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Original Article
Increased sensitivity of BCR-ABL-induced B-ALL to imatinib by releasing leukemia B cell differentiation blockage

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Abstract: Aims: In B cell acute lymphocytic leukemia (B-ALL), B cells are blocked mainly at the pro/pre-B phase, making them poorly responsive to imatinib. We aimed to investigate whether it was possible to promote pro/pre-B cell maturation beyond this phase and make them sensitive to imatinib treatment by overexpressing immunoregulatory tyrosine activation motif (ITAM) with BCR-ABL in a Ph+ B-ALL mouse model. Materials & Methods: Ph+ B-ALL mouse models were induced by BCR-ABL using retroviral transduction/transplantation. Results: Overexpression of ITAM promoted the differentiation of blocked pro/pre-B cells to B220+IgM+ and increased disease sensitivity to imatinib in mice. Btk deficiency accelerated the progression of BCR-ABL-induced B-ALL. Conclusion: B-cell development blockage released by ITAM renders leukemia cells sensitive to imatinib treatment in BCR-ABL-induced B-ALL.

Keywords: BCR-ABL, B-cell acute lymphocytic leukemia, BCR, Bruton’s tyrosine kinase, immunoregulatory tyrosine activation motif, imatinib

Introduction

B cell development can be divided into distinct phases that can be characterized by surface antigens [1]. Interleukin-7 receptor alpha (IL-7Rα) is the first to be expressed on the cell membrane of the B lymphocyte lineage [2]. In IL-7Rα-deficient mice, B-cell development is blocked at pre/pro B cell stage [3]. Immunoglobulin M (IgM) is crucial for pre-B cell to immature B cell transitioning. The μ chains together with surrogate light chains λ5 form a complex with vpre-B on the pre-B cell membrane to process itself through differentiation and maturation [4]. IgM is associated with the Igα/Igβ complex, which contains ITAMs in its cytoplasmic tail that mediates signal transduction [5]. Previously, numerous studies had shown that the ITAMs of the Igα/Igβ complex could activate spleen-associated tyrosine kinase (Syk), Bruton’s tyrosine kinase (Btk), and the Src kinase family, such as Fyn, Lyn, and Blk [6]. Furthermore, the loss of Btk or B cell linker (Blnk) protein, as a junction protein of Syk-dependent activation of Btk phosphorylation, has been found in some cases of human B-cell leukemia [7, 8]. Additionally, Lyn deficiency can accelerate disease progression in BCR-ABL-induced B-ALL [9], while overexpression of ITAM promotes B-cell development [10]. Based on these previous reports, ITAM signaling plays important roles in B cell maturation.

In Philadelphia chromosome-positive (Ph+) human leukemia, approximately 5% of cases are B-ALL. Moreover, most B cells are blocked at the pre-B stage [11]. We predict that BCR-ABL disorders the signaling pathways involved in B cell differentiation and maturation. It is established that various signaling pathways are implicated in the regulation of B-cell proliferation and differentiation [12]. Investigation of the signal pathways involved in the blockage of B-cell development by BCR-ABL would help us to understand the mechanism of BCR-ABL transforming normal cells to leukemia cells and identify effective therapeutic targets.
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Materials and methods

Reagents and small molecule drugs

Gibco® RPMI1640 culture medium and fetal bovine serum were purchased from Thermo Fisher Scientific (Shanghai, China). Imatinib (STI571 Gleevec; Novartis) was obtained from Smart Med (Canada). Flow antibodies B220-Blue (48-0451-82), IgM-APC (17-5790-82), and Cd43-PE-Cy7 (25-0439-42) were purchased from Thermo Fisher Scientific (Shanghai, China).

Generation of retrovirus and lentivirus stocks

The retroviral constructs of MSCV-IRES-GFP, MSCV-BCR-ABL-IRES-GFP, MSCV-BCR-ABL-IRES-ITAM-IRES-GFP, and MSCV-BCR-ABL-IRES-MAhB-IRES-GFP were used to generate high-titer, helper-free, replication-defective ecotropic virus stock by transient transfection of 293T cells as previously described [13].

Whitlock-Witte cultivation

Whitlock-Witte assay was performed according to the methods reported in previous literature [14]. WT and Btk-/- mouse BM cells were collected. Transfection medium containing 2 ml BCR-ABL viruses and 2 ml lymphatic culture medium (RPMI1640 containing 5% fetal bovine serum, 1% of the double resistance, 1% of glutamine, and 50 μm beta-mercaptoethanol) were used to resuspend BM cells. The mix was centrifugated at 1,000 g, 37°C for 1.5 h. After centrifugation, cells were incubated for another 3 h. Transfected cells were seeded into well of 24-well plates at 1,000 k, 500 k, and 300 k cell serial density. All wells were supplied with normal BM cells to 1×10^6 cells. Each density was conducted in triplicate. Living cells were counted on day 7 post virus transduction [15].

