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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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JFL and JTB coordinated the study.

Running head: PI3K/Akt pathway activation in adult ALL

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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.

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 that 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 cases 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 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, 2mM L-glutamine and penicillin/streptomycin and cultured at 37ºC in 5% CO_2.

Intracellular phospho-specific flow cytometry. Cells were fixed with Cytofix buffer, pelleted by centrifugation and 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 supplementary data (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's correlation coefficient. *P* values lower than 0.05 were 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 confirmed that adult leukemia cases displayed constitutive hyperactivation of the JAK/STAT pathway (**Figure 1A;** Supplementary **Figures S1-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 upregulated in B-ALL cells as compared to controls (**Figures 1B-C;** Supplementary **Figures S1-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,C**, red labels).

PTEN lipid phosphatase activity is decreased in adult B-ALL cases despite upregulated 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;** Supplementary **Figures S1, 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,D**). Of note, we did not find evidence for significant associations between PTEN expression and parameters such as B-ALL maturation stage, sex, age (Supplementary **Figure S4)** or prognostic factors such as BCR-ABL expression (**Figures 2A** and Supplementary **S3A**, red versus 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 hematological tumors (19, 21-24). Moreover, it is well established that PTEN phosphorylation by CK2 downregulates 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 (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 10μM

CX-4945 and viability was assessed after 48h by Annexin V/PI staining. Both ALL cell lines were sensitive to CK2 inhibition, although to different extent (**Figure 4A**). Moroever, CX-4945 induced apoptosis in primary leukemia samples (**Figure 4B,D**) without significantly affecting healthy control cells (**Figure 4C-D**). These data indicate that ALL cells, which display high levels of CK2 expression and activation (**Figure 3B**), are clearly more dependent 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 two 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 which are not exclusive to BCR-ABL oncogenic activation. In our current studies we present evidence that CK2-mediated PTEN non-deletional posttranslational 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 two adolescent cases (Patients #02 and #12; Table 1) that were enrolled as ‘adults’ at Hospital dos Capuchos. Importantly, exclusion of these two 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 versus Figures S2 and S3). None of the two cases constituted ‘outliers’ in what regards 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 hematological 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 that will benefit the most from targeted therapies against PI3K/Akt signaling pathway.

Authorship and Disclosures

AMG performed most of the experiments, analyzed data, and drafted the manuscript; MVS, VP, LRM, AM and AS-C performed experiments and analyzed data; PR, ABS and JFL recruited patients; JC performed patient immunophenotypical characterization and classification; JFL contributed to the coordination of the study; JTB coordinated the study, designed the research and wrote the manuscript. The authors have no conflicts of interest to declare.

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Table1. Immunophenotype and cytogenetics features of B-ALL patient samples.

Patient no.	Gender	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		-	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-.

Figure Legends

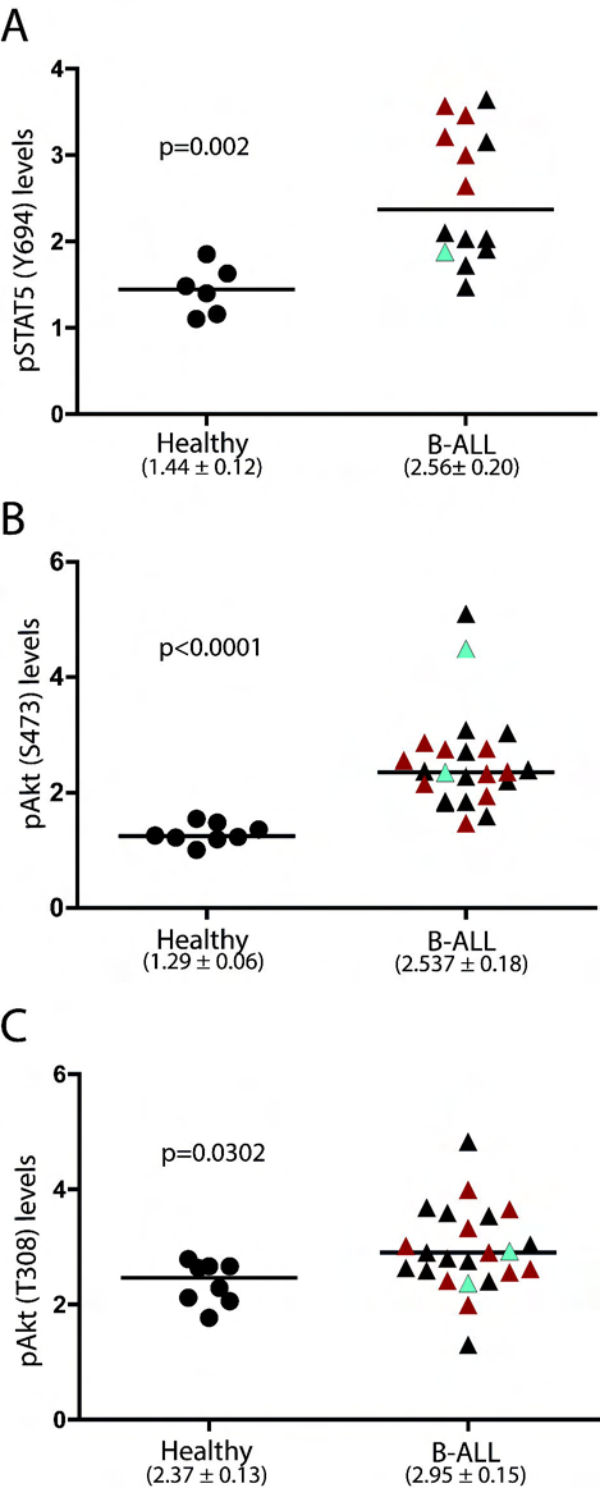
Figure 1. PI3K/Akt and JAK/STAT pathways are constitutively hyperactivated in primary B-ALL cells. Levels of phosphorylated **(A)** STAT5 (Y694), **(B)**Akt (S473), and **(C)** Akt (T308) in bone marrow cells from healthy individuals and B-ALL samples were quantified by flow cytometry analysis using 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 2-tailed Mann Whitney test. Corresponding data analysis focused strictly on the adult ALL cases is presented in Supplementary Figure 2.

