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Characterization of aspects of leukemia biology and association with patient prognosis

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List of abbreviations

ac	activated caspase-3
AIEOP-BFM	Assoziacione Italiana Ematologia Oncologia Pediatrica and Berlin-Frankfurt-Münster study group
AKT	serine/threonine kinase involved in different cellular functions
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
APAF-1	apoptotic protease activating factor-1
APC	adenomatous polyposis coli
B2M	beta-2-microglobulin
BCA	bicinchoninic acid
BCL-2	B-cell lymphoma 2
BCL-XL	B-cell lymphoma-extra large
BCP	B-cell precursor
BCR-ABL	fusion gene expressed from a t(9;22) chromosomal translocation, also known as Philadelphia chromosome, encoding a tyrosine kinase
BID	BH3 interacting domain death agonist
BSA	bovine serum albumin
bp	base pairs
CAD	caspase-activated deoxyribonuclease
cAMP	cyclic adenosine monophosphate
cc	cytochrome c-release
cd	cell death
CD	cluster of differentiation
cDNA	complementary DNA
CI	confidence interval
CLL	chronic lymphocytic leukemia
CML	chronic myeloid leukemia
CNS	central nervous system
COX2	cytochrome c oxidase subunit 2

CO_2	carbon dioxide
CRAC	cytochrome c-related activation of caspase-3
CRLF2	cytokine receptor-like factor 2
DAB	diaminobenzidine
DAPK1	death-associated protein kinase 1
ddH_2O	double-distilled water
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
dsDNA	double-stranded DNA
DTT	dithiothreitol
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ETV6-RUNX1	formerly known as *TEL/AML1*, fusion gene expressed from a t(12;21) chromosomal translocation
FAB	French-American-British classification system of hematologic diseases
FADD	Fas-associated protein with death domain
FBS	fetal bovine serum
FDR	false discovery rate
FITC	fluorescein isothiocyanate, fluorochrome
FOXO3A	forkhead box O3
FSC	forward scatter
GWAS	genome-wide association study
HPB-ALL	human T cell leukemia cell line
HRP	horseradish peroxidase
IAPs	inhibitor of apoptosis proteins
IKZF1	IKAROS family zinc finger 1
JAK2	Janus kinase 2
LIVIN	also known as *BIRC7* (baculoviral IAP repeat containing 7), encoding a member of the inhibitor of apoptosis (IAP) proteins
MCL-1	myeloid cell leukemia sequence 1
MLL	mixed-lineage leukemia
MOMP	mitochondrial outer membrane permeabilization

MOPC	mouse IgG1 kappa from plasmacytoma cells
MRD	minimal residual disease
mTOR	mammalian target of rapamycin
NaCl	sodium chloride
NF-1	neurofibromatosis type 1
NOD	non-obese diabetic
NSCLC	non-small cell lung cancer
NSG	NOD/SCID/IL2Rcγnull
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDE4A	phosphodiesterase 4A
PE	phycoerythrin, fluorochrome
PerCp	peridinin chlorophyll protein, fluorochrome
PFA	paraformaldehyde
PKC	protein kinase C
p-value	probability value
q-value	FDR adjusted p-value
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RPPA	reverse phase protein array, also RPMA (reverse phase protein microarray)
r_s	spearman's rho, also spearman's rank correlation coefficient
RT	reverse transcription
SCID	severe combined immunodeficiency
SDHA	succinate dehydrogenase complex, subunit A
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SSC	side scatter
T_A	annealing temperature
TBP	TATA box binding protein
TIFF	tagged image file format
TTL	time to leukemia

WBC	white blood cell count
WGS	whole-genome sequencing
XIAP	X-linked inhibitor of apoptosis protein, also known as *BIRC4*

1. Introduction

Acute lymphoblastic leukemia (ALL) is a disease of aberrantly proliferating lymphoid progenitor cells and it is the most common cancer in childhood and adolescence [124]. The word leukemia is formed from the two Greek expressions *leukos*, for 'white' and *haima*, for 'blood' and the disease was first described by Rudolf Virchow in 1845. The first case of acute leukemia in children was described by Michael Anton Biermer in 1860 [104].

1.1 Epidemiology of leukemia

Leukemia accounts for approximately one third of all pediatric malignancies, of which 78.6 % are ALL and the incidence in Europe is 3.4/ 100 000 per year in children below 15 years of age. The median age at diagnosis is 4 years and 9 months and the occurrence is more frequent in boys than in girls [72]. Although therapy efforts have improved cure rates to above 80 %, every fifth patient still encounters relapse, associated with inferior survival [25, 102, 123].

1.2 Etiology

Malignant transformation of lymphoid progenitor cells leads to blocked differentiation, altered response to growth signals, clonal expansion and evasion of cells from apoptosis, resulting in accumulation of leukemia cells in the bone marrow [52, 121]. The origin of the disease is dependent on a number of factors, involving genetic predisposition and environmental factors [46]. Studies in concordant twins with leukemia [42] or one preleukemic and a leukemic twin [60] indicate fetal origin leading to predisposition for leukemic transformation. Furthermore, an increased risk exists for children with hereditary syndromes such as trisomy 21 [41] and type 1 neurofibromatosis (*NF-1*) [145]. Supposed environmental factors are certain types of chemicals, notably benzene metabolites and anti-cancer drugs (particularly inhibitors of topoisomerase II), exposure to ionizing radiation, infections and exposure of parents to toxins [6, 46, 67, 82]. Overall, the etiology is not finally clarified.

1.3 Clinical presentation

Clinical signs are predominantly related to suppression of normal hematopoiesis and extramedullary infiltration by leukemic cells. Typical symptoms are fever,

fatigue, weight loss, and bone pain. Moreover, patients frequently demonstrate anemia, thrombocytopenia and neutropenia, leading to pallor, an increased risk of bleeding and infections. Further characteristics are lymphadenopathy and hepatosplenomegaly. CNS involvement is present in 5 % of patients at diagnosis and can cause neurological symptoms including headache and vomiting [36].
The diagnosis of ALL is based on bone marrow aspiration, showing at least 25 % of the nucleated cells to be lymphoblasts [169].

1.4 Classification

Pediatric ALL is a heterogeneous disease and can be further classified based on morphologic, immunologic or genetic criteria.
Morphologic classification is performed by microscopic assessment of leukemic blasts according to the French-American-British (FAB) classification system, classifying ALL into three distinct groups (L1, L2 and L3) [16]. Immunophenotyping, using a panel of antibodies directed to cell surface and cytoplasmatic molecules, enables immunologic classification of ALL cells. ALL is divided into B- and T-lymphoblastic leukemia and can be further classified according to specific markers [15]. B-cell precursor ALL is more frequently in children than T-ALL or mature B-ALL. The latest update of the World Health Organization (WHO) classification of ALL distinguishes different subgroups, defined by genetic abnormalities [169]. Moreover, immunologic and cytogenetic ALL subgroups can be distinguished by specific gene expression profiles, however this technique is not used in routine diagnostics [51, 179].

