RESEARCH ARTICLE

Kinase activity profiling reveals active signal transduction pathways in pediatric acute lymphoblastic leukemia: A new approach for target discovery

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Still about 20% of patients with acute lymphoblastic leukemia (ALL) struggle with relapse, despite intensive chemotherapy. We and others have shown that kinase activity profiling is able to give more insights in active signal transduction pathways and point out interesting signaling hubs as well as new potential druggable targets. With this technique the gap between newly designed drugs and ALL may be bridged. The aim of this study was to perform kinome profiling on 20 pediatric ALL samples (14 BCP-ALL and six T-ALL) to identify signaling proteins relevant to ALL. We defined 250 peptides commonly activated in both BCP-ALL and T-ALL representing major signal transduction pathways including MAPK, PI3K/Akt, and regulators of the cell cycle/p53 pathway. For 27 peptides, differentially phosphorylation between BCP-ALL and T-ALL was observed. Among these, ten peptides were more highly phosphorylated in BCP-ALL while 17 peptides showed increased phosphorylation in T-ALL. Furthermore we selected one lead of the list of commonly activated peptides (HGFR_Y1235) in order to test its efficacy as a potential target and provide proof of principle for this approach. In conclusion kinome profiling is an elegant approach to study active signaling and identify interesting potential druggable targets.

Keywords:
Acute lymphoblastic leukemia / HGFR / Kinome profiling / Protein arrays / RON / Signal transduction

Additional supporting information may be found in the online version of this article at the publisher's web-site

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Abbreviations: ALL, acute lymphoblastic leukemia; **BCP-ALL**, B-cell progenitor acute lymphoblastic leukemia; **HGFR**, hepatocyte growth factor receptor (also known as c-Met); **IL-7R**, interleukin-7 receptor; **JAK**, janus kinase; **MAPK**, mitogen-activated protein kinase; **Pre-B cells**, precursor B-cells; **Pre-BCR**, precursor B-cell receptor; **Pre-TCRA**, precursor T-cell receptor alpha; **RTK**, receptor tyrosine kinase; **T-ALL**, T-cell acute lymphoblastic leukemia; **TCR**, T-cell receptor; **TK**, tyrosine kinase

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1 Introduction

The gap between newly designed drugs such as receptor tyrosine kinase (RTK) and tyrosine kinase (TK) inhibitors and the applicability of these drugs for the treatment of acute lymphoblastic leukemia (ALL) can be bridged by studying active signal transduction pathways. A cancer cell is distinguished from a normal cell by an uncontrolled cell proliferation resulting from the self-sufficiency in growth signals and the insensitivity toward growth inhibitory signals and avoiding apoptosis to prevent cell death, resulting together in an immortal phenotype [1]. These characteristic features are the net result of various genetic, epigenetic, and microenvironmental abnormalities, leading to the activation of signal transduction pathways enabling crucial cellular processes [2]. This phenomenon can also be applied to leukemias.

Traditionally ALL is classified according to the immunophenotype into B-cell progenitor ALL (BCP-ALL) and T-cell ALL (T-ALL). Nowadays, ALL can be subclassified based on cytogenetic and molecular genetic abnormalities involved in prognosis and development of leukemia [3–5].

A number of important mechanisms in ALL cells have recently been identified. For instance, the survival and proliferation of precursor B-cells (pre-B-cells) appears to be dependent on the function of the precursor B-cell receptor (pre-BCR) and interleukin-7 receptor (IL-7R) [6, 7]. In the early stage of development these receptors are required for proliferation and survival of precursor B-cells (pre-B cells) via the mitogen-activated protein kinase (MAPK), PI3K/Akt, and Janus kinase (JAK)/STAT signal transduction pathways [7]. Substantial changes in signal transduction pathways occur during the transition of pre-B cells into mature B-cells [7–9]. The differentiation arrest of pre-B cells in BCP-ALL suggests an unsuccessful switch from the proliferative stage towards B cell differentiation [7]. In T-ALL cells, Notch1 is the most prevalent pathway promoting cell growth via transcriptional upregulation of *MYC* working in concert with the PI3K/Akt pathway to reinforce cell growth [5]. In addition, Notch1 transcriptional activity is able to upregulate the IL-7R, precursor T-cell receptor alpha (pre-TCRA), and the NF-kappaB signaling stimulating cell growth and survival [5]. Furthermore, translocations involving the *TCR* loci may lead to an aberrant expression of *TCR* genes and unsuccessful rearrangements of the *TCR* loci leading to the absence of a mature TCR resulting into a differentiation block at various stages of development [10].

Although current chemotherapy strategies are intensified, still about 20% of ALL patients struggle with relapse. Therefore, new therapeutic strategies are warranted. Previously, we generated more insights in active signal transduction pathways using kinase activity profiling in pediatric acute myeloid leukemia and brain tumors [11–13]. We were able to point out interesting signaling hubs as well as new potential druggable targets for future investigations.

The present study shows that kinome profiling in pediatric BCP-ALL and T-ALL is a suitable approach to obtain more insights into active signal transduction pathways by describing commonly active signal transduction pathways and revealing unknown signaling proteins relevant in ALL.

2 Materials and methods

2.1 Patient material and cell lines

Primary blood and bone marrow samples from 20 newly diagnosed ALL patients (two proB-ALL, four common B-ALL, eight preB-ALL, and six T-ALL) were collected after getting written informed consent in accordance with the regulations and protocols of the medical ethics committee. Patients' characteristics are shown (Supporting Information Table 1). Patient samples were included if the age at diagnosis was between 9 and 18 years. Mononuclear cells were isolated by Lympho-prep (Nycomed, Zürich, Switzerland) density gradients and cryopreserved in liquid nitrogen until use. The cryopreserved leukemia cells were thawed rapidly at 37°C and diluted in a 6 mL volume of newborn calf serum, as described earlier [14]. All samples contained more than 95% blast cells.

