Adult B-cell acute lymphoblastic leukemia cells display decreased PTEN activity and constitutive hyperactivation of PI3K/Akt pathway despite high PTEN protein levels

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ABSTRACT

Adult B-cell acute lymphoblastic leukemia remains a major therapeutic challenge, requiring a better characterization of the molecular determinants underlying disease progression and resistance to treatment. Here, using a phospho-flow cytometry approach we show that adult diagnostic B-cell acute lymphoblastic leukemia specimens display PI3K/Akt pathway hyperactivation, irrespective of their BCR-ABL status and despite paradoxically high basal expression of PTEN, the major negative regulator of the pathway. Protein kinase CK2 is known to phosphorylate PTEN thereby driving PTEN protein stabilization and concomitant PTEN functional inactivation. In agreement, we found that adult B-cell acute lymphoblastic leukemia samples show significantly higher CK2 kinase activity and lower PTEN lipid phosphatase activity than healthy controls. Moreover, the clinical-grade CK2 inhibitor CX-4945 (Silmitasertib) reversed PTEN levels in leukemia cells to those observed in healthy controls, and promoted leukemia cell death without significantly affecting normal bone marrow cells. Our studies indicate that CK2-mediated PTEN posttranslational inactivation, associated with PI3K/Akt pathway hyperactivation, are a common event in adult B-cell acute lymphoblastic leukemia and suggest that CK2 inhibition may constitute a valid, novel therapeutic tool in this malignancy.

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Introduction

B-cell acute lymphoblastic leukemia (B-ALL), the most common type of ALL, is characterized by clonal expansion of developmentally arrested malignant B-cell precursors. Currently, up to 85% of children and 40% of adults with ALL can be cured with the use of risk-adjusted multi-agent therapeutic regimens.[1-3] However, patients who do not respond to treatment or who develop resistance have extremely poor prognosis.[4,5] Therefore, novel therapeutic strategies targeting leukemia-specific molecular determinants hold significant potential and are urgently required. They may be all the more critical in adult ALL, where conventional treatment options are considerably less effective, with the majority of the cases relapsing.[1,5]

The PI3K/Akt signaling pathway is involved in a wide array of physiological processes whose deregulation is frequently associated with tumorigenesis. In fact, PI3K/Akt pathway activation is extremely common in different cancers. However, little is known about the levels of activation of this pathway in B-ALL, especially in adult cases,[6,7] with most studies focusing on the PI3K/Akt downstream target mTOR and its respective effectors.[8-10] Yet, mTOR is part of a complex network that does not necessarily reflect the levels of activation of PI3K/Akt pathway, since it can be regulated by other upstream events.[11,12]

The activity of PI3K/Akt signaling can be inhibited by the tumor suppressor phosphatase and tensin homologue (PTEN), which converts phosphatidylinositol 3,4,5-trisphosphate (PIP3) into phosphatidylinositol 4,5-bisphosphate (PIP2). We have shown that constitutive hyperactivation of PI3K/Akt pathway in diagnostic childhood T-ALL results in most cases from casein kinase 2 (CK2)-mediated PTEN phosphorylation and consequent PTEN non-deletional inactivation.[13]

In the present studies, we used phospho-flow cytometry, a convenient methodology to study signaling activation at the single-cell level using relatively limited cell numbers,[14] to determine the activation status of the PI3K/Akt pathway in adult ALL specimens collected at diagnosis.

Methods

Primary samples and cell lines

Bone marrow samples from adult (n=21) or adolescent (n=2) B-ALL patients (Table 1) and healthy individuals (n=8), collected after informed consent and ethical committee approval in accordance with the Declaration of Helsinki, were enriched in mononuclear cells by density centrifugation over Ficoll-Paque. Human B-ALL cell lines were maintained in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine and penicillin/streptomycin and cultured at 37°C in 5% CO_2.

Intracellular phospho-specific flow cytometry

Cells were fixed with Cytofix buffer, pelleted by centrifugation and

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Table 1. Immunophenotype and cytogenetics features of B-ALL patient samples.

