PTEN is a tumor suppressor in CML stem cells and BCR-ABL–induced leukemias in mice

Cong Peng,1 Yaoyu Chen,1 Zhongfa Yang,1 Haojian Zhang,1 Lori Osterby,1 Alan G. Rosmarin,1 and Shaoguang Li1

1Division of Hematology/Oncology, Department of Medicine, University of Massachusetts Medical School, Worcester

The tumor suppressor gene phosphatase and tensin homolog (PTEN) is inactivated in many human cancers. However, it is unknown whether PTEN functions as a tumor suppressor in human Philadelphia chromosome–positive leukemia that includes chronic myeloid leukemia (CML) and B-cell acute lymphoblastic leukemia (B-ALL) and is induced by the BCR-ABL oncogene. By using our mouse model of BCR-ABL–induced leukemias, we show that Pten is down-regulated by BCR-ABL in leukemia stem cells in CML and that PTEN deletion causes acceleration of CML development. In addition, overexpression of PTEN delays the development of CML and B-ALL and prolongs survival of leukemia mice. PTEN suppresses leukemia stem cells and induces cell-cycle arrest of leukemia cells. Moreover, PTEN suppresses B-ALL development through regulating its downstream gene Akt1. These results demonstrate a critical role of PTEN in BCR-ABL–induced leukemias and suggest a potential strategy for the treatment of Philadelphia chromosome–positive leukemia. (Blood. 2010;115:626-635)

Introduction

The human Philadelphia chromosome (Ph) arises from a reciprocal translocation between chromosome 9 and 22, resulting in the formation of chimeric BCR-ABL oncogene. BCR-ABL encodes a constitutively activated, oncopgenic tyrosine kinase.1 Ph+ leukemia induced by BCR-ABL includes chronic myeloid leukemia (CML) and B-cell acute lymphoblastic leukemia (B-ALL). The BCR-ABL kinase inhibitor imatinib mesylate induces a complete hematologic remission of leukemia cells. Moreover, B-ALL is less sensitive to imatinib, suggesting that leukemia stem cells are not eliminated. Over time, patients frequently become drug-resistant and develop progressive disease despite continued treatment.5-7 Moreover, B-ALL is less sensitive to imatinib, suggesting that inhibition of BCR-ABL kinase activity is not enough to suppress B-ALL development. New therapeutic strategies need to be developed for Ph+ leukemia.

Tumors progress to more advanced stages after acquiring additional genetic alterations, and inactivation of tumor suppressor genes are common in human cancers. Phosphatase and tensin homolog (PTEN)9 is often deleted or inactivated in many tumor types, including glioblastoma,9 endometrial carcinoma,10 and lymphoid malignancies.11 PTEN is a phosphatase that dephosphorylates phosphatidylinositol-3-phosphate.12,13 Phosphatidylinositol-3-trisphosphate is a direct product of phosphoinositide 3-kinase (PI3K) activity and plays a critical role in the regulation of cell survival and growth by activating the Ser/Thr protein kinase PDK1 and its downstream target Akt.14,15 Activated Akt mediates several well-described PI3K responses that include cell survival and growth, cellular metabolism, angiogenesis, and cell migration.

Mice with a complete null mutation of Pten develop early embryonic lethality at E9.5.16-18 Pten-heterozygous mice die within 1 year after birth, and survivors develop a broad range of tumors, including mammary, thyroid, endometrial, and prostate cancers,16-18 as well as autoimmunity related to Fas-mediated response.19 Mice with the tissue-specific deletion of Pten using the Cre-loxP system have become available for studying physiologic functions of Pten in adult tissues and organs.20,21 For example, mice with Pten deletion in adult hematopoietic cells develop and die of acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL).22

Akt1 is a major downstream signaling molecule of PTEN and is activated after PTEN is mutated in human cancers. Investigators in a recent study23 showed that the deficiency of Akt1 is sufficient to suppress the development of several types of tumors in Pten-heterozygous mice, including prostate cancer, endometrial carcinoma, thyroid neoplasia, intestinal polyps, and lymphoid hyperplasia. Moreover, rapamycin, which directly inhibits the Akt downstream molecule mammalian target of rapamycin (mTOR), effectively inhibits survival and proliferation of AML cells from Ptenfl/fl;Mx-1-Cre AML mice and prolongs the survival of these diseased mice.24 Together, these results demonstrate a crucial role of the PI3K-PTEN-Akt pathway in cancer development. In this study, we investigated the role of Pten in the development of BCR-ABL–induced CML and B-ALL in mice. We also tested the effect of Pten on leukemia stem cells (LSCs) and studied the role of Akt1 as a Pten downstream signaling molecule in B-ALL development. Furthermore, we evaluated the potential role of targeting the Akt1-mTOR pathway in the treatment of BCR-ABL–induced leukemia.

