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Original Article

Prognostic value, biological role, and mechanisms of LCN2 in childhood acute lymphoblastic leukemia

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Abstract: Resistance to glucocorticoids (GC) is associated with poor prognosis in childhood acute lymphoblastic leukemia (ALL). Lipocalin 2 (LCN2) plays a pro-tumorigenic role in solid tumors and chronic myeloid leukemia by promoting initiation, invasion, metastasis and drug resistance, and has gained increasing attentions as a therapeutic target. However, ALL cells show a low expression status of LCN2. Meanwhile, the clinical significance and biological role of LCN2 remain unclear in childhood ALL. Therefore, we collected bone marrow, peripheral blood, and cerebrospinal fluid samples from children with ALL and control individuals to assess LCN2 expression. Lentiviral transduction was used to establish stable LCN2 overexpression in Nalm6, CEM-C1, CEM-C7, and Molt4 cell lines. The cell growth, proliferation, cell cycle, apoptosis, ferroptosis, and sensitivity to dexamethasone were detected to clarify the function of LCN2. Compared with healthy individuals, non-tumor patients and intracranial solid tumors, LCN2 expression was down-regulated in patients with childhood ALL at diagnosis. Lower LCN2 expression in the bone marrow was associated with poor prognostic features and a lower disease relapse-free rate. Effective chemotherapy could restore the expression of LCN2. Overexpression of LCN2 led to an inhibition of cell growth and an induction of ferroptosis in GC sensitive ALL cells (Nalm6 and CEM-C7), and reversed GC resistance by up-regulating the expression of glucocorticoid receptor (GR) and phosphorylated-GR (p-GR) and inhibiting the Notch signaling pathway. On the contrary to solid tumors, our results suggest that inducing the expression of LCN2 might be a novel therapeutic protocol in childhood ALL.

Keywords: Lipocalin 2 (LCN2), acute lymphoblastic leukemia, pediatric cancer, glucocorticoid resistance, ferroptosis, prognosis

Introduction

Acute lymphoblastic leukemia (ALL) is a malignant hematological disease caused by the blockage of lymphocyte differentiation at an early stage, leading to the infiltration of bone marrow, blood, and extramedullary sites [1]. ALL is the most common malignant cancer in childhood, with more than 50000 new cases diagnosed every year worldwide [2]. In recent decades, the 5-year survival rate for children with ALL in developed countries has exceeded 90% due to the optimization of chemotherapy regimens and the improvement of supportive care [3, 4]. However, relapse and glucocorticoids (GC) resistance remain significant challenges. Additionally, as survival rates improve, chemotherapy-related toxicities have become a major concern. Therefore, further advancement in the prognosis and quality of life for children with ALL depends on the development of safe and effective targeted therapies. These thera-

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pies could potentially reduce the intensity of chemotherapy or replace allogeneic hematopoietic stem cell transplantation [3, 5-7].

Lipocalin 2 (LCN2), also known as neutrophil gelatinase-associated lipocalin (NGAL), siderocalin, and oncoprotein 24p3, was first discovered in neutrophils. It inhibits bacterial growth by chelating iron in bacteria, playing a role in non-specific immune responses [8]. LCN2 has a bidirectional role in regulating intracellular iron content. When bound with extracellular iron, it can transport this iron into the cell, increasing intracellular iron levels. Conversely, LCN2, which is not bound to iron, enters the cell, binds to siderophore, and transports intracellular iron to the extracellular environment, decreasing intracellular iron levels [9]. Beyond its roles in immune response and iron metabolism, LCN2 acts as an oncogene, promoting tumor cell proliferation, migration, invasion, metastasis, epithelial-mesenchymal transition, and angiogenesis while inhibiting apoptosis and ferroptosis and influencing the tumor microenvironment in solid tumors [10, 11]. The expression of LCN2 is up-regulated in solid tumors, where high expression of LCN2 indicates poor prognosis [12-15]. With the exception of chronic myeloid leukemia (CML) [16, 17], LCN2 expression is down-regulated in most types of leukemia [18], and its potential mechanisms and clinical significance remain unclear. Unlike solid tumors, only one study to date has found that LCN2 enhances oxidative stress-induced cell apoptosis in acute myeloid leukemia (AML) [19]. Given the limited understanding of LCN2's role in leukemia, particularly ALL, this study aimed to investigate its prognostic value, biological function, and underlying mechanisms.

Methods

Collection of clinical samples

Clinical samples from patients with ALL were obtained from West China Second University Hospital of Sichuan University between October 2020 to December 2023. The experimental group comprised 151 bone marrow (BM), 57 peripheral blood (PB), and 50 cerebrospinal fluid (CSF) samples from newly diagnosed children with ALL. The control group included 97 BM samples (62 from non-tumor children and 25 from healthy donors), 14 PB samples from healthy individuals, and 10 CSF samples from children with intracranial solid tumors. Additionally, samples were collected on the 19th and 46th day of chemotherapy: 51 BM samples, 37 PB samples, and 33 CSF on the 19th day, 44 BM samples, 29 PB samples, and 18 CSF samples on the 46th day. This study was approved by the Ethics Committee of the Hospital (No. 335) and written informed consent was obtained from the participants' legal guardians.

Collection of clinical data

Clinical data from pediatric ALL patients included demographic and disease-specific factors such as age, gender, ethnicity, blast cell percentage in BM, white blood cell (WBC) count in PB, immunophenotype, fusion genes, chromosomal karyotypes, blood tumor transcriptome sequencing, initial CSF results (central nervous system (CNS)-1, CNS-2, and CNS-3), minimal residual disease (MRD) levels on the 19th and 46th day of chemotherapy, and clinical risk stratification. CNS-1 was defined by a CSF WBC count < 5 cells/μl with no blast cells on flow cytometry. CNS-2 and CNS-3 had < 5 or ≥ 5 cells/μl, respectively. MRD positivity was ≥ 0.01% and negativity was < 0.01%. Patients followed the Chinese Children Cancer Group's 2020 protocol for ALL (CCCG-ALL-2020) (ChiCTR1900024837), starting with dexamethasone (Dex) for the initial 4 days, then either Dex or prednisone. We introduced a GC sensitivity test based on the international Berlin-Frank-Munster (BFM) protocol's prednisone sensitivity test. We defined GC insensitivity in childhood ALL as a blast cell count in PB ≥ 1×10^9/L on day 7 of treatment, and sensitivity as a count < 1×10^9/L. A followed 52 children for over 2 years until January 1, 2024. Total survival time was from diagnosis to follow-up endpoint or death, while relapse-free survival time was from diagnosis to recurrence or follow-up endpoint.

Cell culture

Leukemia cell lines Nalm6 and Molt4 were acquired from Shanghai Research Institute and St. Jude Children's Research Hospital, respectively, while CEM-C1 and CEM-C7 came from the University of Texas. All cells were cultured in RPMI 1640 with 10% fetal bovine serum (FBS) (Gibco) at 37°C in a 5% CO_2 incubator.

RNA isolation and quantitative real-time PCR

Total RNA was extracted Trizol (Thermo) and further purified using chloroform, isopropanol, and 75% alcohol. cDNA was synthesized from 1 μg of RNA using the Transcriptor cDNA Synthesis Kit (Roche). Quantitative real-time PCR (qRT-PCR) was carried out on a Real-Time PCR System (BIO-RAD) with GoTaq qPCR Master Mix and mRNA expression was analyzed using the 2-ΔΔCt method, normalized to GAPDH. Experiments were replicated three times. Primer sequences used were: LCN2 (sense: 5-GACAACCAATTCCAGGGGAAG-3, antisense 5-GCATACATCTTTTGCGGGTCT-3) GAPDH (sense 5-GAAGGTGAAGGTCGGAGTC-3, antisense 5-GAAGATGGTGATGGGATTTC-3).

Western blot

Cells were lysed with RIPA buffer (Beyotime) containing phosphatase and protease inhibitor cocktail (Roche). Proteins were separated by SDS-PAGE, transferred to PVDF membranes (Roche) and immunoblots were incubated with primary antibodies overnight at 4°C, followed by secondary antibodies for 2 h at room temperature. The PVDF membrane was incubated with Chemiluminescent Substrate (Immobilon) and analyzed using the Gene Company Limited's Gel electrophoresis imaging system. Western blot analysis used primary antibodies for β-actin (1:2000, 66009-1-Ig, Proteintech), LCN2 (1:1000, ab23477, Abcam), GR (1:500, sc-393232, Santa Cruz Biotech), p-GR(Ser211) (1:500, 4161S, Cell Signaling Technology), p-GR(Ser226) (1:200, ab228972, Abcam), Notch1 (1:1000, 4380S, Cell Signaling Technology), AKT (1:1000, 4691S, Cell Signaling Technology), p-AKT (1:1000, 4060S, Cell Signaling Technology), P38 (1:1000, 8690S, Cell Signaling Technology), p-P38 (1:1000, 4511S, Cell Signaling Technology), JNK1+JNK2+JNK3 (1:500, ab179461, Abcam), p-JNK1+JNK2+JNK3 (1:500, ab124956, Abcam), Bim (1:1000, 2933S, Cell Signaling Technology), Caspase-3 (1:1000, 9662S, Cell Signaling Technology) and Cleaved Caspase-3 (1:500, 9661, Cell Signaling Technology).