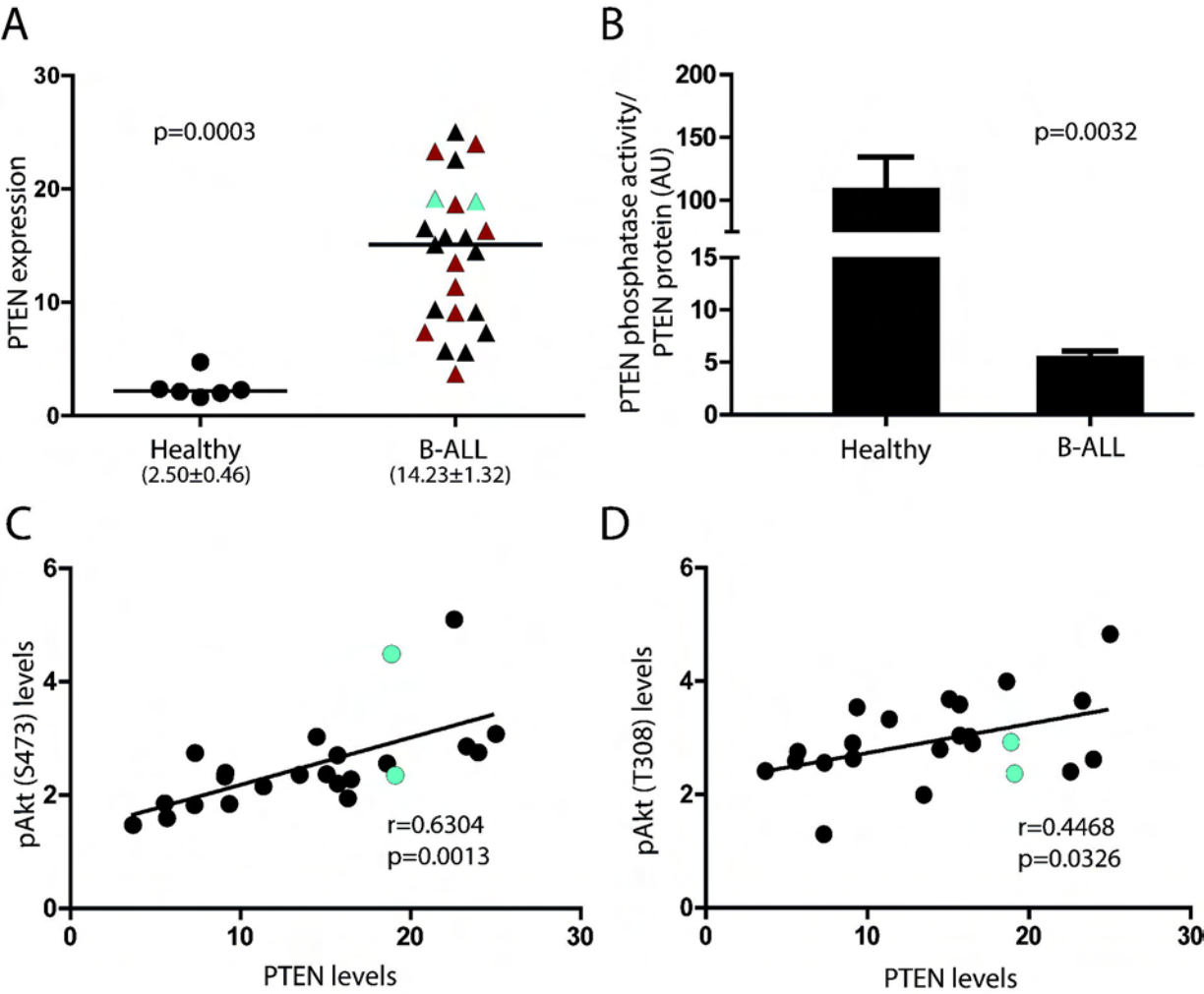
Figure 2. PTEN lipid phosphatase activity is downregulated in ~~adult~~ B-ALL cases despite increased protein expression. (A) PTEN levels in normal bone marrow cells (n= 6) and B-ALL primary cells (n= 23) were quantified by flow cytometry. Points represent individual samples, Ph+ patients are indicated 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 2-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 is presented in Supplementary Figure 3.

