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RUNX1 transactivates BCR-ABL1 expression in Philadelphia chromosome positive acute lymphoblastic leukemia

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Abstract
The emergence of tyrosine kinase inhibitors as part of a front-line treatment has greatly improved the clinical outcome of the patients with Ph+ acute lymphoblastic leukemia (ALL). However, a portion of them still become refractory to the therapy mainly through acquiring mutations in the BCR-ABL1 gene, necessitating a novel strategy to treat tyrosine kinase inhibitor (TKI)-resistant Ph+ ALL cases. In this report, we show evidence that RUNX1 transcription factor stringently controls the expression of BCR-ABL1, which can strategically be targeted by our novel RUNX inhibitor, Chb-M'. Through a series of in vitro experiments, we identified that RUNX1 binds to the promoter of BCR and directly transactivates BCR-ABL1 expression in Ph+ ALL cell lines. These cells showed significantly reduced expression of BCR-ABL1 with suppressed proliferation upon RUNX1 knockdown. Moreover, treatment with Chb-M' consistently downregulated the expression of BCR-ABL1 in these cells and this drug was highly...
**1 | INTRODUCTION**

Acute lymphoblastic leukemia (ALL) is an acute form of leukemia characterized by the emergence of highly proliferative immature white blood cells, known as lymphoblasts. Approximately 6000 new cases are reported yearly in the United States and ALL is the most frequently encountered malignancy in childhood.\(^1\) ALL is one of the first cancers for which an effective chemotherapeutic treatment was developed and its cure is now a realistic goal and is achieved in more than 90% of affected children,\(^2\)-\(^6\) while only 20%-40% of adults respond to and survive courses of intensified chemotherapies.\(^7\),\(^8\) This difference is supposed to originate from the vulnerability of elderly patients who have weakened immune and circulatory organ systems. Philadelphia chromosome positive ALL (Ph\(^+\) ALL) marks a subset of leukemia with distinctive treatment strategy and outcomes due to the existence of the BCR-ABL1 pathogenic fusion gene that is created by juxtaposing the ABL1 gene on chromosome 9 to part of the BCR gene on chromosome 22.\(^3\),\(^9\) The emergence of imatinib mesylate, a tyrosine kinase inhibitor (TKI) that inhibits ABL1, KIT and PDGFR, entirely changed the game of anti-leukemia strategy toward Ph\(^+\) ALL.\(^10\),\(^11\) Adding imatinib to standard therapy improved the outcomes for adults with Ph\(^+\) ALL, at least in part, by facilitating allogeneic stem cell transplant.\(^12\) However, a portion of adults steadily develop resistance to TKI therapy, mainly through acquiring point mutations in the kinase domain of BCR-ABL1 in ALL cells.\(^13\) These patients can be treated by the next generation of tyrosine kinase inhibitors such as nilotinib, dasatinib, or ponatinib. In particular, the third-generation TKI, ponatinib, is a potent orally bioavailable pan BCR-ABL1 inhibitor that inhibits both wild-type and mutant BCR-ABL1 kinase, including the ‘gatekeeper’ T315I mutation, which is resistant to all other currently available TKIs.\(^14\),\(^15\) However, because of the risk of cardiovascular side effects, the risk/benefit balance must be evaluated for each patient.\(^14\) Therefore, a new treatment modality against TKI treatment-resistant Ph\(^+\) ALL with no side effects is highly needed.