ELISA

An ELISA for LCN2 was conducted using the RayBio® (ELH-Lipocalin2-1) commercial kit, diluting plasma samples 1:100, while CSF and culture medium (CM) samples were undiluted.

Lentiviral transduction

LCN2 overexpression and control lentivirals were procured from Shanghai GeneChem Co., Ltd., using the GV513 vector (details at http://www.genechem.com.cn/index/supports/tool_search.html?keywords=GV513). LCN2 fragments were amplified with primers LCN2-p1 and LCN2-p2: LCN2-p1: AGGTCGACTCTAGAGGATCCCGCCACCATGCCCCTAGGTCTCCTGTG; LCN2-p2: ACCGTAAGTTATGTGCTAGCTCAGCCGTCGATACACTGGTCGATTG. Naml6, CEM-C7, CEM-C1, and Molt4 cells were transfected with LCN2-overexpression (oe) and empty vector (EV) control lentivirus. Puromycin selection began 72 h after infection, with fresh doses every 2-3 days for a month. Green fluorescent protein (GFP)-positive cells were identified via flow cytometry using a Beckman FC500. LCN2 overexpression (LCN2-oe) was confirmed by western blot and ELISA.

Diagram of cell growth curve

LCN2-oe and EV controls of Nalm6, CEM-C7, CEM-C1, and Molt4 cell lines were seeded in 6-well plates at 4×10^4 cells/ml in 2 ml RPMI 1640 medium. Cell counts were taken every 24 h using a cell counting board, averaging three measurements for the final result.

CCK-8 assay

Cell viability was assessed using the CCK-8 assay (Dogesce, CK04). Nalm6 cells were seeded in 6-well plates at 3.5×10^5 cells/ml, while CEM-C7, CEM-C1, and Molt4 cells were seeded in 6-well plates at 2×10^5 cells/ml, all in 2 ml RPMI 1640 medium. After 24 h, 48 h, and 72 h post-seeding, 100 μl of cell suspension was transferred to 96-well plates with 10 μl of CCK-8 incubated at 37°C for 2 h, and absorbance at 450 nm was measured using a Biotek synergy h1Microplate Reader (Bio Tek).

Flow cytometry analyses

For apoptosis evaluation, cells were stained with Annexin V and 633 by the manufacturer (DOJINDO, AD11), the analyzed by flow cytometry 1 h later. For cell cycle analysis, cells were stained with PI (DOJINDO, C543) for 30 min and

analyzed by flow cytometry with an excitation wavelength of 488 nm.

Iron, ferrous iron, malondialdehyde, reactive oxygen species and glutathione measurement

Leukemia cells were analyzed for intracellular iron and ferrous iron by Iron Assay Kit (Elabscience, E-BC-K880-M) and ferrous iron Assay Kit (Elabscience, E-BC-K881-M), according to the manufacturer's protocol. One million leukemia cells were collected and exposed to 10 μmol/L of 2',7'-dichlorodihydrofluorescein diacetate (Sigma-Aldrich, D6883) for 20 min, and fluorescence intensity was measured with a Biotek Synergy H1 Microplate Reader (Bio Tek) to assess reactive oxygen species (ROS) levels. Three million cells were used to measure malondialdehyde (MDA) levels with MDA Assay Kit (Elabscience, E-BC-K028-M) and one million cells were analyzed for glutathione (GSH) levels using a GSH Assay Kit (Elabscience, E-BC-K030-M), all following the manufacturer's protocols.

Exogenous LCN2

Exogenous LCN2 (MCE, HY-P70658A) was introduced to the cell CM of at concentrations of 0.5, 5, 25, 50, and 100 ng/mL. Cell viability was assessed at 24 h, 48 h, and 72 h post-seeding with varying LCN2 concentrations.

Cell viability was evaluated at 24, 48, and 72 hours post-seeding with varying LCN2 concentrations. Data were shown as median (range), and group differences were analyzed using T-test or Mann-Whitney test. Spearman correlation assessed the link between LCN2 expression and blast cell percentage in pediatric ALL patients' BM. Kaplan-Meier curves and log-rank analysis were used for survival analysis, with significance set at $P < 0.05$. Data and ROC curve analysis were conducted using SPSS 22.0 and GraphPad Prism 8. Asterisks indicated significant differences. LCN2 expression in ALL patients' BM was compared to healthy donors and non-tumor pediatric patients by analyzing transcription levels.

Statistical analysis

Data were presented as median (range) and group differences were evaluated using T-test or Mann-Whitney test. Spearman correlation assessed the link between LCN2 expression and blast cell percentage in pediatric ALL patients' BM. Kaplan-Meier curves and log-rank analysis were used for survival analysis, with significance set at $P < 0.05$. Data and receiver operating characteristic (ROC) curve analysis were conducted using SPSS 22.0 (IBM® SPSS® software, USA) and GraphPad Prism version 8 (GraphPad Software, San Diego, USA). Asterisks denoted statistically significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

Results

Down-regulation of LCN2 in patients with childhood ALL at diagnosis and restoration of LCN2 expression after chemotherapy

To examine LCN2 expression in the BM of patients with ALL, we first analyzed the transcription levels of LCN2 in the BM of healthy donors and non-tumor pediatric patients. Our analysis showed no differences in the mRNA expression of LCN2 between healthy donors (median value 0.3984, range 0.1333-0.9324, n = 35) and non-tumor patients (median value 0.4501, range 0.006014-2.013, n = 62) ($P = 0.1238$) (**Figure 1A** and **1B**). However, the transcription level of LCN2 in BM at diagnosis was significantly down-regulated in pediatric patients with ALL (median value 0.005091, range 0.00002247-1.459, n = 151) compared to both healthy donors and non-tumor patients ($P < 0.0001$) (**Figure 1C**). Similarly, at diagnosis, the protein expression level of LCN2 in BM was down-regulated in patients with ALL compared to healthy donors ($P = 0.023$) (**Figure 1D**).

LCN2 is a secreted protein, and its expression in plasma and CSF was detected by enzyme-linked immunosorbent assay. The median concentration of LCN2 in CSF (30.49 pg/mL) from children with ALL at initial diagnosis was approximately 1.7% of the LCN2 concentration in plasma (1716 pg/mL). Plasma LCN2 levels in patients with ALL at diagnosis (median value 1716 pg/mL, range 343.7-26969 pg/mL, n = 57) were significantly lower than in healthy individuals (median value 49865 pg/mL, range 5040-158609 pg/mL, n = 14) ($P < 0.0001$) (**Figure 1E**). Additionally, CSF LCN2 levels in children with ALL at diagnosis (median value 30.49 pg/mL, range 2.833-253.0 pg/mL, n =