Methods

Cell lines

Ba/F3 pre-B cells were grown in RPMI 1640 medium containing 10% fetal calf serum (FCS), 10% WEHI medium, and 50μM 2-mercaptoethanol.
Mice
C57BL/6J, B6.129S4-Pten<sup>tm1Hova</sup>/J (Pten<sup>fl</sup>) and B6.129P2-Akt<sup>1Mibb</sup>/J (Akt<sup>1-/-</sup>) mice were obtained from The Jackson Laboratory. Mice were maintained in a temperature- and humidity-controlled environment and given unrestricted access to 6% chow diet and acidified water. All animal studies were approved by the University of Massachusetts Institutional Animal Use and Care Committee.

Antibodies and Western blot analysis
Antibodies against c-Abl (sc-131), p-Tyr (sc-508), PTEN (sc-7974), p53 (sc-22-243), and actin (sc-1616-R) were purchased from Santa Cruz Biotechnology. Cre (cat. no. 69 050) antibody was ordered from Novagen. Protein lysates were prepared by lysing cells in radioimmunoprecipitation buffer, and immunoprecipitation and Western blotting were carried out as described previously.

Construction of triple-gene coexpression plasmids
The original MSCV-IRES-GFP vector was first modified to add new cloning sites for the restriction enzymes MfeI, NotI, and MluI. To do so, the internal ribosomal entry segment (IRES) sequence was first amplified by the murine stem cell virus (MSCV) primer (GGCTCTCCCTTGGACGCTCCTCGG) and the IRES-MfeI primer (CATGCGCATGGCAATGAGCGGGCGTTTGGCCATATTACATC), which contains the new MfeI, NotI, and the existing NcoI sites (underlined). This step allowed us to synthesize a new IRES fragment containing the MfeI, NotI, and NcoI sites. To replace the original IRES sequence in the original MSCV-IRES-GFP vector with the newly synthesized IRES, this vector was cut with EcoRI and NcoI, and then the new IRES fragment was cloned into the MSCV-IRES-GFP vector with EcoRI and NcoI, forming a new MSCV-IRES-GFP vector that contains 2 additional sites, MfeI and NotI. To add the MluI site to the new MSCV-IRES-GFP vector, an IRES-MluI fragment was amplified from the vector by the MSCV primer and the GFP-MluI primer (CCATCGGATCAGGTGAATGTTGCTGAGTCTGG), which contains the existing ClaI and the new MluI sites (underlined). The synthesized IRES-GFP fragment was digested with EcoRI and ClaI and then cloned into new MSCV-IRES-GFP vector between the EcoRI and ClaI sites to generate the final MSCV-IRES-GFP vector. Compared with the original MSCV-IRES-GFP vector, this final MSCV-IRES-GFP vector contains additional sites MfeI, NotI (before the GFP sequence), and MluI (after the GFP sequence). To clone the BCR-ABL cDNA into this final MSCV-IRES-GFP vector, BCR-ABL was cloned into it at the EcoRI site.

To make the MSCV-BCR-ABL-Pten-GFP construct, total RNA was isolated from C57BL/6J mouse liver tissue to synthesize the Pten cDNA by reverse-transcription polymerase chain reaction (RT-PCR). The Pten cDNA was amplified by Pten-NotI (5′-AGCCGGCGCATGACAGGATCATCAAAGAGAGAGA) and BCR-ABL-XhoI (5′-GCGCTACCGGTAGATCGATTAATGGGTG) primers. The cDNA was sequenced from both ends to confirm the sequence. The Pten cDNA was cloned into the MSCV-BCR-ABL-GFP vector between NotI and MluI sites. The IRES-GFP fragment was amplified by MluI-MluI (ccacggtgAAAAAGAACGAAGAAGG) and MluI-MluI (ccgctgtaaagttctgccggcatgatcagtctg) primers by use of MSCV-GFP as a template, and the IRES-GFP fragment was inserted after the Pten sequence at the MluI site.

To make MSCV-BCR-ABL-cre-GFP construct, the cre (improved Cre) open reading frame (ORF) was amplified by cre-MfeI (CGGAAATCATGATCCCCATCTTCCCTCC) and cre-MluI (ccacggtgAAAAAGAACGAAGAAGG) and cre-MluI (ccgctgtaaagttctgccggcatgatcagtctg) by use of the pBOB-CAG-cre-SD (Addgene) as a template. The cre ORF was cloned into the MSCV-BCR-ABL vector between NotI and MluI sites, and the IRES-GFP fragment was cloned at the MluI site after the cre ORF.