Runt-related transcription factor 1 (RUNX1), also known as acute myeloid leukemia 1 protein (AML1), is an essential master transcription factor implicated in the differentiation and the maintenance of hematopoietic stem cells.\(^16\) In ALL, a well known t(12;21)(p13.1;q22) translocation causes the fusion of the ETS variant 6 (ETV6) and RUNX1 genes (ETV6-RUNX1, formerly TEL-AML1). It is the most common translocation in childhood ALL,\(^17\) suggesting a fundamental involvement of RUNX1 in the pathogenesis of ALL cells. Intriguingly, Yamamoto K et al.\(^18\) reported that the elevated expressions of wild-type RUNX1 closely correlates with worse outcomes in chronic myeloid leukemia (CML) patients, another type of leukemia caused by the same chimeric protein BCR-ABL1 as Ph\(^+\) ALL but with a different break point. The molecular mechanisms underlying the possible interaction of RUNX1 and BCR-ABL1, however, have poorly been elucidated so far. We have previously reported the requirement of RUNX1 in the development and the maintenance of AML,\(^19\)-\(^24\) another form of acute leukemia originating in myeloid progenitor cells. In this report, we addressed the leukemogenic role of RUNX1 in Ph\(^+\) ALL and elaborated to elucidate the molecular mechanisms in the regulation of BCR-ABL1 expression and in the proliferation of Ph\(^+\) leukemia cells.

**2 | MATERIALS AND METHODS**

**2.1 | Cell lines and plasmids**

SU-Ph2 is an imatinib-sensitive cell line established from a patient with Ph\(^+\) ALL. SU/SR is an imatinib-resistant subline of SU-Ph2 obtained after long-term exposure to imatinib until they finally acquired the T315I mutation in BCR-ABL1 gene. These cells were kindly gifted from Dr. A. Kanamaru (Department of Internal Medicine, Kinki University School of Medicine, Osaka, Japan). ALL-derived BALL-1, KOC-45, SUP-B15, SU-Ph2 and SU/SR cells as well as CML-derived MYL, BV173 and KS62 cells were maintained in RPMI 1640 medium with 10% heat-inactivated FBS and 1% penicillin-streptomycin at 37°C in 5% CO\(_2\). Human BCR-ABL1 was a kind gift from Nora Heisterkamp (Addgene plasmid # 31 285). pENTR1A Dual Selection vector (Thermo Fisher Scientific), CSIV-TRE-RFA-Ubc-KT and CSII-EF-MCS-ires-hKO1 (RIKEN BRC) were used to construct expression vectors. All of the products were verified by DNA sequencing.
2.2 | Dual luciferase reporter assay

HEK293T cells were seeded in 10 mL DMEM supplemented with 10% heat-inactivated FBS and 1% PS 1 d before transfection. Cells were transfected with 10 μg of pGL4.20 harboring the BCR promoter and 1 μg pRL-CMV with polyethylenimine (PEI; Sigma-Aldrich). The BCR promoter region was amplified from the genomic DNA of SU/SR cells using specific primers (F 5′-TTCCTCGGACGCTAAGCTC-3′ and R 5′-TCCTCGGACGCTAAGCTC-3′). At 24 h after transfection, doxycycline was added at 3 μg/mL and incubated for another 24 h. The cells were then rinsed twice with PBS and lysed with 1x lysis buffer as supplied in the PicaGene® Dual Sea Pansy Luminescence kit (TOYO B-net). The luciferase and Renilla luciferase activity were measured using ARVO X5 (PerkinElmer).

2.3 | IC50 evaluation

For cell survival assay, 3 x 10^4 cells were seeded onto 96-well flat plates. The indicated concentrations of PI polyamides or drugs were added to the culture medium and cells were incubated for 48 h. Cell viability was then assessed using the Cell Count Reagent SF (nacalai tesque, Inc) and the Infinite 200 PRO multimode reader (TECAN). The results were represented as the average ± SD values obtained from 3 independent experiments.

2.4 | Statistics

Statistical significance of differences between control and experimental groups was assessed using a 2-tailed unpaired Student t test. The results were represented as the average ± SD values obtained from 3 independent experiments.

2.5 | Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was conducted as previously described.26 Briefly, total RNA was extracted from cultured cells using the RNeasy mini kit (Qiagen) and reverse transcribed using the ReverTra Ace® qPCR RT Master Mix (TOYOBO) to generate cDNA. qRT-PCR was conducted on the StepOne™ real-time PCR system (Applied Biosystems). Relative expression levels were calculated using the 2−ΔΔCt method. Primers used for qRT-PCR are listed in Table S1.