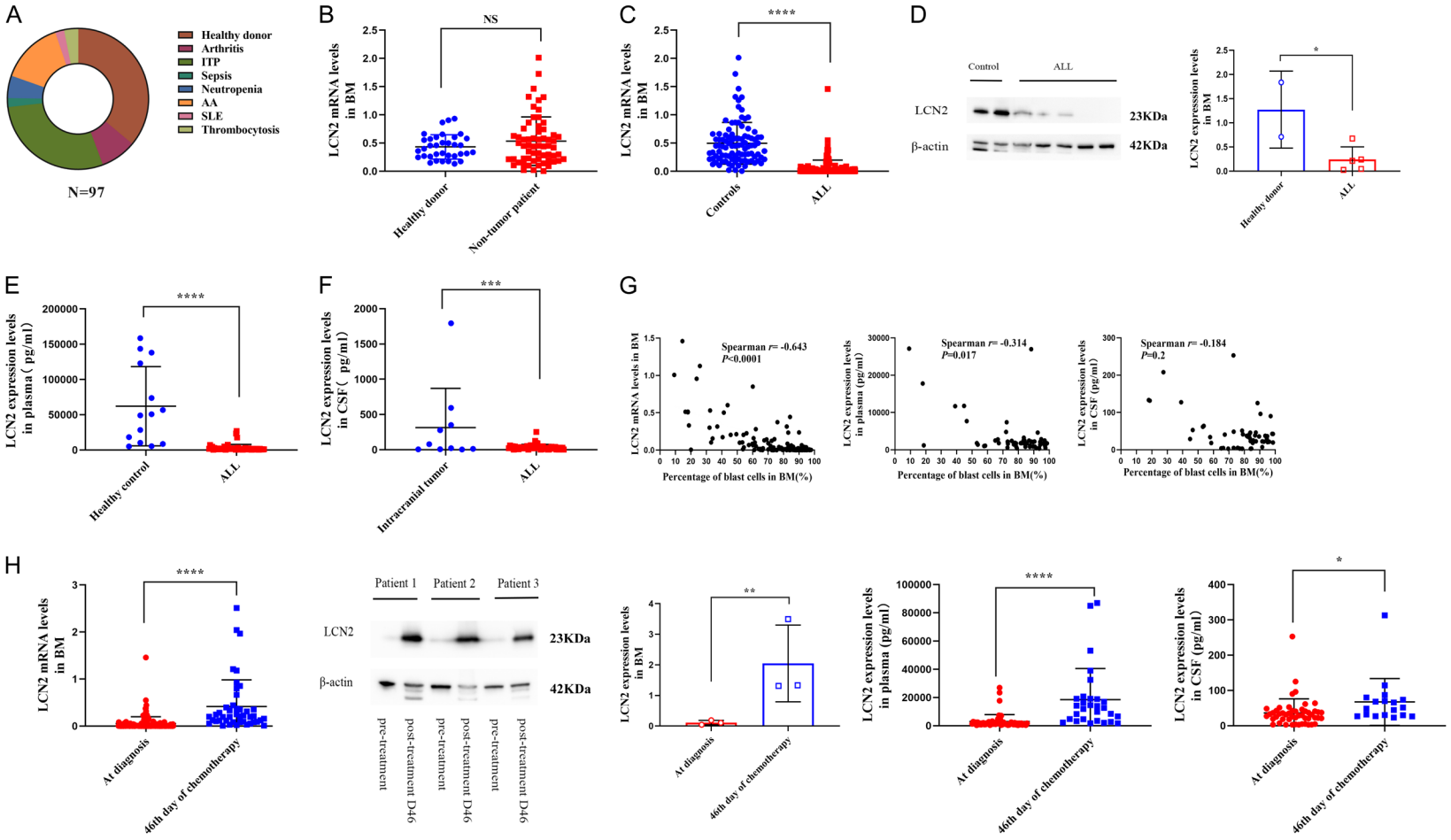


Figure 1. Lipocalin 2 (LCN2) is down-regulated in childhood acute lymphoblastic leukemia (ALL) at diagnosis and restored followed by chemotherapy. A. The composition of diseases in the bone marrow (BM) of non-tumor patients in children. B. There was no significant difference in LCN2 mRNA expression between healthy donors and non-tumor patients in the BM (P = 0.1238). C. The LCN2 mRNA expression in children with ALL at diagnosis was found to be significantly lower than in control individuals (P < 0.0001). D. The expression of LCN2 in the BM of children diagnosed with ALL was found to be significantly lower compared to that of healthy individuals (P = 0.023). E. The LCN2 expression in plasma at diagnosis was significantly lower in children with ALL compared to healthy individuals (P < 0.0001). F. The LCN2 expression in cerebrospinal fluid (CSF) was lower in children with ALL compared to those with intracranial tumors (P = 0.00062). G. LCN2 mRNA expression in BM and its plasma levels are inversely related to blast cell percentage in BM, while cerebrospinal fluid (CSF) levels show no correlation (Spearman r = -0.643, P < 0.001; Spearman r = -0.314, P = 0.017), while CSF levels show no correlation (Sperman r = -0.184, P = 0.2). H. On day 46 of chemotherapy, LCN2 levels in BM, plasma, and CSF were significantly higher than at diagnosis (P < 0.0001, P = 0.0021, P < 0.0001, P = 0.0219).

50) were significantly lower compared to children with intracranial solid tumors (median value 50.75 pg/mL, range 4.906-1794 pg/mL, n = 10) (P = 0.00062) (**Figure 1F**).

We observed a negative correlation between LCN2 expression (both mRNA in BM and protein in plasma) and the percentage of blast cells in BM at diagnosis (Sperman r = -0.643, P < 0.001 and Sperman r = -0.314, P = 0.017 for BM and plasma, respectively). However, CSF LCN2 expression did not correlate with the percentage of blast cells in BM (Sperman r = -0.184, P = 0.2) (**Figure 1G**). Interestingly, upon receiving chemotherapy, LCN2 expression in BM, plasma, and CSF, was restored by day 46 (P < 0.0001, P = 0.0021, P < 0.0001, and P = 0.0219, respectively) (**Figure 1H**). In contrast, there was no difference in LCN2 expression on day 19 of chemotherapy compared to diagnosis.

Relationship between LCN2 expression and clinical features of childhood ALL

LCN2 mRNA expression in the BM was analyzed for correlation with clinical features, molecular abnormalities, and therapeutic responses of childhood ALL. The LCN2 expression did not correlate with age, gender, ethnicity, or chromosome karyotype. However, it was corelated with the counts of WBC in PB, immunophenotype, CNS involvement, and clinical risk stratification. The LCN2 mRNA expression in BM of patients with ALL was lower in those: with WBC ≥ 50×10^9/L than WBC < 50×10^9/L (P = 0.014); with T-cell ALL (T-ALL) than B-cell ALL (B-ALL) (P = 0.047); with CNS-2 and CNS-3 than CNS-1 involvement (P = 0.008); and with medium-high risk than low risk (P = 0.009) (**Table 1**). The LCN2 mRNA expression in BM only correlated with *RAS* mutations. It was lower in patients with *RAS* mutations compared to those without (P = 0.048) (**Table 2**). The LCN2 mRNA expression in BM was not correlated with *ETV6/RUNX1* fusion gene, *BCR-ABL*, Ph-like ALL, *FLT3* mutations, *NOTCH1* mutations, *PTEN* mutations, or *CDKN2A/B* mutations. Pairwise comparisons with various molecular abnormalities found that LCN2 mRNA expression in BM of patients with *RAS* mutation (median value 0.0047, n = 45) was lower than in patients with the *ETV6/RUNX1* fusion gene (median value 0.0178, n = 26) (P = 0.014). In terms of therapeutic response, LCN2 mRNA expression correlated with GC sensitivity testing and MRD on day 19 if treatment but not to MRD on day 46 or relapse. Patients with GC insensitivity and positive MRD on day 19 had lower LCN2 mRNA expression compared to those with GC sensitivity and negative MRD on the same day (P = 0.047 and P = 0.0039, respectively) (**Table 3**). In section 3.1, LCN2 mRNA expression in BM negatively correlated with the percentage of blast cells. There were no significant differences in blast cell percentage between patient groups stratified by clinical risk, molecular abnormalities, and therapeutic response (Figure S1). This suggests that the observed associations between LCN2 expression and these factors are not due to variations in blast cell burden. The ROC curve (**Figure 2A**) showed that area under curve (AUC) for LCN2 mRNA levels in BM identifying T-ALL was 0.953 (95% CI 0.92-0.985), with P < 0.001. The best threshold for T-ALL was 0.00135, offering 87% sensitivity and 95% specificity.

However, plasma LCN2 expression only correlated with WBC count and immunophenotype (**Table 4**). It was lower in patients with ALL with lower WBC counts (< 50×10^9/L) and B-ALL compared to those with higher WBC counts (≥ 50×10^9/L) and T-ALL (P = 0.018 and P = 0.003, respectively). This is in contrast to the expression in BM. Moreover, CSF LCN2 expression was solely associated with CNS involvement (**Table 5**). LCN2 expression in CSF was lower in patients with CNS-2 and CNS-3 compared to CNS-1 (P = 0.005).

Relationship between LCN2 expression and survival rate in ALL

We followed 52 out of 151 children with ALL for more than 2 years (median follow-up: 30 months, range: 24-38 months). Five patients relapsed, and one patient died during this period. All relapsed patients belonged to the medium-risk group and had very low LCN2 mRNA expression (0.0004, 0.0002, 0.0005, 0.000043, and 0.0001). Four of the relapsed patients survived after receiving allogeneic hematopoietic stem cell transplantation, while the fifth patient died without treatment following relapse. The median LCN2 mRNA expression in BM of the 52 patients was 0.01165. These patients were then divided into a LCN2 high expression (n = 26) and a low expression

Table 1. The correlation between Lipocalin 2 (LCN2) mRNA expression in bone marrow (BM) at diagnosis of childhood acute lymphoblastic leukemia (ALL) and clinical features

Clinical features	Number	LCN2 mRNA level (median, range)	*P* value
Age group			
1-10	129	0.0064 (0.000022-1.459)	0.548
≥ 10	22	0.0825 (0.0001-0.2137)	
Gender			
Male	98	0.0067 (0.000022-0.5482)	0.165
Female	53	0.0060 (0.001-1.459)	
Race			
Han	136	0.0625 (0.000022-1.459)	0.563
Non-Han	15	0.0144 (0.0001-0.159)	
White blood cell (WBC) count			
$< 50\times10^9$/L	111	0.0150 (0.0001-1.459)	0.014
$\geq 50\times10^9$/L	40	0.00115 (0.000022-0.043)	
Immunophenotype			
B-ALL	131	0.0144 (0.0001-1.459)	0.047
T-ALL	20	0.0002 (0.000022-0.0017)	
Karyotype[#]			
Hyperdiploid	22	0.0064 (0.000022-1.459)	0.247
Non-hyperdiploid	109	0.0040 (0.0001-0.1473)	
Central nervous system (CNS) involvement			
CNS-1	127	0.0309 (0.000022-1.459)	0.008
CNS-2 and CNS-3[&]	24	0.0017 (0.0001-0.0237)	
Clinical risk stratification			
Low risk group	73	0.0120 (0.0001-1.459)	0.009
Medium-high risk group[*]	78	0.0028 (0.000022-0.2346)	

Note: [#]20 ALL patients without mitotic phase. [&]2 ALL patients with CNS-3 involvement. [*]3 ALL patients classified as high risk.

group (n = 26) based on this median value. While there was no difference in overall survival rate between the high and low LCN2 expression groups ($P = 0.456$), the disease relapse-free survival rate was lower in the expression group than the high expression group ($P = 0.019$) (**Figure 2B**).

