Leukemia and Lymphoma

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Marie Fridberg a, Anna Servin a, Lola Anagnostaki b, Johan Linderoth c, Mattias Berglund d, Ola Söderberg d, Gunilla Enblad d, Anders Rosén d, Tomas Mustelin g, Mats Jerkeman c, Jenny L. Persson b, Anette Gjörloff Wingren a

a Department of Tumor Biology, Lund University, Malmö University Hospital, Malmö, Sweden
b Department of Pathology, Lund University, Malmö University Hospital, Malmö, Sweden
c Department of Oncology, Lund University, Lund, Sweden
d Department of Oncology, Radiology and Clinical Immunology, Section of Oncology, Uppsala University, Uppsala, Sweden
e Department of Genetics and Pathology, Uppsala University, Uppsala, Sweden
f Department of Biomedicine and Surgery, Division of Cell Biology, Linköping University, Linköping, Sweden
g Laboratory of Signal Transduction, The Burnham Institute, La Jolla, CA, USA

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Protein expression and cellular localization in two prognostic subgroups of diffuse large B-cell lymphoma: Higher expression of ZAP70 and PKC-β II in the non-germinal center group and poor survival in patients deficient in nuclear PTEN

MARIE FRIDBERG¹, ANNA SERVIN¹, LOLA ANAGNOSTAKI², JOHAN LINDEROTH³, MATTIAS BERGLUND⁴, OLA SÖDERBERG⁵, GUNILLA ENBLAD⁴, ANDERS ROSEN⁶, TOMAS MUSTELIN⁷, MATS JERKEMAN³, JENNY L. PERSSON², & ANETTE GJÖRLOFF WINGREN¹

¹Department of Tumor Biology, Lund University, Malmö University Hospital, Malmö, Sweden, ²Department of Pathology, Lund University, Malmö University Hospital, Malmö, Sweden, ³Department of Oncology, Lund University, Lund, Sweden, ⁴Department of Oncology, Radiology and Clinical Immunology, Section of Oncology, Uppsala University, Uppsala, Sweden, ⁵Department of Genetics and Pathology, Uppsala University, Uppsala, Sweden, ⁶Department of Biomedicine and Surgery, Division of Cell Biology, Linköping University, Linköping, Sweden, and ⁷Laboratory of Signal Transduction, The Burnham Institute, La Jolla, CA, USA

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Abstract
Patients diagnosed with diffuse large B-cell lymphoma (DLBCL) show varying responses to conventional therapy, and this might be contributed to the differentiation stage of the tumor B-cells. The aim of the current study was to evaluate a panel of kinases (ZAP70, PKC-β I and II and phosphorylated PKB/Akt) and phosphatases (PTEN, SHP1 and SHP2) known to be frequently deregulated in lymphoid malignancies. De novo DLBCL cases were divided into two subgroups, the germinal center (GC) group (14/28) and the non-germinal center (non-GC) or activated B-cell (ABC) group (14/28). ZAP70 and PKC-β II were expressed in a significantly higher percentage of tumor cells in the clinically more aggressive non-GC group compared with the prognostically favourable GC group. Also, the subcellular localization of PKC-β I and II differed in DLBCL cells, with the PKC-β I isoform being expressed in both the cytoplasm and nucleus, while PKC-β II was found exclusively in the cytoplasm. Loss of nuclear PTEN correlated with poor survival in cases from both subgroups. In addition, five cell lines of DLBCL origin were analyzed for protein expression and for mRNA levels of PTEN and SHP1. For the first time, we show that ZAP70 is expressed in a higher percentage of tumor cells in the aggressive non-GC subgroup of DLBCL and that PKC-β I and II are differently distributed in the two prognostic subgroups of de novo DLBCL.

Keywords: B cell, lymphoma, germinal center, DLBCL, ZAP70, PKC-β

Introduction
Diffuse large B-cell lymphomas (DLBCL) represent the most common type of non-Hodgkin lymphomas in Western countries and are characterized by heterogeneous clinical, immunophenotypic and genetic features [1–6]. Although DLBCL is one of the most chemotherapy-responsive human malignancies, many newly diagnosed patients will not be cured with conventional anthracycline-based chemotherapy [1]. The variability in response to therapy suggests underlying heterogeneity in a disease that is largely treated with a homogeneous approach.

Using cDNA microarrays, two subgroups of DLBCL have recently been identified, which differ in their ability to be cured by the multi-agent
chemotherapy that is used at present [7–9]. The
germinal center (GC) subgroup expresses genes
characteristic of normal GC B-cells and has been
associated with a better outcome, whereas the
activated B-cell like (ABC) group expresses genes
characteristic of activated blood B-cells and has been
associated with a poor outcome. Moreover, a type 3
(a group of unclassified cases) group was also
identified, with similar outcome as the ABC like-
group [8]. The ABC group and the type 3 group
together, are commonly termed the non-germinal
center (non-GC) group. Bcl6, CD10 and IRF4
(MUM1) have been shown to be differentially ex-
pressed in the GC and non-GC subgroups using
gene expression arrays as well as immunohistochem-
istry [7–10]. As shown by others, these three
marks might successfully subdivide GC and non-
GC DLBCL [11]. Both CD10 and Bcl6 are
considered as GC markers while IRF4 has been
found to be expressed in plasma cells and a subset of
cells in the apical light zone of the GC [12–14].