Bone marrow cell lines were transfected with BCR-ABL retrovirus and then cultured in a 6-well plate in RPMI 1640 medium containing 10% FCS and 50μM 2-mercaptopoethanol for 1 week. Protein lysates were collected and analyzed by Western blotting.

Bone marrow transduction/transplantation
The retroviral constructs MSCV-GFP, BCR-ABL-PTEN-GFP, or BCR-ABL-cre-GFP carrying the BCR-ABL cDNA were used to make high-titer, helper-free, replication-defective ectropic viral stocks by transient transfection of 293T cells by use of the kat system as previously described. Then, 6- to 10-week-old wild-type C57BL/6 and Pten<sup>fl</sup> (The Jackson Laboratory) mice were used for leukemogenesis experiments. Induction of CML and B-ALL was described previously. In brief, to induce CML, bone marrow cells from 5-FU–treated (200 mg/kg) donor mice were transduced twice with BCR-ABL retrovirus by cocultivation in the presence of interleukin-3, interleukin-6, and stem cell factor. To induce B-ALL, bone marrow cells from non–5-FU–treated donors were transduced with BCR-ABL in the absence of any cytokines. Wild-type recipient mice were prepared by 1150 cGy gamma irradiation. A dose of 0.5 × 10<sup>6</sup> (CML) or 1.0 × 10<sup>6</sup> (B-ALL) cells was transplanted via tail vein injection. Diseased mice were analyzed by histopathologic and biochemical analyses as described previously.

Flow cytometry
Hematopoietic cell lines were isolated from peripheral blood and bone marrow of the disease mice, and red blood cells were lysed with NH<sub>4</sub>Cl red blood cell lysis buffer (pH 7.4). The cells were washed with phosphate-buffered saline and stained with B220-PE for B cells and Gr1-APC for neutrophils, Sca1-APC/c-Kit-PE for hematopoietic stem cells, and Hoechst blue for DNA. After staining, the cells were washed once with phosphate-buffered saline and subjected to fluorescence-activated cell sorting (FACS) analysis.

Chip
Chromatin immunoprecipitation (Chip) assay was performed by use of the Chip-Ti Express kit following the manufacturer’s instructions (Active Motif). In brief, BaF3 cells were lysed and chromatin was broken from 200–hp into 500–hp fragments by sonication. Next, genomic DNA was incubated with anti-rabbit immune IgG (a negative control) or anti-p53 antibody overnight, and then protein G Sepharose beads preincubated with bovine serum albumin were added to pull DNA fragments bound to anti-p53 antibody. Recovered bound DNA was used as templates for PCR amplification of p53 binding site on the Pten promoter with the primers 5′-CAACGGCGGCGTAAGCTC-3′ and 5′-ACAAAGATCCCGCCCATAT-3′.

In vitro culture of LSCs
Bone marrow cells isolated from CML mice were cultured in vitro in the presence of stemspan SFEM, stem cell factor, insulin-like growth factor-2, thrombopoietin, heparin, and α-fibrinogenbinding factor as described previously.

Drug treatment
Imatinib was dissolved in water directly at a concentration of 10 mg/mL and administered orally by gavage in a volume less than 0.5 mL twice a day at 100 mg/kg body weight, beginning 8 days after bone marrow transplantation (BMT) and continuing until the morbidity or death of leukemic mice. Rapamycin (Calbiochem) was dissolved in dimethyl sulfoxide (DMSO) to make 1mM stock solution before it was diluted in culture medium.

Results
PTEN expression is down-regulated by BCR-ABL
We performed a global gene expression analysis by using DNA microarray to identify genes regulated by BCR-ABL in a BCR-ABL–expressing mouse pre–B-cell line (ENU-BCR-ABL cells).
Methods section.

In mouse, p53 transcriptional activation and promotes p53 export and in BaF3 cells to show the binding of p53 to the binding sequence. (D) p53 binds to

Comparing with non–BCR-ABL–expressing parental cells, we observed a 1.43-fold decrease in the level of Pten mRNA (Figure 1A). The down-regulation of Pten by BCR-ABL was further confirmed by Western blotting in BCR-ABL–expressing Ba/F3 cells (Ba/F3-BCR-ABL; Figure 1B). We also treated Ba/F3 and Ba/F3-BCR-ABL cells with the BCR-ABL kinase inhibitor imatinib and found that imatinib did not have an effect on Pten expression in Ba/F3 cells but caused the restoration of Pten protein expression back to its endogenous level in Ba/F3-BCR-ABL cells (Figure 1B), indicating that this Pten down-regulation is dependent upon BCR-ABL kinase activity.