1.5 Prognostic factors

In current clinical trials risk stratification of B-cell precursor ALL is based on characteristics of the patients and of the disease at diagnosis and on response to initial treatment [102, 123]. In the AIEOP-BFM ALL 2000 protocol, patients are classified as standard-risk (SR), medium-risk (MR) or high-risk (HR) [26]. Stratification criteria in this protocol are cytogenetics (positivity for t(4;11) or t(9;22)), blast cell count in peripheral blood on day 8 after treatment with prednisone and one intrathecal dose of methotrexate and minimal residual disease (MRD) levels at different time points during therapy in order to detect remaining ALL cells at submicroscopic level by quantitative PCR [167].

Others have addressed further prognostic factors. For example, gender, age, ethnicity and CNS disease have been evaluated for risk stratification in the Total Therapy Study XIIIB at St Jude Children's Research Hospital, but did not have prognostic significance [122].

Cytogenetic alterations, including numerical and structural chromosomal changes are frequent in ALL and associated with treatment response and outcome. Hyperdiploidy of 50 or more chromosomes and combined trisomies 4 and 10 are associated with favorable prognosis [53, 173], in contrast, hypodiploidy of less than 45 chromosomes is indicative of poor outcome [54]. Down's syndrome (trisomy 21) is also reported to be associated with inferior outcome [33, 172]. The gene fusion of *ETV6-RUNX1* (formerly designated as *TEL-AML1*), resulting from translocation t(12;21) is present in 22 % of pediatric ALL and associated with favorable prognosis [149]. Fusions of *BCR-ABL1* (corresponding to t(9;22), Philadelphia chromosome, encoding a constitutively active tyrosine kinase) are present in 3 % to 5 % of children with ALL and are associated with poor prognosis [9, 141]. However, targeted therapies by tyrosine-kinase inhibitors (TKI) are currently tested in clinical trials and are expected to improve treatment outcomes in the future [66, 144]. Furthermore, rearrangements of the *MLL* gene are predominantly present in infant leukemia and associated with a poor prognosis [14].

Recently, measurement of MRD on day 15 by flow cytometry in bone barrow, after 14 days of steroids, one intrathecal dose of methotrexate and treatment with vincristine, daunorubicine and asparaginase, was shown to be a powerful predictor of relapse [12] and is currently evaluated in the ongoing BFM trials. Furthermore, genome-wide association studies (GWAS) might be an important tool to predict outcome in the future. Germline single-nucleotide polymorphisms (SNPs) have been shown to be associated with treatment response in pediatric ALL [147, 176, 177] and patients with high risk for relapse were shown to have a distinct gene expression profile [95, 179]. Moreover, genomic analyses have successfully identified alterations of single genes, which are associated with prognosis. Deletion or mutation of *IKZF1* (IKAROS family zinc finger protein 1) leads to expression of aberrant isoforms of the transcription factor IKAROS and is associated with poor prognosis of these patients [106]. Over-expression of *CRLF2* (Cytokine receptor-like factor 2) is a major feature of Down's syndrome ALL and

associated with activating mutations in *JAK2* (janus kinase 2) and poor outcome [56, 105, 132]. Moreover, attempts by whole-genome sequencing (WGS) have been undertaken like the “Pediatric Cancer Genome Project”, which started in 2010, analyzing a large number of pediatric tumor samples and matched germline samples [34]. Meanwhile, several genetic alterations in different types of pediatric cancer have been identified [49, 61, 180, 181]. These findings might contribute to new therapeutic options in the near future.

1.6 Apoptosis signaling

Programmed cell death (apoptosis) plays an important role in cell turnover and it is critical for embryonic development [28, 76]. However, deregulation of apoptosis signaling contributes to numerous diseases including cancer, autoimmune disorders or degenerative diseases [157]. Apoptosis might be induced by activation of the extrinsic and/ or intrinsic pathway [44, 55] (Figure 1). The extrinsic pathway is activated by signals from the cell’s environment which are detected by death receptors on the cell surface [11]. The intrinsic pathway of apoptosis is mainly triggered by cellular stresses such as DNA damage or lack of oxygen [47]. A critical protein, released from mitochondria in the intrinsic pathway, is cytochrome c [88]. When released to the cytosol, it binds to a molecule named apoptotic protease activating factor-1 (Apaf-1), leading to formation of a multimeric complex, called apoptosome and to subsequent activation of procaspase-9 [69, 83]. Conclusively, effector caspases, such as caspase-3 are cleaved and thereby activated, leading to DNA degradation by activation of caspase-activated deoxyribonucleases (CAD) [135, 151, 165]. The apoptosis pathway is complexly regulated by various mechanisms. Critical regulators of apoptosis signaling are molecules of the BCL-2 (B-cell lymphoma 2) protein family, with members showing pro-apoptotic activity on the one side and anti-apoptotic activity on the other side [8, 48, 129]. Furthermore, inhibitor of apoptosis proteins (IAPs) suppress apoptosis by inhibition of caspases [32]. It has been shown that chemotherapeutic drugs induce apoptosis signaling in cancer cells [29, 75] and that chemotherapy-induced apoptosis of leukemia cells involves both the extrinsic and intrinsic pathways [43, 99]. However, the ability to suppress apoptosis is considered to be a feature of certain cancer cells and responsible for treatment failure and relapse in cancer patients [38, 52]. During the last years, the expression levels of certain apoptosis

regulating molecules have been addressed in several studies on different types of leukemia concerning a potential prognostic impact. For example, the anti-apoptotic molecule BCL-XL (B-cell lymphoma-extra large) was investigated in childhood ALL and it has been reported to be associated with good prognosis [4] or not to have prognostic significance [136]. However, the expression level of single apoptosis regulating molecules might not reflect the ability of proficient apoptosis signaling of leukemia cells. In previous studies of our group, the ability of leukemia cells to undergo apoptosis was determined on a functional level by analyzing two key apoptogenic events: (i) mitochondrial release of cytochrome c and (ii) activation of the effector caspase-3. Analysis of primary patient ALL and AML leukemia cells revealed that proficient apoptosis signaling was exclusively found in patients with favorable outcome [92, 93].

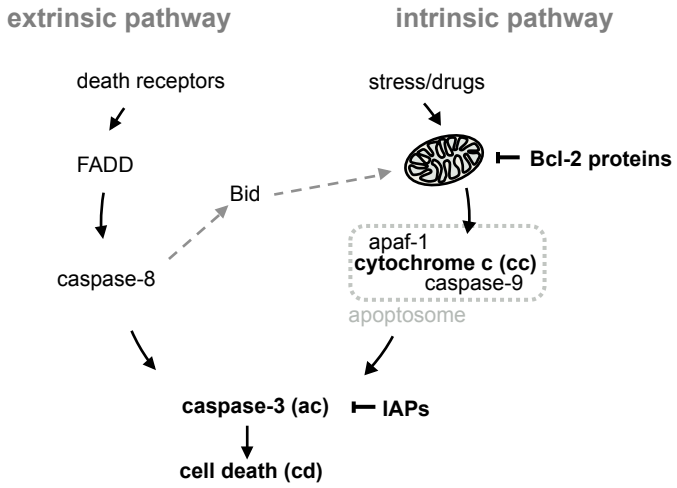

Figure 1. Apoptosis signaling
Apoptosis signal transduction is activated for example by cytotoxic drugs or cellular stress. Extrinsic pathway: activation of death receptors. Binding of FADD (Fas-associated protein with death domain) leads to activation of caspases. Intrinsic pathway: release of cytochrome c (cc) from the mitochondria to the cytosol leads to binding to apoptotic protease activating factor-1 (Apaf-1) and to activation of caspase-9. Downstream effector caspases, such as caspase-3 (ac) are cleaved and thereby activated, ultimately leading to cell death (cd). BCL-2 proteins and inhibitor of apoptosis proteins (IAPs) regulate the apoptosis pathway. BID (BH3 interacting domain death agonist) connects the extrinsic and intrinsic pathway.