In a study by Bai et al., bcl6 and CD10 expression
was positively correlated with the apoptotic index and
the proliferation-associated protein Ki67 [15]. The
authors proposed several lines of evidence indicating
a positive correlation of CD10 expression with
proliferation and apoptosis in both normal and
malignant B-cells. Indeed, GC-cells are character-
ized by high proliferation and have the propensity to
undergo apoptosis and upregulate CD10 expression
on apoptotic induction, whereas CD10 is absent on
other subsets of mature B-cells that are not char-
acterized by high apoptosis [16–19]. The protein
tyrosine kinase (PTK) ZAP70 is a key mediator of
T-cell receptor (TCR) signalling and was until
recently believed to be restricted to B-cell chronic
lymphocytic leukaemia (B-CLL), where its expres-
sion is an important negative prognostic factor.
ZAP70 has now been reported to be expressed in
normal and malignant B-cells of various differentia-
tion stages but its function in B-cell receptor (BCR)
signalling is so far not well understood [20,21].

The protein kinase C-β (PKC-β) gene encodes two
mRNAs that originate from alternative splicing of the
C-terminal exons [22]. Little is known about the
potentially different biological roles of the two
isoforms of PKC-β, I and II, but in a recent report
they were shown to exhibit different membrane
translocation patterns in response to angiotensin II
type IA receptor agonist activation [23]. The PKC-β
gene is over-expressed in refractory rather than in
curable DLBCL [24] and the PKC-β activity en-
hances B cell proliferation and survival. PKC-β II has
further been implicated to be involved in poor
response to chemotherapy and survival in DLBCL
[25,26]. B-cells that are deficient of the tumour
suppressor PTEN are hyperproliferative in response
to mitogenic stimuli [27] and exhibit a lower thresh-
old for activation through the BCR. Loss of PTEN
function is one of many different genetic alterations
used by tumors to activate PI3K/Akt pathway and
confers selective growth and survival advantage [28].
In a recent paper, nuclear PTEN was proposed to
exert tumor suppressor functions by maintaining
chromosomal integrity [29]. The protein tyrosine
phosphatase (PTP) SHP1 can also be a tumour
suppressor in hematopoietic cells where it antago-
nizes growth-promoting and potentially oncogenic
effects of tyrosine kinases [30].

In this study, two prognostic subgroups of DLBCL
were divided by the use of immunohistochemistry in
a GC and a non-GC group, based on the expression of
CD10, Bcl6 and IRF4 [12,31]. We provide novel
data showing that de novo DLBCL cases express
ZAP70 and PKC-β II in a significantly higher
amount in the prognostically unfavourable non-GC
group, compared to the GC group. No differences
in PTEN, SHP1, SHP2, PKC-β I or phosphorylated
Akt expression were found between the groups, but
interestingly, the localization pattern of the two
PKC-β isoforms differed. Further, we show that
deficiency of nuclear PTEN expression correlates
with poor survival in de novo DLBCL cases. 5
DLBCL cell lines, with different origin, were also
analyzed for their expression of CD10, Bcl6, IRF4,
ZAP70, PKC-β I and II, phosphorylated Akt, PTEN,
SHP1 and SHP2. Studies of the expression of the
most commonly mutated tumor suppressor and
proliferation genes is of outmost importance to
clarify the molecular background of DLBCL and
may provide new insight for future treatment of this
disease.

Materials and methods

Tissue array and immunohistochemistry

A total of 28 pieces of paraffin embedded de novo
DLBCL tissues from 8 men and 20 women with a
median age of 70 and diagnosed between 2001 and
2006, were selected by a pathologist from the
Department of Pathology, University Hospital in
Malmö, Sweden (Ethical approval No. LU 210/
2006). Representative areas in all paraffin blocks
were chosen and tissue arrays were constructed as
described earlier [32]. In brief, punches of 0.6 mm
were taken from each block and mounted manually
in a recipient block. Four micrometer sections were
dried, deparaffinized, rehydrated and treated in a
microwave for 10 min with target retrieval solution
pH 9.9. This was followed by incubation in an
automated immunohistochemical staining machine
Western blot

