ABL1 and Cofilin1 promote T-cell acute lymphoblastic leukemia cell migration

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Running title: Novel mechanism of ABL1 in T-cell acute lymphoblastic leukemia

Abstract

The fusion gene of *ABL1* is closely related to tumor proliferation, invasion and migration. It has been reported recently that ABL1 itself has been required for T-cell acute lymphoblastic leukemia cell migration towards CXCL12. Further experiments revealed that ABL1 inhibitor Nilotinib inhibited leukemia cell migration towards CXCL12, indicating the possible application of Nilotinib in T-ALL leukemia treatment. To further explore the specific mechanism of this process, in the present study ABL1 interacting proteins were characterized and their roles in the process of leukemia cell migration induced by CXCL12 were investigated. Co-immunoprecipitation in combination with MS analysis identified 333 proteins that interact with ABL1, which included Cofilin1. Gene ontology analyses revealed that many of them were enriched in the intracellular organelle or cytoplasm, including nucleic acid binding components, transfectors or co-transfectors. Kyoto Encyclopedia of Gene and genome analysis showed that the top three enriched pathways were translation, glycan biosynthesis and metabolism, together with human diseases. ABL1 and Cofilin1 were in the same complex. Cofilin1 binds the SH3 domain of ABL1 directly; however, ABL1 is not required for the phosphorylation of Cofilin1. Molecular docking analysis shows that ABL1 interacts with Cofilin1 mainly through hydrogen bonds and ionic interaction between amino acids residues. The mobility of leukemic cells was significantly decreased by Cofilin1 siRNA. These results demonstrate Cofilin1 as a novel ABL1 binding partner. Furthermore, Cofilin1 participates in the migration of leukemia cells towards CXCL12 , indicating possible targeting to ABL1 and Cofilin1 in T-ALL leukemia treatment.

Keywords: chemokine receptor; non-receptor tyrosine kinase; cytoskeleton; migration

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is a malignant blood disease, characterized by a complex pathogenesis [1]. The primary cause of death is infiltration to organs, such as liver and lung, as well as secondary lymphatic organs. Cell migration is a prerequisite for T-ALL infiltration [2-5]. For example, it has been reported that protein tyrosine phosphatase 4 A3 (PTP4 A3 or PRL-3) plays a key role in the initiation and development of T-ALL by promoting the migration of leukemia cells [2]. Carma1 in the CBM complex (Carma1, Bcl10 and MALT1 together called CBM complex) regulates the migration of T-ALL to the CNS (central nervous system), and the expression level of Carma1 is positively related to the seriousness of T-ALL, since Carma1-deficent T-ALL cells very rarely migrate to the CNS, compared with wildtype T-ALL cells [3]. Therefore, basic knowledges about the mechanism of migration is necessary for the treatment of leukemia due to infiltration.

In the past few decades, most research on the role of ABL1 in leukemia has focused on its transformation form, including the *BCR-ABL* fusion gene, one of the characteristic changes of chronic myeloid leukemia (CML) [6-8] and the *NUP214-ABL1* fusion gene in T-ALL [9-11]. Specific tyrosine kinase inhibitors (TKIs) for ABL1, such as imatinib and Nilotinib have been developed and have greatly improved the survival status of CML patients [6, 7]; however the mechanism of ABL1 and the possible application of ABL1 inhibitor in T-ALL patient treatment remains unclear. To better understand the mechanisms of how ABL1 functions in the CXCR4-mediated leukemia cell migration, we characterized the ABL1 binding partners using Co-immunoprecipitation (Co-IP) followed by mass spectrometry (MS), and further investigated the roles of the binding partners of interest in the migration of the leukemia cell towards CXCL12. The results of this paper provide greater understanding of the molecular mechanism of leukemia migration and provide a novel target of intervention therapy caused by leukemia cell migration.