The cell lines, Jurkat (T-ALL), Molt 4 (T-ALL), Nalm 6 (BCP-ALL), and RCH-ACV (BCP-ALL) were cultured in RPMI-1640 medium (Cambrex Bio Science, East Rutherford, NJ, USA) supplemented with 10% fetal calf serum (FCS, Bodinco, Alkmaar, The Netherlands). All cultures contained 1% penicillin/streptomycin (Gibco, Invitrogen, Carlsbad, CA, USA).

2.2 PepChip

Kinase activity profiles of 20 primary ALL patients were determined using the PepChip™ Kinomics microarray system (Pepscan, Lelystad, The Netherlands). The experimental procedures and data analysis were performed as described earlier [13]. A detailed description of the method and data analysis as well as the file containing the processed raw data can be found in the Supporting Information.

The phospho-ELM database (version 9.0) and the NCBI blast database were used to verify corresponding proteins and phosphorylation sites of active peptides.

The minimal kinome approach determined the set of phosphorylated peptides that shared phosphorylation in ≥ 90% of all samples within BCP-ALL samples ($n = 14$) or T-ALL ($n = 6$), as described previously [11, 12]. Using the minimal kinome approach, peptides are active or inactive, individual phosphorylation intensities are neglected. Protein–protein interactions and the protein networks were created using the STRING database (Version 9.1, Jensen et al. Nucleic Acids Res 2009) and Cytoscape (Version 3.1.1, Cline MS, Nat Protoc, 2007). For functional analysis, active peptides were aligned to corresponding signaling pathways using the online available pathways of the Kyoto encyclopedia of genes and genomes (KEGG) and the PhosphoSite databases. Differences between phosphorylation intensities were determined using Mann–Whitney U analysis (SPSS, Version 20,

IBM, Armonk, NY, USA). $p \leq 0.05$ was considered statistically significant.

2.3 Antibody microarrays

Acute lymphoid leukemia patient samples ($N = 8$) were subjected to a human phosphokinase microarray (Catalog Number ARY003, R&D Systems, Minneapolis, MN, USA) following manufacturers protocol and described previously [13]. Per patient 50 μg of protein lysate was added to the array.

Cell lines transduced with shRON or shControl were subjected to a stress and apoptosis signaling antibody array (Catolog number 12856, Cell Signaling, Danvers, MA, USA) following manufacturers protocol. Per sample 25 μg of protein lysate was added.

Scanned images were processed using Image J software (version 1.41) followed by relative quantification of signal intensities using ScanAlyze array analysis software (Eisen Lab Software). Phosphorylation intensities were determined by subtracting the background intensity from the measured spot intensity, after which the mean intensity and the standard deviation of the duplicate proteins were determined. For the human phosphokinase microarray relative phosphorylation intensities were determined by normalizing the mean spot intensity to the mean membrane intensity (membrane A or B). For the stress and apoptosis signaling array α-tubulin was used as a loading control for the normalization.

2.4 Flow cytometric analysis

For the membrane protein expression of c-Met and RON, cells were blocked in PBS 1% Bovine Serum Albumin (BSA), and stained with c-Met-FITC or RON-APC antibody (R&D systems, Abingdon, United Kingdom). To determine intracellular protein expression, cells were permeabilized and blocked using a PBS 5% BSA and 0.5% Triton X-100 solution (Sigma, St. Louis, MO, USA). IgG-FITC and IgG-APC were used as isotype controls. Fluorescence was determined with a LSRII flow cytometer (BD FACS DIVA software, BD Bioscience, Breda, The Netherlands) and data was analyzed using FlowJo software (Tree Star, Ashland, OR, USA). Since all tested ALL cell lines were negative for membrane and intracellular c-Met expression, we used the brain tumor cell line RES259 as a positive control.

2.5 shRNA mediated knockdown

Lentiviral vector (pLKO.1) containing shRNA sequences targeting RON were obtained from Open Biosystems, ordered (Waltham, MA, USA) and inserted in a pLKO1-mCherry vector. Sequences are available upon request. Lentiviral particles were generated by cotransfection of pLKO1-mCherry-shRON or scrambled vector with packaging plasmid psPAX2 and the envelope plasmid pMD2.g into 293T cells using FuGENE HD transfection reagent (Roche, Woerden, The Netherlands). Leukemic cells were incubated with lentiviral supernatants for one or two consecutive days after which stably transduced cells were expanded. Transduction efficiency was measured by flow cytometry and the downregulation of RON was confirmed by Western blot analysis. Subsequently, transduced cells were cultured and absolute cell counts were measured every 48 h using a Coulter Counter (Beckman Coulter, Fullerton, CA, USA) to assess cell growth. Viable cell percentages were obtained by FACS analysis based upon the forward side scatter.

2.6 Western blot analysis

Western blots were performed as previously described [13]. Antibodies were from Abgent (RON, San Diego, CA, USA) and Santa Cruz Biotechnology (β-actin, Dallas, TX, USA).