Patient n.	Sex	Age (years)	% Blasts	Cytogenetics	Immunophenotype (EGIL classification)
01	Female	64	80	BCR-ABL	B-II (Common B)
02	Male	15	88	–	B-II (Common B)
03	Female	67	80	BCR-ABL	B-II (Common B)
04	Female	53	96	–	B-I (Pro-B)
05	Male	52	22	–	B-II (Common B)
06	Female	46	85	–	B-I (Pro-B)
07	Female	35	80	BCR-ABL	B-III (Pre-B)
08	Female	29	85	MLL-AF4	B-I (Pro-B)
09	Male	59	95	BCR-ABL	B-II (Common B)
10	Male	50	75	–	B-III (Pre-B)
11	Female	61	80	–	B-II (Common B)
12	Male	15	40	–	B-III (Pre-B)
13	Female	64	80	BCR-ABL	B-II (Common B)
14	Female	64	n.d.	BCR-ABL	n.d.
15	Female	57	63	–	B-III (Pre-B)
16	Male	75	70	BCR-ABL	B-III (Pre-B)
17	Male	41	35	BCR-ABL	B-II (Common B)
18	Female	47	70	BCR-ABL	B-III (Pre-B)
19	Female	47	20	–	B-II (Common B)
20	Female	51	43	n.d.	B-III (Pre-B)
21	Male	73	45	Hiperdiploidy	B-II (Common B)
22	Female	56	75	–	B-III (Pre-B)
23	Female	65	n.d.	–	B-II (Common B)

-: not detected; n.d.: not determined; B-ALL maturation stages were defined according to the European Group for the immunological classification of leukemias (EGIL) criteria: stage I (Pro-B-ALL), cCD79a+, CD19+, HLA-DR+, TdT+, CD10-, CD20-, cyIgM-, sIg- ; stage II (Common-B-ALL), cCD79a+, CD19+, HLA-DR+, TdT+, CD10+, CD20-, cyIgM-, sIg- and stage III (Pre-B-ALL), cCD79a+, CD19+, HLA-DR+, TdT+, CD10-, CD20+/-, cyIgM+, sIg-.

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permeabilized in ice-cold PERM buffer III, washed in staining buffer and stained with the following antibodies: CD79a-APC; Akt-Alexa Fluor 488, PTEN-PE, phospho-Akt (S473)-Alexa Fluor 488, phospho-Akt (T308)-PE, and phospho-STAT5 (Y694)-Alexa Fluor 488. Samples were analyzed on a FACSAria or LSRFortessa using the gating strategy indicated in the *Online Supplementary Appendix* (*Online Supplementary Figure S1*).

Endogenous PTEN in vitro *lipid phosphatase assay*

PTEN phosphatase activity was measured *in vitro* as previously described.[13]

Endogenous CK2 in vitro *kinase assay*

CK2 activity was measured *in vitro* as previously described.[15]

Treatment with signaling inhibitors

Cells were cultured in control medium or in the presence of CX-4945 or LY294002 for the indicated time points and used for protein and viability analysis.

Immunoblotting

Cell lysates were resolved by SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblotted with the antibodies against actin, phospho-PTEN (S380), PTEN, CK2α and CK2α'.

Analysis of cell viability and apoptosis

Cell viability was determined by double-staining with APC or FITC-conjugated Annexin V and propidium iodide (PI) and flow cytometry analysis, as previously described.[16]

Statistical analysis

Differences between populations were calculated using unpaired two-tailed Students's t-test or Mann-Whitney test, as appropriate. Correlations were analyzed using the Pearson correlation coefficient. $P<0.05$ was considered significant.

Results

JAK/STAT and PI3K/Akt pathways are hyperactivated in adult B-ALL cells

Hyperactivation of signaling pathways involved in promotion of proliferation and survival is commonly associated with cancer progression. Previous studies have shown that one of these signaling cascades, the JAK/STAT pathway, is constitutively activated in B-ALL patients displaying the BCR-ABL fusion (also known as Philadelphia chromosome (Ph)-positive cases) or CRFL2 overexpression in combination or not with activating mutations in JAK1 and JAK2.[3,17] Using phospho-specific flow cytometry and a gating strategy that enabled us to focus on blast cells (*Online Supplementary Figure S1*) to compare primary bone marrow cells from healthy donors with B-ALL blasts collected from leukemia patients at diagnosis (Table 1), we con-

firmed that adult leukemia cases displayed constitutive hyperactivation of the JAK/STAT pathway (Figure 1A; *Online Supplementary Figures S1 and S2*). Moreover, we found a discrete subgroup of samples that presented very high levels of STAT5 phosphorylation. In line with the knowledge that BCR-ABL drives STAT5 activation,[17,18] this group was enriched in Ph-positive cases (Figure 1A, red labels).