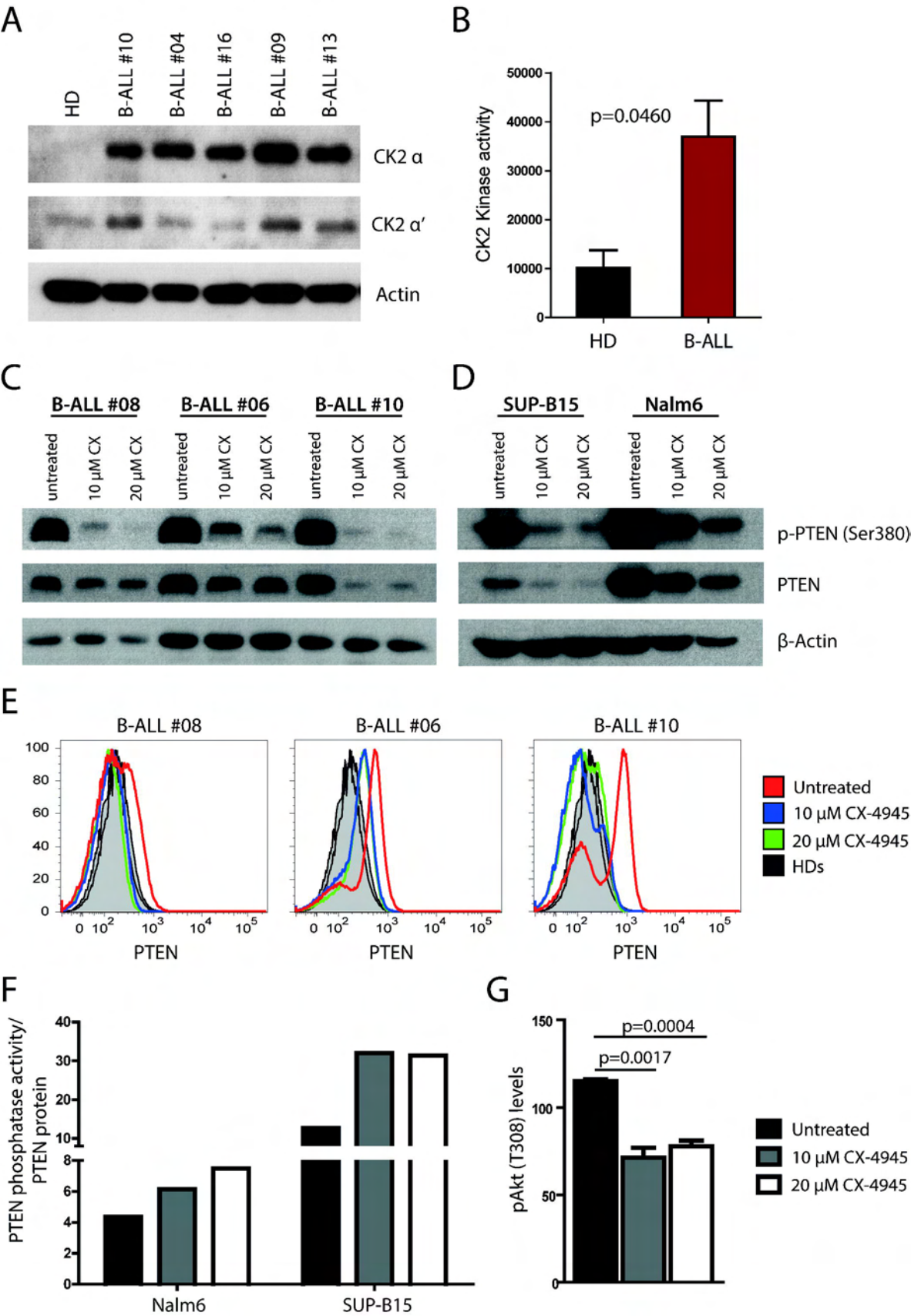
Figure 3. CK2 regulates PTEN expression and activity in B-ALL cells. (A) Levels of CK2α and CK2α' expression were evaluated in bone marrow mononuclear cells from one healthy donor (HD) and primary B-ALL cells. β-actin was included as loading control. **(B)** CK2 kinase activity in healthy donors (n=3) 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 24h 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 24h were also analyzed by flow cytometry. PTEN levels from 2 representative healthy donors (grey 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 (grey) or 20μM (white) CX-4945. Statistical analysis was performed by two-tailed unpaired Student's t-test.