Materials and Methods

Reagents and Antibodies

Recombinant Human/Feline/RhesusMacaque (Cat. no. 350-NS) and Recombinant Mouse CXCL12/SDF-1α (Cat. no.460-SD) were purchased from R&D systems. Poly-D-lysine (C0312) was purchased from Beyotime Biotechnology. ATP (disodiumsalt) (IA0590) was purchased from Solarbio. Monoclonal antibody (mAb) against ABL1(sc-56887) and monoclonal antibody (mAb) against Cofilin1(E-8) (sc-376476) were purchased from Santa Cruz. Monoclonal antibody to phosphotyrosine

produced in mouse (PY20, P4110) was purchased from Sigma-Aldrich. Antibody against ACTB (D110001) was purchased from Sangon Biotech Company in China. Antibodies against GST-Tag (AE006) and His-tag (AE003) were purchased from Abclonal. Ammonium bicarbonate, dithiothreitol (DTT), iodoacetamide (IAA), and sodium carbonate were purchased from Sigma-Aldrich (St. Louis, MO). Urea and Sodium dodecyl sulfate (SDS) were purchased from Bio-Rad (Hercules, CA). Acetonitrile and water for nano-LC-MS/MS were purchased from J. T. Baker (Phillipsburg, NJ). Trypsin was purchased from Promega (Madison, WI). All other chemical reagents were purchased at analytical grade.

Plasmids The plasmid that expresses GST-Crk-CTD was constructed and owned by our laboratory. The sequence encoding the SH3, SH2, or SH3-SH2 domain of ABL1 was amplified by PCR, using the cDNA of Jurkat cells as the template, and then subcloned into the pGEX-4T-1 vector with *Bam*HI and *Xho*I. The sequence that encodes Cofilin1 was amplified by PCR and subcloned into the pET-32a vector with *Bam*HI and *Xho*I. siRNA sequences and primers designed to construct the corresponding plasmids are shown in Table 1.

Cell culture and siRNA transfection Jurkat and L1210 cells were kindly provided by the Institute of Cell Biology (Shanghai, China). Jurkat cells were maintained at 37 °C with 5% CO_2 in RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin mixture. L1210 cells were maintained at 37 °C with 5% CO_2 in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. For small interfering RNA transfection, 1.5×10^6 Jurkat or L1210 cells were seeded in each well of a 24 well plate pre-coated with 0.1 mg/ml poly-D-Lysine, cultured in 500 µl RPMI1640 or DMEM medium without antibiotics in a 37℃ incubator containing 5% CO2 overnight. Then cells attached to poly-D-Lysine-coated wells were washed three times with PBS, and transiently transfected with small interfering RNAs (siRNAs) oligos targeting Cofilin1 using Lipofectamine 2000 following the manufacturer's instructions for adherent cells. Four to six hours after transfection, the medium was replaced with complete medium. After 24 h, cells were collected and cell lysate was used in the following Western blot assay to detect interfering efficiency or transwell assay.

Transwell assay This was performed using 3.0 µm (for Jurkat) or 8.0 µm (for L1210) pore-sized 24-well transwell chambers and plates (Corning). Before the assay, cells were starved overnight in medium with 0.5% FBS, then washed and re-suspended in serum-free medium at a density of 3×10^6 cells/ml. 100 µl suspension was seeded into each upper chamber and 500 µl of medium supplemented

with CXCL12 (25 ng/ml) or equal volume of medium was added to the lower one. The transwell plates were maintained at 37℃ in a humidified atmosphere with 5% CO_2. After 4 h (for Jurkat) or 8 h (for L1210), each upper well was gently taken away, and 5 pictures of the cells that had migrated to the lower wells were taken randomly under a 4 × objective of a phase contrast microscope. Relative migration rates were the results of dividing the cell number of each group by that of the control group.

Co-immunoprecipitation (Co-IP) 2×10^7 of cells per sample were stimulated with CXCL12 at 37℃ for the indicated time periods and then lysed in the lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 2.5 mM sodium pyrophosphate, 1 mM NaF, 1 mM Na3VO4, 1 mM-glycerophosphate, and 10 μg/ml aprotin/leupeptin/PMSF]. After incubation on ice for 30 min, the lysates were centrifuged at 11000 rpm for 30 min. 500 μg protein supernatants of each sample was incubated on ice with 10 μl protein A/G-Sepharose beads and 3 μg mouse IgG for 2 h and centrifugated at 500 g for 5 min. The supernatants were incubated on ice with 3 μg ABL1 antibody overnight. Then 30 μl protein A/G-Sepharose beads (50% slurry) were added and incubated on ice with rotation for 4 h. After three times of washing with Co-IP lysis buffer, the samples were used for Western blot assay or MS analysis.