2.6 | ChIP-PCR

ChIP assay was performed using SimpleChIP® Plus enzymatic Chromatin IP Kit (Cell Signaling Technology) according to the manufacturer’s instructions. Chromatin preparation was processed for immunoprecipitation with anti-RUNX1 antibody (ab23980, abcam) at 4°C overnight. Following ChIP, DNA was amplified with specific primers listed in Table S2 using Ex Taq® polymerase (Takara Bio Inc). Obtained DNA was analyzed using agarose gel electrophoresis.

2.7 | Immunoblotting

Cells were washed twice in ice-cold PBS and lysed in lysis buffer as previously described.21 Equal amounts of protein samples were loaded onto the gels for each target proteins, separated using SDS-PAGE and electrotransferred onto 45-μm pore size polyvinylidene difluoride membranes (Millipore, IPVH00010). Membranes were probed with the following primary antibodies: anti-c-abl (Cell Signaling Technology, 2862), anti-RUNX1 (Santa Cruz Biotechnology, clone A-2), anti-GAPDH (Santa Cruz Biotechnology, clone 0411), anti-phospho-AKT(Ser473; Cell Signaling Technology, 9271), anti-AKT (Cell Signaling Technology, 9272) and anti-p53 (Santa Cruz Biotechnology, clone DO-1) antibodies. For secondary antibodies, HRP-conjugated anti-rabbit IgG and anti-mouse IgG (Cell Signaling Technology, 7074 and 7076) were used. Primary antibodies and secondary antibodies were diluted to 1:1000 and 1:5000. Blots were visualized using Chemi-Lumi One Super (Nacalai Tesque) and the ChemiDoc XRS + Imager (Bio-Rad Laboratories).

2.8 | shRNA interference

shRNA targeting human RUNX1, BCR-ABL1, and p53 were designed and sub-cloned into pENTR4-H1tetOx1, CS-RfA-ETV, and CS-RfA-ETBsd vectors (RIKEN BRC). Non-targeting control shRNA was designed against luciferase (sh_Luc). The target sequences were provided in Table S3.

2.9 | Xenograft mouse model

NOD/Shi-Scid, IL-2RKO (NOG) mice were purchased from the Central Institute for Experimental Animals, Japan and were used as controls in all experiments. For leukemia cell lines mouse xenograft models, 2 x 10^6 cells/body of SU/SR cells with doxycycline-inducible shRNA expression vector targeting Luciferase or RUNX1 were injected intravenously into NOG mice. At 7 d after transplantation, 1 mg/mL doxycycline (Sigma) and 30 mg/mL sucrose (Wako) were added to the drinking water and started to be given orally. Peripheral blood was then collected every week and chimerism was checked by a flow cytometer. For the patient-derived xenograft (PDX) study, PDX cells were provided by Dr. Itaru Kato’s group. Appropriate informed consent was obtained from this patient. At the age of 6, she was diagnosed with Ph1-positive BCP-ALL (minor BCR-ABL1-positive), and was in remission with multidrug chemotherapy including imatinib. At 1 y and 6 mo

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2.4 | Statistics

Statistical significance of differences between control and experimental groups was assessed using a 2-tailed unpaired Student t test and was declared if the P-value was less than .05. Equality of variances in 2 populations was calculated using the F test. The results were represented as the average ± SD values obtained from 3 independent experiments.

2.5 | Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was conducted as previously described.26 Briefly, total RNA was extracted from cultured cells using the RNeasy mini kit (Qiagen) and reverse transcribed using the ReverTra Ace® qPCR RT Master Mix (TOYOBO) to generate cDNA. qRT-PCR was conducted on the StepOne™ real-time PCR system (Applied Biosystems). Relative expression levels were calculated using the 2−ΔΔCt method. Primers used for qRT-PCR are listed in Table S1.