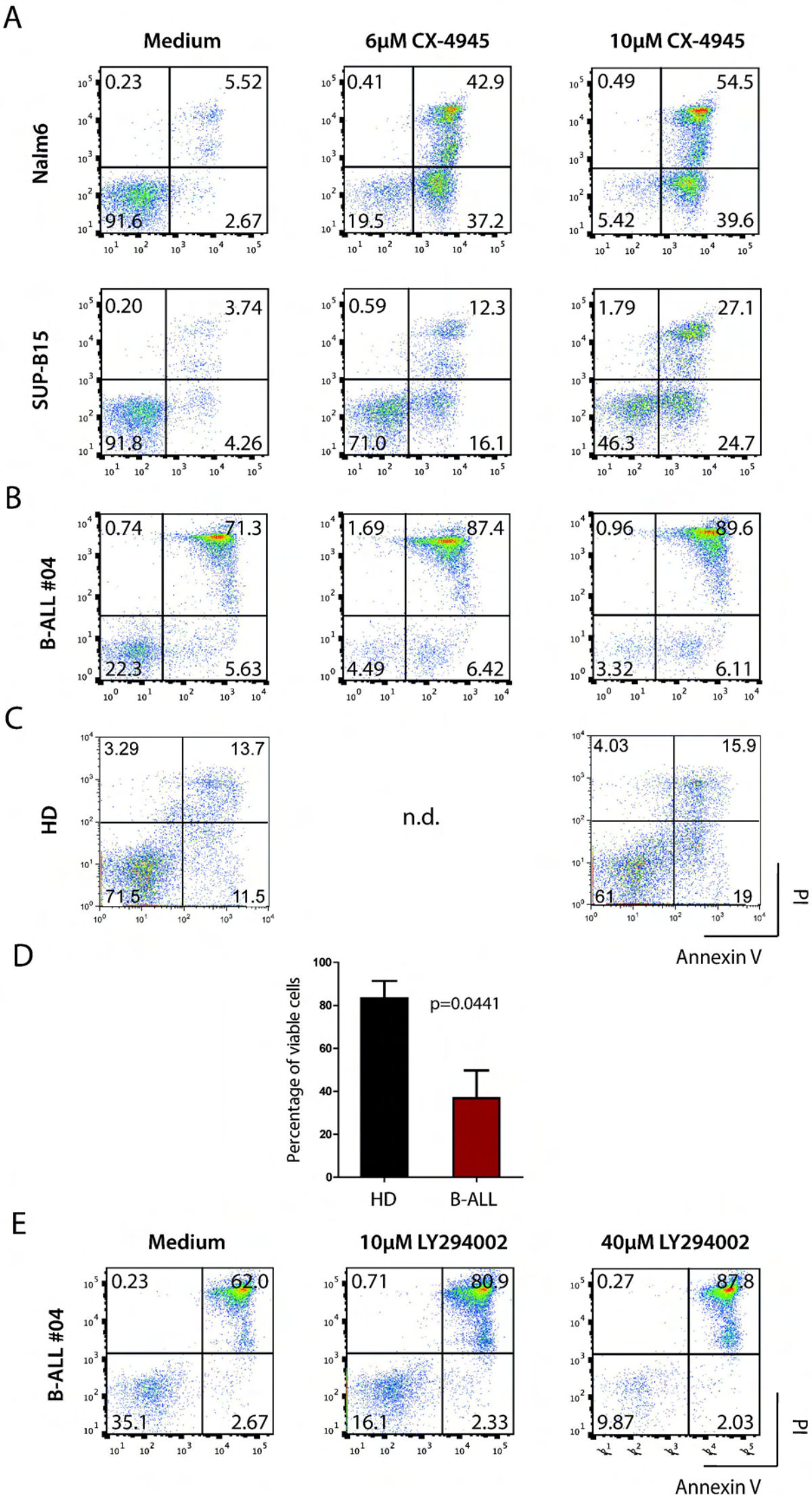
Figure 4. CK2 and PI3K inhibition induce apoptosis of B-ALL cells. B-ALL cell lines **(A)**, primary leukemia cells collected at diagnosis **(B)** or healthy bone marrow mononuclear cells **(C)** were cultured for 48h with vehicle control, 6μM or 10μM of CX-4945 and cell viability was evaluated by flow cytometry after annexinV/PI staining. **(D)** Comparison of the effect of CK2 inhibition on viability at 72h 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 48h. 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 three analyzed. Data are representative of 3 healthy controls and 2-5 B-ALL patients.


A
pSTAT5 (Y694) levels
p=0.002
Healthy
(1.44 ± 0.12)
B-ALL
(2.56± 0.20)
B
pAkt (S473) levels
p<0.0001
Healthy
(1.29 ± 0.06)
B-ALL
(2.537 ± 0.18)
C
pAkt (T308) levels
p=0.0302
Healthy
(2.37 ± 0.13)
B-ALL
(2.95 ± 0.15)


A
PTEN expression
30
20
10
0
p=0.0003
Healthy
(2.50±0.46)
B-ALL
(14.23±1.32)
B
PTEN phosphatase activity/
PTEN protein (AU)
200
150
100
15
10
5
0
p=0.0032
Healthy
B-ALL
C
pAkt (S473) levels
6
4
2
0
r=0.6304
p=0.0013
0
10
20
30
PTEN levels
D
pAkt (T308) levels
6
4
2
0
r=0.4468
p=0.0326
0
10
20
30
PTEN levels


A
HD
B-ALL #10
B-ALL #04
B-ALL #16
B-ALL #09
B-ALL #13
CK2 α
CK2 α'
Actin
B
CK2 Kinase activity
50000
40000
30000
20000
10000
0
p=0.0460
HD
B-ALL
C
B-ALL #08
B-ALL #06
B-ALL #10
untreated
10 μM CX
20 μM CX
D
SUP-B15
Nalm6
p-PTEN (Ser380)
PTEN
β-Actin
E
B-ALL #08
B-ALL #06
B-ALL #10
PTEN
Untreated
10 μM CX-4945
20 μM CX-4945
HDs
F
PTEN phosphatase activity/
PTEN protein
Nalm6
SUP-B15
G
pAkt (T308) levels
p=0.0004
p=0.0017
Untreated
10 μM CX-4945
20 μM CX-4945


A
Medium
6µM CX-4945
10µM CX-4945
Nalm6
0.23
5.52
91.6
2.67
0.41
42.9
19.5
37.2
0.49
54.5
5.42
39.6
SUP-B15
0.20
3.74
91.8
4.26
0.59
12.3
71.0
16.1
1.79
27.1
46.3
24.7
B
B-ALL #04
0.74
71.3
22.3
5.63
1.69
87.4
4.49
6.42
0.96
89.6
3.32
6.11
C
HD
3.29
13.7
71.5
11.5
n.d.
4.03
15.9
61
19
PI
Annexin V
D
Percentage of viable cells
p=0.0441
HD
B-ALL
E
Medium
10µM LY294002
40µM LY294002
B-ALL #04
0.23
62.0
35.1
2.67
0.71
80.9
16.1
2.33
0.27
87.8
9.87
2.03
PI
Annexin V