We next tested whether Pten down-regulation by BCR-ABL correlates with p53 degradation. In BCR-ABL–expressing Ba/F3 cells, the level of p53 was lower than that in parental Ba/F3 cells, and this reduced p53 level was reversed after imatinib treatment (Figure 1B). This result suggests that BCR-ABL might down-regulate Pten through P53. There are 2 p53 binding sites on human Pten promoter, and p53 positively regulates Pten by binding to these 2 sites. BCR-ABL causes down-regulation of p53 in leukemia cells through the up-regulation of MDM2 that inhibits p53 transcriptional activation and promotes p53 export and proteasome-dependent degradation in the cytoplasm. In mouse, we only found one p53 binding site on Pten promoter (Figure 1C), and we performed the Chip assay and showed that p53 bound to Pten promoter in Ba/F3 cells (Figure 1D).

PTEN overexpression delays CML development

CML developed faster in the absence of Pten (Figure 2), indicating that Pten is a tumor suppressor in BCR-ABL–induced leukemia. To further test this idea, we examined whether overexpression of PTEN delays CML development. We cloned the Pten gene into the BCR-ABL-GFP construct for simultaneous expression of the 3 genes, BCR-ABL, Pten, and GFP (Figure 3A). Western blot analysis showed that this triple-gene retroviral construct allowed overexpression of Pten in cells (Figure 3B). We next transduced donor bone marrow cells from wild-type mice with BCR-ABL-PTEN-GFP or BCR-ABL-GFP retrovirus, followed by transplantation of the transduced cells into lethally irradiated recipient mice. Mice receiving donor bone marrow cells transduced with BCR-ABL-iCre-GFP retrovirus developed CML much faster than those receiving bone marrow cells transduced with BCR-ABL-GFP (Figure 2C; \( P < .005 \)). In these CML disease mice, the majority of GFP cells were Gr1+ but not B220+ leukemia cells (supplemental Figure 1, available on the Blood website; see the Supplemental Materials link at the top of the online article). The accelerated death of CML mice in the absence of Pten correlated with a greater percentage of GFP+Gr1+ myeloid leukemia cells (Figure 2D) and a greater number of leukemia cells (Figure 2E) in peripheral blood of the mice. Accelerated CML development in the absence of Pten also correlated with more severe infiltration of leukemia cells in the lungs (Figure 2F,H) and splenomegaly (Figure 2G-H). These results demonstrated that Pten is a potent tumor suppressor in BCR-ABL–induced CML.

Pten\(^{fl/fl}\);Mx-1-Cre mice develop AML 20 days after PIPC treatment that initiates the deletion of Pten. We wondered whether the mice receiving donor bone marrow cells transduced with BCR-ABL-iCre-GFP developed AML, which may contribute to the accelerated death of CML mice in the absence of Pten (Figure 1C). We found that these mice developed typical CML (Figure 2), and we did not observe any signs for AML development (data not shown). To further rule out the possible contribution of AML to the accelerated death of CML mice in the absence of Pten, we transduced normal bone marrow cells from PTEN\(^{fl/fl}\) mice with MSCV-iCre-GFP retrovirus to delete Pten, followed by transplantation of the transduced cells into the lethally irradiated recipient mice. Although 20% white blood cells in peripheral blood of the recipient mice were GFP\(^{+}\), indicating that the iCre gene was expressed in the cells; none of these mice developed AML, and all mice survived (supplemental Figure 2). This result suggests that the deletion of Pten in non–BCR-ABL–expressing bone marrow cells is insufficient to induce AML in our bone marrow transduction/transplantation model system.

PTEN deletion causes acceleration of CML development

Because Pten was down-regulated by BCR-ABL (Figure 1), we tested whether Pten functions as a tumor suppressor in CML development by using Pten conditional knock mice (Pten\(^{fl/fl}\)). To delete Pten from bone marrow cells of Pten\(^{fl/fl}\) mice, we transduced the cells with BCR-ABL-iCre-GFP retrovirus or BCR-ABL-GFP retrovirus as a control (Figure 2A). Western blot analysis showed expression of iCre and a significant decrease of the Pten protein level (Figure 2B), indicating that the Pten gene was deleted from the cells. To test whether deletion of Pten affects CML development, we transduced bone marrow cells from Pten\(^{fl/fl}\) mice with BCR-ABL-iCre-GFP or BCR-ABL-GFP retrovirus, followed by transplantation of the transduced cells into lethally irradiated recipient mice. Mice receiving donor bone marrow cells transduced with BCR-ABL-iCre-GFP developed CML much faster than those receiving bone marrow cells transduced with BCR-ABL-GFP (Figure 2C; \( P < .005 \)). In these CML disease mice, the majority of GFP cells were Gr1+ but not B220+ leukemia cells (supplemental Figure 1, available on the Blood website; see the Supplemental Materials link at the top of the online article). The accelerated death of CML mice in the absence of Pten correlated with a greater percentage of GFP+Gr1+ myeloid leukemia cells (Figure 2D) and a greater number of leukemia cells (Figure 2E) in peripheral blood of the mice. Accelerated CML development in the absence of Pten also correlated with more severe infiltration of leukemia cells in the lungs (Figure 2F,H) and splenomegaly (Figure 2G-H). These results demonstrated that Pten is a potent tumor suppressor in BCR-ABL–induced CML.