Modified figure originally published in [125]. doi:10.1038/cddis.2012.107. Reproduced with kind permission from Nature Publishing Group.

1.7 NOD/SCID xenograft mouse model

In another recent study carried out in our research group, different engraftment phenotypes of patient ALL samples were identified in the NOD/SCID/huALL (non-obese diabetic/severe combined immunodeficiency/human acute lymphoblastic leukemia) mouse model [95]. Time after transplantation to clinical manifestation of leukemia in the recipients has been identified to be associated with patient

outcome. Time to leukemia (TTL) indicates the period from transplantation to clinical manifestation of leukemia in the recipients. Leukemia samples can be classified as TTL^{short}, indicating manifestation of leukemia in the recipients within ten weeks after transplantation and TTL^{long}, which is characterized by an engraftment time of more than ten weeks. Importantly, the TTL^{short}-phenotype is associated with early relapse and inferior relapse free survival of the corresponding patients compared to samples showing a TTL^{long}-phenotype. Moreover, our research group further characterized the TTL-phenotypes by gene expression profiling (GEP), identifying signatures, which point to distinctive survival/ cell death pathways.

1.8 Aims of the study

In this thesis, two different investigations were carried out with respect to further characterization of the TTL-engraftment phenotypes in pediatric BCP-ALL xenograft samples in order to elucidate molecular mechanisms accompanying for the distinct TTL-phenotypes and identifying potential markers for outcome prognostication:

(i) Analysis of apoptosis signaling parameters and of selected apoptosis regulating molecules and (ii) screening for differentially regulated proteins between the TTL subgroups were performed.

(i) The aim of the first part of this study was to characterize the impact of apoptosis signaling of human leukemia cells on engraftment properties in NOD/SCID mice and on treatment response and the clinical outcome of the corresponding patients by analyzing key steps of the apoptosis pathway, namely mitochondrial release of cytochrome c and activation of caspase-3, using a functional assay in *ex vivo* experiments. Furthermore, expression of transcript and protein levels of apoptosis signaling molecules, previously identified by GEP analysis in our microarray study, were evaluated in larger sample cohorts. In addition, apoptosis regulating molecules of the BCL-2 family and of the inhibitor of apoptosis proteins (IAPs) were addressed.

(ii) In the second part, a screening approach was employed in order to identify molecules differentially expressed on protein level comparing both TTL subgroups. Therefore, a reverse phase protein array (RPPA) and subsequent validation by western blot analysis were used.

2. Materials and methods

2.1 Materials

2.1.1 Solutions

Aprotinin	Sigma-Aldrich, Italy
Biocoll separating solution	Biochrom AG, Germany
Bovine serum albumin (BSA)	Serva, Germany
Fast Green FCF	Sigma-Aldrich, Italy
Fetal Bovine Serum (FBS)	Gibco Invitrogen, Germany
Glycine	Sigma-Aldrich, Germany
HBSS (Hank's Balanced Salt Solution)	Gibco Invitrogen, Germany
I-BLOCK	Tropix, USA
Leupeptin hydrochloride	Sigma-Aldrich, Italy
L-Glutamine 200 mM	Gibco Invitrogen, Germany
Methanol	Sigma-Aldrich, Germany
MOPC (Clone Number MOPC 21)	Sigma-Aldrich, Germany
Novex Tris-Glycine SDS Sample Buffer	Invitrogen, Italy
PageRuler Prestained Protein Ladder	Fermentas, Germany
Paraformaldehyde (PFA)	Sigma-Aldrich, Germany
PBS	Biochrom, Germany
Pefabloc AEBSF	Roche, Italy
Penicillin-Streptomycin	Gibco Invitrogen, Germany
Pepstatin A	Sigma-Aldrich, Germany
Powdered milk	Roth, Germany
RPMI 1640 medium	Gibco Invitrogen, Germany
Saponin	Sigma-Aldrich, Germany
Sodium azide	Sigma-Aldrich, Germany
Sodium dodecyl sulfate	Sigma-Aldrich, Germany
T-PER	Pierce, Italy
Trizma base	Sigma-Aldrich, Germany
TWEEN 20	Sigma-Aldrich, Germany

2.1.2 Cell lysates

A431 + EGF Cell Lysate	BD Biosciences, Europe

HeLa + Pervanadate Cell Lysate	BD Biosciences, Europe
Jurkat Cell Lysate	BD Biosciences, Europe
Jurkat Apoptotic Lysate	BD Biosciences, Europe

2.1.3 Antibodies used for flow cytometry

CD19 PerCp	BD Biosciences, Europe
Goat F(ab')$_2$ Anti-Mouse IgG$_{2b}$ FITC	SouthernBiotech, Germany
Mouse IgG$_1$ PE	BD Biosciences, Europe
Mouse IgG$_1$ PerCp	BD Biosciences, Europe
Mouse IgG$_{2b}$	Dako, Germany
PE Rabbit Anti-Active Caspase-3	BD Pharmingen, Europe
Purified Mouse Anti-Cytochrome C	BD Pharmingen, Europe

2.1.4 Antibodies used for reverse phase protein array

2.1.4.1 Primary antibodies

In this study, 51 primary antibodies directed to key signaling molecules involved in different pathways such as proliferation, differentiation and apoptosis were used. All antibodies have previously been validated by western blots (performed by Benedetta Accordi, Gloria Milani and Luisa Galla, Department of Pediatrics, Padova University, Italy). For further information of the primary antibodies used for RPPA see Table A1 in the Appendix.

2.1.4.2 Secondary antibodies

Biotinylated Goat Anti-Rabbit IgG Antibody	Vector Laboratories, USA
Polyclonal Rabbit Anti-Mouse Immunoglobulins	Dako, Italy

2.1.5 Antibodies used for western blot analysis

Purified Mouse Anti-Bcl-2	BD Biosciences, Europe
Polyclonal Rabbit Anti-Mcl-1	Stressgen, Canada
Monoclonal Mouse Anti-XIAP	BD Biosciences, Europe
Monoclonal Mouse Anti-Livin	Imgenex, USA
Anti-PKCα, clone M4	Upstate, Italy
Anti-Phospho-PKCα S657	Upstate, Italy
Purified Mouse Anti-Annexin I	BD Biosciences, Europe
Purified Mouse Anti-ß-Catenin	BD Biosciences, Europe

Purified Mouse Anti-Cyclin B	BD Biosciences, Europe
Monoclonal Anti-ß-Actin antibody	Sigma-Aldrich, Germany
Goat anti-mouse IgG-HRP	Santa Cruz Biotechnology, Europe
Goat anti-rabbit IgG-HRP	Santa Cruz Biotechnology, Europe

2.1.6 Consumption items

24-well Cell Culture Plate	BD Biosciences, Europe
384-Well Low Profile Microplates	Genetix, Italy
Amersham Hyperfilm ECL	GE Healthcare, Germany
Criterion Tris-HCl Precast Gels	Bio-Rad, Germany
FAST slides	Whatman Schleicher & Schuell, USA