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

A. Margarida Gomes, Maria V. Soares, Patrícia Ribeiro, Joana Caldas, Vanda Póvoa, Leila R. Martins, Alice Melão, Ana Serra-Caetano, Aida B. de Sousa, João F. Lacerda and João T. Barata

SUPPLEMENTARY DATA

(Methods, Supplementary Figures 1-4)

Methods

Primary samples and B-ALL cell lines. Bone marrow samples from adult (n=21) or adolescent (n=2) B-ALL patients were collected in accordance with the Declaration of Helsinki, with informed consent after Ethical Committee approval from Hospital Santo António dos Capuchos (Lisbon, Portugal) and Hospital Santa Maria (Lisbon, Portugal). Patient characteristics are summarized in Table 1. Normal bone marrow samples were obtained from healthy individuals donating bone marrow for patients at Hospital Santa Maria. Samples were enriched by density centrifugation over Ficoll-Paque (GE Healthcare) and washed twice in culture medium (RPMI-1640 supplemented with 10% FBS, 2mM L-glutamine and penicillin/streptomycin). Human B-ALL cell lines, SUP-B15 (Ph+) and Nalm6 (Ph-), were maintained in RPMI 1640 supplemented with 10% FBS, 2mM L-glutamine and penicillin/streptomycin and cultured at 37ºC in 5% CO_2.

Intracellular phospho-specific flow cytometry. To determine the phosphorylation status of PI3K/Akt and JAK/STAT pathways, cells were washed twice with PBS, pelleted by centrifugation, and fixed with Cytofix buffer (BD Biosciences) for 10min at 37ºC. Cells were then pelleted and permeabilized in ice-cold PERM buffer III (BD Biosciences) for 30min on ice. The cells were washed twice in staining buffer (BD Bioscience), and stained with the following antibodies (all from BD Biosciences): CD79a-APC; Akt-Alexa Fluor 488, PTEN-PE, pAkt S473-Alexa Fluor 488, pAkt T308-PE, and pSTAT5 Y694-Alexa Fluor 488. Following incubation for 30 min at room temperature, cells were washed in staining buffer and analyzed on a FACSAria or LSRFortessa (BD Biosciences). At least 100,000 events were collected for all samples.

Data were collected using DIVA software (BD Bioscience) and analyzed with FlowJo software (Tree Star). For each sample, live lymphocytes were gated based on their forward scatter (FSC) versus side scatter (SSC) profile. Single cells were then selected on a plot of forward scatter area (FSC-A) vs forward scatter width (FSC-W) to exclude signaling data from doublets. Lymphoblastic population was identified based on cCD79a staining and analysis of individual phospho-proteins was then performed in this cell population. Normalized basal phospho-protein levels were calculated as the ratio between the Mean Fluorescence Intensity (MFI) of cells stained with a phospho-specific antibody with MFI of control cells stained only with CD79a.

Endogenous PTEN *in vitro* lipid phosphatase assay. PTEN phosphatase activity was measured *in vitro*. Briefly, immunoprecipitations were carried out with an anti-PTEN antibody (Santa Cruz Biotechnology) overnight and a secondary agarose-conjugate antibody for 3 hours at 4ºC. Immunoprecipitated protein was washed, resuspended in enzyme reaction buffer (50mM Tris, pH 8; 50mM NaCl; 10mM DTT and 10mM $MgCl_2$), and incubated with 10µM PIP3 (Echelon) for 30 min at 37ºC, after which phosphatase reaction was stopped with 100µL malachite green reagent (Echelon). Free phosphatase levels were measured in an ELISA plate reader at 630nm. Absorbance was converted into pmol phosphate using a phosphate standard curve.

Endogenous CK2 *in vitro* kinase assay. CK2 activity was measured using the Casein Kinase 2 Assay Kit (Millipore), according to the manufacturer's instructions. Briefly, protein lysates were incubated for 10 minutes at 30°C in a reaction mixture containing a CK2-specific peptide, [γ-^{32}P]ATP (PerkinElmer) and a PKA inhibitor cocktail. The radioactivity incorporated into the substrate was determined by scintillation counting. The kinase activity was calculated by subtracting the background for each sample (without substrate).

Immunoblotting. Cells were lysed in 50mM Tris-HCl pH 8.0, 150mM NaCl, 5mM EDTA, 1% (v/v) NP-40, 1mM Na3VO4, 10mM NaF, 10mM NaPyroph, 1mM 4-(2-aminoethyl) benzenesulfonyl (AEBSF), 10μg/ml leupeptin, 10μg/ml aprotinin, 1μg/ml Pepstatin, resolved by 10% SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblotted with the following antibodies: p-PTEN (S380), PTEN (Cell Signaling Techonology), actin, CK2α, and CK2α´ (Santa Cruz Biotechonology).