3 Results

3.1 Kinome profiling in primary pediatric ALL

To identify signaling proteins relevant to ALL, we performed kinome profiling on 20 pediatric ALL samples (14 BCP-ALL and 6 T-ALL). A representative image of a PepChip™ Kinomics microarray is shown in which 976 unique evaluable target peptides are spotted (Supporting Information Fig. 1). For kinome profiling in pediatric ALL we analyzed our data using two methods (Supporting Information Fig. 2). On one hand, commonly activated peptides within the BCP-ALL and T-ALL sample population could be determined using the minimal kinome approach [11, 12] (Supporting Information Fig. 2.I). In the minimal kinome, a peptide is considered to be commonly active if the phosphorylation of the peptide is shared by ≥90% of all samples within BCP-ALL and T-ALL. On the other hand, differences in peptide phosphorylation intensities between BCP-ALL and T-ALL were assessed using the nonparametric Mann-Whitney U test (Supporting Information Fig. 2.II).

We started the analysis by describing a set of commonly activated peptides of potential interest for both BCP-ALL and T-ALL. Activation of 250 peptides derived from 220 different proteins was found to be common for all samples (Supporting Information Table 3). We created a disease network of these 220 proteins based on known protein–protein interactions, showing interactions between 208 different proteins (Fig. 1). For functional analysis, proteins were aligned to known signal transduction pathways, using the KEGG and PhosphoSite pathway databases. As expected based on the active signaling pathways described in pediatric leukemias, many peptides could be aligned to the MAPK-signaling pathway (downstream of the pre-BCR and pre-TCR), the PI3K/Akt-signaling pathway (downstream of the pre-BCR in BCP-ALL

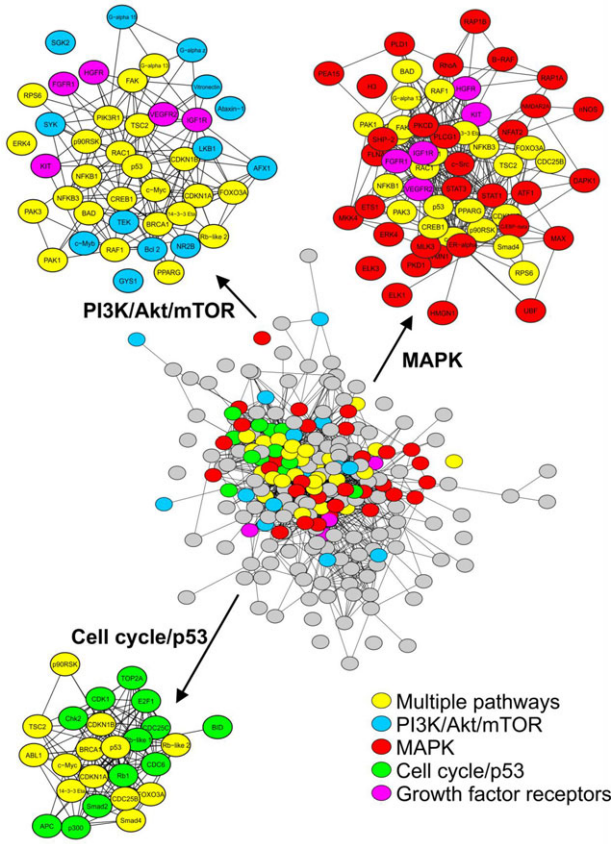

Figure 1. Protein–protein interaction network of commonly activated peptides. Disease network of the 220 different proteins regarding the 250 commonly activated peptides as identified by the minimal kinome surrounded by the proteins aligned to the three most commonly activated signaling pathways. Shown are known protein–protein interactions according to the STRING database. For functional analysis, proteins were aligned to known signal transduction pathways using the KEGG and PhosphoSite pathway databases. Proteins involved in the three major signaling pathways and the growth factor receptors are highlighted. Yellow: proteins aligned to multiple pathways, blue: PI3K/Akt/mTOR pathway, red: MAPK pathway, green: regulators of the cell cycle including components of the p53 pathway, pink: growth factor receptors.

and activated by Notch1 in T-ALL), and regulators of the cell cycle including components of the p53 pathway (Fig. 1).

The MAPK-signaling pathway could be associated with 66 activated peptides derived from 60 different proteins. These included peptides derived from protein kinases including RAF as well as RAS related peptides (RAP1A and RAP1B), MAPK related peptides (MKK4 and MLK3), and transcription factors or transcription coactivators downstream of the MAPK pathway (e.g. ELK1, ETS1, and CREB1, Fig. 1). A total of 49 peptides, derived from 42 proteins, correspond to the PI3K/Akt-signaling pathway including the peptides derived from protein kinases related to phosphatidylinositol 3 kinase (PIK3R1) and ribosomal S6 kinases (RPS6 and p90RSK, Fig. 1). Phosphorylation of 33 peptides and 27 corresponding proteins could be aligned to regulators of the cell cycle including components of the p53 pathway. Among these, peptides were derived from checkpoint kinase 2, cyclin dependent kinase inhibitors (CDKN1A and CDKN1B) and the tumor suppressor retinoblastoma 1 (Rb1, Fig. 1).

Complementary to the kinome results we performed a human phosphokinase microarray on eight primary ALL samples (see Supporting Information Table 4 for mean relative protein phosphorylation intensities). Phosphorylation profiles between BCP-ALL and T-ALL were comparable (Fig. 2B). Phosphorylation of proteins associated with the three major active signaling pathways could be appreciated, including the phosphorylation of hub proteins, which were not observed in the minimal kinome set (e.g. ERK1/2, p38, JNK, Akt1, mTOR, and GSK3β, Fig. 2A and B). Activity of peptides derived from these hub proteins could not be detected in the minimal kinome data set due to the stringent data analysis (e.g. a peptide is considered to be commonly active if the phosphorylation of the peptide is shared by ≥90% of all samples within BCP-ALL and T-ALL). A peptide derived from ERK1/2 (ERK1/2_T185) was activated in 70% of the primary ALL samples, p38 (p38_T180) in 75% of the samples, JNK1/2 (JNK1/2/3_T183/Y185/Y223) in 45% of the samples, Akt1 (Akt1_S473, Akt1_T308, and Akt1_Y326) in 35, 75, and 55% of the samples, respectively, mTOR (mTOR_S2448 and mTOR_S2481) both in 60% of the samples, and GSK3β (GSK3 β _S9, and GSK3 β _S21) in 50 and 35% of the samples, respectively.