10 × 10⁶ cells were lysed with 50 μl lysis buffer (9 mM Hepes, 0.08 mM EDTA, 0.08 mM EGTA, 9 mM KCL, 5%NP40, complete protease inhibitor (Roche, Mannheim, Germany)). Routinely 15 μl protein lysates/lane were run on 10% SDS gels. Gels were blotted on PVDF membranes (Techmate 500, Dako, Copenhagen, Denmark) with primary antibodies: mouse anti-human CD10 and mouse anti-human ZAP70 (Serotec, Oxford, UK), mouse anti-human Bcl6 (Chemicon/Millipore, Bedford, MA), mouse anti-human MUM1/IRF4 (Abcam, Cambridge, UK), rabbit anti-human p-Akt 1/2/3, rabbit anti-human SHP1, rabbit anti-human SHP2, mouse anti-human PKC-β I and rabbit anti-human PKC-β II (Santa Cruz Biotechnology, Santa Cruz), and rabbit monoclonal anti-human PTEN (Cell Signaling, Danvers, MA). Dako real envision detection system peroxidase/DAB (Dako, Copenhagen, Denmark) was used for incubation with secondary antibodies and visualisation. Slides were counterstained with haematoxylin. Analyses were performed independently by one pathologist and two researchers. There was a 90% agreement between the observers, and in cases of variance, the pathologist determined the outcome. To classify patients into the GC- or non-GC group, we used the three marker-model previously described by Hans et al. and Berglund et al. [12,31]. In brief, cases were considered GC-positive if CD10 alone or both CD10 and Bcl6 were positive. If both CD10 and Bcl6 were negative, the cases were considered GC-negative. If CD10 was negative and Bcl6 positive, IRF4 determined the outcome: Positivity for IRF4 designated the patient material to the non-GC group while negative IRF4 staining ment the GC group. The staining intensity for ZAP70, PKC-β I and II, p-Akt, PTEN, SHP1 and SHP2 was graded from 0 to 3. 0 = no staining, 1 = weak staining, 2 = moderate staining and 3 = strong staining. For measuring the fraction of positive cells, we used a 25% cut off to classify patients as positive or negative for all markers. Further, proportion of tumor cells less than 25% positive were scored as 1; greater than 25%, but less than 50% positive were scored as 2; greater than 50%, but less than 100% positive were scored as 3. Cell lines were fixed in paraformaldehyde and dehydrated before being paraffin embedded and prepared as described for the patient material. The cell lines were also placed in the GC/ non-GC group by use of the three marker model. Pearson Chi-Square tests and Mann Whitney tests were performed and Statistical calculations analyzed, with SPSS for Windows 12.0.1 (2002, SPSS Inc.).

Protein expression in prognostic subgroups of DLBCL

The following B cell lines have been described elsewhere [33]. In brief, FLEB 14–4 (pro-B) is established by EBV transformation of B cells from an ALL child, Nalm-6 (pre-B) is EBV-negative, containing intracellular μ-chains, Rael and Daudi (small mature B) are derived from Burkitt lymphoma (BL) patients and express surface IgG and IgM, respectively, U-698 (B-blast) is derived from a non-Hodgkin lymphoma and LP-1 is a plasmacytoma representing plasma cells near terminal differentiation. The cells were cultured in RPMI 1640 medium (Invitrogen, San Diego, CA) supplemented with 10% fetal calf serum and garamycin 0.1 g/ml.

The EBV-negative DLBCL cell lines U-2932, U-2904, U-2940, U-2946 and ULA were from Department of Oncology, Genetics and Pathology, Uppsala, Sweden. The DLBCL cell lines used in this study represent (i) de novo DLBCL: U-2946 and ULA [34], (ii) transformed DLBCL from Hodgkin lymphoma: U-2932 [35] and U-2940 [36] and (iii) transformed DLBCL from follicular lymphoma: U-2904 [37]. Amini et al. previously showed that U-2932 carries high-level amplifications of the chromosomal bands 18q21 (BCL2-loci) and 3q27 (BCL6-loci) and overexpression of these genes. Also, p53 is overexpressed in U-2932 due to a point mutation [35]. U-2940 display microinstability and multiple chromosomal rearrangements described in both Hodgkin and non-Hodgkin lymphoma, and some of these aberrations are shared by U-2932 [36]. For U-2904, Sambade reported translocation of t(8;14) typically seen in Burkitt lymphoma where c-myc is overexpressed, and of t(14;18) associated with follicular lymphoma and Bcl2 overexpression [37]. The de novo DLBCL cell line U-2946 is unpublished. The cells were cultured in RPMI 1640 medium (Invitrogen, San Diego, CA) supplemented with 10% fetal calf serum and garamycin 0.1 g/ml.

Following washing (PBS in 0.05% Tween20 PBS) and incubation with HRP-conjugated secondary Ab for 45 min at RT, membranes were developed with the ECL chemiluminescent method (Amersham Biosciences, UK) and Fluor-S-Max detector. Membranes were routinely stripped of primary Ab and reprobed with actin as a protein-loading control. The Abs used for Western blot were rabbit anti-actin (I-19) and rabbit anti-SHP1 (C-19) from Santa Cruz Biotechnology. Rabbit anti-IgG-HRP used as secondary Ab was from Dako (Glostrup, Denmark).
with 10% fetal calf serum and garamycin 0.1 g/ml, except from ULA which was cultured in equal amounts of IMDM + glutaMax and Opti-MEM I (Invitrogen, San Diego, CA), 10% fetal calf serum and garamycin 0.1 g/ml.

