control group.) ALL – acute lymphoblastic leukemia; PBMC – peripheral blood mononuclear cells; RT-PCR – real time polymerase chain reaction; SD – standard deviation; BM – bone marrow.

Table 1. Clinicopathological characteristics of patient samples (N=70).

Characteristics	Number of cases (%)	
Age (years)		
15–39	57	(81.4%)
>40	13	(18.6%)
Gender		
Male	51	(72.9%)
Female	19	(27.1%)
Immunotype		
B	34	(48.6%)
T	36	(51.4%)

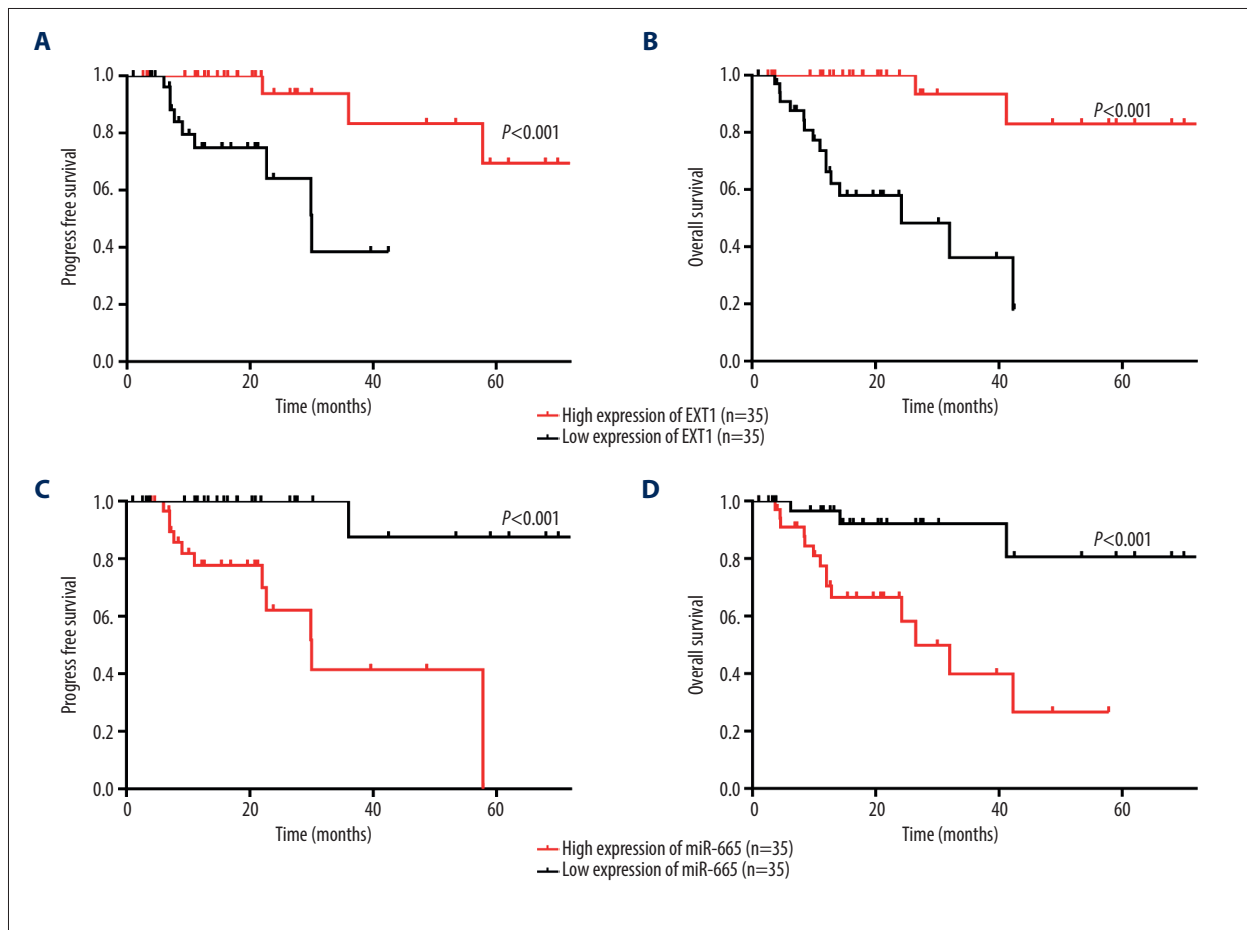| Characteristics	Number of cases (%)	
Risk stratification		
Standard risk	52	(74.3%)
High risk	18	(25.7%)
Serum LDH level		
Normal	16	(22.9%)
High	54	(77.1%)

LDH – lactate dehydrogenase.