In addition to major signaling pathways we observed phosphorylation of peptides derived from various growth factor receptors in our minimal kinome data set, including fibroblast growth factor receptor 1 (FGFR1), hepatocyte growth factor receptor (HGFR) also known as c-Met or Met, insulin-like growth factor 1 receptor, stem cell growth factor receptor KIT also known as c-Kit, and vascular endothelial growth factor receptor 2 (VEGFR2, Fig. 1).

Subsequently, differences in peptide phosphorylation intensities on the kinome array between BCP-ALL and T-ALL were investigated. Twenty-seven peptides were differentially expressed between BCP-ALL and T-ALL (Fig. 3). As shown in Fig. 3A, ten peptides were more highly phosphorylated in BCP-ALL compared to T-ALL. An increase in phosphorylation of peptides derived from well known protein kinases, JNK1/2/3_T183/Y185/Y223 and Lyn_Y508, could be observed in BCP-ALL compared to T-ALL (Red boxes, Fig. 3A). We could also find phosphorylation of peptides derived from less well known kinases like BTEB2_S153 (also known as KLF5), CD32_Y281, p73_Y99, and Osteopontin_S148 (OPN_S148) (Blue boxes, Fig. 3A). In T-ALL, 17 peptides showed an increased phosphorylation compared to BCP-ALL (Fig. 3B). Among these 17 peptides, peptides derived from proteins comprehensively described in T-ALL e.g. BIM_S118, glucocorticoid receptor_S608 (GR_S608), Shc_Y350, and TAL1_S122 could be observed (Red boxes, Fig. 3B) as well as less frequently described peptides e.g. CDX2_S60, and

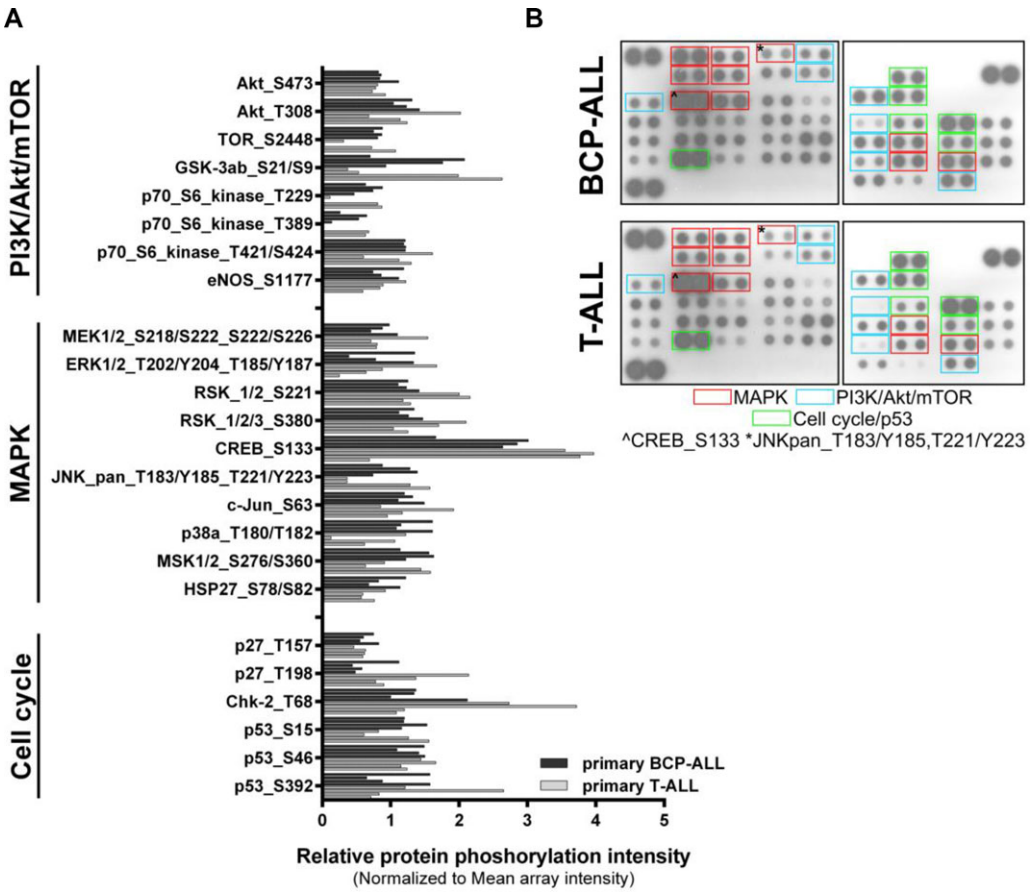

Figure 2. Phosphoproteome profiles of primary BCP-ALL and T-ALL samples. Human phosphokinase microarray analysis indicates phosphorylation of the proteins associated with the three commonly activated signaling pathways. The graph (A) and the representative human phosphokinase microarrays for BCP-ALL and T-ALL (B) clearly show the phosphorylation of the hub proteins associated with the three important pathways. Proteins involved in the three major signaling pathways and CREB_S133 and JNKpan_T183/Y185,T221/Y223 phosphorylation are highlighted (Fig. 2B). Relative phosphorylation intensities were determined by normalizing the mean spot intensity to the mean membrane intensity.

