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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

effective even in an imatinib-resistant Ph$^+$ ALL cell line. In good agreement with these findings, forced expression of *BCR-ABL1* in these cells conferred relative resistance to Chb-M'. In addition, in vivo experiments with the Ph$^+$ ALL patient-derived xenograft cells showed similar results. In summary, targeting RUNX1 therapeutically in Ph$^+$ ALL cells may lead to overcoming TKI resistance through the transcriptional regulation of *BCR-ABL1*. Chb-M' could be a novel drug for patients with TKI-resistant refractory Ph$^+$ ALL.

KEYWORDS
bcr-abl, fusion proteins, gene expression regulation, leukemia, lymphoid, Philadelphia chromosome, RUNX1 protein, human

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.[1-3] 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,[3-6] 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 a subset of ALL cases. 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, KOCL-45, SUP-B15, SU-Ph2 and SU/SR cells as well as CML-derived MYL, BV173 and K562 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′-TTAGAGGGAGGCTAATCAGGG-3′ and R 5′-TCCTCGGACGCTAAGCTC-3′). At 24 h after transfection, doxycycline was added at 3 μmol L^{-1} and incubated for another 24 h. The cells were then rinsed twice with PBS and lysed with 1× 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 | IC$_{50}$ evaluation

For cell survival assay, 3×10^4 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). Percent inhibition curves were drawn and IC$_{50}$ of the indicated compounds was calculated based on median-effect method.[25]

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 (Quiagen) 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$^{-\Delta\Delta Ct}$ 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, 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-2RγKO (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, 2×10^6 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

after the diagnosis, she had a isolated central nervous system (CNS) recurrence. She achieved remission again after switching to dasatinib, Hyper-CVAD, and intensified intrathecal injections. Bone marrow transplantation was performed from an HLA-matched relative donor, but she had the second relapse in the CNS. At the second CNS recurrence, the T315I mutation was tested and was negative. She became refractory to treatment and died 1 y and 4 mo after transplantation. The PDX cells used in this study were established using leukemia cells collected from cerebrospinal fluid at the time of the first relapse of the CNS alone. These PDX cells were intravenously transplanted into NOG mice. At 2 wk after transplantation, Chb-M' (320 µg/kg body weight, twice per week) or DMSO (the equivalent amount, twice per week) administration was intravenously started, and oral administration of imatinib mesylate (Tokyo Chemical Industry Co., Ltd., 100 mg/kg body weight, daily) was started. Bone marrow was then collected every week and chimerism was checked using a flow cytometer and an anti-human CD45 antibody and an anti-mouse CD45 antibody (BD Biosciences). Overall survival was monitored until the mice succumbed to their disease. For the bone marrow of 1 representative of each group at day 36, H&E staining and immunohistochemical staining with anti-human CD45 antibody (Thermo Fisher Scientific, MA5-13197), anti-Ki-67 antibody (Agilent, M7240), anti-RUNX1 antibody (Abcam, ab35962) and anti-BCR (BCR-ABL1 p190/p210) antibody (Santa Cruz Biotechnology, G6) were done.

2.10 | Study approval

All animal studies were properly conducted according to the Regulations on Animal Experimentation at Kyoto University, based on International Guiding Principles for Biomedical Research Involving Animals. All procedures used in this study were approved by the Kyoto University Animal Experimentation Committee (Permit Number: Med Kyo 14 332). PDX analysis was approved by the Kyoto University Hospital Ethical Board (Approval number: G-1030).