Pten\(^{fl/fl}\);Mx-1-Cre mice develop AML 20 days after PIPC treatment that initiates the deletion of Pten. We wondered whether the mice receiving donor bone marrow cells transduced with BCR-ABL-iCre-GFP developed AML, which may contribute to the accelerated death of CML mice in the absence of Pten (Figure 1C). We found that these mice developed typical CML (Figure 2), and we did not observe any signs for AML development (data not shown). To further rule out the possible contribution of AML to the accelerated death of CML mice in the absence of Pten, we transduced normal bone marrow cells from PTEN\(^{fl/fl}\) mice with MSCV-iCre-GFP retrovirus to delete Pten, followed by transplantation of the transduced cells into the lethal irradiated recipient mice. Although 20% white blood cells in peripheral blood of the recipient mice were GFP\(^{+}\), indicating that the iCre gene was expressed in the cells; none of these mice developed AML, and all mice survived (supplemental Figure 2). This result suggests that the deletion of Pten in non–BCR-ABL–expressing bone marrow cells is insufficient to induce AML in our bone marrow transduction/transplantation model system.

PTEN overexpression delays CML development

CML developed faster in the absence of Pten (Figure 2), indicating that Pten is a tumor suppressor in BCR-ABL–induced leukemia. To further test this idea, we examined whether overexpression of PTEN delays CML development. We cloned the Pten gene into the BCR-ABL-GFP construct for simultaneous expression of the 3 genes, BCR-ABL, Pten, and GFP (Figure 3A). Western blot analysis showed that this triple-gene retroviral construct allowed overexpression of Pten in cells (Figure 3B). We next transduced donor bone marrow cells from wild-type mice with BCR-ABL-PTEN-GFP or BCR-ABL-GFP retrovirus, followed by transplantation of the transduced cells into recipient mice. CML development was significantly slower in mice receiving bone marrow cells transduced with BCR-ABL-PTEN-GFP than in those receiving bone marrow cells transduced with BCR-ABL-GFP (Figure 3C, \( P < .001 \)), indicating that Pten overexpression caused a delay of CML development. The delayed CML development correlated with a less percentage and number of leukemia cells in peripheral blood (Figures 3D,E), and also with less severe splenomegaly (Figure 3F) and infiltration of leukemia cells in the lungs (Figure 3G). These
results further support the role of Pten as a tumor suppressor in CML development.

To evaluate whether PTEN overexpression in BCR-ABL–expressing cells synergizes with the therapeutic effect of imatinib on CML, we treated mice receiving bone marrow cells transduced with BCR-ABL-PTEN-GFP or BCR-ABL-GFP retrovirus with imatinib. As expected, imatinib treatment prolonged survival of CML mice (Figure 3G; P < .001). However, imatinib-treated CML mice receiving bone marrow cells transduced with BCR-ABL-PTEN-GFP lived significantly longer than those not treated with imatinib (Figure 3G; P < .001). The synergistic effect of Pten overexpression with imatinib treatment correlated with less leukemia cells in peripheral blood of the mice (Figure 3E). To explain how Pten reduced proliferation of leukemia cells, we performed the DNA content analysis to examine the effect of Pten overexpression on cell-cycle progression of these cells. We showed that the percentage of leukemia cells in the S + G2M phase was much lower in leukemia cells with Pten overexpression than in those without Pten overexpression (Figure 3H; P < .01), indicating that Pten inhibits the proliferation of leukemia cells by inducing a cell-cycle arrest. Furthermore, we examined whether Pten induces apoptosis of leukemia cells by staining the cells with PI and annexin V. Corresponding to the result in the cell-cycle analysis, apoptosis in leukemia cells with Pten overexpression was more severe than in those without Pten overexpression (Figure 3I; P < .05).