2.1.7 Instruments

2470 Arrayer	Aushon Biosystems, USA
EL800 Microplate Reader	BioTek, Germany
Criterion Cell	Bio-Rad, Germany
Dako Autostainer Plus	Dako, USA
FACSCalibur flow cytometer	BD Biosciences, Europe
LightCycler 2.0	Roche, Germany
MJ Research PTC-200 Thermo Cycler	Bio-Rad, Germany
OPTIMAX X-ray Processor	PROTECT medical systems, Germany
Photometer GeneQuant 1300	GE Healthcare, Germany
ScanArray 4000	Packard Biochip Technologies, USA
Trans-Blot SD Semi-Dry Transfer Cell	Bio-Rad, Germany

2.1.8 Kits

Amersham ECL Advance	GE Healthcare, Germany
BCA-Protein Assay Kit	Pierce, Europe
Biotin Blocking System	Dako, Italy
CSA, Catalyzed Signal Amplification System	Dako, Italy
LightCycler DNA Master SYBR Green I	Roche, Germany
RNeasy Mini Kit	Qiagen, Germany

Superscript II Reverse Transcriptase	Invitrogen, Germany
Taq PCR Core Kit	Qiagen, Germany

2.1.9 Software

BD CellQuest Pro	BD Biosciences, Germany
Excel 2011	Microsoft, USA
GeNorm	Biogazelle, Belgium
Prism 6.0	Graphpad, USA
ImageJ 1.45s	NIH Image, USA
MicroVigene	VigeneTech, USA
PASW Statistics 18.0.0	IBM, Germany

2.2 Culture of NOD/SCID/huALL xenograft samples

Leukemia xenograft samples derived from pediatric BCP-ALL patients were isolated from spleens of engrafted mice and cultured *ex vivo*. Mononuclear cells were separated from spleens of recipients by ficoll density gradient centrifugation for 15 min with 1300 rpm at room temperature and purity of minimum 80 % leukemic cells was reached in all samples. Cells were cultured *ex vivo* for 16 hours in RPMI 1640 cell culture medium containing 10 % FBS, L-Glutamine and Penicillin-Streptomycin at 37°C in a humidified atmosphere with 5 % CO_2. Culture was performed in 24-well plates each with $1x10^6$ cells in 1 ml of volume.

2.3 Flow cytometry

Cells were stained with antibodies against surface leukemia markers and intracellular apoptosis molecules. After centrifugation for 7 min with 1300 rpm at 4°C in washing buffer (1 % BSA and 0.1 % sodium azide in PBS), surface staining with anti-CD19-PerCp was performed, incubating cells for 20 min at 4°C in the dark, followed by another washing step. Thereafter, cells were fixed with 4 % PFA (paraformaldehyde) for 20 min at 4°C and permeabilized with 0.2 % saponine supplemented with MOPC (IgG1 kappa from murine myeloma) in order to block unspecific binding. Levels of intracellular cytochrome c and active caspase-3 were studied. Anti-cytochrome c antibody, binding exclusively to its mitochondrial form and PE-conjugated rabbit anti-active caspase-3 antibody were added and incubated for 20 min at 4°C. After another washing step, staining with FITC-conjugated secondary goat $F(ab')_2$ anti-mouse IgG_{2b} was performed. After

incubation and a last washing step, cells were kept in 100 µl 4 % PFA. As negative isotype controls, cells were stained with unspecific mouse IgG_{2b} antibody followed by FITC-conjugated secondary goat $F(ab')_2$ anti-mouse IgG_{2b}, PE-conjugated mouse IgG1 and PerCp-conjugated mouse IgG1, respectively. Furthermore, cell death was detected by forward/side scatter criteria. Flow cytometric measuring was performed on a BD FACSCalibur flow cytometer, acquiring 30 000 events and the CellQuest software was used for analyzing the data.

2.4 Analysis of gene expression

Transcript levels of pro-apoptotic *DAPK1 (*death-associated protein kinase 1) and of the anti-apoptotic genes *MCL-1 (*myeloid cell leukemia sequence 1), *BCL-2* (B-cell lymphoma 2), *XIAP (*X-linked inhibitor of apoptosis protein), *LIVIN* (also known as *BIRC7* baculoviral IAP repeat containing 7) and *PDE4A (*phosphodiesterase 4A) were detected by quantitative real-time PCR.

2.4.1 RNA extraction

Ribonucleic acid (RNA) was extracted from isolated leukemia cells, using RNeasy Mini Kit (Qiagen, Germany). Cells were disrupted by dilution with a lysis buffer and homogenized by centrifugation on a spin column in order to decrease the viscosity of the lysate. After three washing steps, DNase (deoxyribonuclease) digestion was performed in order to eliminate DNA (deoxyribonucleic acid) contamination, followed by three further washing steps and dilution of the RNA in RNase (ribonuclease) free water. RNA concentration was determined by spectrophotometric analysis, measuring absorbance of ultraviolet light at 260 nm of each sample. We checked RNA quality by calculating the ratio of absorbance at 260 and 280 nm and exclusively samples with high purity were processed further.

2.4.2 Reverse transcription

Synthesis of complementary DNA (cDNA) was performed, employing Superscript II Reverse Transcriptase (Invitrogen, Germany). 500 ng of total RNA were denaturized for 3 min at 85°C in presence of random primers and sterile, distilled water. After supplementation with further components of the Superscript II Reverse Transcriptase kit (First-Strand Buffer, DTT, dNTP, H_2O, Superscript, RNAseOUT), conditions for incubation were 37°C for 15 min, 45°C for 30 min, 50°C for 15 min and 90°C for 2 min.

2.4.3 PCR

Quality of cDNA was checked by PCR on a MJ Research PTC-200 Thermo Cycler with the Taq PCR Core Kit (Qiagen, Germany) employing the following conditions. Pre-incubation took place at 95°C for 1 min, amplification for 40 cycles each for 1 min at 94°C, for 1 min at a primer dependant temperature and for 1 min at 72°C, followed by 5 min at 72°C and finally cooling down to 4°C.

2.4.4 Gel electrophoresis

PCR products were separated by agarose gel electrophoresis in agarose gels between 1 % and 2 %.

2.4.5 Quantitative real-time PCR

Quantitative real-time PCR was performed on a LightCycler 2.0 (Roche, Germany), using LightCycler DNA Master SYBR Green I (Roche, Germany). SYBR Green I binds to double-stranded DNA (dsDNA), leading to enhanced fluorescence upon binding [182], and thereby allowing determination of the amount of generated dsDNA. The procedure was performed according to a standard protocol, using the following conditions. Pre-incubation for 10 min at 95°C to activate FastStart Taq DNA polymerase and denaturize the DNA, amplification for 40 cycles each for 10 s at 95°C, for 5 s at a primer dependent annealing temperature (T_A) and for 15 s at 72°C, subsequently melting curves were generated followed by cooling to 4°C. Expression levels of target genes were normalized by geometric averaging of different control genes, using GeNorm [168]. *SDHA*, *TBP* and *B2M* were identified as most stably expressed within six control genes tested and used throughout the study. The primer sequences used for PCR are summarized in Table 1.