Figure 2. Low EXT1 and high miR-665 expression in adult ALL BM correlates with poor patient survival. The PFS and OS times of patients with high EXT1 (**A, B**) or low miR-665 (**C, D**) levels were significantly longer than those of patients with low EXT1 or high miR-665 levels (*P*<0.001). ALL – acute lymphoblastic leukemia; BM – bone marrow; PFS – progression free survival; OS – overall survival.

Table 2. Correlation between EXT1/miR-665 and clinicopathological characteristics of ALL patients (N=70).

Characteristics	miR-665		P value	EXT1		P value
	Low	High		Low	High	
Age (years)						
15–39	26	31	0.124	28	29	0.759
>40	9	4		7	6	
Gender						
Male	27	24	0.42	24	27	0.42
Female	8	11		11	8	
Immunotype						
B	13	21	0.056	21	13	0.056
T	22	14		14	22	
Risk stratification						
Standard risk	30	21	0.006*	21	30	0.029*
High risk	5	14		14	5	
Serum LDH level						
Normal	11	5	0.088	5	11	0.088
High	24	30		30	24	

ALL – acute lymphoblastic leukemia; LDH – lactate dehydrogenase.

Table 3. Multivariate survival analysis in patients with ALL.

Characteristics	PFS			OS		
	Univariate analysis	Multivariate analysis		Univariate analysis	Multivariate analysis	
	P value	RR (95%CI)	P value	P value	RR (95%CI)	P value
Age	0.084			0.02*		
Gender	0.913			0.97		
Immunotype	0.537			0.288		
Risk stratification	0.002*	3.010 (1.060–8.543)	0.038*	0.008*	4.197 (1.247–14.125)	0.021*
Serum LDH level	0.071			0.211		
EXT1	0.002*	0.074 (0.014–0.396)	0.002*	0.001*	0.041 (0.007–0.259)	0.001*

ALL – acute lymphoblastic leukemia; LDH – lactate dehydrogenase; PFS – progression free survival; OS – overall survival; LDH – lactate dehydrogenase.

MiR-665 promotes cell growth and inhibits apoptosis by suppressing EXT1

To further investigate the mechanism underlying the regulation of EXT1 expression, a miR-665 inhibitor was transfected into Reh cells (Figure 5A). EXT1 expression was appreciably upregulated compared with that in the NC cells (Figure 5B).