Treatment with CX-4945. In order to assess the effect of CK2 inhibition in PTEN expression, leukemia cells were cultured in 24-well plates as 2 x 10^6 cells/ml at 37ºC with 5% CO2 in control medium, or in the presence of 10 or 20μM of CX-4945 (Adooq.com Bioscience). After 24h cells were harvested and fixed immediately with Cytofix buffer (BD Biosciences) for 10 min at 37ºC. The cells were then pelleted, resuspended in ice-cold PERM buffer III (BD Biosciences), and incubated for 30 min on ice. Cells were washed twice with staining buffer (BD Bioscience), resuspended at 1x10^7cells/mL, and stained with CD79a-APC, PTEN-PE, or pAkt T308-PE for 30min at room temperature. The cells were washed with staining buffer and analyzed in LSRFortessa (BD Bioscience).

Analysis of cell viability and apoptosis. Cells were cultured in 24-well plates as 2 x 10^6 cells/ml at 37ºC with 5% CO2 in control medium or with the indicated concentrations of LY294002 (Calbiochem) and CX-4945. After 48 hours, cells were harvested and viability was determined by double-staining with APC or FITC-conjugated Annexin V (R&D systems) and propidium iodide (PI) (Sigma). Briefly, cells were washed with PBS and resuspended in 100μL of binding buffer with annexin V and PI. After 15 min of incubation at room temperature in the dark, 100μL of binding buffer were added and the samples were analyzed by flow cytometry. Viability index was calculated as the ratio of experimental to control conditions.

Statistical analysis. GraphPad Prism version 5.00 for windows (GraphPad Software) was used for statistical analyses. Differences between populations were calculated using unpaired two-tailed Students´s t test, Mann-Whitney test, or One-way ANOVA, as appropriate. Correlations were analyzed using the Pearson's correlation coefficient. *P* values lower than 0.05 were considered significant.