Bone marrow transduction and transplantation

WT (C57BL/6J) and Btk-/- mice (B6; 129S-Btktm1wK/J) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were maintained in a specific pathogen-free animal house, and all animal experiments were approved by The Animal Management Ethics Committee of Sichuan University (Chengdu, China). Eight 8 to 12 week-old C57BL/6 mice were used for bone marrow transduction/transplantation. Retroviral transduction and transplantation of mouse bone marrow cells for inducing CML by BCR-ABL was done as had been described previously [16].

Treatment of B-ALL mice with imatinib

Imatinib was dissolved in sterile water at a concentration of 10 mg/ml, and orally administered at a dose of 100 mg/kg twice daily until the end of point. Treatment was initiated on day 7 post BM transduction/transplantation.

Flow cytometry

Cells isolated from mouse peripheral blood (PB), spleen (SPL), and bone marrow (BM) removed were prepared as previously described [17]. The differentiation stages of B-cell were analyzed with specific a combination as: B220+Cd43+IgM- for pro-B cell; B220+Cd43-IgM- for pre-B cell; B220+Cd43-IgM+ for mature B cell. All leukemia cells were labeled with GFP.

Statistical analysis

All data are expressed as the mean ± SD and analyzed using the GraphPad Prism version 7.0 software, and differences among groups are examined using the ANOVA and t-test. P<0.05 denotes statistical significance (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

Results

ITAM promotes leukemia B cell differentiation

In the BCR-ABL-induced B-ALL mouse model, B leukemia cells were transformed from normal B cell progenitors [18]. As shown in Figure 1A, most of the leukemia cells in the bone marrow were blocked in the early B cell stage (98.8% of these cells were IgM negative). Further analysis showed that 92.2% of these leukemia cells were pro-B cells (B220+Cd43-) and 7.8% were pre-B cells (B220+Cd43+). BCR plays an important role in the process of B cell differentiation and maturation. The heavy chain constant region of BCR interacts with Iga/Igβ complexes on the B cell membrane. ITAM, the Iga/Igβ active motif, sends signals into the nucleus through phosphorylated substrates Blk, Syk, Lyn, and Btk [19, 20]. However, the role of ITAM
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Figure 1. ITAM blocks B-cell development in the pro-/pre-B stage in BCR-ABL-induced B-ALL. A. The B-ALL mouse model was induced by BCR-ABL; after 3 weeks, we analyzed the leukemic cells in the bone marrow and most of them were IgM- cells, including pro- and pre-B cells (pro-B cells: GFP+IgM-B220+CD43+; pre-B cells: GFP+IgM-B220+CD43-). B. The donor cells from WT mice infected with BCR-ABL+ITAM and BCR-ABL+MAHB virus, respectively, were subsequently injected into the recipients' induced B-ALL, at day 14, and FACS was used to analyze the leukemia cell differentiation in BM. C. Kaplan-Meier survival curves for recipients of BCR-ABL+ITAM and BCR-ABL+MAHB-transduced BM cells from WT donor mice. D. The total number of white cells was analyzed in the peripheral blood of all recipients induced by BCR-ABL+ITAM and BCR-ABL+MAHB on day 11, 14, 18, 21, and 29 post BMT. (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001). Results represent the mean ± SEM. All experiments were repeated twice. B-ALL stands for B-cell acute lymphocytic leukemia; BM stands for bone marrow; BMT stands for bone marrow transplantation; FACS stands for fluorescence-activated cell sorting; GFP stands for green fluorescent protein; IgM stands for immunoglobulin M; ITAM stands for immunoregulatory tyrosine activation motif; SEM stands for standard error of the mean; WT stands for wild-type.
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in Ph+ B-ALL has not been reported. We co-expressed ITAM or its non-functional mutants MAHB with BCR-ABL respectively to induce the B-ALL mouse model. On day 14, we analyzed the differentiation of B cells in BM of the disease mice. As shown in Figure 1B, compared to MAHB, overexpression of ITAM significantly promoted the differentiation of B cells. The B220+IgM+ cells were increased from 8.17% to 16.3%. This indicated that overexpression of ITAM in Ph+ B-ALL promoted B cells from pro-/pre-B to a more mature stage. Moreover, overexpression of ITAM prolonged the mean survival time (31.5 days) compared to the non-functional MAHB control group (27.5 days), although there was no statistically significant difference (P=0.0501) (Figure 1C). Leukemia cells in peripheral blood were regularly examined on days 11, 14, 18, 21 and 29 post bone marrow transplantation. Overexpression of ITAM significantly decreased the tumor burden in peripheral blood on days 14 and 18 (Figure 1D). In summary, leukemia cell differentiation in the Ph+ B-ALL mouse model was mainly blocked at the pro-/pre-B stage, and overexpression of ITAM promoted tumor cell differentiation to the more mature stage (B220+IgM+), subsequently preventing the progression of B-ALL.