In contrast to JAK/STAT pathway, the activation status of PI3K/Akt signaling pathway and its potential role in adult ALL remain less well characterized. We analyzed the basal phosphorylation levels of Akt S473 and T308 residues, which are mandatory for Akt full kinase activation. The levels of Akt phosphorylation at both residues were generally up-regulated in B-ALL cells as compared to controls (Figures 1B and C and *Online Supplementary Figures S1* and *S2*), indicating that PI3K/Akt pathway is constitutively activated in adult ALL cells. Notably, we did not find evidence for increased PI3K/Akt signaling pathway activation specifically in BCR-ABL-positive cases (Figure 1B and C, red labels).

PTEN lipid phosphatase activity is decreased in adult B-ALL cases despite up-regulated PTEN protein expression

The tumor suppressor PTEN is the major negative regulator of PI3K/Akt pathway, antagonizing PI3K signaling by dephosphorylating PIP3 into PIP2. Although PTEN mutation is relatively infrequent in leukemia,[19,20] we have previously shown that non-deletional PTEN posttranslational inactivation is very common in pediatric T-ALL.[13] Thus, we next sought to evaluate whether PTEN protein levels were affected in adult B-ALL patient samples. Similar to T-ALL, most B-ALL cases displayed increased PTEN levels as compared with normal bone marrow cells from healthy individuals (Figures 2A and *Online Supplementary Figures S1 and S3*). This 'paradoxical' upregulation of PTEN was paralleled by decreased PTEN *in vitro* lipid phosphatase activity in leukemia cells (Figure 2B). These findings indicate that the overexpression of PTEN in adult B-ALL cases was not associated with increased activity, but rather with overall PTEN inactivation. In support of this conclusion, we found that there was a significant positive correlation between total PTEN protein levels and phosphorylation of Akt, indicative of PI3K/Akt signaling pathway activation and thus an indirect measure of decreased PTEN activity (Figure 2C and D). Interestingly, we did not find evidence for significant associations between PTEN expression and parameters such as B-ALL maturation stage, sex, age (*Online Supplementary Figure S4*) or prognostic factors such as BCR-ABL expression (Figures 2A and *Online Supplementary S3A*, red *vs.* black labels).

CK2 regulates PTEN expression and activity in B-ALL cells

The serine/threonine kinase CK2 is frequently overexpressed and hyperactivated in cancer, including in hematologic tumors.[19,21-14] Moreover, it is well established that PTEN phosphorylation by CK2 down-regulates its activity while increasing PTEN stability.[19,25] Therefore, we analyzed whether CK2 expression and/or activity was altered in B-ALL patient cells as compared to those of healthy controls. We found that, similar to T-ALL cases,13 primary adult B-ALL samples displayed higher expression levels of both catalytic subunits (α and α') of CK2 (Figure 3A), as well as higher CK2 kinase activity (Figure 3B), than healthy control cells. Next, we evaluated whether CK2 could modulate PTEN expression and activity in B-ALL cells. Treatment of B-ALL primary cells (n=3) and cell lines (n=2) with the highly specific CK2 inhibitor CX-4945 resulted in decreased PTEN phosphorylation at the CK2 target residue S380 and concomitant downregulation of PTEN protein expression (Figure 3C-E) to levels comparable to those presented by healthy controls (Figure 3E), which was associated with increased PTEN lipid phosphatase activity (Figure 3F). Accordingly, treatment of primary B-ALL cells (Figure 3G) and cell lines