Anti-proliferation and pro-ferroptotic effects of LCN2

LCN2 expression in the leukemia cell lines and its secretion into the CM were low ($P < 0.0001$) (Figure S2). In section 3.2, LCN2 expression was correlated with immunophenotype and therapeutic response to GC. Therefore, Nalm6 (human acute B lymphoblastic leukemia cell line, GC-sensitive), CEM-C7 (human acute T lymphoblastic leukemia cell line, GC-sensitive), CEM-C1 (human acute T lymphoblastic leukemia cell line, GC-resistant), and Molt4 (human acute T lymphoblastic leukemia cell line, GC-resistant) were chosen to establish LCN2-overexpressing stable lines. Using lentiviral transduction, we successfully established LCN2-overexpressing (oeLCN2) and control (oeEV) lines from each of these leukemia lines (**Figure 3A**).

The growth of LCN2-overexpressing Nalm6 and CEM-C7 (Nalm6-oeLCN2 and CEM-C7-oeLCN2) was inhibited compared to their corresponding control lines (Nalm6-oeEV and CEM-C7-oeEV); however, the growth of CEM-C1 and Molt4 was not affected (**Figure 3B**). The proliferation of Nalm6 and CEM-C7 was decreased after LCN2 overexpression compared to the control lines; however, the proliferation of CEM-C1 and Molt4 was not affected (**Figure 3C**). Cell cycle progression and apoptosis were not altered by LCN2 overexpression in any cell line (Figure S3). The results also revealed exogenous LCN2, at 50 ng/mL, inhibited approximately 40-50% of Nalm6 and

Table 2. The correlation between LCN2 mRNA expression in BM at diagnosis of childhood ALL and molecular abnormalities

Molecular abnormalities	Number	LCN2 mRNA level (median, range)	*P* Value
ETV6/RUNX1 fusion gene			
Positive	26	0.0178 (0.0001-0.2369)	0.772
Negative	125	0.0051 (0.000022-1.459)	
Ph$^+$ALL and Ph-like ALL*			
Positive	27	0.0061 (0.0001-0.3160)	0.878
Negative	124	0.0066 (0.000022-1.459)	
RAS mutation			
Positive	45	0.0047 (0.000022-0.1443)	0.048
Negative	106	0.01005 (0.000046-1.459)	
FLT3 mutation			
Positive	18	0.0054 (0.0001-0.0618)	0.240
Negative	133	0.0066 (0.000022-1.459)	
NOTCH1 mutation			
Positive	14	0.00255 (0.000022-0.2807)	0.652
Negative	137	0.0070 (0.000046-1.459)	
PTEN mutation			
Positive	12	0.0037 (0.000046-0.0413)	0.280
Negative	139	0.0068 (0.000022-1.459)	
CDKN2A, *CDKN2B* mutation			
Positive	9	0.0024 (0.0001-0.3260)	0.699
Negative	142	0.0077 (0.000022-1.459)	

Note: *10 patients with Ph$^+$ALL, 17 patients with Ph-like ALL.

Table 3. The correlation between LCN2 mRNA expression in BM at diagnosis of childhood ALL and treatment response

Treatment response	Number	LCN2 mRNA level (median, range)	*P* Value
Glucocorticoids (GC) sensitivity test			
Sensitivity	121	0.0119 (0.0001-1.459)	0.047
Insensitivity	30	0.0007 (0.000022-0.0565)	
D19-minimal residual disease (MRD)			
Negative	72	0.0079 (0.000022-1.459)	0.0039
Positive	79	0.0005 (0.000043-0.2137)	
D46-MRD			
Negative	143	0.0064 (0.000022-1.459)	0.866
Positive	8	0.02195 (0.000043-0.1437)	
Relapse$^\#$			
No	47	0.0156 (0.0002-1.459)	0.356
Yes	5	0.00002 (0.000043-0.005)	

Note: $^\#$52 ALL patients with a follow-up period exceeding 2 years.

CEM-C7 proliferation at 48 h, while it had no effect on CEM-C1 and Molt4 proliferation (**Figure 3D**).

LCN2 overexpression increased intracellular iron and ferrous iron levels in Nalm6 and CEM-C7 cells (P = 0.0026, P = 0.025; P = 0.027, P = 0.0005), but not in CME-C1 and Molt4 (P = 0.25, P = 0.34; P = 0.17, P = 0.27) (**Figure 3E**). Levels of ROS and MDA, markers of ferroptosis, were assessed. Intracellular levels of ROS and MDA in Nalm6-oeLCN2 and CEM-

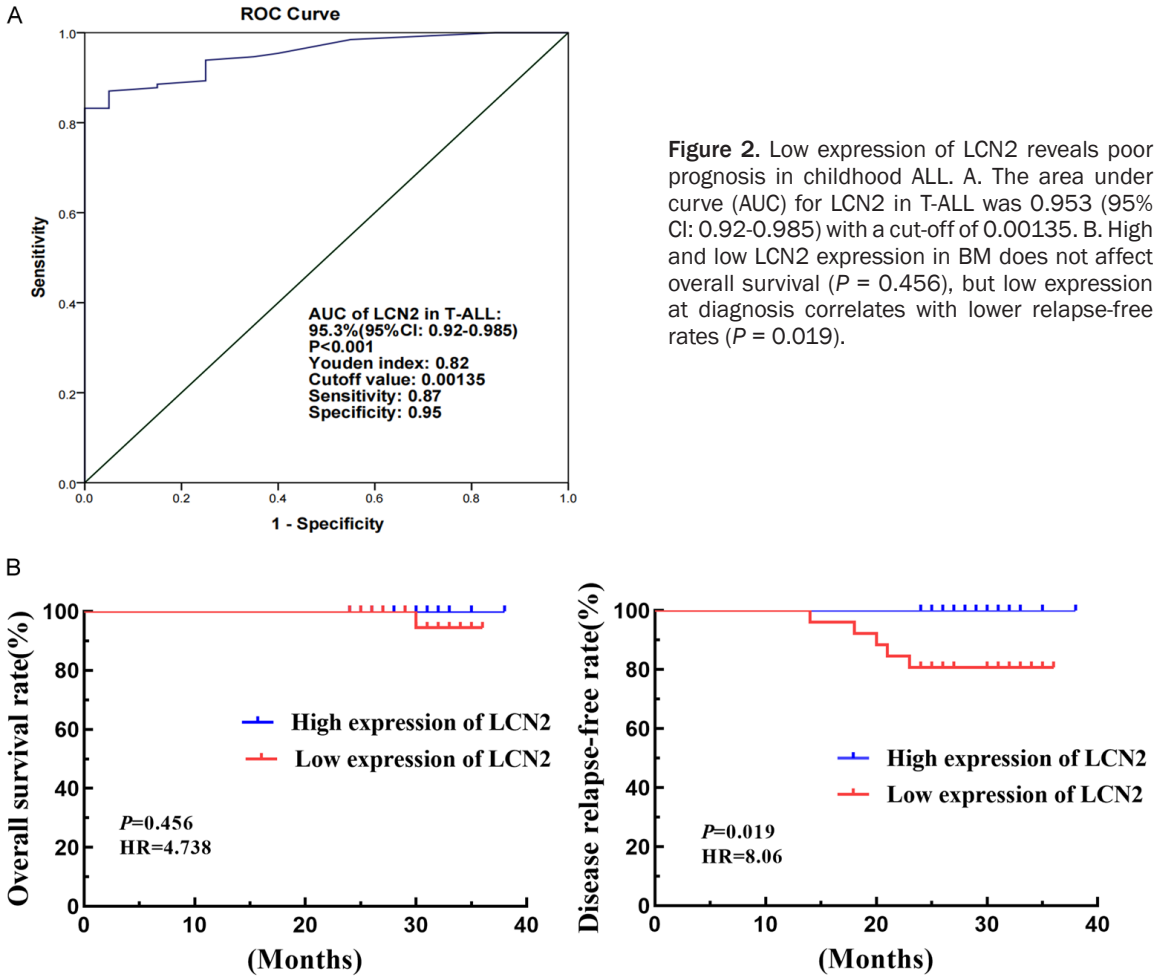


Figure 2. Low expression of LCN2 reveals poor prognosis in childhood ALL. A. The area under curve (AUC) for LCN2 in T-ALL was 0.953 (95% CI: 0.92-0.985) with a cut-off of 0.00135. B. High and low LCN2 expression in BM does not affect overall survival (P = 0.456), but low expression at diagnosis correlates with lower relapse-free rates (P = 0.019).