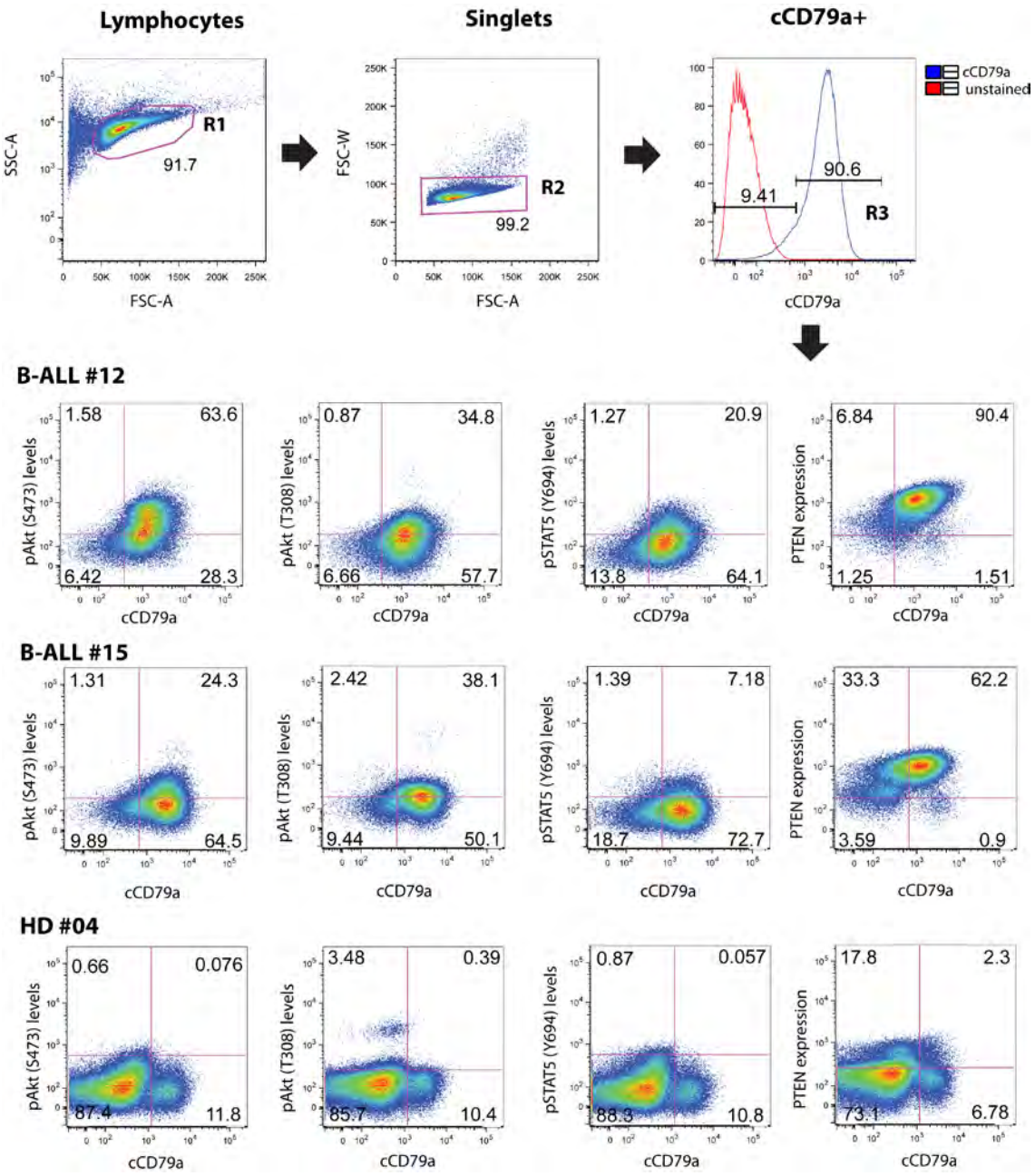


Figure S1. Gating strategy for phospho-flow cytometry analysis. Debris were excluded, and lymphocytes included, using a forward scatter area (FSC-A) *versus* side scatter area (SSC-A) gate (R1). Single cells (singlets) were then selected on a FSC-A *versus* FSC-W plot (R2) to exclude signaling data from doublets. Cytoplasmic CD79a (cCD79a)+ cells (R3) were selected on a histogram of cCD79a staining and analysis of individual phosphoproteins was then performed in this cell population. Examples of phospho-Akt (S473), phospho-Akt (T308), phospho-STAT5 (Y694) and total PTEN *versus* cCD79a stainings for the two individual B-ALL samples and one healthy donor are presented. Background fluorescences from unstained cells (equivalent to irrelevant isotypic controls) were used as negative controls to define positivity in each channel.

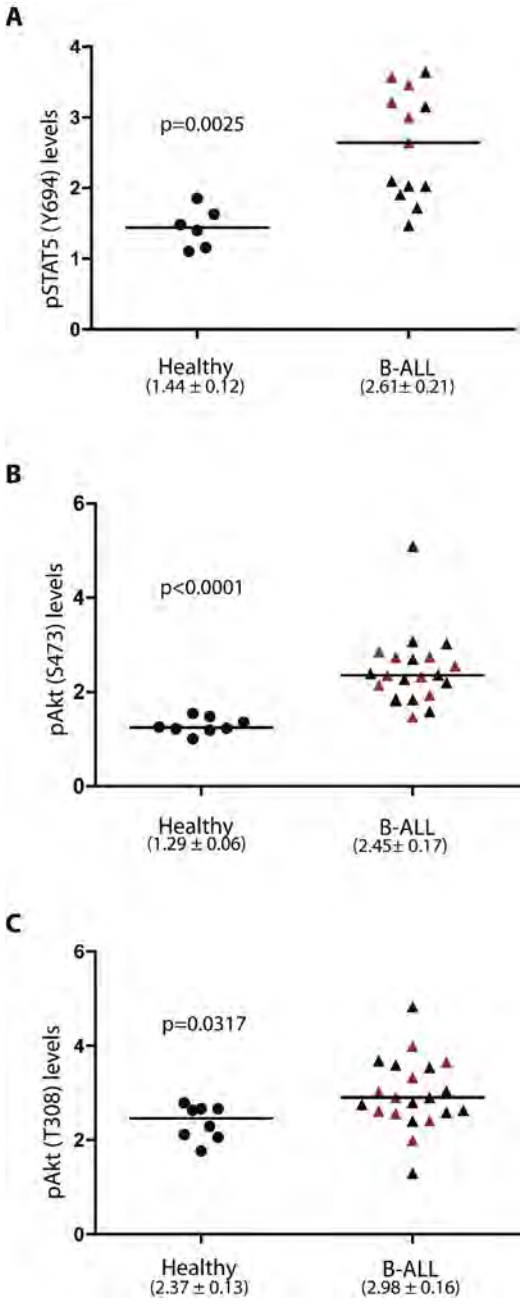


Figure S2. PI3K/Akt and JAK/STAT pathways are constitutively hyperactivated in primary adult B-ALL cells. Levels of phosphorylated **(A)** STAT5 (Y694), **(B)** Akt (S473), and **(C)** Akt (T308) in bone marrow cells from healthy individuals and adult B-ALL samples were quantified by flow cytometry analysis using phospho-specific antibodies. Points represent individual samples and Ph+ patients are indicated in red. Horizontal bars denote median. Mean ± SEM is shown in parentheses. Statistical analysis was performed by 2-tailed Mann Whitney test.