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The expression of RUNX1 is required in the maintenance of Ph+ ALL cells. A. Cell growth curves of SU/SR cells transduced with shRNAs targeting RUNX1 (sh_RUNX1 #1 and sh_RUNX1 #2) or luciferase (sh_Luc). B. Immunoblot of RUNX1 and GAPDH in SU/ SR cells transfected with sh_Luc, sh_RUNX1 #1 and sh_RUNX1 #2. Cells were treated with 3 μmol L⁻¹ doxycycline for 24 h. C. Schema of xenotransplantation assay in NOG mice with SU/SR cells (sh_Luc or sh_RUNX1 #1). D. Chimerism of transplanted leukemia cells in (C; n = 5). E. Overall survival of NOG mice in (C; n = 11). F, G. Organ images of the livers (F) and the spleens (G) with the weight boxplots at day 40 in (C; n = 5). H. Representative histology pictures of the bone marrow at day 40 in (C). H&E staining and immunohistochemical staining with anti-human CD45 antibody were done for each slide (original magnification; ×200).
To test our hypothesis, we first examined the expression of BCR-ABL1 upon RUNX1 knockdown in SU/SR cells. As shown in Figure 2B,C, the expression of BCR-ABL1 was significantly downregulated in RUNX1-silenced SU/SR cells relative to the control both at mRNA and protein levels. In addition, the phosphorylation level of AKT, one of the most important downstream targets of BCR-ABL1, was also significantly reduced upon knockdown of RUNX1 in SU/SR cells (Figure 2C). Of note, the growth rate of SU/SR cells was attenuated upon BCR-ABL1 knockdown to the extent of RUNX1-silencing, underpinning the importance of RUNX1 in the regulation of BCR-ABL1 expression (Figure S3). To address whether RUNX1 directly transactivates BCR-ABL1 expression, we next conducted luciferase reporter assays using the BCR promoter in HEK293T cells. We prepared HEK293T cells that were stably transduced with shRNAs targeting RUNX1 or lentivirus expressing RUNX1. These cells were transiently transfected with a vector harboring a luciferase reporter fused to the BCR promoter (located at −1000 to +200 bp relative to the transcription start site [TSS] of BCR gene), and the expression of shRNAs or RUNX1 was induced by doxycycline. As shown in Figure 2D, while inhibition of RUNX1 downregulated the activity of the BCR promoter, additional RUNX1 expression consistently upregulated its activity. Close inspection of the BCR promoter uncovered the RUNX1 consensus binding site of 5′-TGTGGT-3′ at 802 bp upstream of the TSS of BCR. ChIP experiments confirmed the actual binding of RUNX1 in this region (Figure 2E). These results collectively suggested that RUNX1 binds to the promoter of BCR-ABL1 in Ph⁺ ALL cells and positively regulates it, which could potentially be targeted in anti-leukemia therapy toward this cancer.

3.3 Novel RUNX inhibitor, Chb-M’, induces Ph⁺ALL cell death BCR-ABL1-dependently

To further investigate the role of RUNX1 in Ph⁺ALL cells, we next pharmacologically inhibited RUNX1 by our novel RUNX inhibitor Chb-M’ and examined its anti-leukemia effect on Ph⁺ ALL cells.
Chb-M’ is a pyrrole-imidazole polyamide interlocked with a hairpin conjugated with alkylating reagent chlorambucil that specifically recognizes DNA sequences containing 5’-TGTTGGT-3’, a canonical RUNX1 recognition site. To start with, we examined the specificity of the pyrrole-imidazole polyamide to the 5’-TGTTGGT-3’ region in the BCR promoter by ChIP assay. For this purpose, we prepared alkylating agent-free Chb-M’ (Simple-M’) and tested whether the binding of RUNX1 to the 5’-TGTTGGT-3’ site in the BCR promoter was competitively inhibited by adding Simple-M’. As shown in Figure 3A, Simple-M’ apparently removed RUNX1 from the BCR promoter in our ChIP experiment dose dependently.