TrkA_Y496 (Blue boxes, Fig. 3B). Furthermore, we found high phosphorylation intensities of the peptides derived from CREB1_S133 and Vitronectin_S397 (VTN_S397) in both BCP-ALL and T-ALL but significantly higher in T-ALL compared to BCP-ALL (Green boxes, Fig. 3B). Of these 27 peptides, CREB_S133 and JNKpan_T183/Y185,T221/Y223 were spotted on the human phosphokinase microarray. CREB_S133 was highly phosphorylated in all BCP-ALL and T-ALL samples (Fig. 2B). Furthermore, the relative protein phosphorylation intensity was higher in T-ALL compared to BCP-ALL (2.99 versus 2.54, $P = 0.343$, Supporting Information Table 4). Although phosphorylation of the peptide derived from JNK1/2/3_T183/Y185/Y223 could not be observed in T-ALL, phosphorylation of JNKpan_T183/Y185,T221/Y223 could be detected in the 4 T-ALL samples. However, the mean phosphorylation intensity of these samples was below the mean array intensity (Supporting Information Table 4). In accordance with the kinome profiling results, JNKpan_T183/Y185,T221/Y223 protein phosphorylation was higher in the BCP-ALL samples compared to T-ALL samples (1.07 versus 0.89, $P = 0.686$, Fig. 2B and Supporting Information Table 4). The results of the human phosphokinase microarray are in concordance with the observed peptide phosphorylation events as observed using kinome profiling, however, differences between BCP-ALL and T-ALL are more pronounced in the kinome results.

3.2 Phosphorylation of peptides representing key pathways in ALL

In both normal and malignant B cell progenitor cells, Src-like TKs Lyn, Fyn, Blk, and Syk transmit the signal from the pre-BCR toward the downstream signaling pathways. Peptides derived from Lyn_Y508, Syk_Y323, and Syk_Y526 were present on the kinome array. Lyn_Y508 was more highly phosphorylated in BCP-ALL compared to T-ALL (Fig. 3A). Of these peptides, no specific phosphorylation sites were present on