3 | RESULTS

3.1 | Knockdown of *RUNX1* suppresses the proliferation of Ph⁺ ALL cell lines

To explore the role of RUNX1 in the maintenance of Ph⁺ ALL cells, we first modulated the expression of *RUNX1* in human Ph⁺ ALL-derived

SU/SR cells with doxycycline-inducible shRNA. SU/SR cells are genetically identical to SU-Ph2 cells except for the T315I point mutation in the ABL1 protein, which confers major resistance to TKI treatment (Figure S1).[27-30] As shown in Figure 1A,B, silencing of *RUNX1* significantly suppressed the cell growth of SU/SR imatinib-resistant Ph⁺ ALL cells in vitro. Intriguingly, this *RUNX1* inhibition-mediated suppression of tumor growth was observed not only in Ph⁺ ALL-derived SU/SR and SU/Ph2 cells, but also in CML-derived MYL and K562 cells (Figure S2). As widely known, while *BCR-ABL1* p190 occurs in the majority of Ph⁺ ALL cases, *BCR-ABL1* p210 is the hallmark of CML, and both fusion genes are thought to be under the control of the *BCR* promoter. These results prompted us to further investigate the role of RUNX1 in *BCR-ABL1*-dependent hematologic malignancies.

We next investigated the effect of *RUNX1* inhibition in Ph⁺ ALL cells in vivo, and prepared a Ph⁺ ALL xenograft model. We transplanted SU/SR cells that had been stably transduced with lentivirus expressing control sh_*Luc* or sh_*RUNX1* into immunodeficient NOG mice. At 7 d after the transplantation, doxycycline administration was started to induce in vivo *RUNX1* knockdown (Figure 1C). Peripheral blood was collected every week to check the chimerism of transplanted ALL cells (Figure 1D). Overall survival periods were monitored until they succumbed to their disease. Thoroughly consistent with the results observed in the in vitro experiments, NOG mice transplanted with *RUNX1*-silenced SU/SR cells exhibited prolonged survival with statistical significance (Figure 1E). These mice showed lessened tumor burdens in the spleen and the bone marrow relative to the control (Figure 1F-H).

3.2 | RUNX1 directly transactivates the expression of *BCR-ABL1*

As we found that RUNX1 expression is a prerequisite for the proliferation of Ph⁺ ALL cell lines, we assumed that its expression might be elevated in Ph⁺ ALL patients. As shown in Figure 2A, analysis of a microarray dataset elucidated that the expression of *RUNX1* indeed increased in the bone marrow cells and peripheral blood cells derived from Ph⁺ ALL patients relative to those from the healthy donors and non-leukemic patients. In this data set (GSE13204), non-leukemic patients included those with megaloblastic anemia, hemolysis, iron deficiency, or idiopathic thrombocytopenic purpura. As the expression of the oncogenic *BCR-ABL1* fusion gene is regulated under the *BCR* promoter, as we have mentioned, this finding led us to hypothesize that the expression of the *BCR-ABL1* fusion gene might be transcriptionally controlled by RUNX1.