Sample preparation and protein digestion Bound proteins were extracted from Co-IP beads using SDT lysis buffer (4% SDS, 100 mM DTT, 100 mM Tris-HCl pH 8.0). Beads were boiled for 5 min, ultrasonicated, and boiled for another 5 min. After centrifugation at 12 000g for 15 min, the supernatant containing proteins were collected and digested with FASP method as described previously [12]. Briefly, the detergent, DTT and IAA in UA buffer (8 M Urea, 150 mM Tris-HCl, pH8.0) were added. Finally, the protein suspension was digested with 2 μg trypsin (Promega) in NH4HCO3 buffer overnight at 37 °C. The peptides were collected by centrifugation at 16 000g for 15 min, desalted with C18 StageTip, vacuum dried and dissolved with 0.1% TFA for LC-MS analysis.

LC-MS/MS analysis Analysis was performed on a Q Exactive HF-X mass spectrometer coupled to Easy nLC (Thermo Scientific). Peptides were loaded to a trap column (100 μm*20 mm, 5 μm, C18, Dr. Maisch GmbH, Ammerbuch, Germany) in buffer A (0.1% Formic acid in water). Reverse-phase high-performance liquid chromatography (RP-HPLC) separation was performed using a self-packed column (75 μm×150 mm; 3 μm ReproSil-Pur C18 beads, 120 Å, Dr. Maisch GmbH, Ammerbuch, Germany) at a flow rate of 300 nL/min. The RP-HPLC mobile phase A was 0.1% formic acid in water, and B was 0.1% formic acid in 95% acetonitrile. The gradient was set as follows: 2%-8% buffer B from 0 min to 2

min, 8% to 28% buffer B from 2 min to 42 min, 28% to 40% buffer B from 42 min to 50 min, 28% to 40% buffer B from 50 min to 51 min, 40% to 100% buffer B from 51 to 60 min. MS data was acquired using a data-dependent top 20 method dynamically choosing the most abundant precursor ions from the survey scan (300-1500 m/z) for HCD fragmentation. A lock mass of 445.120025 Da was used as internal standard for mass calibration. The full MS scans were acquired at a resolution of 60,000 at m/z 200, and 15,000 at m/z 200 for MS/MS scan. The maximum injection time was set to for 50 ms for MS and 50 ms for MS/MS. Normalized collision energy was 28 and the isolation window was set to 1.6 Th. Dynamic exclusion duration was 60 s.

Sequence database searching and data analysis The MS data were analyzed using MaxQuant software version 1.6.0.16. MS data were searched against the *Homo sapiens* UniProtKB database (www.uniprot.org). The maximal two missed cleavage sites and the mass tolerance of 4.5 ppm for precursor ions and 20 ppm for fragment ions were defined for the database search. Carbamidomethylation of cysteines was defined as fixed modification, while acetylation of protein N-terminal, oxidation of Methionine was set as variable modifications for database searching. The database search results were filtered and exported with <1% false discovery rate (FDR) at peptide-spectrum-matched level, and protein level, respectively.

Bioinformatic analysis To annotate the sequences, information was extracted from UniProtKB/Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG), and Gene Ontology (GO). GO and KEGG enrichment analyses were carried out with the Fisher's exact test, and FDR correction for multiple testing was also performed. GO terms were grouped into three categories: biological process (BP), molecular function (MF), and cellular component (CC). Construction of protein–protein interaction (PPI) networks were conducted by using the STRING database with the cytoscape software.

Protein molecular docking To investigate the binding mode between human Cofilin1 and ABL1, molecular docking was performed by Schrodinger 2019 software as previously described [13]. The 3D structure of the human Cofilin1 (PDB ID: 4BEX) and ABL1-SH3(PDB ID: 5OAZ) were downloaded from the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). For docking, the default parameters were used as described. Cofilin1 was set as the receptor. ABL1-SH3 was set as the ligand. The distances between amino acids and possible forces in each group were analyzed. The global surface binding and the interactions between amino acids were exported.