The proliferation assays showed that miR-665 inhibition significantly attenuated cell proliferation in the Reh cell line (Figure 5C, 5D). Further experiments showed that miR-665 inhibition significantly increased cell apoptosis in the Reh cell line (Figure 5E, 5G). We used TargetScan (*http://www.targetscan.org*) to predict the miR-665 binding site in EXT1 and showed that miR-665 could interact with the conserved 3'UTR
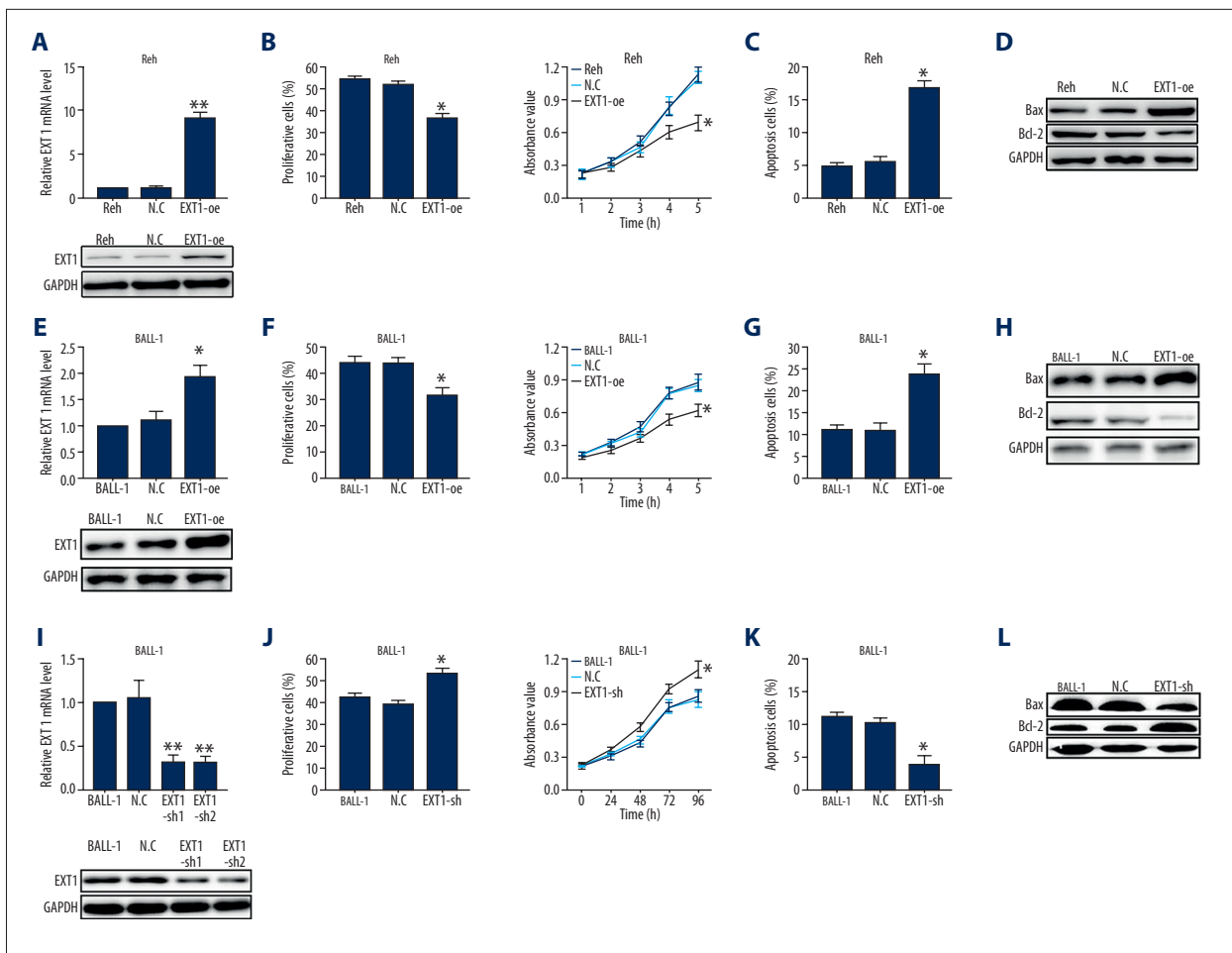
Figure 3. EXT1 modulates cell proliferation and apoptosis of ALL. (**A**) The expression levels of EXT1 were confirmed by RT-PCR (**upper**) and western blotting (**lower**) after the generation of EXT1-overexpressing Reh cells. (**B**) EdU (**left**) and CCK-8 (**right**) assays revealed that overexpression of EXT1 markedly inhibited Reh cell proliferation. Data represent the mean±SD of 3independent experiments. (**C**) The apoptosis of each group of cells was monitored by flow cytometry with FITC-annexin V/propidium iodide (PI) staining; the apoptosis of Reh EXT1-overexpressing cells was increased compared with that of Reh or NC cells. Data represent the mean±SD of 3 independent experiments. (**D**) Apoptosis-related proteins such as Bax were upregulated, and Bcl-2 was downregulated in Reh EXT1-overexpressing cells. (**E**) The expression levels of EXT1 were confirmed by real-time PCR (**upper**) and western blotting (**lower**) after the generation of EXT1-overexpressing BALL-1 cells. (**F**) EdU (**left**) and CCK-8 (**right**) assays revealed that overexpression of EXT1 markedly inhibited BALL-1 cell proliferation. Data represent the mean±SD of 3 independent experiments. (**G**) The apoptosis of each group of cells was monitored by flow cytometry with FITC-annexin V/PI staining; the apoptosis of BALL-1 EXT1-overexpressing cells was increased compared with that of BALL-1 or NC cells. Data represent the mean±SD of 3 independent experiments. (**H**) Apoptosis-related proteins such as Bax were upregulated, and Bcl-2 was downregulated in BALL-1 EXT1-overexpressing cells. (**I**) The expression levels of EXT1 were confirmed by real-time PCR (**upper**) and Western Blotting (**lower**) after the generation of EXT1-sh ALL cells. (**J**) EdU (**left**) and CCK-8 (**right**) assays revealed that EXT1 downregulation markedly increased BALL-1 cell proliferation. Data represent the mean±SD of 3 independent experiments. (**K**) Apoptosis in each group of cells was monitored by flow cytometry with FITC-annexin V/PI staining; apoptosis in BALL-1 EXT1-silenced cells were decreased compared with that in BALL-1 or NC cells. Data represent the mean±SD of 3 independent experiments. (**L**) Apoptosis-related proteins such as Bax were downregulated, and Bcl-2 was upregulated in BALL-1 EXT1-knockdown cells. (Human EXT1 mRNA was normalized to that of human GAPDH; * $P<0.05$, ** $P<0.01$, compared with the control group). ALL – acute lymphoblastic leukemia; RT-PCR – real time polymerase chain reaction; CCK-8 – Cell Counting Kit-8; SD – standard deviation; FITC – fluorescein isothiocyanate; PI – propidium iodide.
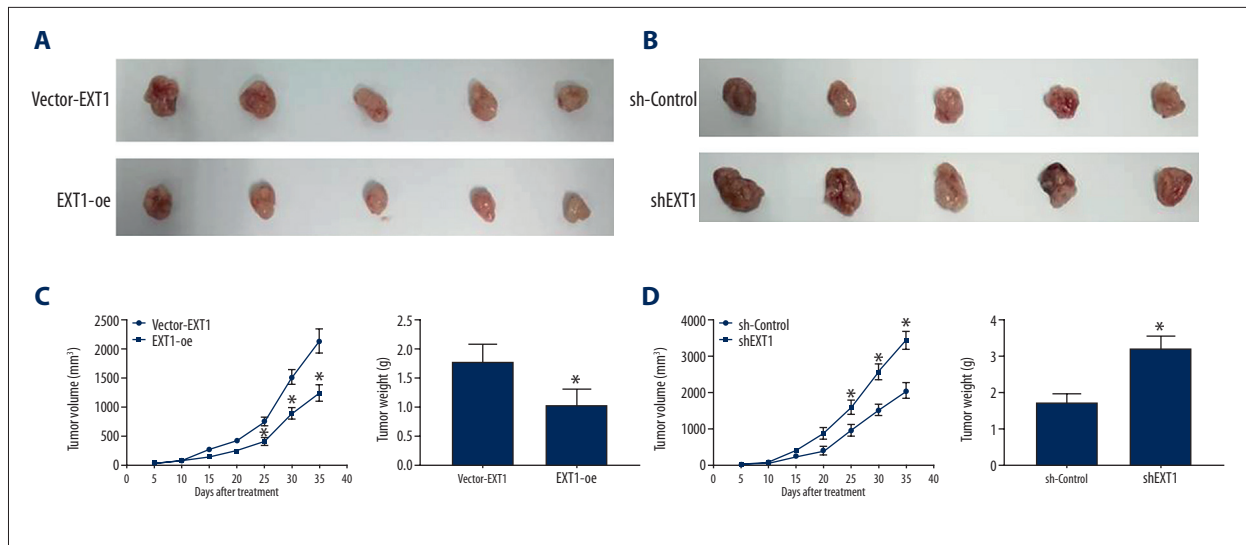
Figure 4. EXT1 modulates tumor growth *in vivo*. (**A**) Images of the xenograft tumors formed in the EXT1-oe and Vector-EXT1 groups of NOD/SCID mice. (**B**) Images of the xenograft tumors formed in the shEXT1 and sh-Control groups of NOD/SCID mice. (**C**) **Left** shows the tumor volumes in the EXT1-oe and Vector-EXT1 groups monitored over time (Reh cells), and the **right** shows the tumor weights in the oe-EXT1 and oe-Control groups. (**D**) **Left** shows the tumor volumes in the shEXT1 and sh-Control groups monitored over time (Reh cells), and the **right** shows the tumor weights in the shEXT1 and sh-Control groups (* *P*<0.05, compared with the control group).