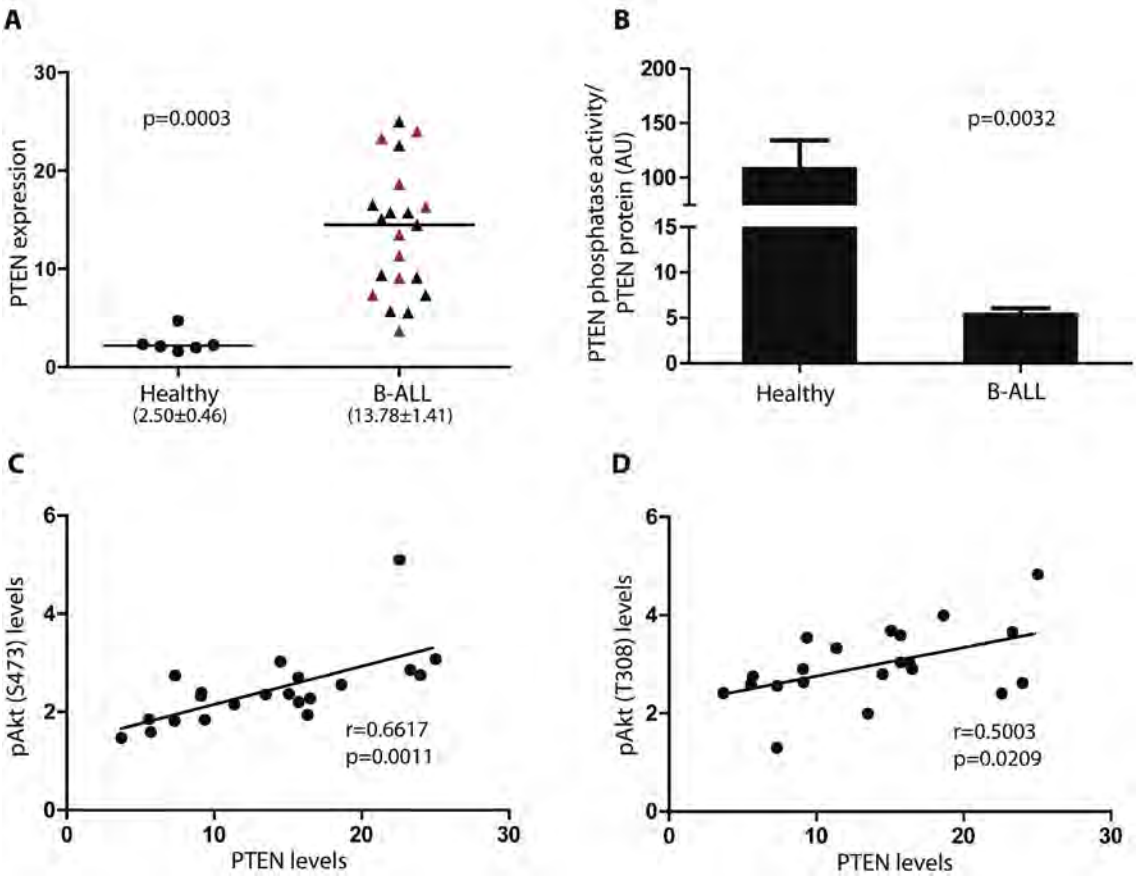


Figure S3. PTEN lipid phosphatase activity is downregulated in adult B-ALL cases despite increased protein expression. (A) PTEN levels in normal bone marrow cells (n= 6) and adult B-ALL primary cells (n= 21) were quantified by flow cytometry. Points represent individual samples, Ph+ patients are indicated in red, 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). Statistical analyses were performed by 2-tailed Mann Whitney (**A**) or Student's t (**B**) tests; or by Pearson's correlation analysis (**C,D**).

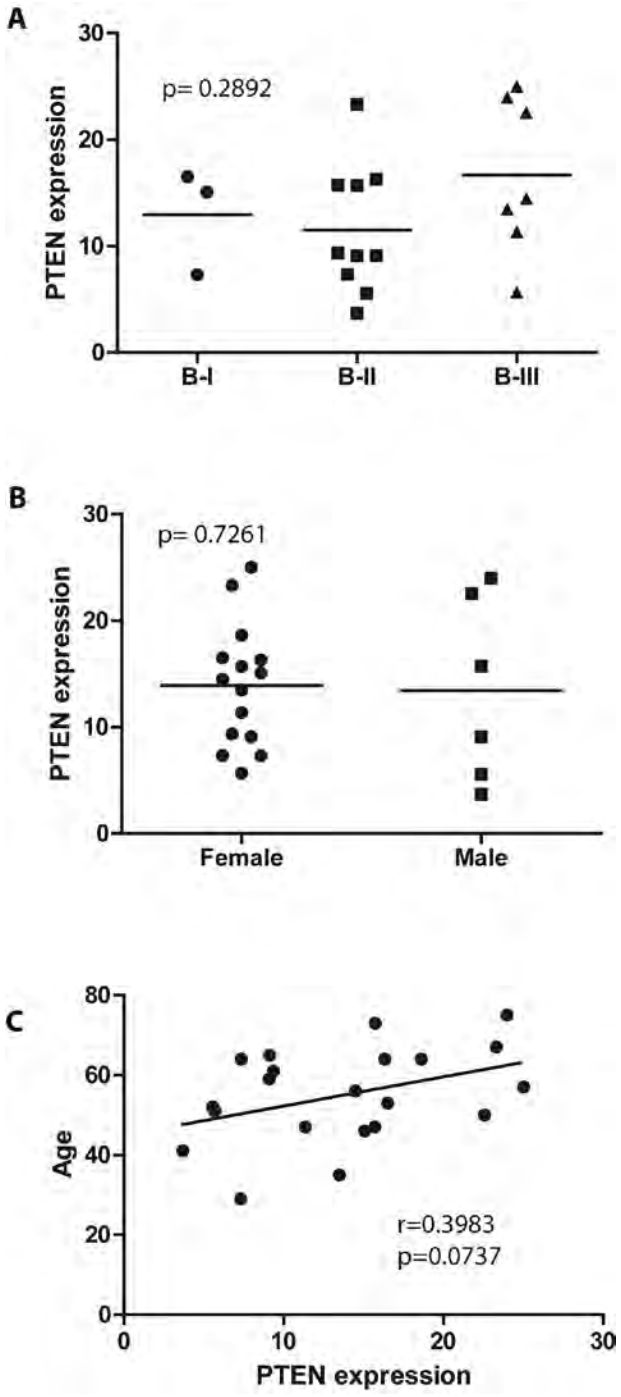


Figure S4. PTEN expression does not appear to correlate with clinical parameters in adult B-ALL patients. (A) Association between PTEN expression levels and B-ALL maturation stage, as defined by EGIL (B-I, B-II and B-III). Statistical analysis was performed by One-Way ANOVA. **(B)** Association between PTEN levels and gender. Statistical analysis was performed by 2-tailed Mann Whitney test. **(C)** Correlation between PTEN expression levels and age. Statistical analysis was performed by Pearson´s correlation analysis.