**ITAM increases imatinib treatment response**

Our previous results showed that ITAM promoted the differentiation of leukemia cells and prolonged the survival of diseased mice. Therefore, we further investigated whether overexpression of ITAM promoted the Ph+ B-ALL response to imatinib. We co-expressed BCR-ABL with ITAM or MAHB to make Ph+ B-ALL disease mouse models. Imatinib (100 mg/kg, oral, twice daily) was administered on day 7 post BM transplantation and administered continuously until the experimental endpoint. We found that the average survival time of MAHB group mice was 32 days and all recipients died out within 50 days post transplantation. In the ITAM overexpression group, only 2 (2/7) mice died of B-ALL. The remaining five mice (5/7) were in good condition without obvious disease-related symptoms (Figure 2A). Leukemia cells in recipient peripheral blood were checked regularly. Compared to the MAHB group, the number of leukocytes in peripheral blood was significantly lower in ITAM overexpression group on day 14, 18, and 21 post bone marrow transplantation (Figure 2B). We also found that during Imatinib treatment, the percentage of total B cells (most of them were leukemia cells) was significantly lower in peripheral blood (PB), spleen (SPL), and bone marrow (BM), while the percentage of IgM+ cells (mature B cells) was higher in ITAM expressing group compared to that in Imatinib-treated MAHB (control) group recipients (Figure 3A and 3B). These results showed that overexpression of ITAM significantly increased Ph+ B-ALL sensitivity to Imatinib and Imatinib significantly improved these recipients’ survival rate.
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Btk deficiency promoted BCR-ABL inducing of B-ALL progression

Following stimulation of B cells with a proliferation or differentiation signal, Lyn phosphorylates ITAM regions to recruit and phosphorylate Syk. Subsequently, the activated Syk phosphorylates Btk, Cin85, and Btk complexes, which bind to phospholipase C-2 (PLC-2). Activated PLC-γ2 hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP2) and produces second messages—IP3 and diacylglycerol. Previous results showed that overexpression of ITAM could promote B-cell differentiation to the mature stage and increase their sensitivity to imatinib. Therefore, it was hypothesized that inhibition of ITAM and its downstream signaling pathways may promote the development of Ph+ B-ALL. To test our hypothesis, we used Btk deficiency, one of the key downstream substrates of ITAM, to investigate the effect of Btk deficiency on Ph+ B-ALL.

Our in vitro and in vivo data are consistent in that Btk deficiency promoted BCR-ABL-transformed cells growing faster in dish culture and disease progression in mice. To investigate the effect of Btk on the influence of imatinib to the differentiation and survival of leukemia cells, BCR-ABL-transformed pro/pre-B cells were cultured in vitro and treated with Imatinib at 2 μM and 5 μM for 48 h, respectively. Compared to their respective vehicle-treated cells, cells with Btk deficiency were more resistant to Imatinib (Figure 4). Therefore, we speculated that we would obtain similar results such that recipients receiving BCR-ABL-transformed Btk deficient cells could not respond well to Imatinib. The Ph+ B-ALL mouse model was induced by BCR-ABL retrovirus in WT and Btk-/- mice (as donors). The survival of model mice is shown in...
Figure 5A: the average median survival of WT mice was 38.5 days, while that of Btk-/- mice was only 27 days. These findings indicated that knockout of Btk in the Ph+ B-ALL model could significantly promote tumor progression. For further verification of the role of Btk as a tumor suppressor gene in the Ph+ B-ALL model, we performed the Whitlock-Witte experiment in vitro to investigate the effect of Btk deletion on the proliferation of BCR-ABL-transduced B cells. As shown in Figure 5B, BCR-ABL-transduced BM cells with Btk loss proliferated significantly faster than those in the control group at 1,000 k, 500 k, and 300 k concentrations. In conclusion, both in vitro and in vivo results showed that inhibition of Btk, the downstream substrate of the ITAM signaling pathway, significantly promoted the development of Ph+ B-ALL.