Fusion protein expression and in vitro protein binding assay Plasmids that expressed GST-ABL1-SH3, GST-ABL1-SH2, GST-ABL1-SH3-SH2 or His-Cofilin1 were transformed into *E. coli* BL21, and expression was induced with isopropyl β-D-thiogalactoside (1 mM) for 4 h. After induction, cells were sonicated and proteins were purified using glutathione-Sepharose 4B beads or Ni^{2+} Sepharose beads according to the manufacturer's instructions.

The *in vitro* protein binding assay was conducted as previously described [14]. Approximately 1μg of GST, GST-ABL1-SH3, GST-ABL1-SH2, or GST-ABL1-SH3-2 (fusion protein bound to beads) were respectively incubated with 1μg of eluted His-Cofilin1 fusion protein in 500 μl of modified GBT buffer [10% glycerol, 50 mM HEPES-NaOH (pH 8.0), 175mM KCl, 7.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 1% Triton X-100]. After 4 h, the proteins bound to beads were collected by centrifugation, washed with GBT buffer, separated by 12% SDS-PAGE and detected by Western Blot assay with anti-His Ab to detect the bound His-Cofilin1 in each sample. SDS-PAGE gel was stained with coomassie blue to show the amount of GST-tagged fusion protein used the binding assay.

In vitro kinase assay 800 ng of recombinant human active ABL1 (mutated Y253 F) protein was co-incubated with 200 μM ATP and 1.2 μg of His-Cofilin1 in 40 μl of kinase buffer [5 mM β-glycerophosphate, 25 mM Tris (pH7.5), 2 mM DTT, 1 mM Na3VO4, 10 mM $MgCl_2$] at 30℃ for 30min. The samples were terminated by boiling with 10 μl of 5×Loading buffer together at 98℃ for 10 min, then resolved by SDS-PAGE and detected by Western Blot assay with PY20 (antibody to pan-phosphorylated tyrosines) to detect the tyrosine-phosphorylation of Cofilin1.

Western Blot Assay Proteins were separated by SDS-PAGE, and then transferred to nitrocellulose membranes. The membranes were blocked with 5% defatted milk in TBST for 1 h at room temperature and incubated with primary antibody overnight at 4°C. After washing, the membranes were incubated with HRP-conjugated secondary antibody at room temperature for 1 h. Finally, the membranes were washed and visualized by using ECL Western blotting reagents.

Xenografts

Six-week-old male DBA/2 mice were purchased and maintained as approved by the governmental animal care and use committees. The mice were injected intravenously with 2.5×10^6 L1210 cells infected with using lentiviral expressing ABL1 shRNAs or control shRNA. Animals were sacrificed 4 weeks later.

Statistical Analysis. All the experiments were performed in triplicate. Data were expressed as the

mean ± SD. One-way ANOVA and t-tests were performed for comparison between two groups. The statistical analysis software package SPSS 21.0 was employed for the statistical comparisons. A p value < 0.05 was considered significant.

Results

ABL1 was required by T-ALL cell migration toward CXCL12 in vitro and infiltration in vivo

To identify the role of ABL1 in T-ALL cell migration, Jurkat and L1210 cell lines were preincubated with ABL1 inhibitor Nilotinib before the transwell assay. Results showed that Nilotinib significantly inhibited T-ALL cell migration towards CXCL12, suggesting the possible application of Nilotinib in T-ALL treatment (Figure 1). Then, L1210 cells were infected with lentiviruses encoding shRNAs to ABL1, and transwell assays were conducted. Results showed that when ABL1 was knocked down by shRNA, L1210 cells were not able to migrate towards CXCL12, demonstrating the involvement of ABL1 in CXCL12-induced leukemia cell migration (Figure 2A-B). To further explore the role of ABL1 *in vivo*, L1210 cells expressing ABL1 shRNAs or control shRNA were injected via tail vein into DBA/2 mice. After 28 days, mice were sacrificed. Spleen index and liver index were calculated. Results showed that spleen index was reduced significantly by ABL1 shRNAs compared with the control group, suggesting the involvement of ABL1 in the infiltration of L1210 cells into spleen (Figure 2C-D).