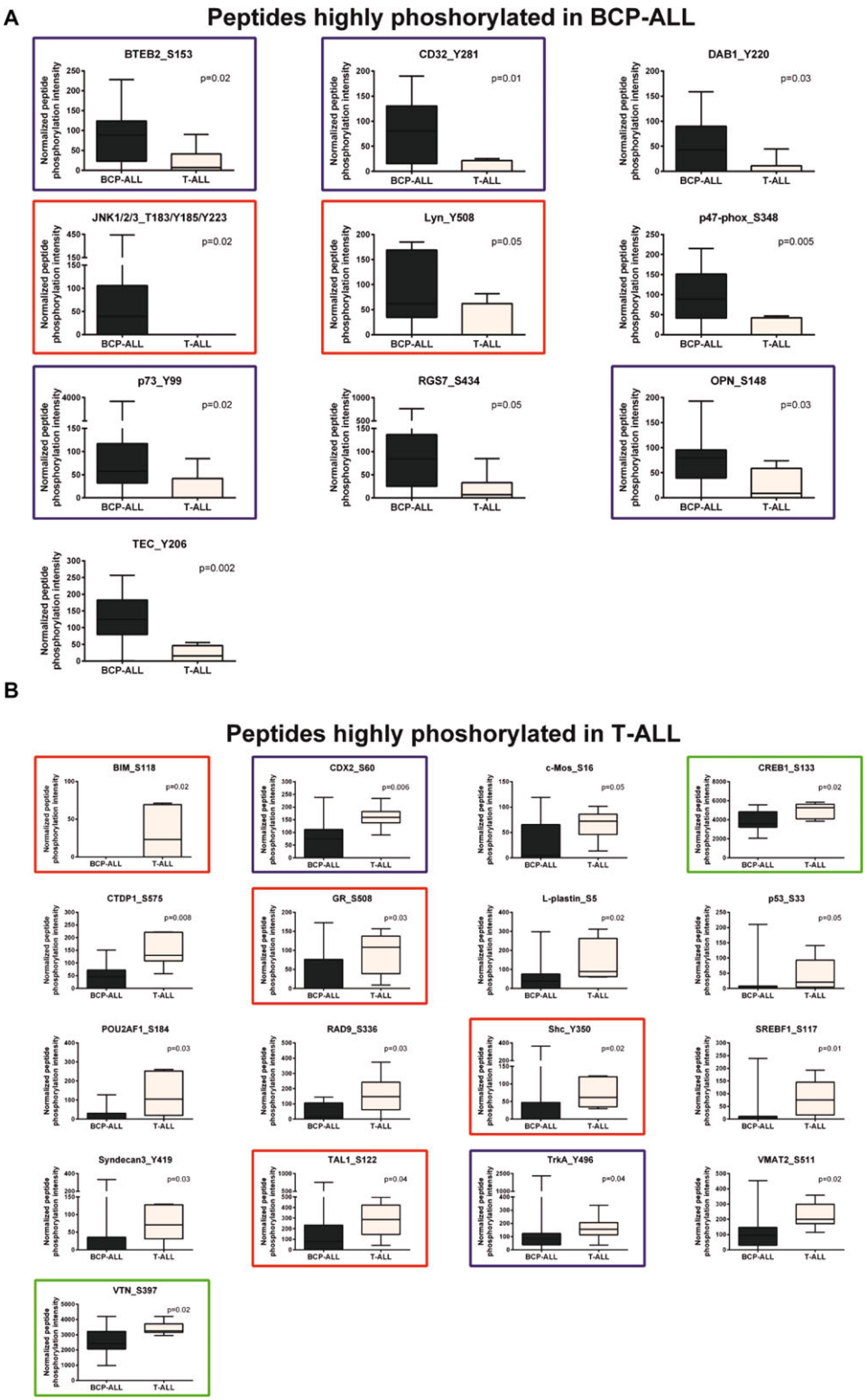

Figure 3. Significant differentially phosphorylated peptides identified by kinome profiling. Mann-Whitney U analysis of the kinome data showed ten peptides highly phosphorylated in BCP-ALL compared to T-ALL (A). In T-ALL, 17 peptides were showed increased phosphorylation compared to BCP-ALL (B). Peptides derived from frequently described proteins are indicated with red boxes, less frequently described peptides, and of potential interest for ALL, are marked with blue boxes. Green boxes show peptides highly phosphorylated in both BCP-ALL and T-ALL, but higher in T-ALL.

Table 1. Phosphorylation of peptides representing key pathways in ALL

Protein_Phosphorylation site	Mean intensity	Frequency (%)
Lyn_Y508	65.85	75.0
Syk_Y323	70.90	85.0
Syk_Y526	237.38	100.0
BLNK_Y96	67.91	80.0
BTK_S180	8980.80	100.0
BTK_Y223	94.52	70.0
STAT5A_S780	7.29	35.0
STAT5A_Y694	93.97	85.0
STAT5B_Y679	185.86	70.0
c-Myc_S62	947.81	100.0
c-Myc_T58	4548.28	100.0
n-Myc_S263	110.04	90.0
Lck_S158	128.67	80.0
Lck_Y192	55.02	60.0
Lck_Y411	60.55	55.0
ZAP70_Y319	78.65	80.0
ZAP70_Y474	123.14	85.0
ZAP70_Y493	62.04	50.0
SLP76_Y113	70.97	75.0
SLP76_Y423	74.57	75.0
NFKB1_S907	949.83	95.0
NFKB1_S932	39.62	40.0
NFKB3_S263	732.92	100.0
NFKB3_S298	1000.81	100.0
NFKB3_S536	23.35	35.0

the human phosphokinase microarray. Of the Src-like TKs involved in the pre-BCR signaling, human phosphokinase data observed phosphorylation of Lyn_Y397 and Fyn_Y420 (Supporting Information Fig. S3A). Phosphorylation of the peptides derived from Syk (Syk_Y323 and Syk_Y526) could be observed in the majority of all samples (85.0% and 100.0%, respectively, Table 1) with no differences between phosphorylation intensities in BCP-ALL and T-ALL (Supporting Information Table 5). The peptide derived from the tumor suppressor BLNK (BLNK_Y96) was phosphorylated in 12 of 14 BCP-ALL samples and in four of six T-ALL samples (Supporting Information Table 5). In BCP-ALL, BLNK (SLP-65) is considered to act as a tumor suppressor and promotes differentiation of pre-B cells [15]. Phosphorylation of the peptides derived from BTK (BTK_S180 and BTK_Y223) could be observed in 100.0% and 70.0% of the ALL samples, respectively (Table 1). As BTK deficiency in B cell precursor leukemia cells results from kinase-deficient dominant-negative BTK splice variants, it was not unexpected to find phosphorylation of peptides derived from known BTK phosphorylation sites [16].

The IL-7R activates the JAK/STAT pathway both in BCP-ALL and T-ALL. Of this pathway only peptides derived from STAT5 were present on the array. Phosphorylation of the STAT5A_S780, STAT5A_Y694, and STAT5B_Y697 could be observed in 35.0%, 85.0%, and 70.0% of the ALL samples, respectively (Table 1). Protein phosphorylation of STAT5A and B proteins could be validated using the human phosphokinase microarray data (Supporting Information Fig. 3B).

In T-ALL cells, Notch1 is the most prevalent pathway, resulting in *MYC* upregulation and pre-TCRA and NF-kappaB activation [5]. No peptides derived from Notch1 were present on the array. Phosphorylation of c-Myc_S62, c-Myc_T58, and N-Myc_S263 could be appreciated in all six T-ALL samples and in 14 of 14, 14 of 14, and 12 of 14 BCP-ALL samples, respectively (Supporting Information Table 5). The transit proteins between the precursor T cell receptor and downstream signaling proteins are Fyn, Lck, ZAP70, and SLP76. Phosphorylation of the peptides derived from Lck, ZAP70, and SLP76 were spotted on the array. Lck_S158, Lck_Y192, and Lck_Y411 were phosphorylated in 80.0%, 60.0%, and 55.0% of the ALL samples, respectively, with no differences in phosphorylation intensity or frequency between BCP-ALL and T-ALL (Table 1, Supporting Information Table 5 and Fig. 3A). Phosphorylation of the peptides ZAP70_Y319, ZAP70_Y474, and ZAP70_Y493 could be detected in 80.0%, 85.0%, and 50.0% of the ALL samples, respectively. The peptides derived from SLP76 (SLP76_Y113 and SLP76_Y423) were both phosphorylated in 75.0% of the samples (Table 1).

Peptides corresponding to the NF-kappaB signaling (NF-kappaB1_S907, NF-kappaB1_S932, NF-kappaB3_S263, NF-kappaB3_S298, and NF-kappaB3_S536) were also commonly phosphorylated in the ALL patients (95.0%, 40.0%, 100.0%, 100.0%, and 35.0%, respectively).