C7-oeLCN2 were higher than that in Nalm6-oeEV and CEM-C7-oeEV (P < 0.0001 and P < 0.0001; P = 0.0139 and P = 0.0116, respectively) (**Figure 3F**). GSH, an antioxidant that inhibits ferroptosis, was decreased in Nalm6 and CEM-C7 with LCN2 overexpression (P < 0.0001 and P < 0.0001, respectively) (**Figure 3F**).

Reversal of GC resistance by LCN2 and underlying molecular mechanisms

We investigated whether LCN2 overexpression could overcome GC resistance in leukemia cells. The half-maximal inhibitory concentration (IC50) of Dex, a commonly used GC drug, in CEM-C1-oeLCN2 and Molt4-oeLCN2 was significantly decreased. After LCN2 overexpression, the IC50 of Dex dropped from 70 μM to 8 μM in CEM-C1 and from 89 μM to 1.5 μM in Molt4 (**Figure 4A**). To assess the functional consequences of this reduction, we treated CEM-1 and Molt4 cells with their respective reduced Dex IC50 concentrations (8 μM for CME-C1-oeLCN2 and 1.5 μM for Molt4-oeLCN2) for 48 h. The percentage of apoptotic cells increased in both CME-C1-oeLCN2 (51.7%) compared to CEM-C1-oeEV (12.7%) and Molt4-oeLCN2 (39.8%) compared to Molt4-oeEV (8.1%) (**Figure 4B**). Furthermore, combining exogenous LCN2 (50 ng/mL) with Dex (1 μM) showed a stronger inhibitory effect on CEM-C1 and Molt4 proliferation compared to either Dex or LCN2 alone (P = 0.00083, P = 0.00019; P = 0.0019, P = 0.002) (**Figure 4C**). These findings collectively suggest that LCN2 can reverse GC resistance in leukemia cells.

To elucidate the mechanism by which LCN2 enhances GC sensitivity, we analyzed the expression of GR, p-GR, and key proteins in GC resistance-related pathways. The expression of GR, p-GR (Ser211), and p-GR (Ser226) were elevated in LCN2-overexpressing CEM-C1 and

Table 4. The correlation between LCN2 expression in plasma at diagnosis of childhood ALL and clinical features and treatment response

Clinical features/Treatment response	Number	LCN2 expression level (median, range) (pg/ml)	*P* Value
Age			
1-10	47	1716 (343.7-26969)	0.321
≥ 10	10	1587 (427.4-2480)	
Gender			
Male	32	1770 (433.1-26969)	0.064
Female	25	1305 (343.7-4453)	
Race			
Han	50	1713 (343.7-26969)	0.648
Non-Han	7	2179 (427.4-17693)	
WBC count			
$< 50\times10^9$/L	44	1713 (343.7-17693)	0.018
$\geq 50\times10^9$/L	13	2056 (808.3-26969)	
Immunophenotype			
B-ALL	46	1704 (343.7-17693)	0.003
T-ALL	11	2376 (808.3-26969)	
Karyotype[#]			
Hyperdiploid	8	604.4 (343.7-1710)	0.171
Non-hyperdiploid	42	1770 (375.3-26929)	
CNS involvement			
CNS-1	43	1725 (343.7-23463)	0.632
CNS-2 and CNS-3[*]	14	1341 (433.1-26969)	
D19-MRD			
Negative	29	1724 (343.7-26969)	0.553
Positive	28	1710 (427.4-23463)	
D46-MRD[&]			
Negative	51	1710 (343.7-26969)	0.502
Positive	5	2027 (427.4-2376)	
GC sensitivity test			
Sensitivity	45	1703 (343.7-23463)	0.099
Insensitivity	12	2150 (489.6-26969)	
Clinical risk stratification			
Low risk group	21	1698 (343.7-2457)	0.067
Medium-high risk group[##]	36	1948 (427.4-26969)	

Note: [#]7 ALL patients without mitotic phase. [&]1 ALL patient one patient succumbing before the D46-MRD test. [*]1 ALL patient with CNS-3 involvement. [##]2 ALL patients classified as high risk.

Molt4 compared to controls (**Figure 4D**). Conversely, the expression of Notch1, a protein associated with GC resistance, was lower in LCN2-overexpressing CEM-C1 and Molt4 than in controls (P = 0.0013 and P = 0.00047, respectively) (**Figure 4D**). However, the key proteins in PI3K/Akt/mTOR and MAPK signaling pathways showed no difference in CEM-C1 and Molt4 after LCN2 overexpression. Finally, we examined the expression of Bim and Cleaved Caspase-3, proteins involved in the apoptotic pathway triggered by Dex. Dex treatment increased the expression of Bim and Cleaved caspase-3 in CEM-C1-oeLCN2 and Molt4-oeLCN2 compared to controls (**Figure 4D**). This suggests that LCN2 overexpression enhances the pro-apoptotic effects of Dex.

Discussion

In this study, we explored the expression, prognostic value, underlying mechanisms, and pos-

Table 5. The correlation between LCN2 expression in cerebrospinal fluid (CSF) at diagnosis of childhood ALL and clinical features and treatment response

Clinical features/Treatment response	Number	LCN2 expression level (median, range) (pg/ml)	*P* Value
Age			
1-10	41	31.63 (2.833-125.1)	0.110
≥ 10	9	29.62 (2.863-253.0)	
Gender			
Male	29	28.90 (2.833-63.77)	0.058
Female	21	36.34 (2.841-253)	
Race			
Han	43	31.36 (2.833-253.0)	0.632
Non-Han	7	33.00 (3.040-96.07)	
WBC count			
$< 50\times10^9$/L	43	33.00 (2.833-253.0)	0.303
$\geq 50\times10^9$/L	7	22.72 (2.863-46.14)	
Immunophenotype			
B-ALL	42	32.32 (2.841-253.0)	0.339
T-ALL	8	22.92 (2.833-46.14)	
Karyotype[#]			
Hyperdiploid	10	12.71 (2.863-253.0)	0.956
Non-hyperdiploid	36	34.58 (2.833-125.1)	
CNS involvement			
CNS-1	40	36.04 (3.040-253.0)	0.005
CNS-2 and CNS-3[*]	10	12.71 (2.833-22.72)	
D19-MRD			
Negative	32	31.31 (2.833-125.1)	0.278
Positive	18	31.50 (3.040-253.0)	
D46-MRD[&]			
Negative	47	31.63 (2.833-253.0)	0.429
Positive	3	5.064 (3.040-48.40)	
GC sensitivity test			
Sensitivity	43	33.00 (2.833-253.0)	0.772
Insensitivity	7	21.30 (2.894-96.7)	
Clinical risk stratification			
Low risk group	23	31.36 (2.841-63.77)	0.244
Medium-high risk group[&]	27	31.63 (2.833-253.0)	

Note: [#]4 ALL patients without mitotic phase. [*]1 ALL patient with CNS-3 involvement. [&]1 ALL patients classified as high risk.

sibility of LCN2 as a targeted therapy in childhood ALL. Patients with ALL exhibited low expression of LCN2 at diagnosis, and its expression restored after chemotherapy. Importantly, the low LCN2 expression in BM at diagnosis was associated with a poorer prognosis, including lower disease relapse-free survival rates. The growth and proliferation of GC-sensitive leukemia cells were inhibited, and ferroptosis was enhanced by LCN2. In GC-resistant cells, the sensitivity to GC was enhanced by LCN2 via the up-regulation of GR and p-GR expression and inhibition of Notch signaling.