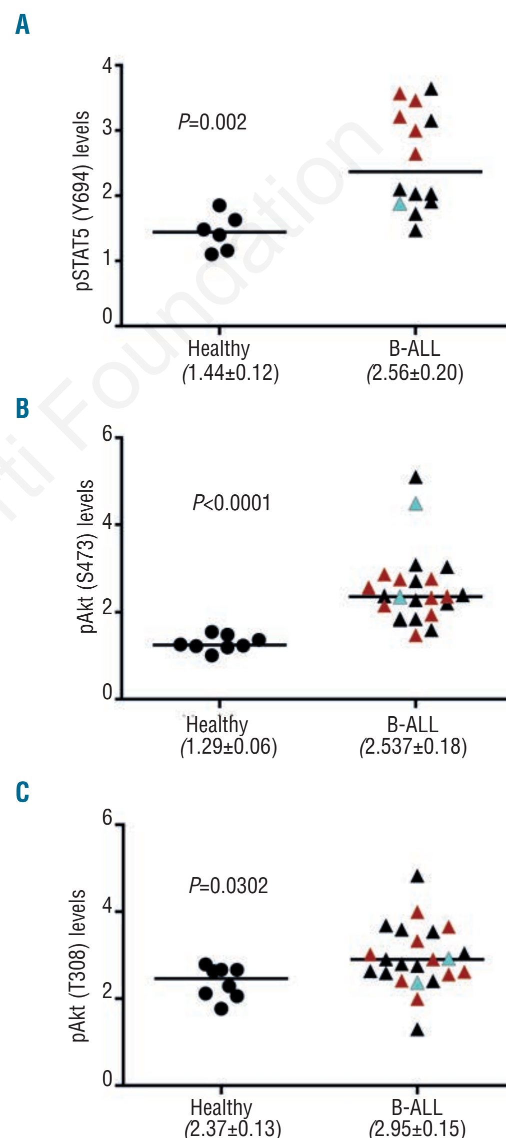

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Figure 1. PI3K/Akt and JAK/STAT pathways are constitutively hyperactivated in primary B-ALL cells. Levels of phosphorylated (A) STAT5 (Y694), (B) Akt (S473), and (C) Akt (T308) in bone marrow cells from healthy individuals and B-ALL samples were quantified by flow cytometry analysis using phospho-specific antibodies. Points represent individual samples and Ph^+ patients are indicated in red. Adolescent cases are highlighted in light blue. Horizontal bars denote median. Mean ± SEM is shown in parentheses. Statistical analysis was performed by two-tailed Mann Whitney test. Corresponding data analysis focused strictly on the adult ALL cases (*Online Supplementary Figure 2*).