FIGURE 1 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; ×10 (upper panels) and ×40 (lower panels), Scale bars; 200 µm). Mean ± SD. *P < .05, **P < .01, ***P < .001, using two-tailed Student t test (A, D), log-rank (E), Mann-Whitney U test (F, G)
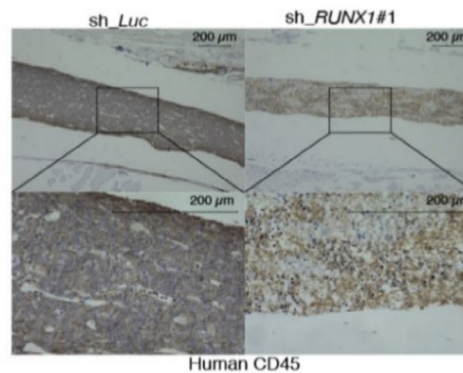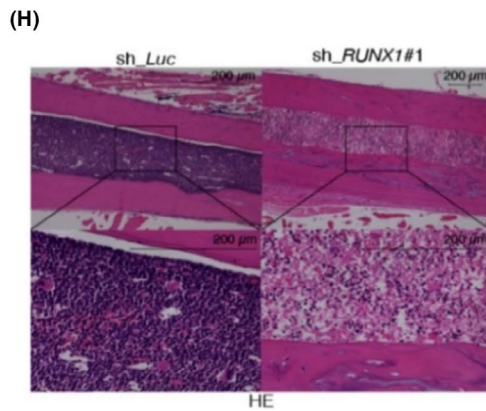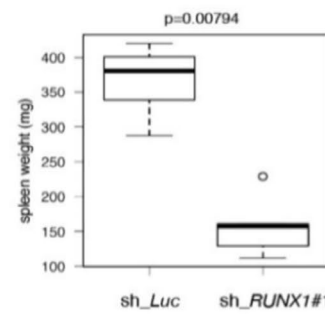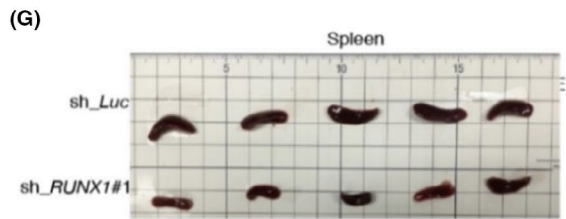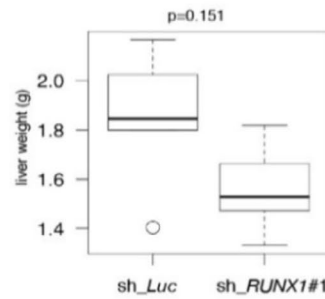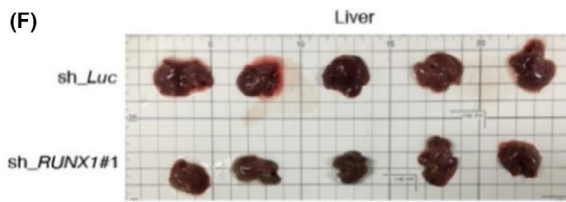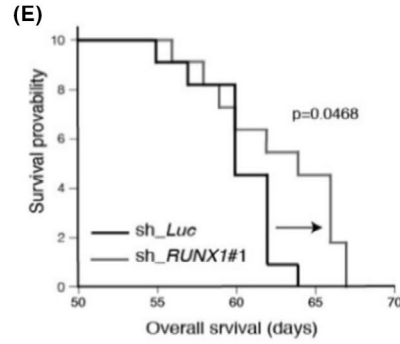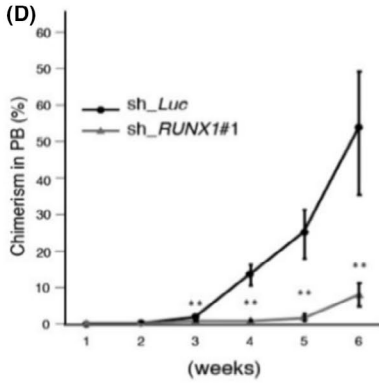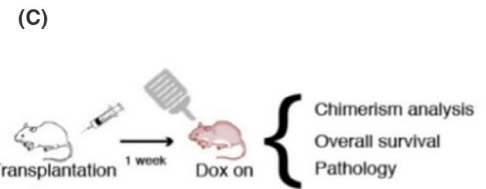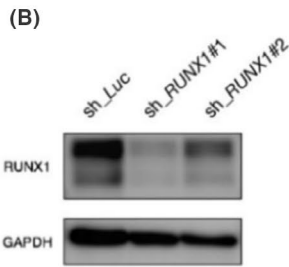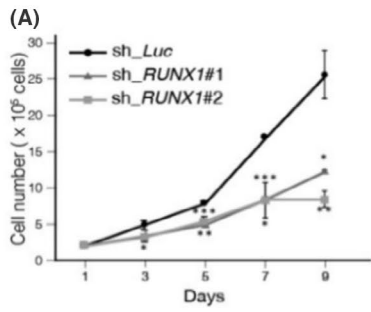
To test our hypothesis, we first examined the expression of *BCR-ABL1* upon *RUNX1* knockdown in SU/SR cells. As shown in Figure 2B,C, the expression of *BCR-ABL1* was significantly downregulated in *RUNX1*-silenced SU/SR cells relative to the control both at mRNA and protein levels. In addition, the 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'[21] and examined its anti-leukemia effect on Ph^{+} ALL cells.
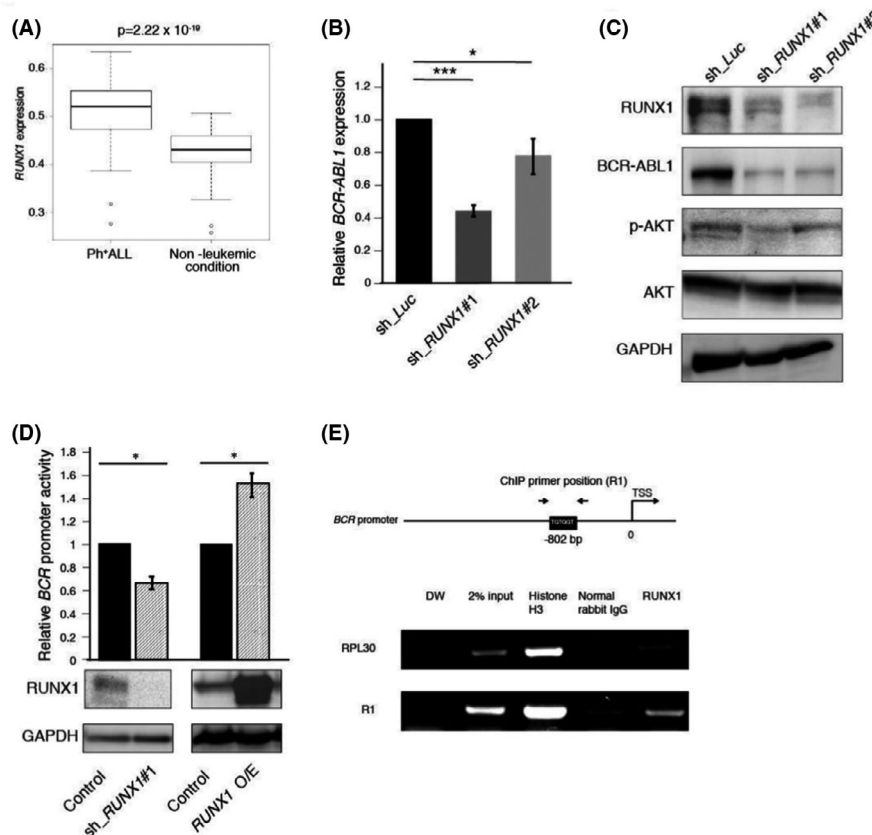