**PCR**

Total RNA from the cell lines (10 × 10^6 cells) were isolated with TRIzol (Invitrogen, San Diego, CA) and cDNA from 2 µg of RNA was prepared using First Strand cDNA Synthesis Kit according to manufacturers instructions (Amersham Biosciences, Amersham, UK). The PCR was performed in a total volume of 50 µl reaction mix, consisting of 5 µl Taq buffer which was added to 4 µl MgCl2 (Fermentas, Burlington, Canada), 2 µl of 10 × dNTP, 2 µl Taq polymerase (Finnzymes, Espoo, Finland), 34 µl of RNase free water, 1 µl cDNA and 1 µl of forward and reverse primer, respectively. For SHP1 and PTEN, the following PCR program was used: Denaturation at 95°C for 1 min followed by 30 cycles of 95°C for 1 min, annealing of primers for 1 min at 57°C and elongation for 1.5 min at 72°C. Final extension was done for 7 min at 72°C. For GAPDH the following PCR program was used: Denaturation at 94°C for 2 min followed by 45 cycles of 94°C for 30 s, annealing of primers for 1 min at 55°C and elongation for 1 min at 72°C. Final extension was done for 10 min at 72°C.

**PCR primers**

Oligonucleotide primers were as follows: **PTEN**, 5'-GAA ACT ATT CCA ATG TTC AGT GGC-3' and 5'-CTG ATC TTC ATC AAA AGG TTC ATT CTC-3' (expected size 587 bp), **SHP1**, 5'-A GTT GAA TTC ATG TGC GAG GGT GGA CGC TAC ACA-3' and 5'-A GTT GAA TTC ATG ACT GGT AAT GCC AGA TCT CCC-3' (expected size 735 bp), **GAPDH**, 5'-CGA CCA CTT TGT CAA GCT CA-3' and 5'-AGG GGT CTA CAT GGC AAC TG-3' (expected size 228 bp).

**PTP assay**

In order to measure the activity of SHP1, 10 × 10^6 cell lysates were incubated with anti-SHP1-antibody (rabbit anti-human SHP1) for 2 h in 4°C, and immunoprecipitated with protein G-beads (Amersham Biosciences, Amersham, UK) for 1 h, in 4°C. A PTP-activity assay was then performed, where 20 µl beads solution were incubated with 20 µl buffer (100 mM Bis-Tris pH 6.0, 150 mM NaCl, DTT 5 mM) and 60 µl ρNPP-solution (phosphorylated substrate), for 45 min in 37°C. As a negative control and background, one sample was immunoprecipitated with mouse anti-human HA-antibody (Roche, Basel, Switzerland). After 45 min, OD was measured at 405 nm. The OD correlates to the degree of free phosphate-groups, and this indicates how active the PTP is in the analyzed cells.

**Results**

**Division into the subgroups and clinical data**

The 28 patient samples were divided into a GC- and a non-GC group based on the expression of GC markers CD10, Bcl6 and post-GC marker IRF4 as analysed by immunohistochemical analysis and as described in “Material and methods”. A 25% cut off to classify cases as positive or negative for all markers was used. Fourteen patients (4 men, 10 women) were classified as belonging to the GC group and 14 patients (4 men, 10 women) constituted the non-GC group (Table IA). Although comprised of a rather small group of DLBCL cases, the sub-group division correlated significantly with Ann Arbor stage classification [38] as analyzed by Pearson Chi-Square test. Indeed, we found that the non-GC group were characterized by a more advanced stage disease (stage III–IV) compared with the GC group (p-value = 0.022) (Table IB). However, no significant differences in elevated S-lactate dehydrogenase (S-LDH) (p-value = 0.450), high International prognostic index, IPI, (p-value = 0.256), high Ki67 levels (p-value = 0.135) or survival (p-value = 0.445) could be found between the two subgroups, when analyzed by Pearson Chi-Square test.

**The cases of the non-GC group displayed significantly higher number of ZAP70 and PKC-βII positive cells compared to the GC group**

First, the DLBCL cases were stained and analyzed for expression of ZAP70, PKC-βI, PKC-βII and

<table>
<thead>
<tr>
<th>Antibody</th>
<th>GC group (N)</th>
<th>Non-GC group (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD10</td>
<td>11/14</td>
<td>0/14</td>
</tr>
<tr>
<td>Bcl6</td>
<td>10/14</td>
<td>6/14</td>
</tr>
<tr>
<td>IRF4</td>
<td>4/14</td>
<td>11/14</td>
</tr>
</tbody>
</table>

Cases were designated to the GC-group if they were positive for CD10 or CD10 and Bcl6. If they were negative for CD10 and Bcl6, they were considered to belong to the non-GC group. If CD10 was negative and Bcl6 positive, the expression of IRF4 determined the outcome. Cases were placed in the GC group if IRF4 was negative and in the non-GC group if IRF4 was positive.
phosphorylated PKB/Akt (p-Akt) (Table II). A Mann Whitney test was performed showing that the non-GC group displayed a significantly higher ZAP70 expression in terms of percent positively stained tumor cells, compared with cases in the GC-group (p-value = 0.039). Also, in the non-GC group, 51 – 100% of PKC-bII positive tumor cells were found in 11 out of 14 cases, compared with in only 2 out of 14 cases in the GC group (Table II). This difference in percentage positively stained PKC-bII tumor cells resulted in a statistical significant difference between the GC and non-GC groups (p-value = 0.001). PKC-bI and p-Akt were expressed in a high percentage of tumor cells in both subgroups (p-value = 0.246 and p-value = 0.769, respectively).