With respect to the antitumor effect on Ph+ ALL cells, Chb-M’ effectively controlled their proliferation in several Ph+ ALL cell lines that we tested in this study (Figure 3B, Figure S4A-D). Furthermore, treatment with Chb-M’ downregulated the expression of BCR-ABL1 both at mRNA and protein levels in these cells (Figures 3C,D and S4E-H). Contrary to Figure 3A, Chb-M’ suppressed BCR-ABL1 expression at lower concentration, suggesting that DNA alkylation by chlorambucil is important for transcriptional regulation, as described in our previous reports.31,32 The phosphorylation of AKT was also consistently reduced in SU/SR cells upon Chb-M’ treatment (Figure 3D). These results were thoroughly consistent with those obtained in the RUNX1 knockout experiments. Of note, additional BCR-ABL1 expression in SU/SR cells and MYL conferred relative resistance to Chb-M’ treatment (Figures 3E,F and S5). Therefore, we found that Chb-M’ preferentially suppresses the growth of ALL cells with BCR-ABL1 relative to those without it (Figure 3G). These results collectively suggested that the anti-leukemia effect of Chb-M’ largely depended on this oncogenic fusion gene.

We have previously found and reported that the growth suppression induced by Chb-M’ is highly dependent on the p53 cell death pathway.21 Therefore, we tested whether p53 significantly contributed to the Chb-M’-mediated growth suppression in SU/SR cells. For this purpose, we prepared p53-knockdowned SU/SR cells and challenged them with Chb-M’. As shown in Figure S6A-F, p53 knockdown indeed conferred relative resistance to Chb-M’ to a certain extent, suggesting a possible involvement of p53 in the Chb-M’-mediated tumor suppression in these cells, however, the growth of p53-silenced SU/SR cells was still effectively controlled by Chb-M’ at submicromolar levels. Considering the significant resistance to Chb-M’ conferred by BCR-ABL1 overexpression in these cells (Figure 3E), these results overall indicated that the growth suppression mediated by Chb-M’ was dependent on both functional p53 and BCR-ABL1, however possibly more on BCR-ABL1 in these Ph+ ALL cells.

3.4 | Chb-M’ significantly suppresses the growth of Ph+ ALL PDX cells by downregulating BCR-ABL1 expression in vivo

We investigated the effects of Chb-M’ on Ph+ ALL PDX cells in vivo. We transplanted Ph+ ALL PDX cells derived from the first relapse patient into NOG mice. At 2 wk after the transplantation, Chb-M’ administration was started to treat these mice. DMSO and imatinib mesylate were injected as controls (Figure 4A). Bone marrow was collected every week to check the chimerism of transplanted ALL cells. Chb-M’ significantly suppressed the cell growth of Ph+ ALL PDX cells in the bone marrow, compared with DMSO at week 5 (Figure 4B). NOG mice treated with Chb-M’ had significantly prolonged overall survival compared with mice treated with DMSO (Figure 4C), which is consistent with the results observed in our previous in vivo experiments with the SU/SR Ph+ ALL cell line.21 The patient sample was negative for the T315I mutation, but imatinib did not prolong survival compared with controls in PDX experiments. To investigate the mechanism of imatinib resistance, we performed mutation analysis on the RNA-seq data of the PDX cells, and the results are listed in Table S4, which showed no mutations in the ABL1 gene, including T315. The underlying mechanism of imatinib resistance in Ph+ leukemia patients, in addition to mutations in the kinase domain of ABL1, has recently been shown to be due to the genomic amplification of BCR-ABL1 or the upregulation of the BCR-ABL1 transcript.33-35 FISH of BCR-ABL1 showed that most leukemic cells at the patient’s initial diagnosis had 3 signals of BCR-ABL1, indicating genomic amplification of BCR-ABL1. In addition, the mRNA expression of RUNX1 and BCR-ABL1 was increased in relapse-derived PDX cells compared with those in primary-derived PDX cells (Figure S7). This is consistent with the previous report that high expression of RUNX1 is associated with disease progression of CML.18 From these results, the imatinib resistance in the PDX cells may be due to the increased expression of BCR-ABL1 associated with increased copy number of BCR-ABL1 and upregulation by RUNX1. As shown in the H&E staining and immunohistochemistry (human CD45 and Ki-67) panels, Chb-M’ lessened the tumor burdens in the bone marrow relative to the controls. In addition, Chb-M’ suppressed RUNX1 and BCR-ABL1 expression of leukemic cells as shown by immunohistochemistry (Figure 4D). Taken together, our RUNX inhibitor, Chb-M’, could be used as a novel drug for patients with TKI-resistant refractory Ph+ ALL through the downregulation of BCR-ABL1 (Figure 4E).