Table 1. Primer sequences used for PCR

gene	primer sequence	T_A [°C]	product size [bp]
DAPK1	sense: 26s: 5'-CGAGGTGATGGTGTATGGTG-3'	65	122
	antisense: 26a: 5'-CTGTGCTTTGCTGGTGGA-3'		
PDE4A	sense: 14s: 5'-GCAGTGTTCACGGACCTGGAGATTCTCGC-3'	67	372
	antisense: 16a: 5'-GCGGTCGGAGTAGTTATCTAGCAGGAGGACCCC-3'		
BCL-2	sense: 1s: 5'-CCTTCACCGCGCGGGGACGCTTTG-3'	51	339
	antisense: 2a: 5'-GATAGGCACCCAGGGTGATGCAAGC-3'		
MCL-1	sense: 2s: 5'-CGAAGACGATGTGAAATCGTTGTCTCGAGTG-3'	63	1078
	antisense: 3a: 5'-GATATGCCAAACCAGCTCCTACTCCAGCAACA-3'		
XIAP	sense: 3s: 5'-GATTGGAAGCCCAGTGAAGACCCTTGGG-3'	56	246
(BIRC4)	antisense: 6a: 5'-CTTAATGTCCTTGAAACTGAACCCCATTCG-3'		
Livin	sense: 1s: 5'-GCATGGGCTCTGAGGAGTTGCGTCTG-3'	67	273
(BIRC7)	antisense: 3a: 5'-GCAGCTGGGAGTGAGTCTCCTGCACACTG-3'		
SDHA	sense: 12s: 5'-CATGCTGCCGTGTTCCGTGTGGG-3'	67	321
	antisense: 14a: 5'-GGACAGGGTGTGCTTCCTCCAGTGCTCC-3'		
TBP	sense: 2s: 5'-GAGGAAGTTGCTGAGAAGAGTGTGCTGGAG-3'	65	4931
	antisense: 3a: 5'-GTCAGTCCAGTGCCATAAGGCATCATTGG-3'		
B2M	sense: 2s: 5'-GTGGAGCATTCAGACTTGTCTTTCAGCAAGGAC-3'	65	140
	antisense: 2a: 5'-CACTTAACTATCTTGGGCTGTGACAAAGTCACATGG-3'		

T_A indicates annealing temperature; bp base pairs; *DAPK1* Death-associated protein kinase 1; *PDE4A* Phosphodiesterase 4A; *BCL-2* B-cell lymphoma 2; *MCL-1* Myeloid cell leukemia sequence 1; *XIAP* X-linked inhibitor of apoptosis protein, also known as *BIRC4*; *LIVIN* also known as *BIRC7* (baculoviral IAP repeat containing 7), encoding a member of the inhibitor of apoptosis proteins (IAP); *SDHA* Succinate dehydrogenase complex, subunit A; *TBP* TATA box binding protein; *B2M* Beta-2-microglobulin

2.5 Protein analysis

Expression and phosphorylation status of proteins were analyzed, employing the reverse phase protein array (RPPA) strategy as performed before [2] and results were validated by western blot.

2.5.1 Protein extraction

For protein analysis $1x10^7$ cells derived from spleens of leukemic NOD/SCID mice were washed with PBS (centrifugation for 2 min at 1150 rpm at 4°C) and lysed on ice for 20 min in T-PER (Tissue Protein Extraction Reagent), supplemented with NaCl 5M and protease and phosphatase inhibitors (Sodium orthovanadate, Pefabloc, Aprotinine, Pepstatin A, Leupeptin). Thereafter, samples were purified by centrifugation for 10 min at 10000 rpm at 4°C. Protein concentration was determined spectrophotometrically with the Pierce BCA (bicinchoninic acid)

Protein Assay [153], following standard protocols and samples were stored at -80°C.

2.5.2 Reverse phase protein array (RPPA)

In this study, xenografted human ALL samples were analyzed by RPPA. The protein samples were immobilized on slides, allowing analysis of multiple samples simultaneously at the same conditions. Each slide was tested for the expression of a single molecule and the protein expression was quantified and compared within the samples.

2.5.2.1 RPPA printing

Extracted proteins were diluted to 1 mg/ml with a Tris-Glycine SDS buffer + β-Mercaptoethanol and boiled at 97°C for 8 min. Thereafter, samples were loaded into a 384-well plate in a four-point dilution curve (from undiluted to 1:8) and printed in duplicates onto nitrocellulose-coated glass slides (FAST slides) using the Aushon 2470 Arrayer. Printed slides were stored desiccated at -20°C until use. Multiple slides were spotted to allow staining with the complete panel of specific antibodies.

2.5.2.2 RPPA staining

Arrays were blocked for 3 hours in blocking solution (2 g I-BLOCK and 0.1 % Tween 20 in 1 l PBS) prior to antibody staining in the Dako Autostainer Plus using the CSA (Catalyzed Signal Amplification) sytem. First, biotin blocking was performed, followed by protein block and incubation with primary antibody. Thereafter, biotinylated secondary antibodies (Biotinlyated Rabbit Anti-Mouse Immunoglobulins or Biotinylated Goat Anti-Rabbit IgG Antibody) were incubated, followed by incubations with Streptavidin-Peroxidase Complex, Amplification Reagent, Streptavidin-HRP (horseradish peroxidase) and finally Diaminobencidine-Chromogen Solution, which reacts with the enzyme HRP and results in a brown-colored product.

2.5.2.3 Total protein staining

Three slides were stained with Fast Green FCF in order to determine the amount of total protein immobilized in each spot on the slides. Fast Green FCF is capable of binding to proteins and absorbing light with a maximum at 625 nm. Slides were washed with PBS for 1 min and subsequently incubated with Fast Green FCF

(0.01% Fast Green FCF, 10% acetic acid, 30% ethanol, in ddH_2O) for 2 hours in the dark, followed by washing for 2 hours at room temperature with a washing solution (10 % acetic acid, 30 % ethanol, in ddH_2O). Slides were then dried in the dark overnight and scanned using the ScanArray 4000.

2.5.2.4 Quantification of protein expression

TIFF images of antibody-stained protein arrays were analysed using commercially available software specifically developed for RPPA analysis, the Microvigene Software. This software identifies the spots on the arrays and estimates the signal intensity of the staining, resulting in numeric values. For each slide the signal intensity of a negative control slide was subtracted and the resulting value was normalized to the total protein staining with Fast Green FCF, thus, a normalized value was calculated for each xenograft leukemia sample for each tested protein.

2.5.3 Western blot

2.5.3.1 Electrophoretic separation of proteins

Protein lysates were separated according to their electrophoretic mobility by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), employing Criterion Tris-HCl Precast Gels. Proteins were supplemented with Loading Dye Solution (60 mM Tris HCl, 2 % SDS, 10 % Glycerol, 5 % Beta-mercaptoethanol, 0.01 % Bromphenol blue) and denaturized for 9 min at 95°C. SDS-PAGE was performed at 120 V and max. 400 mA in Running Buffer (25 mM Tris, 192 mM Glycine, 0.1 % SDS) for approximately 2 h, resulting in movement of proteins towards the anode, according to their size and charge.