Identification of ABL1-interacting proteins

To characterize proteins attached to ABL1, we performed Co-IP using ABL1 antibody and identified the partner proteins using LC-MS/MS. As shown in supplemental Table 1 and Figure 3A, a total of 333 proteins were identified, among which 277 proteins were identified from the treated samples, 279 proteins were identified from the control samples, and 223 proteins were identified from both control and CXCL12-treated samples. Thus, 54 proteins were responsive to the experimental CXCL12 treatment.

Bioinformatics analysis of the ABL1 interacting proteins GO clustering analysis was performed to provide relevant information about their biological processes, molecular functions, and cellular components. Within the biological process category, the majority of the proteins were involved in mRNA metabolic or catabolic processes. For molecular functions, the data indicated that most of the proteins were linked to binding. Regarding cellular components, the ribonucleoprotein complex, cytosolic ribosome, ribosome subunit, vesicle or exosome, membrane-bounded vesicle, extracellular

bounded organelle or other extracellular region elements were the top ranked categories (Figure 3B-C, Table S2). Genes in the cellular component (CC) were mainly distributed in the intracellular organelle or cytoplasm (Figure 3D). The main molecular functions (MF) were nucleic acid binding or transcription factor/cofactor binding (Figure 3E). KEGG pathway enrichment analysis revealed that the ABL1-interacting proteins were related to 170 pathways (Table S3). The 15 highly enriched pathways are shown in Figure 4E, among which 10 pathways have significant p value. The five most significantly enriched were ribosome, spliceosome, DNA replication, mismatch repair, and nucleotide excision repair (Figure 3F).

Novel ABL1-binding proteins related to cytoskeleton regulation

Cell migration is a multi-step dynamic process regulated by many factors, and the cytoskeleton is the most important in cell migration [15, 16]. Therefore, cytoskeletal regulators and cytoskeletal binding proteins that bind ABL1 may play an important role in migration of T-ALL cells. Results showed a large number of proteins that directly or indirectly regulate the cytoskeleton in its immunoprecipitation complex (Table S1). A total of 10 cytoskeleton related proteins that might interact with ABL1 were selected according to the literature (Table 2). Then the network of those cytoskeleton-related proteins and ABL1 were analyzed using STRING software. Results showed that Vinculin (VCL) and 14-3-3 protein epsilon (YWHAE) were connected with ABL1; RhoGDI2 (ARHGDIB) and PREX2 were not present in the network with ABL1. Cofilin1 (CFL1) was connected to ABL1 by 14-3-3 protein epsilon (Figure 3G).

Our recent report has shown that ABL1 is responsible for the phosphorylation of RhoGDI2 (26). Cofilin1 is an F-actin-binding protein that regulates dynamic changes in the cytoskeleton. Therefore, we verified the interaction of RhoGDI2 and Cofilin1 with ABL1 by Co-IP and subsequent Western blot assay. The results showed that ABL1 and Cofilin1 co-existed in the immunoprecipitation complex independent of the stimulation of CXCL12 (Figure 4A-D).

ABL1 combines with Cofilin1 through its SH3 domain

Additionally, the key subunit of ABL1 in its binding to Cofilin1 was investigated using an *in vitro* protein binding assay. The results showed that ABL1 binds Cofilin1 through its SH3 domain directly (Figure 5A). Phosphorylated Cofilin1 is not able to shear the actin filament and does not bind to the actin monomer, thereby increasing the stability of the actin filament [17]. Next, we used an *in vitro* kinase assay to test whether active ABL1 could phosphorylate Cofilin1. Results showed that ABL1 had