FIGURE 2 R Runt-related transcription factor 1 (RUNX1) directly transactivates the expression of *BCR-ABL1*. A, *RUNX1* expression (probe ID:209360_s_at., GSE13204) in pediatric Ph[+] ALL (mean = 0.5123, n = 122) and in the control samples (mean = 0.4278, n = 79). B, Relative mRNA expression of *BCR-ABL1* in SU/SR cells stably transduced with sh_*Luc*, sh_*RUNX1* #1 or sh_*RUNX1* #2. Cells were treated with 3 μmol L^{-1} doxycycline for 24 h. C, Immunoblot of RUNX1, BCR-ABL1, phosphorylated-AKT (p-AKT), AKT and GAPDH in the same SU/SR cells as (B). Cells were treated with 3 μmol L^{-1} doxycycline for 24 h, then lysed for protein extraction. D, Luciferase reporter activity of *BCR* promoter in HEK293T cells upon knockdown (sh_*RUNX1#1*) or overexpression (*RUNX1* O/E) of *RUNX1* with immunoblot images of RUNX1 and GAPDH in the samples. E, Gel image of ChIP-PCR in SU/SR cells with RUNX1 antibody. The binding of RUNX1 transcription factors to the *BCR* promoter was assessed with the primers amplifying the region including the RUNX1 consensus binding site (5′-TGTGGT-3′) located at 802 bp upstream of TSS (R1). RPL30 was used as RUNX1-irrelevant negative control. Mean ± SD. *P < .05, ***P < .001, using Mann-Whitney *U* test (A), two-tailed Student *t* test (B, D)