2.5.3.2 Semi-dry blot

Proteins, previously separated by SDS-PAGE, were transferred to nitrocellulose by semi-dry blotting [81] in a Trans-Blot SD Semi-Dry Transfer Cell. Transfer from Criterion Tris-HCl Precast Gels to Hypond ECL Nitrocellulose Membranes was done in Blotting Buffer (25 mM Tris, 192 mM Glycine, 20 % Methanol) for 1½ h at current density of 1 mA/ cm^2 with max. 20 V. Proteins move towards the anode and thus onto the membrane of nitrocellulose, keeping the same order as in the Criterion Tris-HCl Precast Gel.

Subsequently, membranes were blocked for 2 h in blocking buffer (5 % powdered milk in PBS-Tween) to reduce unspecific binding of the andibodies to the membrane.

2.5.3.3 Immunodetection of proteins

To detect specific proteins, nitrocellulose membranes were incubated with a primary antibody and subsequently with a secondary antibody, labeled to HRP. Nitrocellulose membranes were incubated overnight at 4°C with the primary antibody, diluted in BSAazid (2 % BSA and 0.02 % sodium azide). After washing with PBS-Tween incubation with secondary antibody, diluted in Blocking Buffer, was done for 1 h at room temperature. Goat anti-mouse or anti-rabbit IgG-HRP was used as secondary antibody and after a further washing step, membranes were incubated in Amersham ECL Detection Reagent, resulting in a luminescent reaction, catalyzed by HRP. Luminescent signals were determined on autoradiography films (Amersham Hyperfilm ECL), by OPTIMAX X-ray Processor.

2.5.3.4 Protein quantification

Protein expression was quantified by ImageJ software [143]. The software estimated the intensities of the protein bands and converted them into numeric values, which were normalized to the loading controls.

2.6 Statistics

Statistical analyses were performed using Microsoft Excel, GraphPad Prism and PASW Statistics 18.0.0 software and p-values of < 0.05 were considered significant in all tests throughout the study. Data are shown as means and error bars standard errors of the means (SEM). Survival analyses were performed using the Kaplan-Meier method and the log-rank test. Unless otherwise indicated, Mann-Whitney-U-Test was used to compare values of two groups and spearman correlation to test an association of two variables to each other.

3. Results

Parts of the results have been published (Queudeville, M., Seyfried, F. et al.: Rapid engraftment of human ALL in NOD/SCID mice involves deficient apoptosis signaling. Cell Death Dis *3*: e364 (2012) 3, e364; doi:10.1038/cddis.2012.107, M. Queudeville and F. Seyfried share the first authorship of this work) [125] and have been presented at national and international meetings as posters and oral communications (oral communication at the Annual Meeting of the Kind-Philipp Foundation for Leukemia Research, 2010 in Wilsede, poster at ACUTE LEUKEMIAS XIII, 2011 in Munich, poster at the 53rd ASH Annual Meeting, 2011 in San Diego and oral communication at the Annual Meeting of the Kind-Philipp Foundation for Leukemia Research, 2012 in Wilsede).

3.1 Characteristics of ALL samples

Altogether, 25 leukemia xenograft samples, which were established by transplantation of primary leukemia cells obtained from ALL patients at diagnosis onto NOD/SCID mice were analyzed in this study. In this cohort, we found a distribution of gender, age and immunophenotype reflecting the distribution of subtypes published in large patient cohorts [72, 90, 150].

In the first part of this study, we investigated apoptosis signaling parameters in patient-derived xenograft ALL samples including samples carrying *ETV6-RUNX1*, *BCR/ABL* and gene fusions of *MLL.* Characteristics of this patient cohort and derived xenograft samples are shown in Table 2.

Table 2. Characteristics of ALL samples analyzed on apoptosis signaling parameters

		N	%
total number		23	100
patient characteristics			
gender	female	10	*43*
	male	13	*57*
age at diagnosis	1-9 years	15	*65*
	0-1 and >9 years	8	*35*
fusion genes	*ETV6-RUNX1*	4	*17*
	BCR/ABL	1	*4*
	gene fusions of *MLL*	2	*9*
	none[1]	16	*70*
immunophenotype	pro-B ALL	2	*9*
	c-ALL	13	*57*
	pre-B-ALL	8	*34*
initial white blood cell count (WBC)	≤ 100,000/µl	18	*78*
	> 100,000/µl	5	*22*
prednisone response (PR)[2]	good	22	*96*
	poor	1	*4*
day 15, blast cell persistence	less than 5%	7	*30*
	more than 5%	7	*30*
	not analyzed	9	*39*
minimal residual disease (MRD)[3]	MRD-SR	6	*26*
	MRD-IR	7	*30*
	MRD-HR	0	*0*
	not analyzed	10	*43*
BFM risk groups	non-HR	19	*83*
	HR	4	*17*
xenograft characteristics			
time to leukemia (TTL)	long	16	*70*
	short	7	*30*
NOD/SCID passage used for functional analysis of apoptosis signaling	P 0	1	*4*
	P 1	8	*35*
	P 2	6	*26*
	P 3	4	*17*
	P 4	2	*9*
	P 5	1	*4*
	P 6	1	*4*

ALL indicates acute lymphoblastic leukemia. TTL indicates time to leukemia; SR, standard risk; IR, intermediate risk; HR, high risk.
[1] In this study, only *ETV6-RUNX1*, *BCR/ABL* and gene fusions of *MLL* were assessed.
[2] Prednisone response is good if blast cell count in PB is lower than 1000/µl at day 8.
[3] MRD-SR if MRD is negative at day 33 (sensitivity 10^{-4}); MRD-HR if at least 10^{-3} at day 78 and MRD-IR all others.

3.2 Patient outcome according to the TTL-phenotype

The time period after transplantation of primary ALL samples onto NOD/SCID mice to clinical manifestation of leukemia signs in the recipients was determined (Figure 2A). Accelerated engraftment (< ten weeks, TTL^{short}) was present in seven leukemia samples, while samples of 16 patients displayed prolonged NOD/SCID engraftment (> ten weeks, TTL^{long}). Comparison of the clinical outcome of corresponding patients revealed significantly inferior relapse free survival of

patients showing the TTL^{short} in contrast to the TTL^{long} phenotype also in this cohort of samples (Figure 2B), in line with our previous findings [95].

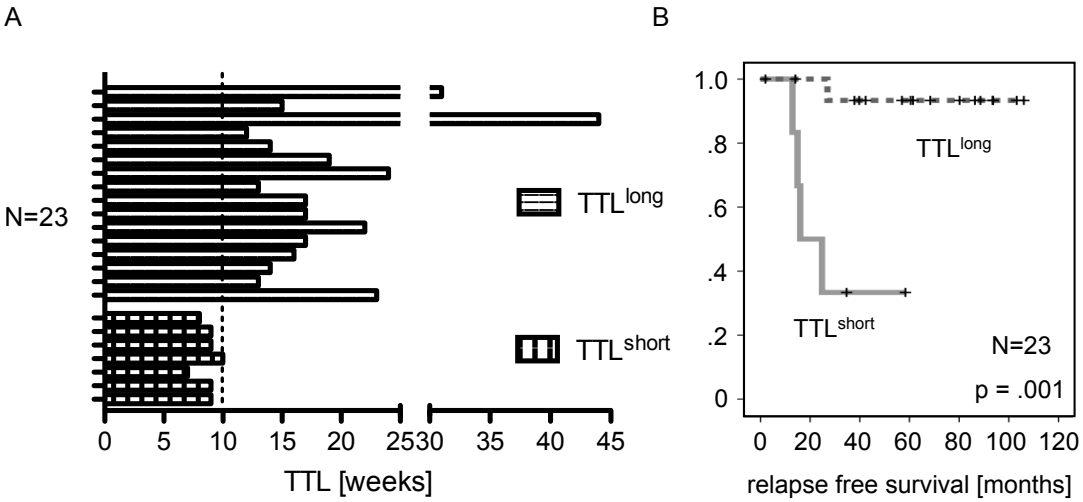

Figure 2. Relapse free survival according to TTL
(A) TTL was determined and classified as TTL^{short} indicating engraftment within 10 weeks after transplantation of leukemia cells or TTL^{long} indicating engraftment after more than 10 weeks.
(B) Relapse free survival of corresponding patients with a TTL^{short}-phenotype compared to TTL^{long}-patients. Kaplan-Meier analysis. N=23. p was calculated by log rank test. TTL indicates time to leukemia.