no effect on the phosphorylation of Cofilin1 (Figure 5B). We also analyzed the specific interaction between ABL1-SH3 domain and Cofilin1 by molecular docking using Schrodinger 2019 software. The results showed that the ABL1-SH3 domain could form a suitable binding mode for Cofilin1 protein (Figure 5C). 18 amino acids in ABL1-SH3, namely Phe72, Ser75, Gly76, Asp77, Asn78, Thr79, Gly92, Tyr93, Asn94, His95, Asn96, Glu98, Trp99, Glu101, Thr110, Pro112, Asn114 and Tyr115, interact with one or more amino acids in Cofilin1; while 21 amino acids in Cofilin1, namely Asp9, Ile12, Lys13, Phe15, Asn16, Asp17, Met18, Lys19, Val20, Arg21, Lys22, Ser23, Arg32, Asp86, Glu97, Asp98, Leu99, Lys127, Thr129, Gly130 and Lys132 interact with one or more amino acids of ABL1-SH3 (Figure 5C).

Knocking down Cofilin1 protein expression via siRNA reduced ALL cell migration toward CXCL12

To further investigate the role of Cofilin1 on T-ALL cell migration toward CXCL12, the expression of Cofilin1 was reduced by siRNA transfection before the transwell assay. The results showed that the decrease of intracellular Cofilin1 protein expression significantly inhibited T-ALL cell migration toward CXCL12 (Figure 6). Together with the above results, this result suggests that ABL1 plays a role in cell migration towards CXCL12 via Cofilin1.

Discussion

ABL1 (*ABL* proto-oncogene 1) is closely related to leukemia. About 8% of T-All patients had the ABL1 fusion gene [18]. In the studies of leukemia, researchers have paid more attention to the role of ABL1 transformation in causing leukemia and ignored the role of ABL1 itself. Our recent work shows that siRNA to ABL1 inhibited T-ALL migration towards CXCL12 [19]. The present study shows that the ABL1-specific inhibitor Nilotinib significantly reduced the migration of Jurkat and L1210 cells toward CXCL12 *in vitro* and infiltration into spleen *in vivo* (Figure 1). Therefore, it would be interesting to explore the possible therapeutic effect of Nilotinib on T-ALL patients in the future. Our current findings improve understanding the molecular basis of anti-leukemic activity by Nilotinib and provide a possible therapeutic strategy in T-ALL therapy.

As a non-receptor tyrosine kinase, ABL1 has a wide range of physiological functions. To reveal the novel mechanisms of ABL1 in CXCR4-mediated migration, cytoskeleton-related proteins that have not been previously reported to be bound by ABL1 were screened based on STRING software, showing that RhoGDI2 and PREX2 were not in the network (Figure 3G). Cofilin1 (CFL1) and ABL1 indirectly interact with each other via YWHAE (Figure 3G). Additionally, it is known that ABL1 kinase in the cytoplasm is able to directly bind F-actin and participate in physiological processes such as

pseudopodia formation, cell adhesion and migration [20, 21]. Here, we found that ABL1 directly binds Cofilin1 via its SH3 domain (Figure 5A and Figure 5C). At this point, our results provide another possibility for ABL1 to regulate F-actin through Cofilin1 besides the classical regulation way through direct binding and the previously reported relationship via YWHAE.

Cofilin1 is an ubiquitous actin-binding protein that can break the fibrous actin filaments into a globular actin monomer by hydrolysis of ATP [22, 23], and thereby participate in numerous cell processes, such as cell structure changes [24], cell cycle inhibition [25], cell growth and differentiation [26]. Cofilin1 is closely related to solid tumors, including prostate cancer [27, 28], liver cancer [29], non-small cell lung cancer [30] and human colorectal cancer progression and chemoresistance [31]. Mice with specific depletion of cofilin1 in thymocytes showed increased steady-state levels of actin filaments, and associated alterations in the pattern of thymocyte migration and adhesion. In a collagen matrix, cofilin1 controlled the speed and resting intervals of migrating thymocytes [32]. Here we reported its role in the migration of malignant blood system tumor cells for the first time based on the literature searching in NCBI-Pubmed and then network analysis using STRING software (Figure 3G).