Different subcellular localization pattern of the PKC-\(\beta\) I and II isoforms in DLBCL cases

Interestingly, the subcellular localization differed between the two PKC-\(\beta\) isoforms, with PKC-\(\beta\) I being expressed in both cytosol and nucleus, whereas PKC-\(\beta\) II was found exclusively in the cytoplasm [Figures 1B and 1C]. Moreover, PTEN, SHP2, Zap70 and p-Akt were detected in both cytoplasm and nucleus, while SHP1 was detected only in the cytoplasm [Figures 1(A) – 1(G)].

Trends towards a stronger PTEN staining intensity in the non-GC group

In the non-GC group, PTEN was found to be expressed in 13 out of 14 cases, as displayed by 26 – 50% and 51 – 100% positively stained tumor cells (Table III). We detected nuclear PTEN in 7 cases in the GC group and in 8 cases in the non-GC group (data not shown for PKC-\(\beta\)I, p-Akt or SHP2). No significant differences were found between the groups in terms of percent positively stained cells for PTEN (p-value = 0.246), SHP1 (p-value = 0.164) or SHP2 (p-value = 0.982) when analyzed by a Mann Whitney test, but a trend towards a stronger PTEN staining intensity (graded 0 – 3) in the non-GC group was found (p-value = 0.094) (Table III).

Absence of nuclear PTEN correlates with poor survival

The de novo DLBCL cases analyzed in this study were related to survival and a higher percentage of cells lacking the expression of nuclear PTEN staining was found to correlate with poor survival (p-value = 0.059) (Table IV). No such correlation was found between any of the other proteins analyzed. The statistical analyses were performed by Mann Whitney tests.
Figure 1. Immunohistochemical stainings of de novo DLBCL tissue. A. ZAP70, B. PKC-β I, C. PKC-β II, D. p-Akt, E. PTEN, F. SHP1, G. SHP2. All tissues A-G were graded “3”, for strong staining intensity. Magnification 40 ×. (Please see colour online)
We further analyzed 5 DLBCL cell lines for protein expression. First, CD10, Bcl6 and IRF4 were analyzed and all DLBCL cell lines were placed in the GC group according to the three marker-model [31]. U-2932 was positive for all three markers, U-2904 and U-2946 were negative for Bcl6, U-2940 was negative for both CD10 and IRF4 and ULA was negative for IRF4 (Table V). Next, we analyzed the cell lines for the staining intensity of ZAP70, PKC-β I and II, p-Akt, PTEN, SHP1 and SHP2 by immunohistochemistry (Table V). U-2904 and U-2946, were strongly positive for ZAP70 when analyzed with immunohistochemistry and Western blot (data not shown), whereas U-2932, U-2940 and ULA were negative. PKC-β II was negative in U-2940 and ULA and rather weakly expressed in the other three cell lines. PKC-β I was strongly expressed in U-2932, U-2946 and ULA and weaker expressed in U-2904 and U-2940. The cytoplasmic and nuclear PKC-β I and cytoplasmic PKC-β II was also seen in all DLBCL cell lines. A strong SHP1 expression was detected in U-2940, U-2946 and ULA, whereas a weak expression and in fewer cells was seen in U-2932 and U-2904 (Table V). SHP2 expression was strong in the two de novo DLBCL cell lines U-2946 and ULA, and weaker in the other three cell lines. The levels of PTEN protein expression as measured by immunohistochemical stainings correlated well with the mRNA levels in the majority of the cell lines tested (Figure 2). A correlation between SHP1 protein and mRNA expression in the cell lines was also found, with an even expression level in all DLBCL cell lines (Figure 2). The exception was U-2904, which showed a SHP1 mRNA level equal to the other cell lines, but displayed a very low protein expression of the PTP.

### Table III. Trend towards a stronger staining intensity of PTEN in the non-GC group of de novo DLBCL cases, but no significant differences between groups regarding percent positive stained cells for PTEN, SHP1 and SHP2.