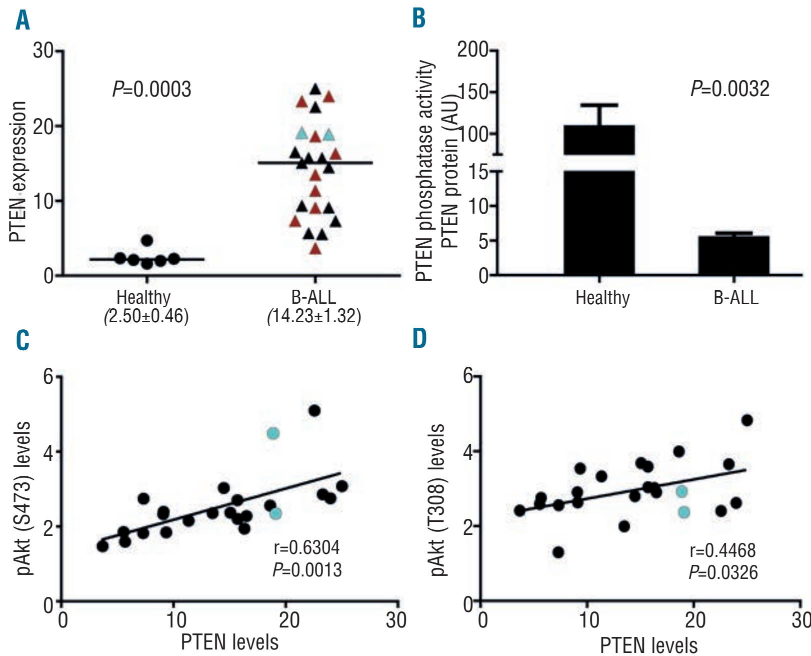

Figure 2. **PTEN lipid phosphatase activity is down-regulated in adult B-ALL cases despite increased protein expression. (A) PTEN levels in normal bone marrow cells (n=6) and B-ALL primary cells (n=23) were quantified by flow cytometry. Points represent individual samples, Ph⁺ patients are indicated in red and adolescent cases in blue, horizontal bars denote median, and mean ± SEM is shown in parentheses. (B) PTEN *in vitro* lipid phosphatase activity was determined after immunoprecipitation of endogenous PTEN from normal bone marrow cells (n=2) and diagnostic adult B-ALL cells (n=4). PTEN activity was normalized to the levels of immunoprecipitated PTEN in each sample. (C,D) Correlation between PTEN expression levels and Akt phosphorylation at S473 (C) and T308 (D). Adolescent cases are highlighted in light blue. Statistical analyses were performed by two-tailed Mann Whitney (A) or Student's t- (B) tests; or by Pearson's correlation analysis (C,D). Corresponding data analysis focused strictly on the adult ALL cases (*Online Supplementary Figure S3*).**