PTEN suppresses CML stem cells

CML is derived from hematopoietic stem cells harboring the BCR-ABL oncogene. 25 It is possible that Pten suppresses CML stem cells, resulting in acceleration of CML when deleted (Figure 2) and delay of CML when overexpressed (Figure 3). We have previously identified BCR-ABL–expressing Lin–c-kit+Sca1+ cells as LSCs in CML induced by BCR-ABL in mice. 26 To test whether Pten expression is affected by BCR-ABL in LSCs, GFP+Lin–c-kit+Sca1+ cells were sorted by FACS from CML mice treated with a placebo or imatinib, and total RNA was isolated for DNA microarray analysis. The microarray study showed that Pten mRNA was significantly down-regulated approximately 3.59-fold by BCR-ABL, and this down-regulation was restored upon imatinib treatment (Figure 4A; P < .001). Correlating with Pten down-regulation in LSCs, p53 was also down-regulated approximately 2.9-fold by BCR-ABL in LSCs (data not shown). These
results further support our observations in BaF3-BCR-ABL cells (Figure 1).

To test whether PTEN functions as a tumor suppressor in LSCs, we transduced bone marrow cells with BCR-ABL-PTEN-GFP or BCR-ABL-GFP retrovirus, followed by transplantation of the transduced cells into recipient mice. At 14 days after the transplantation, bone marrow cells were isolated from CML mice, and LSCs (GFP+/H11001 Lin−/H11002 c-kit+/H11001 Sca1+) were analyzed by flow cytometry. The
percentage of LSCs in mice with CML induced by BCR-ABL-PTEN-GFP was significantly lower than that in mice with CML induced by BCR-ABL-GFP (Figure 4B), indicating that Pten suppresses LSCs.

To determine whether Pten affects the function of LSCs, we compared the ability to induce CML between LSCs that expressed BCR-ABL-GFP or BCR-ABL-PTEN-GFP. At 14 days after BMT, the same number (3 × 10^5) of GFP^+ c-Kit^+ Sca1^+ cells sorted from CML mice were transduced with BCR-ABL-GFP or BCR-ABL-PTEN-GFP and cultured in the presence of DMSO or rapamycin (10μM) for 3 days, followed by FACS analysis of leukemia stem cells (GFP^+ c-Kit^+ Sca1^+).

Pten overexpression delays B-ALL development

We showed previously in this report that Pten functions as a tumor suppressor in CML development. We determined to examine whether Pten also plays a suppressive role in the development of B-ALL induced by BCR-ABL. To induce B-ALL in mice, donor bone marrow cells were transduced with BCR-ABL-PTEN-GFP or BCR-ABL-GFP retrovirus, followed by transplantation of the transduced cells into lethally irradiated recipient mice, as described previously.30 All mice receiving bone marrow cells transduced with BCR-ABL-GFP developed and died of B-ALL within 4 to 5 weeks after transplantation (Figure 5A), whereas mice receiving bone marrow cells transduced with BCR-ABL-PTEN-GFP developed B-ALL with much longer disease latency (Figure 5A, 5B).Because rapamycin suppresses AML stem cells,24 we tested whether rapamycin also inhibits leukemia stem cells in CML. We isolated bone marrow cells from mice with CML induced by BCR-ABL-PTEN-GFP or BCR-ABL-GFP and treated them with rapamycin for 3 days. The survival of Ptenfl/fl;Mx-1-Cre AML mice, likely through inhibition of mTOR by rapamycin also significantly inhibited the survival of leukemia stem cells from CML mice in vitro.
The delayed B-ALL development correlated with a lower percentage and number of BCR-ABL–expressing B-lymphoid cells (GFP⁺\textit{B}220⁺) in peripheral blood of the mice (Figures 5B,C), in which GFP⁺\textit{Gr}1⁺ cells were almost undetectable (supplemental Figure 3).

To evaluate whether PTEN overexpression synergizes with imatinib in treating B-ALL mice, we treated mice receiving bone marrow cells transduced with BCR-ABL-PTEN-GFP or BCR-ABL-GFP retrovirus with imatinib. As expected, imatinib treatment prolonged the survival of B-ALL mice receiving bone marrow cells transduced with BCR-ABL-GFP (Figure 5A). However, imatinib-treated B-ALL mice receiving bone marrow cells transduced with BCR-ABL-PTEN-GFP lived significantly longer than those not treated with imatinib (Figure 5A; \(P = .001\)). The synergistic effect of Pten overexpression and imatinib treatment correlated with fewer leukemia cells in peripheral blood of the mice (Figure 5D).