<table>
<thead>
<tr>
<th></th>
<th>GC group (N)</th>
<th>Non-GC group (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTEN (intensity)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 – 25%</td>
<td>5</td>
<td>36</td>
</tr>
<tr>
<td>26 – 50%</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>51 – 100%</td>
<td>8</td>
<td>57</td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>p-Value</td>
<td>0.094</td>
<td></td>
</tr>
<tr>
<td>SHP1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 – 25%</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>26 – 50%</td>
<td>5</td>
<td>36</td>
</tr>
<tr>
<td>51 – 100%</td>
<td>7</td>
<td>50</td>
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<tr>
<td>Total</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>p-Value</td>
<td>0.164</td>
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<tr>
<td>SHP2</td>
<td></td>
<td></td>
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<tr>
<td>0 – 25%</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>26 – 50%</td>
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<td>0</td>
</tr>
<tr>
<td>51 – 100%</td>
<td>13</td>
<td>93</td>
</tr>
<tr>
<td>Total</td>
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<td>14</td>
</tr>
<tr>
<td>p-Value</td>
<td>0.982</td>
<td></td>
</tr>
</tbody>
</table>

DLBCL tissue was stained by immunohistochemistry and the staining intensity is indicated using a 4-grade system ranging from 0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining. The number of positive cells in percent is indicated for each marker: 0 – 25%, 26 – 50% and 51 – 100%. For statistical analysis, a Mann Whitney test was performed.

### Table IV. Correlation of protein expression and survival in de novo DLBCL.

<table>
<thead>
<tr>
<th></th>
<th>Mors* (N)</th>
<th>%</th>
<th>Survival (N)</th>
<th>%</th>
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<tr>
<td>Nuclear PTEN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 – 25%</td>
<td>9</td>
<td>75</td>
<td>6</td>
<td>37</td>
</tr>
<tr>
<td>26 – 50%</td>
<td>3</td>
<td>25</td>
<td>7</td>
<td>44</td>
</tr>
<tr>
<td>51 – 100%</td>
<td>0</td>
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<tr>
<td>Total</td>
<td>12</td>
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<tr>
<td>p-Value</td>
<td>0.241</td>
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<tr>
<td>Cytoplasmic PTEN</td>
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<tr>
<td>0 – 25%</td>
<td>3</td>
<td>25</td>
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<tr>
<td>51 – 100%</td>
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<td>50</td>
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<td>81</td>
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<tr>
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<tr>
<td>p-Value</td>
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<tr>
<td>PKC-β II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 – 25%</td>
<td>2</td>
<td>17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>26 – 50%</td>
<td>4</td>
<td>33</td>
<td>5</td>
<td>33</td>
</tr>
<tr>
<td>51 – 100%</td>
<td>6</td>
<td>50</td>
<td>7</td>
<td>47</td>
</tr>
<tr>
<td>Total</td>
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<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Value</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHP1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 – 25%</td>
<td>2</td>
<td>17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>26 – 50%</td>
<td>3</td>
<td>25</td>
<td>5</td>
<td>31</td>
</tr>
<tr>
<td>51 – 100%</td>
<td>7</td>
<td>58</td>
<td>11</td>
<td>69</td>
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<tr>
<td>Total</td>
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<td></td>
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</tr>
<tr>
<td>p-Value</td>
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</tr>
<tr>
<td>ZAP70</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 – 25%</td>
<td>3</td>
<td>25</td>
<td>3</td>
<td>19</td>
</tr>
<tr>
<td>26 – 50%</td>
<td>3</td>
<td>25</td>
<td>3</td>
<td>19</td>
</tr>
<tr>
<td>51 – 100%</td>
<td>6</td>
<td>50</td>
<td>10</td>
<td>62</td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Value</td>
<td>0.599</td>
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</tr>
</tbody>
</table>

DLBCL tissue was stained by immunohistochemistry and the number of positive cells in percent is indicated for each marker: 0 – 25%, 26 – 50% and 51 – 100%. For statistical analysis, a Mann Whitney test was performed.

* Mors before 2006-12-31.
PTEN and SHP1 mRNA was detected in all DLBCL cell lines

To investigate if the mRNA levels of SHP1 and PTEN were similar to protein levels, DLBCL cell lines was analyzed by isolating total RNA and preparing cDNA. PTEN and SHP1 mRNA could be detected in all the DLBCL cell lines, as analyzed by PCR [Figure 2(A)]. To compare the mRNA levels between the cell lines, the intensity of the PTEN and SHP1 bands was measured by a volume analysis report. To compensate for any unequal loading and to measure the total RNA content, the GAPDH intensity for each cell line was measured and standardized. Subsequently, the PTEN and SHP1 intensity for the cell lines could be calculated and related to each other, by dividing each PTEN and SHP1-value with their corresponding GAPDH-value, respectively [Figure 2(B)].

Table V. Germinal center marker expression and staining intensity of ZAP70, PKCb I and II, p-Akt, PTEN, SHP1 and SHP2 in 5 DLBCL cell lines.