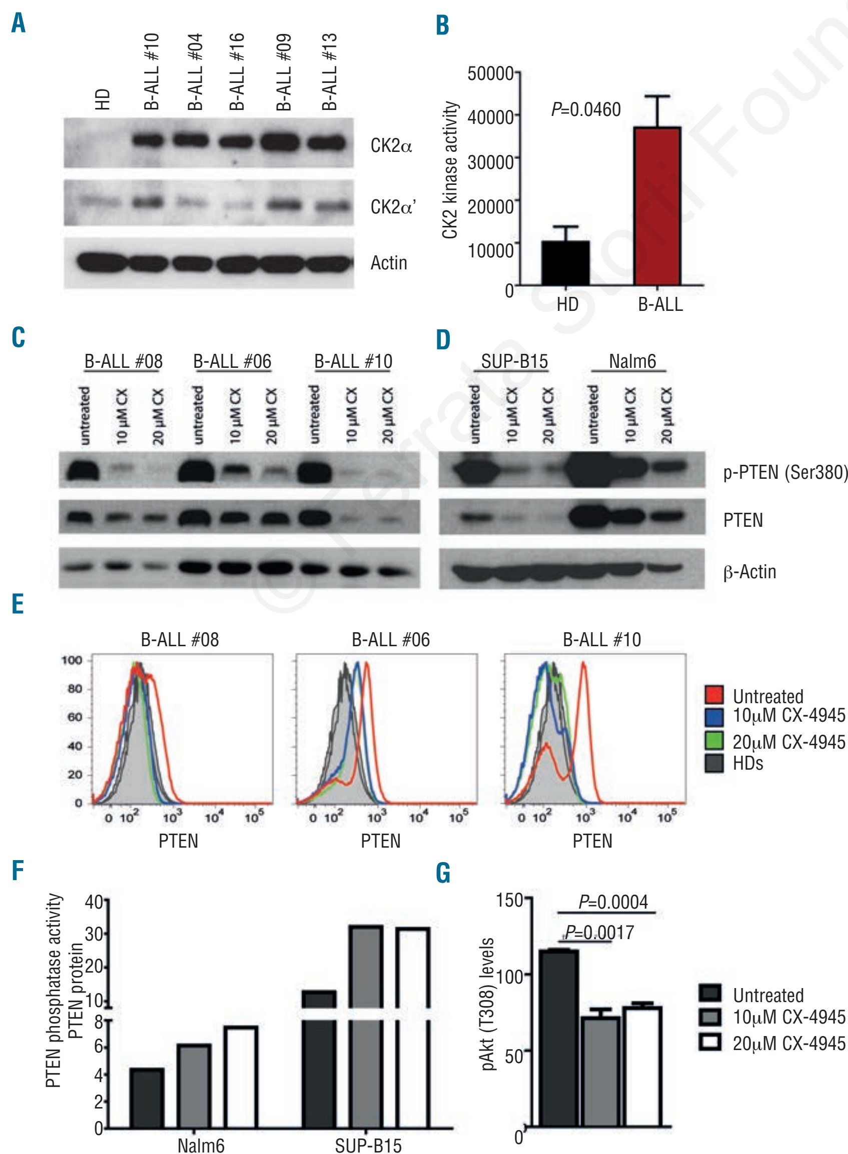

Figure 3. **CK2 regulates PTEN expression and activity in B-ALL cells. (A) Levels of CK2α and CK2α' expression were evaluated in bone marrow mononuclear cells from one healthy donor (HD) and primary B-ALL cells. β-actin was included as loading control. (B) CK2 kinase activity in healthy donors (n=3) and primary B-ALL (n=6) samples lysates was measured *in vitro*. (C) Primary B-ALL cells and (D) B-ALL cell lines (Nalm6 and SUP-B15) were treated for 24 h with vehicle control (untreated), 10 or 20µM CX-4945, and levels of expression and phosphorylation of PTEN were analyzed by immunoblotting. β-actin was included as loading control. (E) PTEN proteins levels in primary B-ALL samples after treatment with CX-4945 for 24 h were also analyzed by flow cytometry. PTEN levels from 2 representative healthy donors (gray shaded histograms) are included as controls for normal PTEN expression. (F) Nalm6 and SUP-B15 cells treated for 24h with vehicle control, 10 or 20µM CX-4945 were lysed, and *in vitro* lipid phosphatase activity of immunoprecipitated PTEN was assessed. PTEN activity was normalized to the level of immunoprecipitated PTEN in each sample. (G) Phosphorylation levels of Akt (T308) was evaluated by flow cytometry in primary B-ALL cells (n=3) after 24h culture with vehicle control (untreated; black column), 10 (gray) or 20 µM (white) CX-4945. Statistical analysis was performed by two-tailed unpaired Student's t-test.**

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(*data not shown*) with CX-4945 resulted in PI3K/Akt pathway inhibition, as determined by the major downregulation of Akt phosphorylation. These results indicate that CK2 activity is responsible for PTEN posttranslational inactivation in B-ALL cells.

Inhibition of PI3K/Akt pathway using CK2 or PI3K antagonists induces B-ALL cell death

To determine the functional relevance of PI3K/Akt activation and CK2 function in B-ALL, we evaluated the effect of CK2 pharmacological inhibition using the highly specific CK2 inhibitor CX-4945, which is currently in clinical trials for different cancers.[26] SUP-B15 and Nalm6 cells were cultured in the presence of 6 and 10 μM CX-4945 and viability was assessed after 48 h by Annexin V/PI staining. Both ALL cell lines were sensitive to CK2 inhibition, although to different extents (Figure 4A). Moreover, CX-4945 induced apoptosis in primary leukemia samples (Figure 4B and D) without significantly affecting healthy control cells (Figure 4C and D). These data indicate that ALL cells, which display high levels of CK2 expression and activation (Figure 3A and B), are clearly more dependent