of EXT1 (Figure 5F). Then, wild-type (WT) and mutant-type (MT) EXT1 were transfected into 293T cells with pre-miR-665 or pre-miR-NC. As shown in Figure 5H, excessive expression of miR-665 reduced the activity of WT EXT1 but did not affect the activity of MT EXT1. We further assessed luciferase activity in Reh cells and demonstrated that the luciferase activity of WT EXT1 was noticeably reduced in miR-665-overexpressing cells, while no changes were observed in the luciferase activity of MT EXT1 (Figure 5I). The results of the luciferase assay suggested that EXT1 is a target of miR-665. In summary, miR-665 promotes cell growth and inhibits apoptosis by suppressing EXT1.

EXT1 promotes cell apoptosis via deactivating the ERK1/2 pathway

To determine differentially expressed proteins in normal and EXT1-overexpressing Reh cells, we used label-free quantification proteomics. The differentially expressed proteins are shown in a heat map (Figure 6A). The expression of 40 proteins was significantly reduced, while the expression of the other 87 proteins was increased. By GO analysis, we found mitochondrial elongation and other pathway changes in the EXT1 overexpression group (Figure 6B). In addition, a STRINGdb analysis showed that protein interactions among 118 genes were significantly enriched (Figure 6C, Table 4). These results indicated that the expression of EXT1 may be related to mitochondrial apoptosis. We further examined the expression of several proteins involved in the ERK1/2 signaling pathway by western blot

analysis and found decreased expression of ERK1/2 and phosphorylated ERK1/2 in EXT1-overexpressing Reh cells (Figure 6D). Compared with dimethyl sulfoxide (DMSO) control, exposure to 50 μM PD98059 alone for 24 hours could induce apoptosis in ALL cells, and the effects of ERK1/2 inhibition on apoptosis could also be observed in EXT1-oe and EXT1-sh cells (Figure 6E, 6F). In conclusion, our results showed that EXT1 overexpression-induced cell apoptosis may be related to the ERK1/2 signaling pathway.