<table>
<thead>
<tr>
<th></th>
<th>U-2932</th>
<th>U-2904</th>
<th>U-2940</th>
<th>U-2946</th>
<th>ULA</th>
</tr>
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<tbody>
<tr>
<td>Positive (+)/negative (−)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD10</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bcl6</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>IRF4</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>GC/non-GC</td>
<td>GC</td>
<td>GC</td>
<td>GC</td>
<td>GC</td>
<td>GC</td>
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<tr>
<td>Staining intensity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZAP70</td>
<td>−</td>
<td>+++</td>
<td>−</td>
<td>+++</td>
<td>−</td>
</tr>
<tr>
<td>PKCβ II</td>
<td>++</td>
<td>+</td>
<td>−</td>
<td>++</td>
<td>−</td>
</tr>
<tr>
<td>PKCβ I</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>p-Akt</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>PTEN</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>SHP1</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>SHP2</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

The 5 DLBCL cell lines were stained for the indicated markers and analyzed for percent stained cells (CD10, Bcl6 and IRF4) and for staining intensity (the PTKs and PTPs). With a 25% cut off, the cell lines were considered positive or negative for the three GC-markers. For staining intensity, − = no staining, + = weak staining, ++ = moderate staining and +++ = strong staining.

Biological activity of SHP1 in the cell lines

To confirm biological activity of the SHP1 protein in the DLBCL cell lines, we performed an in vitro PTP activity assay by immunoprecipitating SHP1 from the same number of cells from each of the DLBCL cell lines and thereafter assaying against the pNPP substrate. The SHP1 activity in U-2940 was about two times higher as compared to the activity in the other DLBCL cell lines tested (Figure 3). U-2904 displayed the weakest SHP1 activity, which is consistent to our observation in immunohistochemical analysis.

Figure 2. SHP1 and PTEN mRNA expression in the 5 DLBCL cell lines. A. PCR was performed on cDNA prepared from the cell lines (1. U-2932, 2. U-2904, 3. U-2940, 4. U-2946, 5. ULA) as described in Material and methods. GAPDH indicates total cDNA level and loading. B. The intensity of the PTEN and SHP1 bands was measured for each cell line and described in a volume analysis report. The PTEN and SHP1 value was divided with the value of the GAPDH-intensity for each cell line, to compensate for any unequal loading. The quotients from one representative experiment for PTEN (left) and SHP1 (right) respectively, are pictured.
SHP1 expression is strong in the early B cell differentiation, but declines with further maturation

SHP1 expression was also investigated at various B cell differentiation stages in cell lines (Figure 4). By Western blot analysis, we show that SHP1 was strongly expressed in the pro-B FLEB 14-4 and the pre-B Nalm-6 cell lines, weakly expressed in the three mature Daudi, Rael and U-698 B cell lines, but not detectable in the plasma cell line LP-1. The biological SHP1 activity as measured by the in vitro PTP activity assay was in line with Western blot results (data not shown).

Discussion

Tyrosine phosphorylation is a rapidly reversible process catalysed by PTKs and reversed by PTPs. Abnormalities in tyrosine phosphorylation play a role in the pathogenesis of numerous inherited or acquired human diseases, from cancer to immune deficiencies. Mutations of PTKs and loss of PTPs have been reported in numerous experimental and clinical cancers [39 – 41]. At least 30 different PTPs have been reported to be affected and loss can occur by genetic or epigenetic mechanisms. Here, we have used immunohistochemistry to analyze the protein expression of ZAP70, PKC-β I and II, p-Akt, PTEN, SHP1 and SHP2, critical for the BCR signaling capacity, in 28 cases of de novo DLBCL and in 5 cell lines of DLBCL origin. Further, we have analyzed the cell lines for mRNA expression of SHP1 and PTEN, and for biological activity of SHP1.