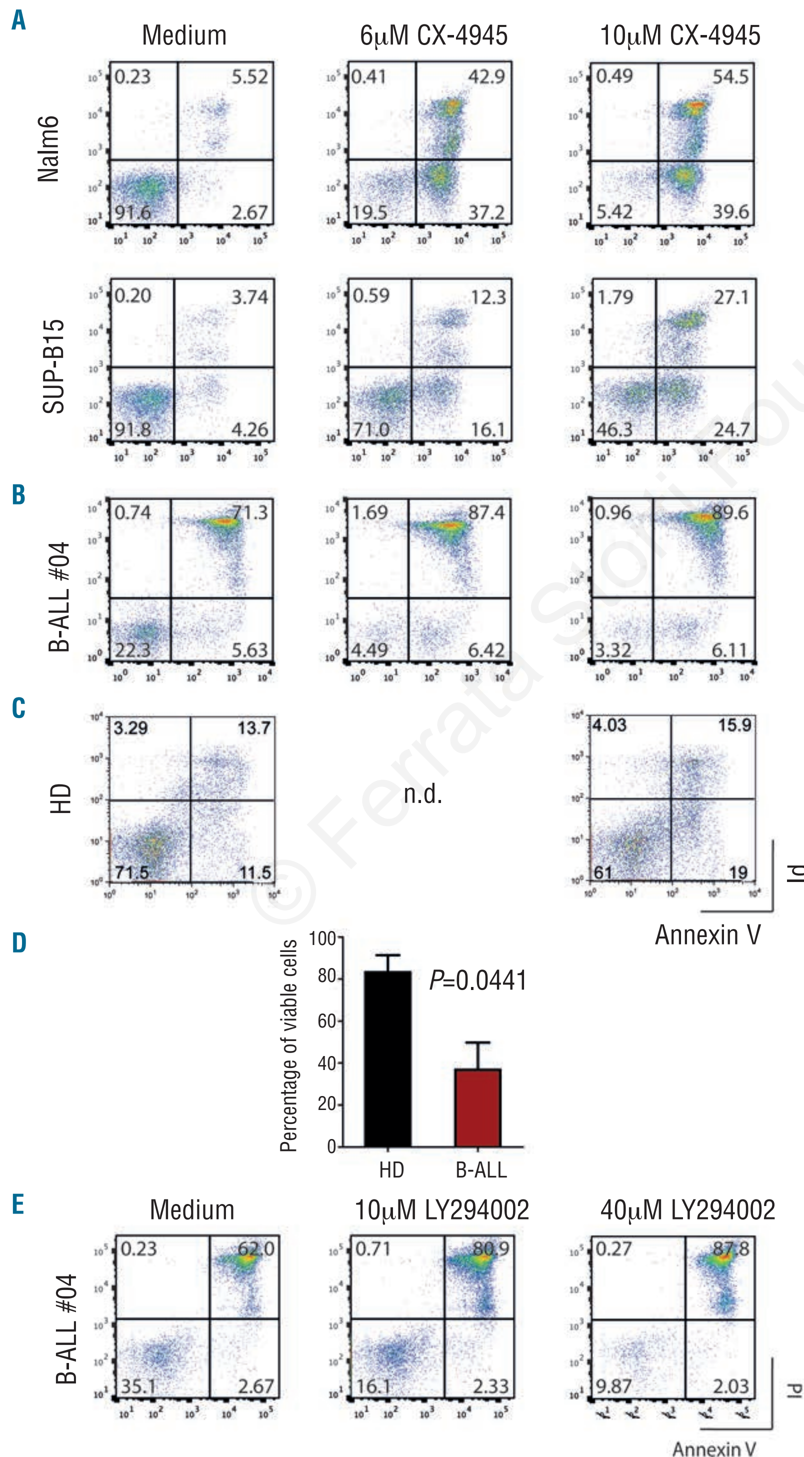

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Figure 4. CK2 and PI3K inhibition induce apoptosis of B-ALL cells. B-ALL cell lines (A), primary leukemia cells collected at diagnosis (B) or healthy bone marrow mononuclear cells (C) were cultured for 48 h with vehicle control, 6μM or 10μM of CX-4945 and cell viability was evaluated by flow cytometry after annexinV/PI staining. (D) Comparison of the effect of CK2 inhibition on viability at 72 h between B-ALL (n=5) and healthy control (n=3) samples. Results are presented as mean±sem of the normalized viability of CX-4945-treated to untreated cells. Statistical analysis was performed by two-tailed unpaired Student's t-test. (E) Primary B-ALL cells were treated with vehicle control, 10 or 40μM LY294002 for 48 h. The percentage of live (bottom left), early apoptotic (bottom right), and late apoptotic/necrotic (top right) is indicated in the respective quadrants. Results from primary cells are from one patient sample representative of 3 analyzed. Data are representative of 3 healthy controls and 2-5 B-ALL patients.

on CK2 activity than normal hematopoietic precursors (Figure 4D). In addition, we specifically analyzed the effect of the PI3K inhibitor LY294002 and confirmed that, as expected, abrogation of PI3K/Akt pathway promotes apoptosis of B-ALL cells (Figure 4E).