Discussion

This study showed that EXT1 expression decreases in ALL and is closely related to clinical prognosis. EXT1 expression was significantly lower in MOLT-4 (derived from T lymphoblastic leukemia) and Reh (derived from non B/non T lymphoblastic leukemia) cells, while normal in BALL-1 (derived from B lymphoblastic leukemia), CCRF-CEM (derived from T lymphoblastic leukemia) and Jurkat (derived from T lymphoblastic leukemia) cells. BM EXT1 expression were not related to immunotype (*P*>0.05) (Table 2). Based on our results at present, we may not draw a conclusion that there were potential EXT1 expression difference between B-ALL and T-ALL. The major finding of our study was that EXT1 overexpression or knockdown influenced cell proliferation and apoptosis and that this change may be related to upstream regulation by miR-665 and downstream targeting of the ERK pathway.
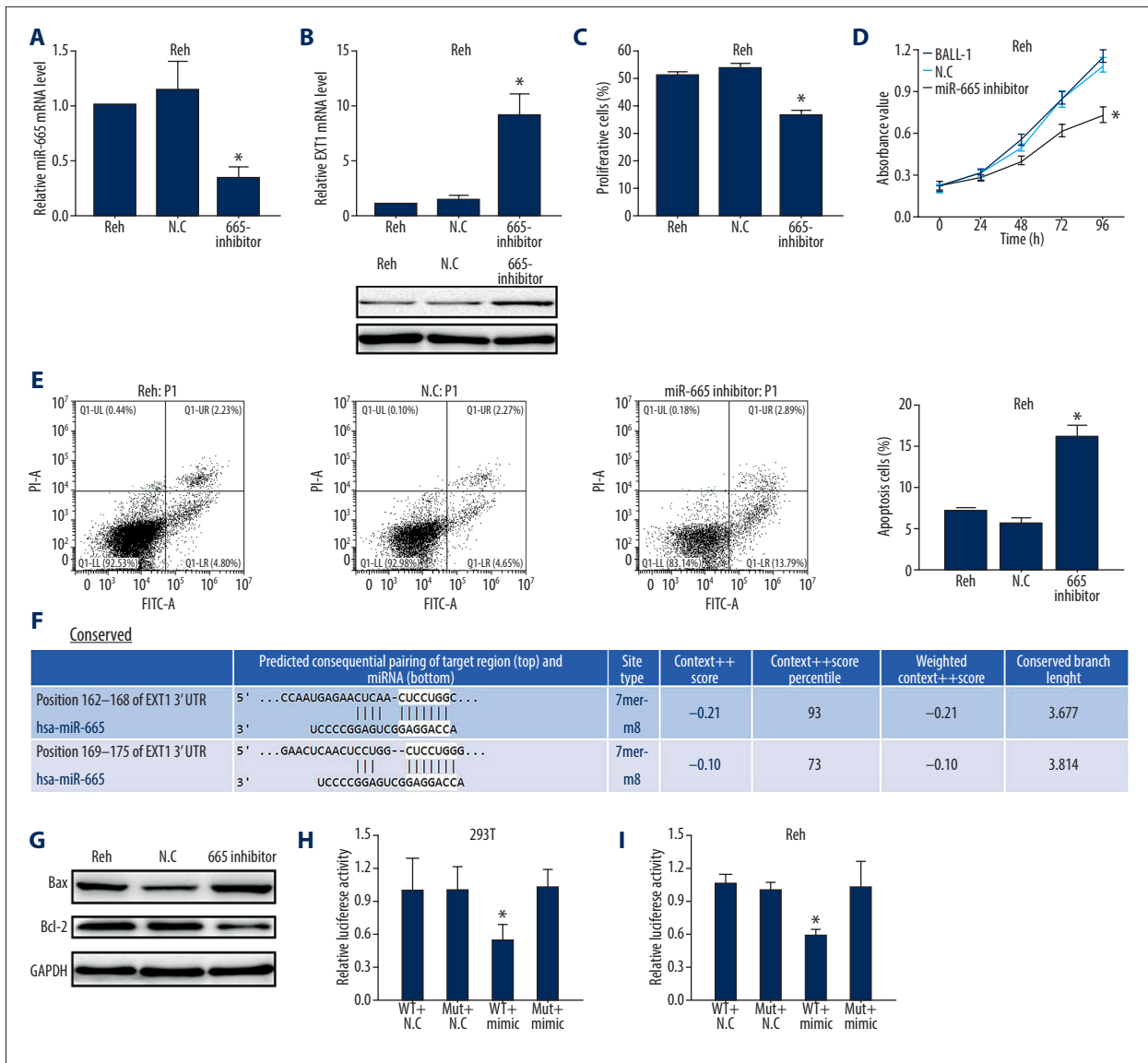
Figure 5. MiR-665 promotes cell growth and inhibits apoptosis by suppressing EXT1. (**A**) The expression levels of miR-665 were confirmed by RT-PCR after establishing miR-665-knockdown ALL cells. Data represent the mean±SD of 3 independent experiments. (**B**) The mRNA expression levels of EXT1 were determined by RT-PCR (**upper**) and western blotting (**lower**) after establishing miR-665-knockdown ALL cells. EdU (**C**) and CCK-8 (**D**) assays revealed that inhibiting miR-665 markedly inhibited Reh cell proliferation. Data represent the mean±SD of 3 independent experiments. (**E**) Cell apoptosis in each group of cells was monitored by flow cytometry and data showed that cell apoptosis was higher in Reh miR-665-knockdown cells than in Reh or NC cells. Data