Chb-M' is a pyrrole-imidazole polyamide interlocked with a hairpin conjugated with alkylating reagent chlorambucil that specifically recognizes DNA sequences containing 5′-TGTGGT-3′, a canonical RUNX1 recognition site. To start with, we examined the specificity of the pyrrole-imidazole polyamide to the 5′-TGTGGT-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′-TGTGGT-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* knockdown 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). Moreover, 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*-knocked down 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 level.[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 that 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 al[18] 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[36], 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. For example, Sharma et al[37] have shown that
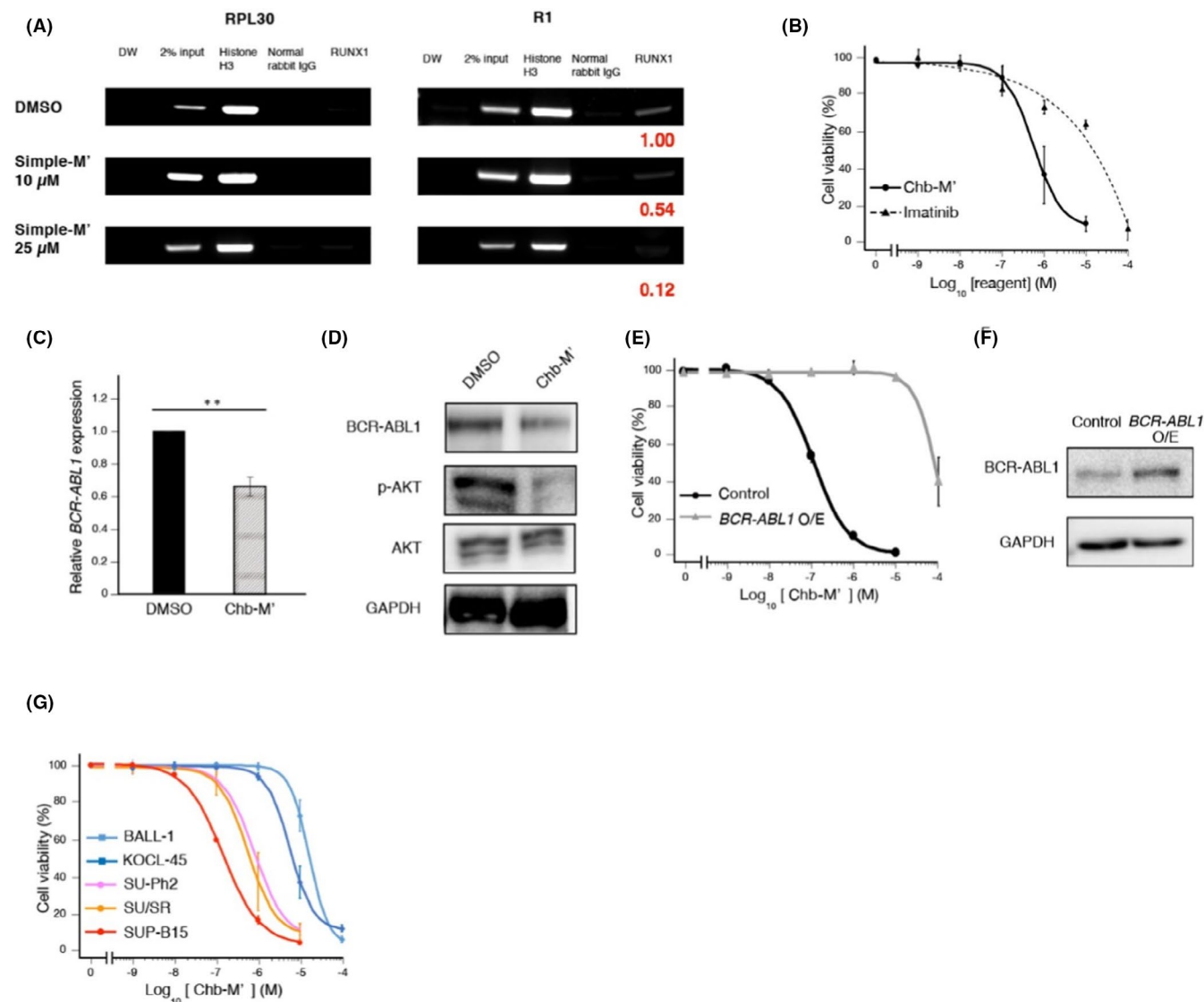
FIGURE 3 Anti-leukemic efficacy of RUNX inhibitor Chb-M' in Ph$^+$ ALL cells. A, Gel image of ChIP-PCR in SU/SR cells treated with DMSO or Simple-M' (10, 25 μmol L^{-1}) for 12 h in the same way as Figure 