4 | DISCUSSION

Runt-related transcription factor 1 (RUNX1) forms a heterodimeric complex with core binding factor-β (CBFβ) on DNA promoter regions and regulates the expression of diverse target genes that are essential for the survival of certain cancers. Yamamoto et al18 have previously reported that functionally deregulated RUNX1 cooperates with BCR-ABL1 and induces a blastic phase-like phenotype of CML in mice. In this study, we found that RUNX1 directly targets BCR-ABL1 in Ph+ ALL cells through regulating the BCR promoter. According to Shah et al,26 a functional promoter of BCR is localized in a region 1000 bp upstream of the BCR exon 1 coding sequence, which includes the RUNX consensus binding sequence we identified in this study. In addition to this study, a few groups have previously studied and reported the functional regulation of the BCR promoter.
MYC and MAX genes interact with the BCR promoter and regulate its transcription. To our knowledge, however, this is the first study that provides evidence for a possibility of pharmacological intervention in the transcriptional regulation of BCR-ABL1 gene. As acquisition of point mutations in the BCR-ABL1 gene is the major mechanism that hampers TKI-mediated tumor suppression in Ph+ ALL patients, therapies that directly modulate the expression of BCR-ABL1 can be a reasonable strategy to overcome the current clinical problems related to TKIs. Together with our previous finding that Chb-M’ is highly effective against T315I mutation positive Ph+ ALL cells even in vivo with minimal side effects,21 our work not only unveiled the novel role of RUNX1 transcription factor in the transcription of BCR-ABL1 expression, but also potentially provides alternative choice for the patients with TKI treatment-resistant Ph+ ALL. Moreover, our study provides pieces of evidence that not only Ph+ ALL cells but also CML cells might be efficiently controlled by RUNX1 inhibition.

Conversely, other RUNX inhibitors that stand on other mechanisms of action (ex. Ro5- 333538) should also be tested in these tumors to further validate our results. In addition, addressing the
roles of other RUNX family members such as RUNX2 and RUNX3 will help elucidate how RUNX family transcription factors generally contribute to the pathogenesis of BCR-ABL1 positive tumors including Ph+ ALL. Although the role of BCR itself has not been fully elucidated in tumorigenesis, we are assuming that the RUNX inhibition strategy can potentially be applied to cancers that are dependent on BCR, such as metastatic colorectal cancer.

The efficacy of available RUNX inhibitors should also be tested in these tumors in future studies. From mutations of PDX cells (Table S4), based on known driver genes in pediatric B-cell precursor ALL, we extracted the 2 driver genes, MSH6 and CREBBP. Of them, CREBBP mutations have been identified as a mechanism of resistance in ALL, and somatic variants in epigenetic modifiers including CREBBP can predict failure of response to imatinib in chronic-phase CML. These suggest that imatinib resistance in PDX cells may be due to the CREBBP mutation in addition to the high expression of BCR-ABL1.

In conclusion, we have discovered a vital role of the RUNX1 transcription factor in the regulation of BCR-ABL1 expression and in the maintenance of Ph+ ALL cells not only in human leukemia cell lines but also in PDX cells. RUNX1 could be an ideal target in the treatment of Ph+ ALL, and future clinical trials with our novel RUNX inhibitor Chb-M' in these patients are awaited.

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SUPPORTING INFORMATION
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