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3.3 Transcript levels of *PDE4A* and *DAPK1*

In an earlier study of our group, several genes have been identified by gene expression profiling to be differentially expressed within the different prognostic subgroups TTL^{short} and TTL^{long} [95]. Of these genes, two are described to be involved in the modulation of apoptosis signaling: *PDE4A* (phosphodiesterase 4A) has been shown to be up-regulated in TTL^{short}, in contrast to down-regulation of pro-apoptotic *DAPK1* (death-associated protein kinase 1). PDE4A is involved in the down-regulation of cAMP levels and it is described to inhibit apoptosis [68], whereas DAPK1 is a protein kinase showing cell death-inducing functions [23].

In this study, quantitative analyses of transcript levels of these genes were performed in a larger cohort of samples including samples carrying the tested fusion genes (*ETV6-RUNX1*, *BCR/ABL* and gene fusions of *MLL*). In line with our previous findings, we found *PDE4A* to be expressed at higher levels in TTL^{short} compared to TTL^{long} in contrast to down-regulation of *DAPK1* transcript levels (Figure 3).

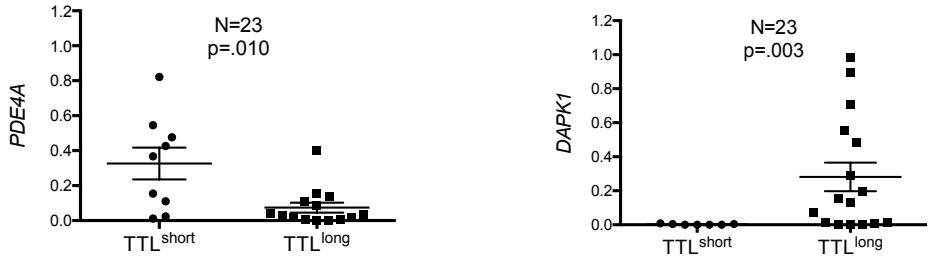

Figure 3. Gene expression of previously identified apoptosis regulating molecules
Relative transcript levels of *PDE4A* and *DAPK1* are presented. Quantitative RT-PCR. U-test, p= significance. Data are presented as the means of all samples ± SEM (standard error of the mean).

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3.4 Expression of anti-apoptotic molecules

Two anti-apoptotic BCL-2 family members, BCL-2 and MCL-1, as well as the anti-apoptotic molecules XIAP and LIVIN were analyzed on transcript and protein level. Gene expression analysis by quantitative RT-PCR did not show differential expression of the molecules between the TTL subgroups. (Figure 4A). For these apoptosis regulators, we also analyzed protein expression. Western blot and subsequent densitometric quantification of BCL-2, MCL-1, XIAP and LIVIN did not show significant differences in protein expression between the TTL subgroups (Figure 4B).

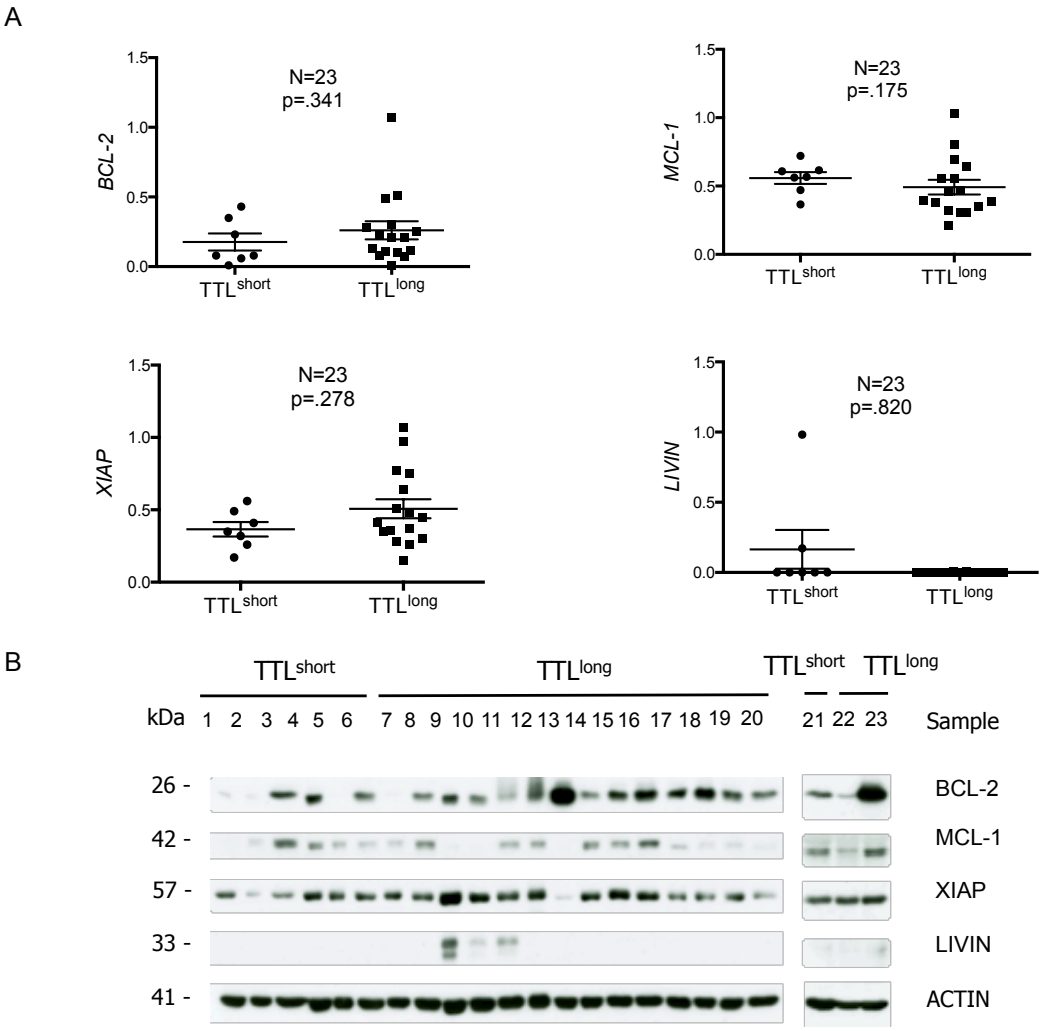

Figure 4. Anti-apoptotic molecules
(A) Relative transcript levels of *BCL-2*, *MCL-1*, *XIAP* and *LIVIN*. Significant differences between samples of time to leukemia short (TTL^{short}) and TTL^{long} phenotype were not found.
(B) Western blot of anti-apoptotic molecules. Densitometric quantification of protein levels relative to beta-Actin did not reveal significant differences between TTL^{short} and TTL^{long}: BCL-2 (p=.135), MCL-1 (p=.769), XIAP (p=.154) and LIVIN (p=.492). U-test, p= significance. Data are presented as the means of all samples ± SEM (standard error of the mean).