3.3 RON as a new potential druggable target in pediatric ALL

RTKs are potent targets for therapeutic interventions. In our list of commonly activated peptides we noticed several growth factor receptors including HGFR. HGFR (c-Met) and its ligand HGF were shown to promote oncogenic transformation of solid tumors as well as myeloid leukemias [17]. Expression of HGFR has not been shown to be associated with ALL. Therefore, we examined the role of HGFR as a potential target in ALL.

The peptide derived from HGFR_Y1235, also known as c-Met, showed high phosphorylation intensity in all primary ALL samples, however, membrane and intracellular c-Met protein expression could not be detected in any of the four ALL cell lines examined, using flow cytometry analysis (Fig. 4A). The peptide derived from HGFR_Y1235 is highly identical to sequences corresponding to other proteins e.g. RON (MST1R), Janus kinase 1 (JAK1), and Janus kinase 2 (JAK2). Since JAK kinases are extensively described in ALL, we sought to examine RON protein expression in these cells. RON membrane and intracellular protein expression could be detected in all four ALL cell lines with membrane expression levels ranging from 42.6% (RCH-ACV) to 96.1% (Jurkat) (Fig. 4A). To test the RON dependency of ALL cells we performed shRNA mediated knockdown of RON expression. Effective knockdown of RON protein expression was observed

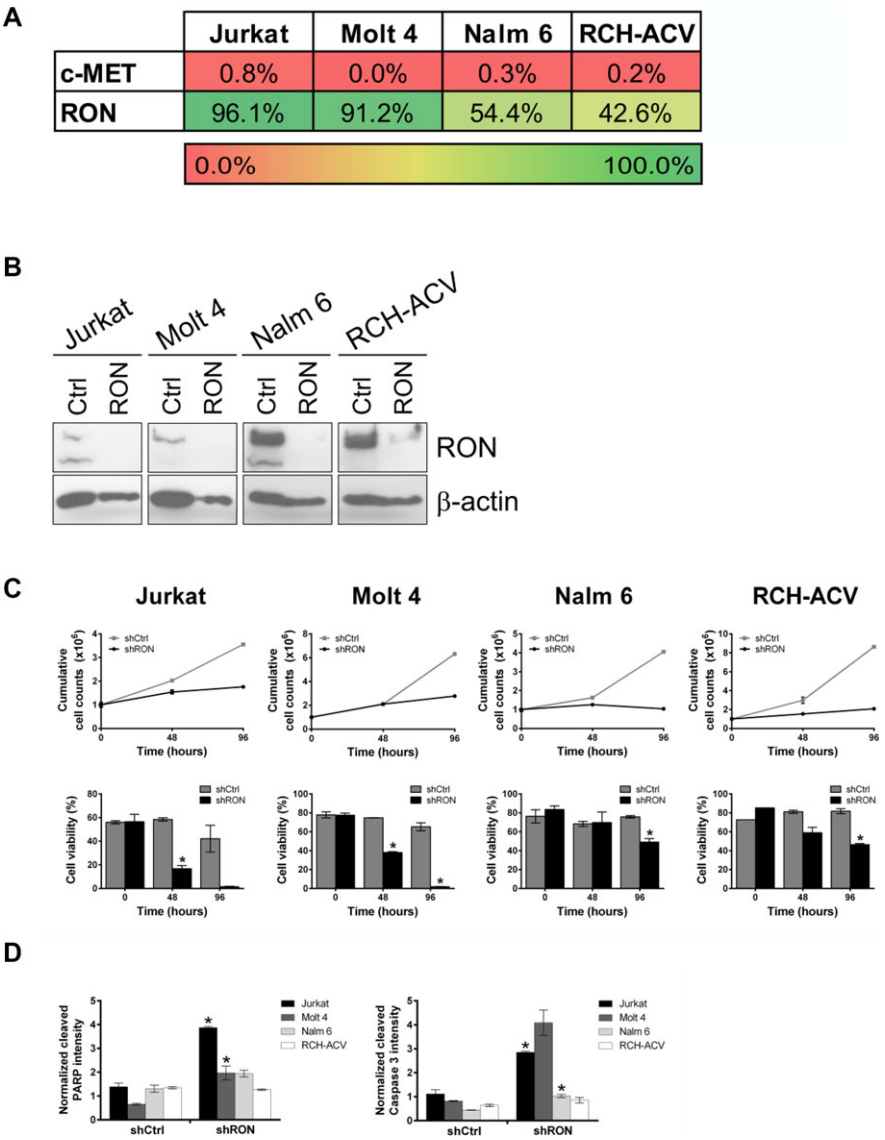

Figure 4. RON as potential druggable target. Membrane c-Met and RON protein expression levels as measured by flow cytometry in four ALL cell lines (A). RON was successfully downregulated in four ALL cell lines using RON and Control shRNAs (shCtrl) as shown by Western blot analysis (B). Growth curve analysis after transduction showed a decrease in cell growth (C, upper panels) and a decrease in cell viability (C, lower panels). Cleaved PARP and Caspase 3 expression in RON transduced cells indicate an increase in apoptotic cells (D). Results are shown as the mean ± standard deviation of two experiments. Asterisks (*) indicate significant differences ($p < 0.05$) by Student's *t*-test in comparison with control.

in all cell lines, as shown by Western blot analysis (Fig. 4B). Growth curve analysis demonstrated a clear effect in cumulative cell counts (Fig. 4C upper panels), accompanied by an increased reduction in cell viability Fig. 4C lower panels). Effects on cell viability were most pronounced in the T-ALL cell lines Jurkat and Molt 4. An increase in cleaved PARP and Caspase 3 indicated cell death based on apoptosis (Fig. 4D). These results indicate that RON expression contributes to leukemic cell growth and survival of ALL cell lines.