Discussion

Analysis of the activation status of oncogenic signaling pathways at the single cell level using phospho-flow cytometry has revealed increased depth in the ability to identify patients at risk of relapse.[14] Moreover, because flow cytometry is currently routinely used in the immunophenotypical subclassification of leukemia patients, phospho-flow cytometry analysis of signaling pathway activation has the potential to be easily translated into clinical applications.

In the present work, we have made use of this technology to evaluate the activation status of JAK/STAT5 and PI3K/Akt pathways in adult B-ALL patients. We showed that all Ph-positive cases that we analyzed displayed high levels of phosphorylation of STAT5, consistent with previous reports showing that BCR-ABL drives STAT5 activation.[17,18] In addition, we also found 2 Ph-negative cases presenting high phospho-STAT5, raising the possibility that they may belong to the recently characterized Ph-like ALL subset.[27] Whether high phospho-STAT5 may serve as a surrogate marker for Ph-positive and Ph-like cases, all of which have poor prognosis, remains an open relevant question.

The few studies that have previously explored the involvement of PI3K/Akt constitutive activation in adult B-ALL did so in a very limited number of patients[6] or focused on mTOR[8-10] rather than Akt. However, mTOR activation is only very indirectly regulated by PI3K and it can be affected by several upstream cues that are PI3K-independent.[10] Therefore, we opted to study Akt phosphorylation, a more direct PI3K effector and central player in the pathway. Our data, using a reasonable number of cases (n=21), clearly revealed that adult B-ALL cells displayed constitutive hyperactivation of PI3K/Akt signaling pathway.

In contrast to a previous report,[6] we did not find evidence that PI3K/Akt hyperactivation is more associated with Ph-positive cases. In agreement, PTEN expression levels, although heterogeneous, were not affected by the BCR-ABL status. This indicates that high PI3K/Akt activity is a general feature of adult B-ALL cells, possibly driven by molecular events that are not exclusive to BCR-ABL oncogenic activation. In our current studies, we present evidence that CK2-mediated PTEN non-deletional post-translational inactivation is one of such mechanisms. Most leukemia samples we analyzed displayed high basal PTEN expression. However, PTEN protein levels did not positively associate with enzymatic activity. On the contrary, irrespectively of their BCR-ABL status, ALL samples showed significantly lower PTEN *in vitro* lipid phosphatase activity than healthy control cells. Because CK2 has been identified as the kinase responsible for PTEN C-terminal phosphorylation and consequent PTEN protein stabilization and functional inactivation,[25,28] we tested the effect of the clinical-grade CK2 inhibitor CX-4945 and showed that it promotes apoptosis of ALL cells, in a manner similar to the PI3K inhibitor LY294002.

Our study included 2 adolescent cases (Patients #02 and #12; Table 1) that were enrolled as 'adults' at Hospital dos Capuchos. Importantly, exclusion of these 2 cases and re-analysis of the remaining 21 cases did not alter any of the results arising from the study of the whole group (Figures 1 and 2 *vs. Online Supplementary Figures S2 and S3*). None of the 2 cases constituted 'outliers' with regards to the expression of PTEN, phospho-Akt (T308) or phospho-STAT5 (Figures 1 and 2), although patient #12 presented phospho-Akt (S473) levels that were in the upper quartile. Our unpublished studies suggest that pediatric ALL cases display significantly higher levels of PI3K/Akt pathway activation than adults (Gomes *et al.*, *manuscript in preparation*). Whether activation of this pathway in adolescent cases resembles more that displayed by childhood or by adult ALL patients, and the biological implications of such putative differences for targeted therapy options, requires further investigation.

Our current observations, which are in line with previous findings in acute myeloid leukemia,[29] chronic lymphocytic leukemia[15,30] and T-ALL,[13,31] support the notion that CK2-mediated PTEN posttranslational inactivation is a frequent event in hematologic tumors and suggest that CK2 inhibition may be a valid therapeutic avenue for the treatment of adult ALL by inactivating PI3K/Akt pathway. Studies are warranted to define whether phospho-flow may help identifying, in an easy and clinically meaningful manner, those patients who will benefit the most from targeted therapies against PI3K/Akt signaling pathway.

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Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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