Discussion

The current study used a BCR-ABL-induced Ph+ B-ALL leukemia mouse model, which mimics the process of human B-ALL initiation and progression. We found that in BCR-ABL-induced B-ALL, most leukemia cells were blocked at the pre-B stage. Overexpression of ITAM significantly promoted pro/pre-B cell transitioning toward the B220+IgM+ stage, resulting in decreasing the tumor burden and increasing the sensitivity of B-ALL to Imatinib treatment. Moreover, Btk is one of the most pivotal downstream regulators of ITAMs. By using the Btk deficient mouse as a donor to make BCR-ABL-induced B-ALL, we found that the loss of Btk accelerated disease progression. Compared to wild-type (WT) B cells, BCR-ABL-transformed B cells had a growth advantage after deletion of Btk under Whitlock-Witte conditions. Collectively, our results demonstrated that releasing the blockage of B-cell development confers sensitivity of Ph+ B-ALL leukemia to therapy with imatinib, which has a significant clinical application for the treatment of Ph+ B-ALL.

The BCR-ABL-induced B-ALL mouse model has irreplaceable advantages in exploring the pathogenesis and developmental mechanisms of Ph+ B-ALL, as well as the discovery of new targets. In the BCR-ABL-induced Ph+ B-ALL mouse model, we found that the development of leukemic B cells was mainly blocked at the pro-/pre-B stage (Figure 1A). In the development and differentiation process of B cells, the BCR on the surface is stimulated by antigens, which transmit signals to the nucleus through the Igα/Igβ complex, thus controlling gene expression in the process of development and differentiation. ITAM in the cytoplasmic region of the Igα/Igβ complex plays a crucial role in this signal transmission [19]. We found that overexpression of ITAM in the Ph+ B-ALL model promoted differentiation of leukemia cells (Figure 1B) and significantly reduced the tumor cell burden (Figure 1D). The peripheral blood tumor burden of MAHB control group mice was increased at 2 weeks; however, the number of leukocytes in the peripheral blood was signifi-
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cantly decreased after 14 days. This decline did not account for a reduction in tumor load. We speculated that the leukemia cells extended into the chest and/or abdominal cavity. In the ITAM overexpression group, this number increased more slowly within 2 weeks, and was maintained at a relatively low level from 2 weeks to 3 weeks. We speculated that tumor cell proliferation was reduced for differentiating into mature B cells with lower proliferation rates. In Ph+ B-ALL, leukemia cells at the early differentiation stage have more malignancy than those at the late stage, and those at the late-stage are more sensitive to treatment. Therefore, inducing further differentiation or trans-differentiation of leukemia cells may also be an effective strategy in therapy against B-ALL [21].

Overexpression of ITAM alone did not significantly improve the survival of mice. However, overexpression of ITAM combined with imatinib treatment significantly prolonged survival and reduced the peripheral blood tumor cell burden (Figure 2). This indicates that inducing differentiation of leukemia B cells may be an effective approach to improve the effectiveness of TKIs in Ph+ B-ALL therapy. Therefore, developing drugs to promote leukemia B cell differentiation and combining it with TKI may improve the effectiveness of treatment against Ph+ B-ALL.

Btk is one of the most important downstream substrates of the BCR signal complex. The role of Btk in Ph+ B-ALL is unclear. Btk is a downstream regulator of the Igα/Igβ complex (ITAM). Btk belongs to the non-receptor protein tyrosine kinases, and its overactivation usually leads to the development of various tumors [22, 23]. Surprisingly, in the Ph+ B-ALL mouse model, knockout of Btk promoted the progression of B-ALL. The exact mechanisms through which BTK deficiency accelerates the progression of Ph+ B-ALL remain unknown and warrant further investigation. Moreover, the results of this study suggest that the Btk inhibitor should be used with caution in the clinical treatment of Ph+ B-ALL, owing to its observed accelerating effect on disease progression.

Ph+ B-ALL is a subtype of B-ALL, characterized by poor treatment response and clinical outcome. Although the use of tyrosine kinase inhibitors (TKIs; imatinib, dasatinib, etc.) has resulted in some benefit, mutations in BCR-ABL lead to drug resistance to these agents, the most common of which is T315I mutation. Recently, the development of third-generation TKIs (ponatinib) has overcome the resistance caused by the T315I mutation; however, clinical trials have shown that treatment with ponatinib has not resulted in long-term remission in patients [24, 25]. In addition, chimeric antigen receptor-T (CAR-T) immunotherapy targeting CD19 is bringing new hope for patients with B-ALL. Nevertheless, the cytokine release syndrome caused by CAR-T therapy in clinical trials also limits its application [26]. Therefore, it is imperative to investigate the pathogenesis mechanisms of Ph+ B-ALL and identify new effective targets for treatment with great clinical significance. Here, we provided a clue to develop new clinical drugs by improving ITAM activity.

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Disclosure of conflict of interest

None.

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