2E. Bands were quantified using Image Lab software (Bio-Rad Laboratories) and normalized to that of the control. B, Dose-response curves of SU/SR cells treated with the indicated doses of Chb-M' (IC50: 658 nmol L^{-1}) and imatinib (IC$_{50}$: 18.2 μmol L^{-1}) for 48 h. C, *BCR-ABL1* mRNA expression in SU/SR cells treated with DMSO or Chb-M' (1 μmol L^{-1}) for 9 h. D, Immunoblot of BCR-ABL1, phosphorylated-AKT (p-AKT), AKT and GAPDH in SU/SR cells treated with DMSO or Chb-M' (1 μmol L^{-1}) for 24 h. E, Dose-response curves of SU/SR cells stably transduced with control (IC$_{50}$: 143 nmol L^{-1}) or *BCR-ABL1* expressing vectors (IC50: 33.1 μmol L^{-1}) for 48 h. F, Immunoblot of BCR-ABL1 and GAPDH in (E). Cells were treated with 3 μmol L^{-1} doxycycline for 48 h. G, Dose-response curves of ALL cell lines with *BCR-ABL1* (SU-Ph2 [IC50: 849 nmol L^{-1}], SU/SR (IC$_{50}$: 658 nmol L^{-1}) and SUP-B15 [IC50: 167 nmol L^{-1}]) and ALL cell lines without *BCR-ABL1* (BALL-1 [IC50: 21.7 μmol L^{-1}] and KOCL-45 [IC50: 6.04 μmol L^{-1}]) treated with the indicated doses of Chb-M' for 48 h. Mean ±SD. **$P < .01$, using two-tailed Student *t* test (C)