The Akt pathway is downstream of Pten because Pten inactivation often results in Akt activation in human cancers.\textsuperscript{31,32} There are 3 mammalian Akt genes that share greater than 85% sequence similarity and encode the Akt isoforms 1 to 3.\textsuperscript{23} It is still unclear whether the 3 Akt isoforms possess different functional specificities in vivo. A recent study has shown that the deletion of the Akt1 gene has a dramatic inhibitory effect on the development of endometrium carcinoma, prostate cancer, thyroid tumor, and adrenal medulla tumors.\textsuperscript{23} Akt1 deficiency also inhibits the proliferation of lymphoid hyperplasia and expansion of both B- and T-cell populations in \textit{Pten}\textsuperscript{−/−} mice.\textsuperscript{23} In addition, the first transforming point mutation in Akt1 (E17K) has been discovered in human breast, colorectal, and ovarian cancers.\textsuperscript{8}

Furthermore, fetal liver cells from Eu-Myc transgenic mouse were transduced with this Akt1 (E17K), followed by transplantation into recipient mice. After 16 weeks, 6 of 10 recipients were low in mice with B-ALL induced by BCR-ABL-PTEN-GFP (Figure 5E).
wild-type or Akt1 were harvested from recipients of BCR-ABL transduced (n/H11005) mice. B-ALL with a significantly longer disease delay (Figure 6A; P<.001). This delayed B-ALL development caused by the Akt1 deficiency correlated with a lesser percentage and number of B-leukemia cells (B220+GFP+) in the peripheral blood of the mice (Figure 6B,C). We examined whether Akt1−/− have a defect in B-cell development because a reduction of bone marrow pro-B cells, the target cells for BCR-ABL to induce B-ALL,26 could lead to a delayed disease development. To rule out this possibility, we analyzed bone marrow cells of Akt1−/− mice by FACS and found that Akt1−/− mice have a normal percentage of pro-B cells (CD43+B220−) in bone marrow compared with wild-type mice (supplemental Figure 4).

Because Pten overexpression synergizes with imatinib in treating B-ALL mice (Figure 5A), we examined whether the Akt1 deficiency also synergizes with imatinib in treating B-ALL. We treated mice receiving wild-type or Akt1−/− bone marrow cells transduced with BCR-ABL-GFP retrovirus with imatinib. As expected, imatinib treatment prolonged survival of B-ALL mice receiving BCR-ABL−transduced wild-type bone marrow cells, whereas imatinib treatment more significantly improved survival of B-ALL mice receiving BCR-ABL−GFP−transduced Akt1−/− bone marrow cells (Figure 6A; P<.001).

Rapamycin inhibits proliferation and induces apoptosis of human CML cells

Recently, down-regulation of PTEN mRNA in CD34+ bone marrow cells of CML patients has been reported,33 supporting our finding that PTEN plays a tumor suppressor role in CML development. We further tested the inhibitory role of PTEN in human K562 CML cells. Western blot analysis showed that the level of PTEN was significantly lower in DMSO-treated K562 cells than in K562 cells treated with imatinib for 24 hours (Figure 7B). Rapamycin also inhibited survival of the cells (Figure 7B). Rapamycin also significantly induced the apoptosis of K562 cells (Figure 7C).

Discussion

Some tumor suppressor genes have been shown to be inactivated or down-regulated by BCR-ABL in Ph+ leukemia, including PP2A,34 p53,35 RB,35 and interferon consensus sequence-binding protein.36 In this study, we show that the tumor suppressor Pten is also down-regulated by BCR-ABL and that overexpression of Pten...
delays the development of CML and B-ALL induced by BCR-ABL. Our DNA microarray study shows that Pten mRNA level was decreased in BCR-ABL–expressing LSCs, indicating that BCR-ABL regulates Pten at a transcriptional level. Our finding that both Pten and p53 are simultaneously down-regulated in BCR-ABL–expressing cells suggests that the Pten down-regulation by BCR-ABL may be mediated by P53, as PTEN transcription is regulated by p53.37,38 p53 has been shown to up-regulate Pten expression, and these potential mechanisms need to be explored further.

Besides p53, other mechanisms might also be involved in the down-regulation of Pten by BCR-ABL. An analysis of the Pten promoter sequence shows potential binding sites for early growth-regulated transcriptional factor 1 (EGFR1), and Pten is up-regulated by EGFR1 in response to radiation treatment.40 EGFR1 also up-regulates Pten, which likely mediates the apoptotic effect of the phosphatase inhibitor calyculin A. There are also pathways that negatively regulate Pten expression. For example, mitogen-activated protein kinase kinase 4 inhibits Pten transcription by activating nuclear factorkB that binds to the Pten promoter.41 In pancreatic cancer cells42 or mesangial cells,43 Pten is down-regulated by transforming growth factor-β. Pten is also regulated at a posttranscriptional level. Phosphorylation of Pten at specific residues in its C-terminal tail is associated with an increase in its stability,44,46 whereas phosphorylation at other sites decreases the protein stability.47 Ubiquitin-dependent degradation of PTEN occurs when human bronchial cells were exposed to zinc ions,48 and the finding of 2 major conserved ubiquitination sites on PTEN supports this regulation.49 BCR-ABL may regulate these pathways to down-regulate Pten expression, and these potential mechanisms need to be explored further.