represent the mean±SD of 3 independent experiments. (**F**) Identification of the miR-665 binding site in the 3′UTR of EXT1 by Targetscan software. The wild type (WT) and mutant sequences in the EXT1 3′UTR are shown. (**G**) Apoptosis-related proteins such as Bax were upregulated, and Bcl-2 was downregulated in Reh miR-665-knockdown cells. Luciferase assays indicated that miR-665 could combine with the WT EXT1 3′UTR in 293T cells (**H**) and Reh cells (**I**). Data represent the mean±SD of 3 independent experiments (human miR-665 mRNA was normalized to that of human U6; human EXT1 mRNA was normalized to that of human GAPDH; * *P*<0.05, compared with the control and NC groups). RT-OCR – real time polymerase chain reaction; ALL – acute lymphoblastic leukemia; CCK-8 – Cell Counting Kit-8; SD – standard deviation.
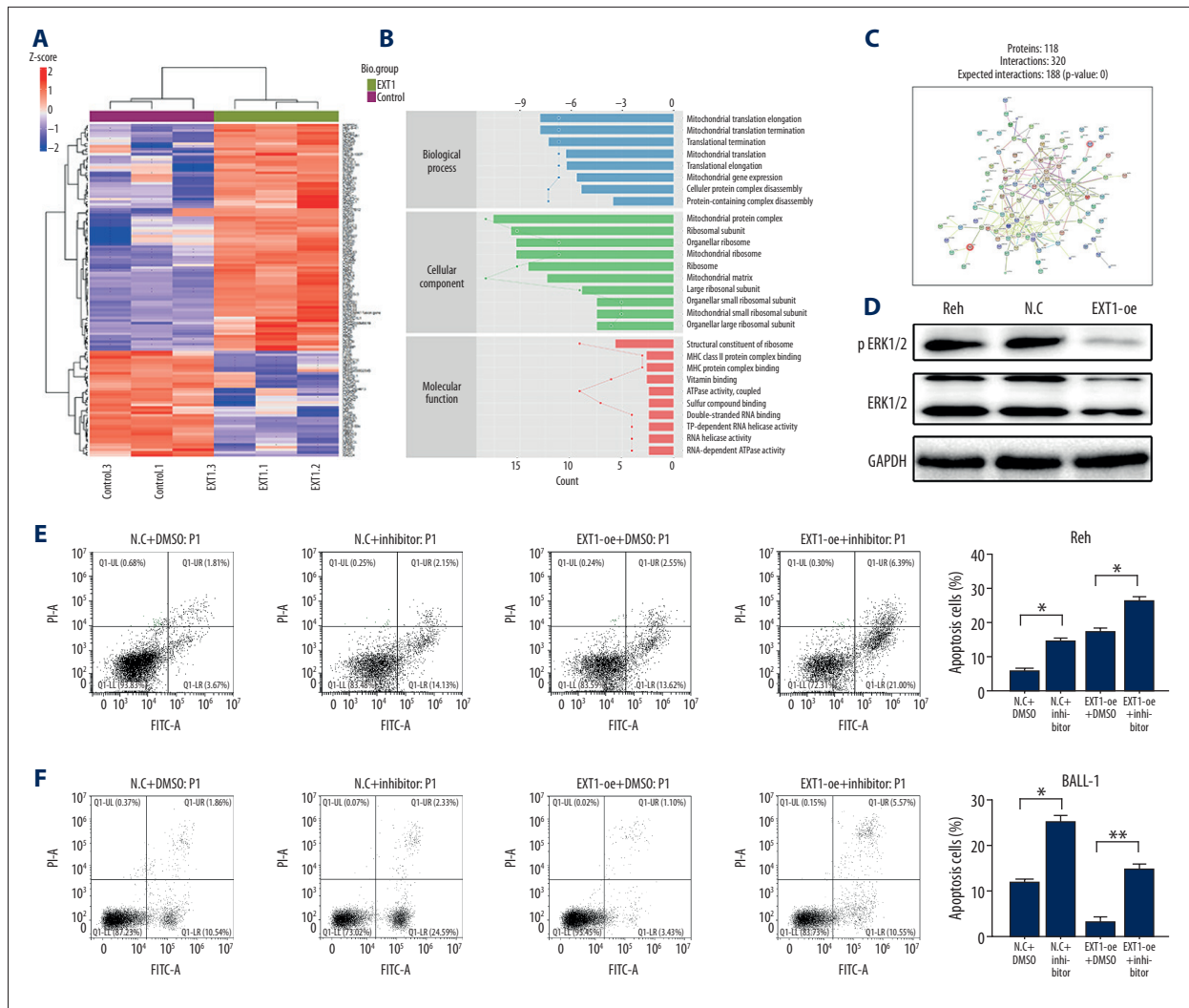
Figure 6. EXT1 promotes cell apoptosis via deactivating the ERK1/2 pathway. (**A**) Differentially expressed proteins in the control and EXT1-overexpressing groups are shown in the heat map. (**B**) The differentially expressed proteins were analyzed by GO analysis. (**C**) STRINGdb analysis showed that protein interactions among 118 genes were significantly enriched. (**D**) The expression levels of GAPDH, ERK1/2 and phosphorylated ERK1/2 in Reh, NC, and Reh EXT1-overexpressing cells. (**E, F**) show the apoptosis of cells in each group detected by flow cytometry. The cells were treated with or without 50 μM PD98059 for 24 hours. PD98059 was used as ERK1/2 inhibitor. Data represent the mean±SD of 3 independent experiments (* $P<0.05$, ** $P<0.01$, compared with the control group). GO – Gene Ontology; SD – standard deviation.