The function of Cofilin1 is precisely regulated by many factors, including pH value, concentration of calcium, inositol phosphate and kinase [33-35]. RhoGTPase family is a key regulatory molecule that links the cell surface receptor and cytoskeleton [36-38]. The mechanisms driving Cofilin1 activation may be different and dependent on RhoGTPase, at least in part. Cofilin1 is specifically phosphorylated by activated LIM domain kinase (LIMK) at the Ser3 residue in response to different stimuli, including TGF-β [39], gamma-secretase [40]. As an actin-binding protein, Cofilin1 regulates actin dynamics through its phosphorylation by the Rac1/PAK1/LIMK1 pathway [41] and the RhoA/Rock2/Limk1 pathway [42]. As for the upstream kinase of RhoGTPases, the Rho-associated protein kinase (ROCK) pathway has been reported to result in the phosphorylation of Cofilin1 in primary cortical neurons [43]. Wnt5A targeted Rho-associated protein serine/threonine kinase (ROCK), leading to phosphorylation of LIM kinase-2 (LIMK2) and inactivation of the actin depolymerization factor cofilin-1 (CFL1) [44]. ABL1 regulates Vav1, activation, and further affects Rac1/PAK1/LIMK1/cofilin signaling pathway [45]. In our previously published papers, ABL1-activated RhoA and RhoC have been shown to play a key regulatory role in CXCR4-mediated T-ALL cell migration [19, 46]. In the present study, it was demonstrated that ABL1 is responsible for the migration of Jurkat and L1210 cells (Figure 1). Therefore, the RhoA and RhoC activated Cofilin1 might be downstream of ABL1 kinase and involved

in the regulation of actin during the process of leukemic cell migration. We also detected whether ABL1 phosphorylates Cofilin1; however, active ABL1 was not responsible for the phosphorylation of Cofilin1 (Figure 5B). Our result is consistent with a previous study showing that the third conserved amino acid residue Ser at the N-terminus of the Cofilin1 is the only phosphorylation site of Cofilin1 [47].

The target sequences of Nilotinib and Cofilin1 were different. In the present study, it was found that 18 amino acids in ABL1 SH3 domain, namely Phe72, Ser75, Gly76, Asp77, Asn78, Thr79, Gly92, Tyr93, Asn94, His95, Asn96, Glu98, Trp99, Glu101, Thr110, Pro112, Asn114 and Tyr115, interact with Cofilin1 (Figure 5C). Nilotinib binds the Glu286, Thr315, Met318 and Asp381 in the inactive kinase domain of ABL1 [48, 49], in a similar manner with imatinib, including a hydrogen bond with the main chain amide of interlobe residue Met318, a hydrogen bond with the gatekeeper residue Thr315, and hydrophobic interactions with Phe382 from the DFG motif in the "out" conformation [50]. It was found that Cofilin1 binds the ABL1 SH3 domain (Figure 5A). Tyrosine phosphorylation in the SH3 domain disrupts negative regulatory interactions within the ABL1 kinase core. Tyr89 localizes to a binding surface of the SH3 domain that engages the SH2-kinase linker in the crystal structure of the ABL1 core. Phosphorylation at Tyr89 by Src-family kinases prevents engagement of the ABL1 SH3 domain with its intramolecular binding partner leading to enhanced ABL1 kinase activity and cellular signaling [51]. Our result suggests that the binding of ABL1 with Cofilin1 neither affects the activation of ABL1 by Src-family kinases nor prevents the binding of ABL1 inhibitor Nilotinib.

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Figure legends

Figure 1 Nilotinib inhibited the migration of L1210 and Jurkat cells induced by CXCL12.

A. L1210 cells were pre-incubated with 10 µM Nilotinib or an equal volume of solvent, washed and added to the upper chamber. Serum-free medium containing 25 ng/ml mouse CXCL12 or an equal volume of PBS was added to the lower chamber. After culture at 37℃ for 8 h, 5 different visual fields were randomly selected to count the relative cell mobility. B. Nilotinib treated cells were used in the transwell assay without CXCL12 in the lower chamber. C. L1210 cells were incubated with Nilotinib or equal volume of solvent. MTT assay was conducted after culture at 37℃ for 4 h. D-F, L1210 cells

were substituted by Jurkat cells and the transwell assay or MTT assay were conducted as described above.