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 trans-activation 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. Ro5-3335[38]) should also be tested in these tumors to further validate our results. In addition, addressing the
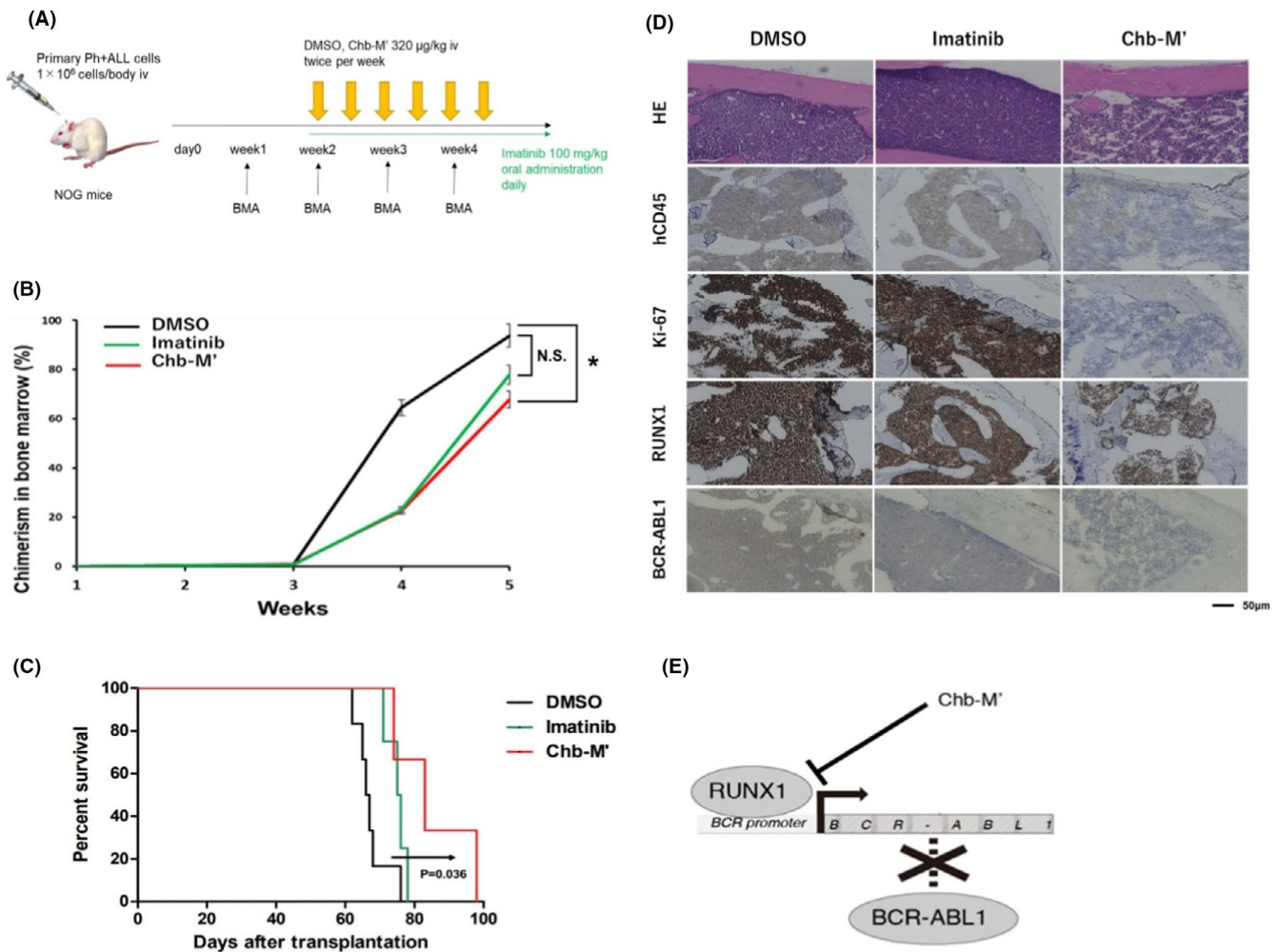
FIGURE 4 Chb-M' significantly suppresses the growth of Ph$^+$ ALL PDX cells by downregulating *BCR-ABL1* expression in vivo. A, Schema of transplantation assay in NOG mice with Ph$^+$ ALL PDX cells. These mice were treated with DMSO, imatinib mesylate or Chb-M'. B, Chimerism of transplanted leukemia cells in bone marrow (n = 7). C, Overall survival of NOG mice transplanted with Ph$^+$ ALL PDX cells (n = 6). D, Representative histology pictures of bone marrow at day 36. H&E staining and immunohistochemical staining with anti-human CD45 antibody, anti-Ki-67 antibody, anti-RUNX1 antibody and anti-BCR (BCR-ABL1) antibody were done for each slide (original magnification; ×10, Scale bars; 50 μm). E, Graphical abstract of this study. *RUNX1*-silencing inhibits the transactivation of *BCR-ABL1* expression and therefore attenuates the proliferation of *BCR-ABL1* fusion gene-dependent leukemia cells. Our RUNX inhibitor, Chb-M', could be potentially a novel drug for Ph$^+$ ALL with TKI resistance through the downregulation of *BCR-ABL1*. Mean ± SD. *$P < .05$, NS; not significant, using two-tailed Student *t* test (B), log-rank (C)

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.[39] 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,[40] we extracted the 2 driver genes, *MSH6* and *CREBBP*. Of them, *CREBBP* mutations have been identified as a mechanism of resistance in ALL,[41] and somatic variants in epigenetic modifiers including *CREBBP* can predict failure of response to imatinib in chronic-phase CML.[42] 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.

DISCLOSURE

The authors have no conflict of interest.

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