Several studies have demonstrated that EXT1 functions as a tumor suppressor[13,24–26]. However, no specific mechanism has yet been proposed. We quantitated EXT1 in adult ALL BM tissues and our results confirmed the results of previous studies showing that the EXT1 level was generally decreased compared with that in normal tissues, and our functional experiments verified that EXT1 might be a tumor suppressor. Although the function of EXT1 is largely undisputed, conflicting observations have been reported. For example, Rogier et al. (2010) showed that EXT1 knockdown promoted apoptosis *in vitro* and inhibited tumor growth in a multiple myeloma model *in vivo* [27]. In this paper, the authors investigated whether

EXT1 knockdown could reduce HS production, thereby causing changes in the BM microenvironment of myeloma and promoting apoptosis. We speculated that the difference in these results is due to the different cancer types studied. At present, the role of HS in leukemia has not been thoroughly studied.

There are a few studies on the role of miR-665 in tumors, for example, miR-665 inhibited the invasion and metastasis of osteosarcoma [28] and cervical cancer [29]. MiR-665 suppressed the growth and migration of ovarian cancer cells by targeting HOXA10 [30]. However, Zhao et al. [31] found that miR-665 may act as an oncogene by targeting NR4A3 and promote tumor

Table 4. Significant proteins in group EXT1 – Control.