4 Discussion

Cancer is the result of various genetic, epigenetic, and/or environmental aberrations, leading to an abnormal activity of signal transduction pathways. Activity of signal transduction pathways could be measured by kinase activity profiling to elucidate interesting signaling hubs to identify potential druggable targets as we showed for pediatric brain tumors and acute myeloid leukemia [11–13]. In this study we provide novel insights into active signal transduction pathways in ALL and especially described more unknown signaling proteins relevant in ALL and of potential interest for future targeted therapy approaches. Furthermore, in order to test the applicability of the list of commonly activated peptides on leukemogenesis, we selected one target (c-Met/RON) for further validation.

Classifying commonly activated peptides in pediatric ALL to their corresponding signal transduction pathways shows that many peptides can be aligned to well known signal transduction pathways downstream of the pre-BCR and pre-TCRA e.g. the MAPK pathway and the PI3K/Akt pathway. Moreover, we observed peptides derived from regulators of the cell cycle including components of the p53 and Rb1 signaling

pathways. This is in line with previous results showing that activation of the CDK6/Rb1 oncogenic pathway is observed in 59% of the ALL patients and is associated with a poor prognosis [18].

We examined the list of differentially phosphorylated peptides in more detail. BTEB2_S163, CD2_Y281, p73_Y99, OPN_S148, CDX2_S60, and TrkA_Y496 have been associated with ALL. *BTEB2, (KLF5)*, mRNA expression is widely expressed in pediatric ALL and regulates the p53-mediated repression of the antiapoptotic protein Survivin, involved in chemotherapeutic induced apoptosis and consequently in drug resistance [19]. Since downregulation of *BTEB2* using siRNAs inhibits Survivin expression and sensitizes ALL cells to chemotherapy, BTEB2 inhibition has been put forward as a potential target for the treatment of drug resistant ALL [19]. CD32, also known as the FcγRII receptor, is an antibody binding receptor expressed on B-cells but also on monocytes, macrophages, neutrophils, and platelets [20, 21]. In BCP-ALL CD32 was found to be expressed by leukemic blast cells in approximately 66% of the childhood BCP-ALL cases [22]. P73 is a member of the p53 tumor suppressor family and has multiple isoforms, with either pro- or anti-apoptotic activities [23]. Mutations in the *p73* gene in ALL are rare but p73 protein expression is observed in the majority (60%) of the ALL patients [24, 25]. Immunohistochemistry showed that OPN expression and the presence of OPN receptors could be detected in adult B-ALL [26]. OPN is thought to be involved in cell adhesion to gain indirect protection from cytotoxic chemotherapy [26]. Recently, it has been demonstrated that the combination of OPN antibodies with arabinofuranosyl cytidine significantly reduces minimal residual disease in adult B-ALL [26]. *CDX2* expression was found previously in the majority of the BCP-ALL and T-ALL samples and high *CDX2* expression levels were associated with an inferior overall survival for both pediatric and adult ALL patients [27, 28]. Furthermore, shRNA mediated downregulation of *CDX2* in the pre-B ALL cell line Nalm 6 resulted in a significantly impaired clonogenic growth potential [28]. Also, our results underscore that *CDX2* may play a pivotal role in leukemogenesis and is an interesting protein to study. TrkA is a neurotrophic tyrosine kinase receptor important for tumor formation and progression [29]. *TrkA* transcription levels are significantly upregulated in ALL, acute myeloid leukemia, chronic myeloid leukemia, and myelodysplastic syndrome [29]. In leukemic cell lines, TrkA regulates the Akt/mTOR pathway and inhibition of TrkA resulted in apoptosis and an impaired proliferation [29]. This short overview of existing literature illustrates that kinome profiling is an elegant approach to identify potential known targets eligible for further targeted therapy studies.

Since the tertiary structure of phosphorylation sites and the spatiotemporal regulatory mechanisms are lost, in vitro kinase activity profiling may reveal phosphorylation of specific peptides which will not occur in vivo [30]. Additionally, considering our kinome profiling platform uses peptides sequences of approximately 11 amino acids in length, crossphosphorylation between family members may occur. We showed that the sequence of the peptide derived from c-Met is 86% identical to sequences of JAK1 and JAK2 and 75% identical to sequences corresponding to RON and since we were not able to detect c-Met protein expression, we concluded that phosphorylation of the peptide derived from HGFR_Y1235 might represent activity of other kinases e.g. RON or JAK1/2. This phenomenon might also explain why peptides derived from B or T-cell lineage specific proteins were phosphorylated in both lineages. For instance Syk, a known B cell lineage specific protein, and ZAP70, a T-cell lineage specific protein, both belong to the Syk family and are highly homologous. The same holds true for BLNK and SLP76, respectively.

RON expression was shown to be required for proliferation of Epstein-Barr virus immortalized primary B lymphoblastoid cell lines cells [31]. Our own results show that RON is expressed in all four leukemic cell lines tested while downregulation of RON protein expression results in a decreased leukemic cell growth and cell survival. Hence it will be interesting to further explore the role of RON in ALL.

In summary, these results show that kinome profiling is an elegant approach to study kinase activity by peptide phosphorylation which allows the rapid identification of known and unknown relevant signaling proteins in ALL. Our list of commonly activated peptides will be able to guide future research into specific proteins as well as targeting options.

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