A large number of studies have confirmed that LCN2 is up-regulated in breast cancer, prostate cancer, pancreatic cancer, and other solid tumors [12-15]. However, this study found that LCN2 was down-regulated in childhood ALL, suggesting that it played a distinct functional role in hematological malignancies compared to solid tumors. Moreover, LCN2 expression was up-regulated in CML [16, 17], but it was

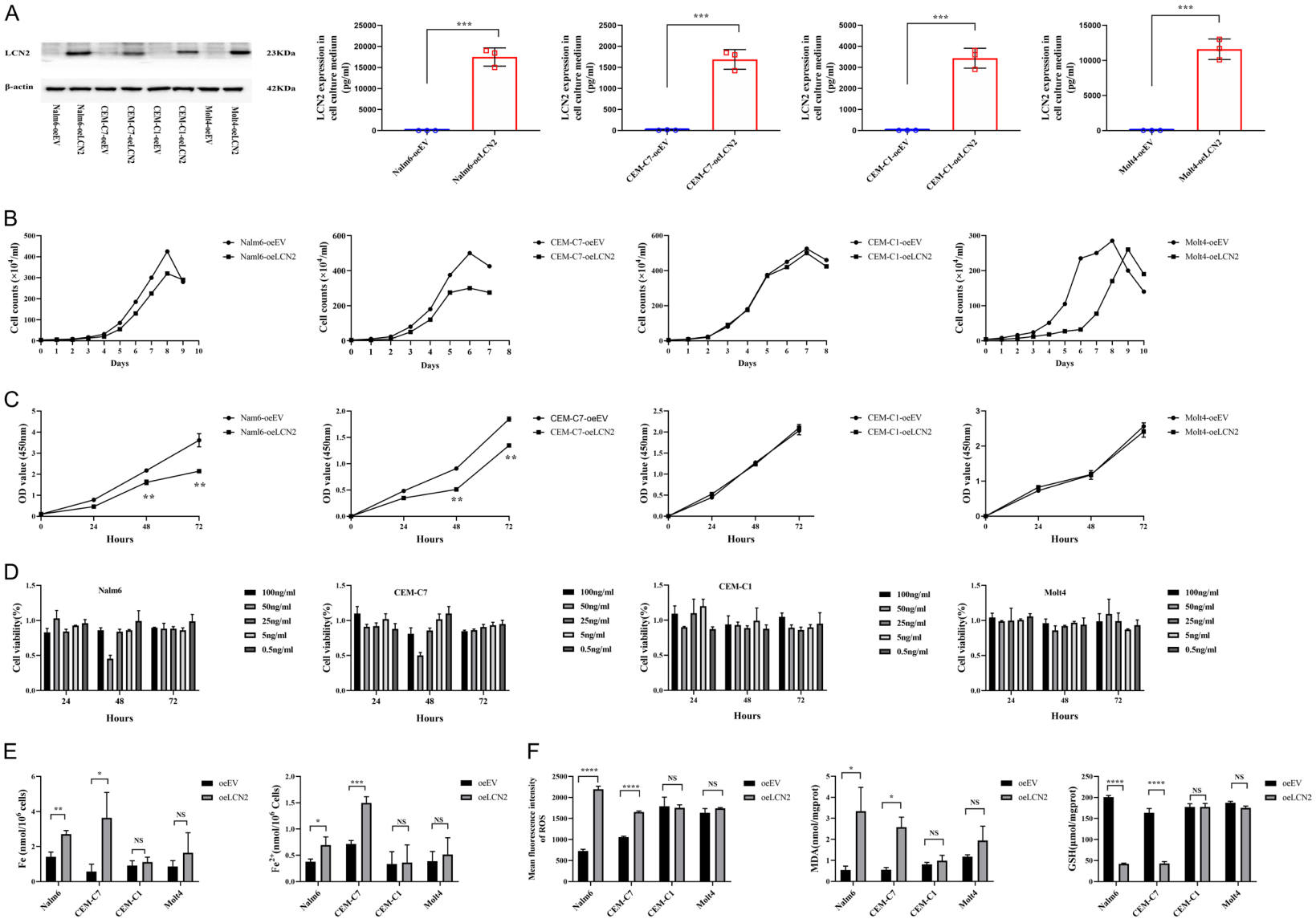
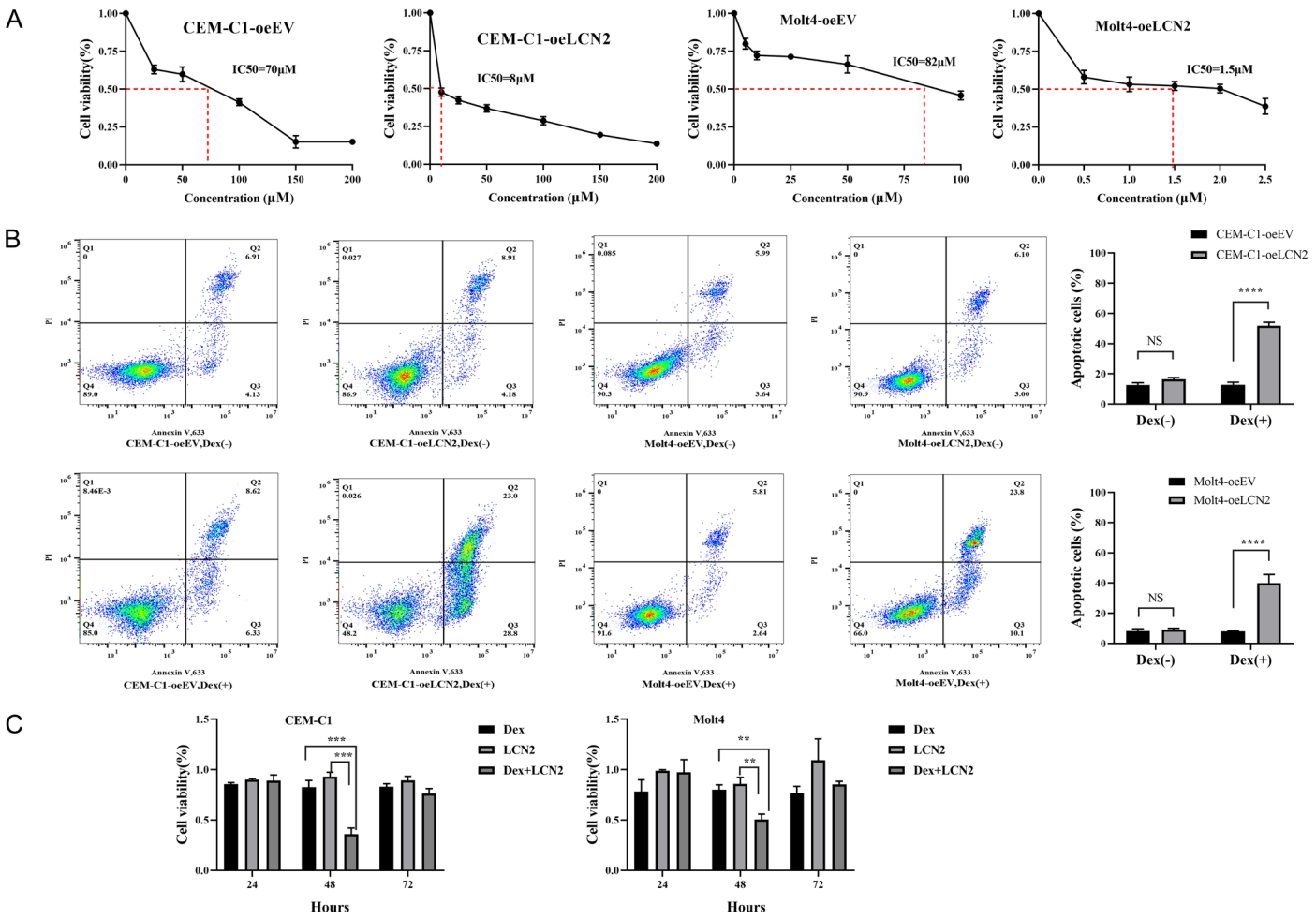


Figure 3. LCN2 inhibits ALL cells growth and proliferation, and promotes ALL cells ferroptosis. A. The up-regulation of LCN2 was observed in Nlam6-oeLCN2, CME-C7-oeLCN2, CME-C1-oeLCN2, and Molt4-oeLCN2 cell lines. B. The overexpression of LCN2 inhibited the growth of Nlam6 and CEM-C7, while it did not affect the growth of CEM-C1 and Molt4. C. The overexpression of LCN2 resulted in inhibition of proliferation in Nlam6 and CEM-C7, while no significant effect was observed on

the proliferation of CEM-C1 and Molt4. D. Following a 48 h intervention with exogenous LCN2 (50 ng/ml) on Nalm6 and CEM-C7, the highest inhibition rate of cell proliferation reached 40-50%, indicating a pronounced impact on these cell lines compared to CEM-C1 and Molt4. E. The overexpression of LCN2 led to an increase in total iron and ferrous iron levels in Nalm6 and CEM-C7 cell lines ($P = 0.0026$, $P = 0.025$; $P = 0.027$, $P = 0.0005$), while no significant changes were observed in CEM-C1 and Molt4. F. LCN2 overexpression resulted in elevated levels of reactive oxygen species (ROS) and malondialdehyde (MDA) ($P < 0.0001$, $P < 0.0001$; $P = 0.0139$, $P = 0.0116$), as well as decreased levels of GSH in Nalm6 and CEM-C7 ($P < 0.0001$, $P < 0.0001$), with no significant changes observed in CEM-C1 and Molt4.