Upregulated proteins	Downregulated proteins
CYC1 MFN2 TMX2 LMAN2 JCHAIN CD79B SRPRB MRPL43 RBP4 DAP3 MRPL4 KEAP1 FCRLA BAZ1A ITGB1 MB2 O94925-3 F13A1 CHD8 IGHM TFRC MRPS31 LRRC59 MRPL15 MRPL44 NOMO3 CD74-Ntrk1 fusion gene SPCS2 VKORC1L1 DKFZp686M0619 CLPTM1 GRSF1 CLPX CD47 RBM3 ILVBL DHX30 CD81 ATL2 BRI3BP STK26 HSD17B12 DDX54 THBS4 RPL19 FDFT1 RTFDC1 PIG60 FAR1 MRPL38 UTP20 MRPS27 TMED5 EMC1 GRPEL1 APOB ITPR3 NDUFB10 FKBP11 ZNF638 ATP5O SPTLC1 derp12 YA61 TYMS DDX41 MRPL48 SYPL ATP5H MRPS18B RPL32 SLFN11 DDX3X CTSZ MANB PPID HEL-S-130P BZW1 SPCS3 MS4A1 NDUFAB1 CD79A NDRG3 FN1 MRPS34 HEL-S-41	hCG_2024613 FN3KRP PDCD5 AP3D1 LSM2 HMGN2 C1orf123 XRCC4 GYG1 PHRF1 NUCB2 PURB PTMA DBI CALM3 GCHFR HEL-S-53e SEPHS1 PITPNA HMBS LSM3 ABRACL PBEF1 GOT1 UBE2V2 PEA15 DHPS EPS15L1 TMED8 ATP6V1H C9orf32 CLASP1 DKFZp686G2045 NECAP2 CBX1 HN1L ANAPC10 NUCKS1 FTH1 LSM6

metastasis in breast cancer. MiR-665 was also reported to promote cell migration, invasion and proliferation by inhibiting PTPRB in hepatocellular carcinoma [32]. MiR-665 significantly upregulated in non-small cell lung cancer (NSLC) extracellular vesicles and may act as a potential oncogene in NSLC [33]. MiR-665 has been shown to predict resistance to neoadjuvant radiochemotherapy in squamous cell carcinoma of the esophagus [34]. We speculated that the conflicting role of miR-665 in these results is due to the different cancer types studied. MiR-665 may act as whether oncogene or tumor-suppressing gene by targeting different genes in different tumors. Thus far, there has been no study on the role of miR-665 in leukemia. As we know, the most classic mechanism of miRNAs is that miRNAs can bind to complementary sequence 3' UTR of target genes, which leads to the degradation of target mRNA and then inhibits the process of protein translation. We demonstrated that miR-665 may play a role in promoting cancer by targeting EXT1 through this classic mechanism in ALL. Firstly, inhibition of miR-665 increased the mRNA and protein levels of EXT1 in Reh cells. Secondly, bioinformatics analysis and luciferase reporter assay provided evidences that miR-665 can bind to the 3' UTR of EXT1.

We studied the signaling pathways downstream of EXT1 overexpression-induced cell apoptosis and found that these networks may be related to the ERK signaling pathway. We examined the expression of several proteins involved in the ERK signaling pathway by WB analysis and found decreased expression of ERK1/2 and phosphorylated ERK1/2 in EXT1-overexpressing ALL cells. As expected, the ERK signaling pathway, which plays an important role in cell proliferation and apoptosis, was noticeably inhibited. There are some similar reports on apoptosis

caused by the inactivation of the ERK pathway in ALL [35,36], which is consistent with our conclusion.

In this study, 70 clinical samples were evaluated, but this may be too few samples. Thus, a larger sample size will be used in the future. In the following work, we should collect more specimens to detect the protein expression of EXT1. In addition, research on the influence of EXT1 on the ERK pathway is preliminary and needs to be further improved in subsequent research.

Conclusions

To the best of our knowledge, this study is the first to confirm the association of low EXT1 levels with several clinical features of ALL. Low BM EXT1 levels independently predict a poor prognosis in adult ALL patients. Our results suggested that the role of EXT1 in ALL may be regulated by upstream miR-665. Moreover, the role of the ERK pathway in the EXT1 regulation of apoptosis was preliminarily explored. We expect that further research on EXT1-, or miR-665-targeted strategies will bring new ideas and possibilities for clinical treatment.

Acknowledgements

Conflicts of interest

None.

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