PTEN maintains normal hematopoietic stem cells in lineage choice and prevents the leukemia development from leukemia stem cells.22,24 Our microarray data show that Pten is down-regulated in BCR-ABL–expressing LSCs, suggesting that BCR-ABL regulates the functions of LSCs through regulating Pten expression. This idea is supported by our finding that LSCs in CML mice grew significantly slower when Pten was overexpressed. The role of Pten in LSCs provides a potential strategy for targeting the Pten and its related PI3K/ AKT pathways in eradication of LSCs.

In this study, we also show that overexpression of PTEN delays B-ALL development and that Akt1 is a major downstream signaling molecule of Pten. Moreover, the inhibition of mTOR by rapamycin significantly inhibits proliferation of human CML leukemia cells K562 and leukemia stem cells from CML mice. These findings support the use of the PTEN-PI3K-AKT-mTOR pathway as a target in treating B-ALL, which is not sensitive to imatinib therapy.

Acknowledgments

This work was supported by the grants from the Leukemia & Lymphoma Society and the National Institutes of Health (R01-CA122142, R01-CA114199) to S.L. S.L. is a Scholar of the Leukemia & Lymphoma Society.

Authorship

Contribution: C.P. and S.L. designed the experiments, analyzed data, and wrote the paper; C.P., Y.C., Z.Y., and S.L. performed the experiments; and H.Z., A. R., and L.P. helped with the experiments.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Shaoguang Li, 364 Plantation St, LRB 315, Worcester, MA 01604; e-mail: Shaoguang.Li@umassmed.edu.

References

10. Pfeffer SL, Herzog TJ, Tribunke DJ, Mutch DG, Gersell DJ, Goodfellow PJ. Allelic loss of Pten in cancers from the long arm of chromosome 10 delays the development of CML and B-ALL induced by BCR-ABL. Our DNA microarray study shows that Pten is down-regulated in BCR-ABL–expressing LSCs, suggesting that BCR-ABL regulates the functions of LSCs through regulating Pten expression. This idea is supported by our finding that LSCs in CML mice grew significantly slower when Pten was overexpressed. The role of Pten in LSCs provides a potential strategy for targeting the Pten and its related PI3K/AKT pathways in eradication of LSCs.

In this study, we also show that overexpression of PTEN delays B-ALL development and that Akt1 is a major downstream signaling molecule of Pten. Moreover, the inhibition of mTOR by rapamycin significantly inhibits proliferation of human CML leukemia cells K562 and leukemia stem cells from CML mice. These findings support the use of the PTEN-PI3K-AKT-mTOR pathway as a target in treating B-ALL, which is not sensitive to imatinib therapy.

Acknowledgments

This work was supported by the grants from the Leukemia & Lymphoma Society and the National Institutes of Health (R01-CA122142, R01-CA114199) to S.L. S.L. is a Scholar of the Leukemia & Lymphoma Society.

Authorship

Contribution: C.P. and S.L. designed the experiments, analyzed data, and wrote the paper; C.P., Y.C., Z.Y., and S.L. performed the experiments; and H.Z., A. R., and L.P. helped with the experiments.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Shaoguang Li, 364 Plantation St, LRB 315, Worcester, MA 01604; e-mail: Shaoguang.Li@umassmed.edu.

References

10. Pfeffer SL, Herzog TJ, Tribunke DJ, Mutch DG, Gersell DJ, Goodfellow PJ. Allelic loss of Pten in cancers from the long arm of chromosome 10 delays the development of CML and B-ALL induced by BCR-ABL. Our DNA microarray study shows that Pten is down-regulated in BCR-ABL–expressing LSCs, suggesting that BCR-ABL regulates the functions of LSCs through regulating Pten expression. This idea is supported by our finding that LSCs in CML mice grew significantly slower when Pten was overexpressed. The role of Pten in LSCs provides a potential strategy for targeting the Pten and its related PI3K/AKT pathways in eradication of LSCs.

In this study, we also show that overexpression of PTEN delays B-ALL development and that Akt1 is a major downstream signaling molecule of Pten. Moreover, the inhibition of mTOR by rapamycin significantly inhibits proliferation of human CML leukemia cells K562 and leukemia stem cells from CML mice. These findings support the use of the PTEN-PI3K-AKT-mTOR pathway as a target in treating B-ALL, which is not sensitive to imatinib therapy.


PTEN is a tumor suppressor in CML stem cells and BCR-ABL–induced leukemias in mice

Cong Peng, Yao Yu Chen, Zhongfa Yang, Haojian Zhang, Lori Osterby, Alan G. Rosmarin and Shaoguang Li