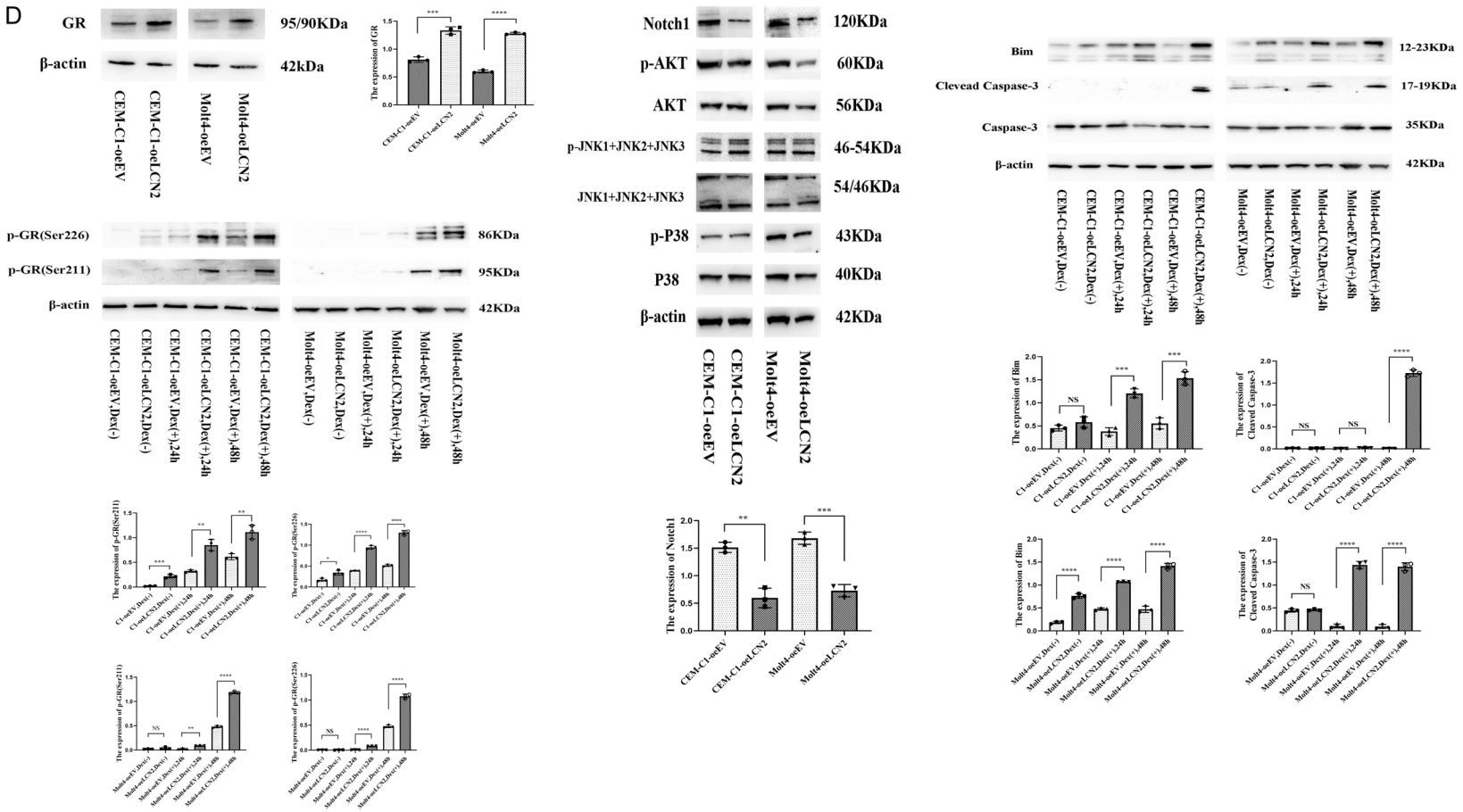


Figure 4. The potential reversal effect and underlying mechanism of LCN2 on GC resistance in ALL cells. A. The up-regulation of LCN2 led to a reduction in the IC50 of dexamethasone (Dex) in CEM-C1 and Molt4 cells. B. Flow cytometry analysis revealed an increased proportion of apoptotic cells in CEM-C1 and Molt4 cells with up-regulated LCN2 expression following treatment with Dex ($P < 0.0001$, $P < 0.0001$). C. The combination of LCN2 inhibition (50 ng/ml) and Dex (1 μM) demonstrated a more significant inhibitory effect on cell proliferation in CEM-C1 and Molt4 compared to either treatment alone ($P = 0.00083$, $P = 0.00019$; $P = 0.0019$, $P = 0.002$). D. LCN2 was found to promote the up-regulation of GR and p-GR (at Ser211 and Ser226) expression in CEM-C1 and Molt4 ($P = 0.0004$, $P < 0.0001$; $P = 0.0016$, $P < 0.0001$, $P = 0.0048$, $P < 0.0001$; $P = 0.0012$, $P < 0.0001$, $P < 0.0001$, $P < 0.0001$). LCN2 was found to inhibit the expression of Notch1 in CEM-C1 and Molt4 cells ($P = 0.0013$, $P = 0.00047$). The overexpression of LCN2 in CEM-C1 and Molt4 cells led to the up-regulation of Bim and Cleared caspase-3 induced by Dex ($P = 0.0004$, $P = 0.0008$, $P < 0.0001$, $P < 0.0001$; $P < 0.0001$, $P < 0.0001$, $P < 0.0001$).

down-regulated in AML [19] and childhood ALL, as observed here. This suggests that LCN2 might be important during the early differentiation stages of hematopoietic stem cells and promote excessive proliferation of pluripotent stem cells and directional progenitor cells. Furthermore, our study found that the expression of LCN2 in BM, PB, and CSF was restored after treatment for childhood ALL. This further suggests that down-regulation of LCN2 plays an important role in the initiation and development of childhood ALL.

In the majority of solid tumors, high expression of LCN2 is associated with a poorer prognosis [15, 20-22]. However, we found that low expression of LCN2 in BM at diagnosis in ALL was correlated with unfavorable clinical features, poor response to therapy, and a lower disease relapse-free survival rate. Moreover, ALL patients with low LCN2 expression in CSF were more likely to develop CNS leukemia. However, the expression of LCN2 in PB at diagnosis was not linked to prognosis. We speculate that this might be due to infections, which are common in patients with ALL at diagnosis, leading to a temporary increase in LCN2 secretion in the PB [23-25]. Notably, we found that LCN2 expression in the BM of patients with *RAS* mutations was lower compared to those without. Recent studies have shown that *RAS* mutations in childhood ALL are associated with a higher risk of induction chemotherapy failure [26] and a poorer prognosis [27, 28]. Therefore, we speculated that LCN2 might be a downstream molecule influenced by *RAS* mutations, potentially contributing to treatment failure and recurrence in ALL. Interestingly, in our study, we did not find an up-regulation of LCN2 expression in the BM of ALL patients with the *BCR-ABL* fusion gene. In contrast, sustained LCN2 secretion has been observed in the BM of patients with CML harboring the fusion gene [16, 29]. This suggests the presence of an upstream mechanism in childhood ALL that specifically inhibits LCN2 up-regulation by the *BCR-ABL* fusion gene.

In solid tumors, LCN2 plays a pro-tumor role by promoting cancer cell proliferation, infiltration, metastasis, and angiogenesis while inhibiting cancer cell apoptosis [16, 21, 30-33]. We found that LCN2 overexpression inhibited the proliferation of Nalm6 and CEM-C7 leukemia cells. Interestingly, it had no effect on their cell cycle or apoptosis. This anti-cancer effect aligns with AML research, where LCN2 was shown to promote apoptosis in cells with a specific *NPM1* mutation [19]. LCN2 is an iron-regulated metabolic protein. In rectal cancer, LCN2 inhibits ferroptosis by reducing intracellular iron levels and up-regulating the expression of proteins that help manage iron (glutathione peroxidase 4 and cysteine glutamate reverse transporter, xCT) [34]. Conversely, in liver cancer, LIFR deficiency resulted in SHP1 activation of NF-κB signaling and up-regulation of LCN2 expression, leading to resistance to iron-based therapies [11]. In contrast, our study found LCN2 promoted ferroptosis in GC-sensitive leukemia cell lines. This suggests that LCN2 could serve as a therapeutic target to induce ferroptosis and improve the prognosis of patients with ALL.