Figure 2. The interference of ABL1 decreased the migration of L1210 cells induced by CXCL12 and infiltration into the spleen.

A. L1210 cells were infected using lentivirus to express ABL1-shRNA. 72 h after infection, cells were lysed and ABL1 was detected via Western blot assay. ACTB was also detected as a loading control. B. ABL1 knockdown L1210 cells were added to the upper chamber in the transwell assay. After incubation at 37℃ for 8 h, five different fields were randomly selected, pictures were taken and cells and relative cell mobilities were counted. C-D. L1210 cells infected by lentivirus expressing ABL1 shRNAs or control shRNA were injected into DBA/2 mice. After 4 weeks, mice were sacrificed. Liver or spleen index was assessed by dividing the weight of liver or spleen by the body weight.

Figure 3. Bioinformatics analysis of the ABL1-interacting proteins. A. Venn diagram shows the numbers of identified proteins from the control and CXCL12-treated Jurkat cells. B. GO categories of the ABL1−interacting proteins. C-E. The identified proteins were classified into biological process (C), cellular component (D), and molecular function (E) by WEGO according to the GO terms. F. KEGG pathway analysis of the ABL1−interacting proteins. G.Protein-protein interaction (PPI) networks of ABL1 and cytoskeleton regulation-related proteins by using the STRING database with the cytoscape software.

Figure 4. RhoGDI2 and Cofilin1 were in the ABL1 Co-immunoprecipitation complex.

Jurkat (A) or L1210 (B) cells were treated with 25 ng/ml CXCL12 for 30 min and cell lysate was used in the immunoprecipitation experiments using ABL1 antibody. RhoGDI2 antibody (GDI2) was used to detect RhoGDI2 in the Western blot assay, with ABL1 as the loading control and ACTB as the input. C-D. Similar experiments were conducted and Cofilin1 in the immunoprecipitation complex was detected.

Figure 5. ABL1 interacts with Cofilin1 through its SH3 domain. A. The *in vitro* binding assay was conducted as described in Materials and Methods His antibody was used to in the Western blot assay. Gel was stained with Commasie blue. B. The *in vitro* kinase assay was conducted as described in the Materials and Methods section. PY20 and Cofilin1 antibody were in the western blot assay. All results are representative of three separate experiments. (C) The crystal structures of ABL1-SH3 and Cofilin1 were imported into Schrodinger 2019 software and the surface binding model was output in the molecular docking experiment (G, green: Cofilin1, purple: ABL1-SH3). The interaction between amino acids from ABL1-SH3 and Cofilin1 are shown in the red dotted frames.

Figure 6. Effect of Cofilin1 interference on migration of T-ALL cells induced by CXCL12. L1210 (A) or Jurkat (C) cells were transfected with Cofilin1 siRNA. 24 h after transfection, cells were counted and 3×10^5 cells were lysed to detect the interference efficiency of siRNA transfection. Cofilin1 and β-actin antibodies were used in the subsequent Western blot assay, with β-actin antibody (ACTB) as the loading control. Equal amounts of transfected L1210 (B) or Jurkat (D) cells were added to the upper chambers in the transwell assay, with serum-free medium containing 25 ng/ml mouse (B), human CXCL12 (D) or nothing in the lower chambers.

Supplementary Materials: Table S1: Identification of ABL1-interacting proteins in control and CXCL12-treated samples using MS; Table S2: Gene ontology annotation of the ABL1 bound proteins based on biological process Gene Ontology terms in Jurkat T-ALL cells; Table S3: Pathway annotation

of the ABL1-bound proteins in Jurkat T-ALL cells. Figure S1: Binding of ABL1 with RhoGDI2 in L1210 and Jurkat T-ALL cells.

Supplemental Table 1. Identification of ABL1-interacting proteins in control and treated samples using LC-MS/MS.
Supplementary Table 2. Gene ontology annotation of the ABL1 bound proteins in Jurkat T-ALL cells-biological processes.
Supplementary Table 3. Pathway annotation of the ABL1-bound proteins in Jurkat cells.