The DLBCL cases in this study were divided into two groups, a GC and a non-GC group, based on their expression of CD10, Bcl6 and IRF4 [12,31]. Only de novo DLBCL cases were included in the study, since other DLBCL cases with earlier history of a low-grade malignant lymphoma can be more similar to the ancestral lymphoma type at the molecular level [31,42]. Fourteen cases were classified as belonging to the GC group (CD10 alone or both CD10 and Bcl6 were positive) and 14 cases to the clinically more aggressive non-GC group. Since the patients were diagnosed rather recently (2001 – 2006), a follow up of 5-year survival was not possible to perform. However, cases of the non-GC group were found to display a more advanced stage disease, stage III-IV, compared to the GC group (CD10 alone or both CD10 and Bcl6 were positive) and 14 cases to the clinically more aggressive non-GC group. Since the patients were diagnosed rather recently (2001 – 2006), a follow up of 5-year survival was not possible to perform. However, cases of the non-GC group were found to display a more advanced stage disease, stage III-IV, compared to the GC group (CD10 alone or both CD10 and Bcl6 were positive) and 14 cases to the clinically more aggressive non-GC group. Between the two groups, there were significant differences in the number of cells positively stained for ZAP70 and PKC-β II. Indeed, the two proteins were expressed in a higher number of cells in the non-GC group, compared to the GC group. This is particularly interesting, since ZAP70 is associated with poor prognosis in B-CLL and was recently found to be expressed in ABCs [20]. In T-cells, ZAP70 is recruited to the TCR upon activation, and from here the protein functions in the activation of downstream targets for proliferation and survival, such as the phosphatidyl inositol-3 kinase. In a recent report, ZAP70 was proposed to function differently in BCR signalling, compared to TCR mediated signaling [43]. It seems to mediate inhibition of events such as BCR internalization that would otherwise terminate the signalling response [43]. In two previous studies, ZAP70 was reported to be absent in DLBCL tissue analyzed by immunohistochemistry [44,45]. Our findings indicate a possible role for ZAP70 in DLBCL development, and it’s importance as a...
possible prognostic marker in DLBCL needs to be further explored. PKC-\(\beta\) I and II showed not only a different distribution between the GC and the non-GC group, but also a different subcellular localization pattern. PKC-\(\beta\) I expression was similar in both groups and was localized to both nucleus and the cytoplasm. Interestingly, PKC-\(\beta\) II was absent in the nucleus, and instead detected in the cytoplasm, with a significant over-expression in the non-GC group which comprises patients with a more advanced disease. This is in line with other studies showing that membrane PKC-\(\beta\) II expression predicts for inferior survival in DLBCL [25,26]. Espinosa et al. reported PKC-\(\beta\) II expression in the membrane, cytoplasm and nucleus in DLBCL cells [26]. We can only detect PKC-\(\beta\) II expression in the cytoplasm and the reason for the difference in PKC-\(\beta\) II localization between our studies can only be speculated. Different antibodies were used for immunohistochemical staining, but in both studies the antibody recognized the C-terminal part of PKC-\(\beta\) II. To our knowledge, our study is the first to show the different distribution of PKC-\(\beta\) I and II in the GC and the non-GC groups. The phosphorylation and activation of PKB/Akt is negatively regulated by PTEN. When analyzed here, no correlation in either expression or staining intensity of p-Akt and PTEN was found and Akt was found to be phosphorylated in a high percentage of cells in both subgroups. However, we report a trend towards a stronger staining intensity of PTEN (cytoplasmic and nuclear) in the non-GC group of DLBCL cases. Moreover, here we show a correlation between the absence of nuclear PTEN expression and inferior survival in de novo DLBCL cases. PTEN has a well-defined role in plasma membrane signaling but is also found in the nucleus in a number of different normal and tumor cell types [46,47]. Recent studies have suggested that phosphatase-independent functions of PTEN modulate the activity and stability of p53 within the nucleus [48,49]. Shen et al. showed recently that PTEN-deficient mouse embryonic fibroblasts contained an increased number of chromosomal fragments, breakage of chromatids and other chromosomal instabilities, suggesting that one of PTENs nuclear tumor suppressor functions might be to guard the genome integrity [29].

In this study of 28 de novo DLBCL cases, we could not detect any significant differences \((p\text{-value}=0.164)\) between SHP1-positive DLBCL cells in the two prognostic subgroups, but the cases in the non-GC group contained a higher percentage of SHP1 positive cells. Previous immunohistochemical stainings of lymphoid tissues have shown that SHP1 is downregulated in B cells forming GCs, while it is expressed in earlier and later stages of B cell differentiation [50]. We also confirm these results by showing that B cell lines of the early stages in the B cell differentiation express higher levels of SHP1, compared to mature B cells and plasma cells. SHP2, which was expressed in both cytoplasm and nucleus in DLBCL tumor cells, was detected in equal amounts in both subgroups.

5 DLBCL cell lines with varying clinical background were analyzed in our study. The cytoplasmic PTEN expression was strong in all DLBCL cell lines with an expression in 90–95% of the cells analyzed. Sambade et al. conclude that chromosomal aberrations in combination with strong CD19 expression may point to a deregulation of the CD19-PI3K pathway in U-2940 [36]. A strong SHP1 expression in a high percentage of cells was also detected in U-2940, U-2946 and ULA, whereas a weaker expression in a fewer number of cells were found in U-2932 and U-2904. The fact that many of the DLBCL cases in our study expressed SHP1 despite previous reports of SHP1 downregulation in many lymphomas [30], led us to further investigate this PTP. The SHP1 expression correlated with biological activity in the DLBCL cell lines. Indeed, the most pronounced SHP1 activity was measured in U-2940 cells and the lowest activity in U-2904, which also displayed the highest and lowest protein expression, respectively.

In conclusion, here we show for the first time a significantly higher expression of ZAP70 in the clinically more aggressive non-GC group, compared to the GC group of de novo DLBCL cases. Further, we report different cellular localization of PKC-\(\beta\) I and II in DLBCL tissue, and over-expression of PKC-\(\beta\) II, but not of PKC-\(\beta\) I, in the non-GC group of DLBCL. We also provide novel information about the tumor suppressors PTEN and SHP1, and report that the absence of nuclear PTEN correlates with poor survival in DLBCL. Given that this disease is cured in only 40% of the cases, analyses of molecules required for cancer cell proliferation or survival are of utmost importance.

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References