In solid tumors, LCN2 promotes resistance to chemotherapy, such as cisplatin resistance in prostate cancer [35], doxorubicin resistance in breast cancer [36], and cisplatin resistance in oral squamous cell carcinoma [37]. In contrast, our study on childhood ALL found that LCN2 reversed resistance to GC, a cornerstone therapy for this disease. We identified that LCN2 overexpression enhances GC sensitivity by up-regulating GR and p-GR expression and down-regulating Notch signaling. In childhood ALL, GC resistance during induction chemotherapy leads to poor treatment response and prognosis, requiring higher-intensity chemotherapy to eliminate MRD. Such aggressive regimens unfortunately come with a higher risk of serious treatment-related complications [38]. Our findings suggest that LCN2 could be a promising target to overcome GC resistance in childhood ALL, potentially leading to improved treatment outcomes.

Our study has limitations. The follow-up time for patients with childhood ALL was relatively short, and longer follow-up studies are needed. Additionally, animal experiments are required to validate the therapeutic potential of LCN2 in ALL. Our research revealed a tumor suppressor role for LCN2 in childhood ALL, which is in contrast to its pro-tumorigenic effects observed in many other cancers. Therefore, further investigation is necessary to determine whether LCN2-based therapies

for ALL could increase the risk of developing secondary tumors later in life. Understanding the dual role of LCN2 in different cancer types could lead to more tailored and effective therapeutic strategies across a range of malignancies.

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Written informed consent was obtained for each patient's guardian.

Disclosure of conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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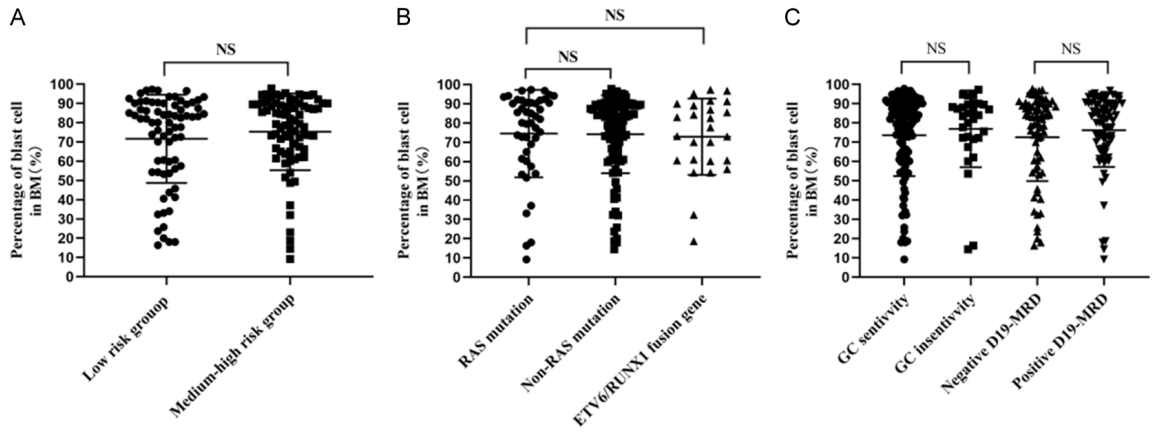

Figure S1. The distribution of blast cells in the BM among different clinical risk groups, molecular changes, and treatment responses in pediatric patients with ALL. A. There was no significant difference in the distribution of blast cells in the BM between low-risk and medium-high-risk ALL groups (P = 0.614). B. ALL groups with *RAS* mutation, non-*RAS* mutation, and *ETV6/RUNX* fusion gene showed no significant differences in blast cell distribution (P = 0.874, P = 0.764). C. The distribution of blast cell percentage in the BM was similar across ALL subgroups, regardless of GC sensitivity, GC insensitivity, negative D19-MRD, or positive D19-MRD (P = 0.495, P = 0.364).

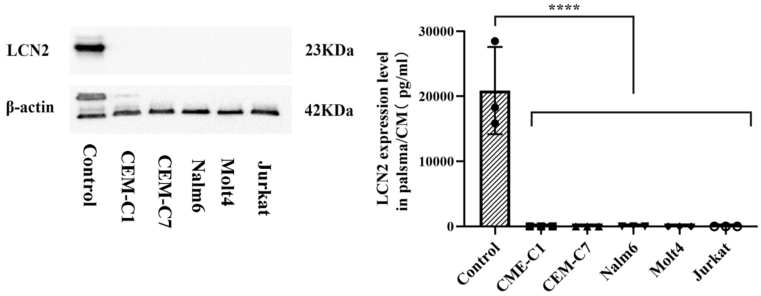

Figure S2. Leukemia cell lines shows a low expression status of LCN2.

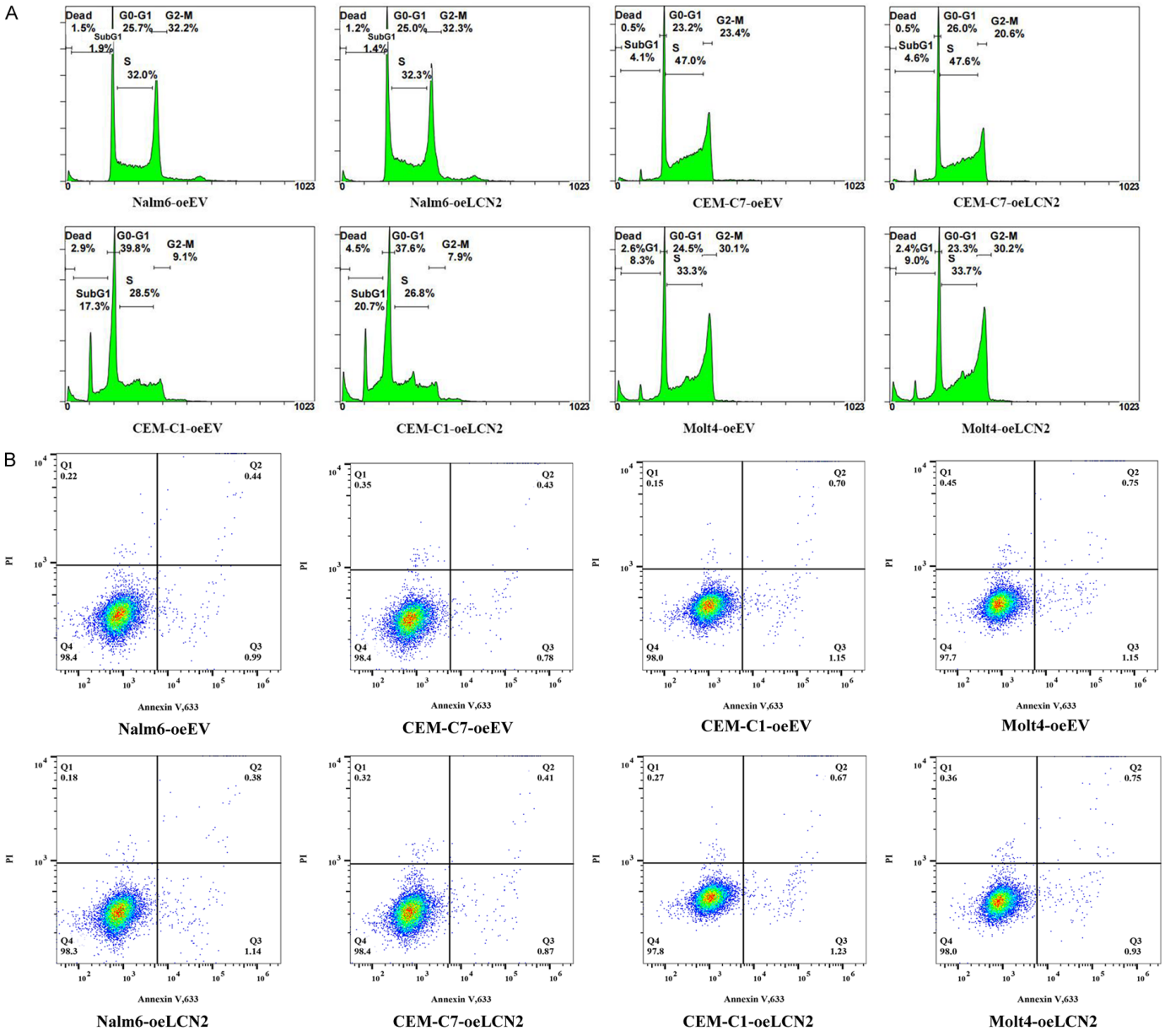

Figure S3. LCN2 has no impact on the cell cycle or apoptosis of ALL cell lines. A. The overexpression of LCN2 did not influence the cell cycle of Naml6, CEM-C7, CEM-C1, and Molt4 cells. B. The overexpression of LCN2 unaffected apoptosis of Naml6, CEM-C7, CEM-C1, and Molt4 cells.