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3.5 Functional analysis of apoptosis signaling parameters

We functionally evaluated apoptosis signaling, analyzing two key apoptogenic events, mitochondrial release of cytochrome c and downstream activation of caspase-3, thereby estimating intact apoptosome function. Xenograft leukemia cells were cultured *ex vivo* and apoptosis signaling was investigated. The

parameters cell death (cd), cytochrome c-release (cc) and activated caspase-3 (ac) were investigated by flow cytometry before (0h) and after 16 hours *ex vivo* culture (16h).

Cytochrome c release was detected using an antibody binding exclusively to the mitochondrial form of cytochrome c. Thus, released cytochrome c was identified by reduction of the cytochome c signal. Caspase-3 activation was identified using an antibody specifically recognizing the active form of caspase-3. Both parameters were evaluated in a plot depicting cytochrome c on the Y-axis and active caspase-3 on the X-axis (Figure 5A). Cytochrome c-release was calculated as percentages of the lower left and lower right quadrant. Active caspase-3 was determined as percentages of the upper right and lower right quadrant.

In previous studies of our research group apoptosis proficiency of individual leukemia samples was determined by estimation of the parameter CRAC (cytochrome c-related activation of caspase-3). CRAC was calculated by substracting cc from ac. In our previous studies, CRAC-positivity has been shown to reflect the functional integrity of apoptosome formation and it was associated with favorable outcome of pediatric ALL and AML patients [92, 93]. In this study, we estimated apoptosis proficiency by estimation of CRAC in primary patient-derived leukemia samples after transplantation on xenografts in the NOD/SCIDhuALL mouse model. Figure 5B shows the analysis of apoptosis signaling in a TTL^{long} sample, showing a positive CRAC-value and thus proficient apoptosis signaling. Figure 5C shows a TTL^{short} sample with a negative CRAC value, indicating apoptosis deficiency.

In addition, cell death was assessed according to forward/side scatter (FSC/SSC) criteria before and after 16 hours of *ex vivo* culture and overall cell death was estimated as the difference of both.

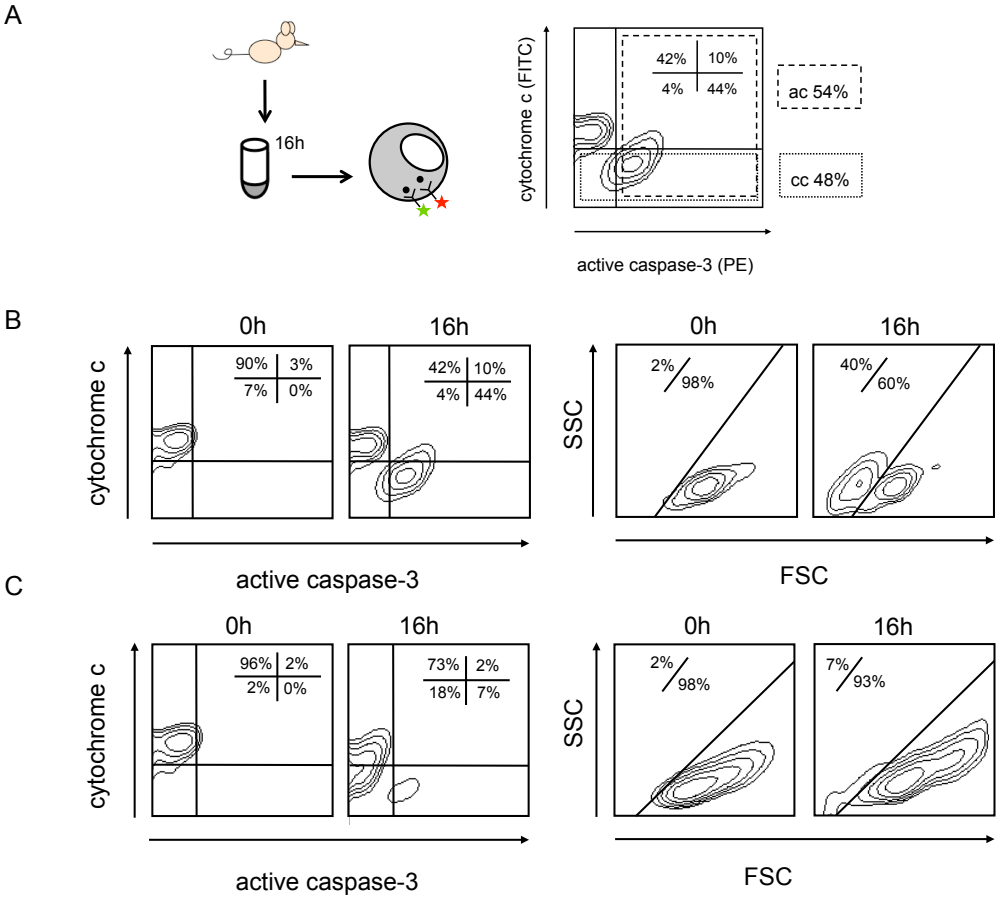

Figure 5. Analysis of cytochrome c-release and activated caspase-3
(A) Xenografted leukemia cells were cultured *ex vivo* for 16 h and cytochrome c-release and activated caspase-3 were determined by intracellular staining before and after *ex vivo* culture. ac indicates active caspase-3 and was calculated as the percentages of the upper and lower right quadrants. cc indicates cytochrome c-release and was calculated as the percentages of the lower right and left quadrants.
(B) Cytochrome c-release and activated caspase-3 in a TTL^{long} sample. ac = $ac^{16h} – ac^{0h}$ = 54% - 3% = 51%; cc = $cc^{16h} – cc^{0h}$ = 48% - 7% = 41%; CRAC indicates cytochrome c-related activation of caspase-3 and was calculated as: CRAC = ac – cc = 51% - 41% = +10. Analysis of cell death in this sample: cd indicates cell death and was determined as: cd = $cd^{16h} – cd^{0h}$ = 40% - 2% = 38%.
(C) Cytochrome c-release and activated caspase-3 in a TTL^{short} sample. ac = $ac^{16h} – ac^{0h}$ = 9% - 2% = 7%; cc = $cc^{16h} – cc^{0h}$ = 25% - 2% = 23%; CRAC = ac – cc = 7% - 23% = -16. Analysis of cell death in this sample: cd = $cd^{16h} – cd^{0h}$ = 7% - 2% = 5%.

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3.6 Apoptosis signaling and NOD/SCID engraftment

We evaluated parameters reflecting active apoptosis signaling with respect to the engraftment phenotype of patient ALL cells after transplantation onto recipient animals. The parameters cell death (cd), cytochrome c-release (cc) and activated caspase-3 (ac) were tested for an association with NOD/SCID engraftment.

We found a positive correlation of prolonged time to leukemia (TTL) with higher amounts of cd detected after *ex vivo* culture according to FSC/SSC criteria (Figure 6). Additionally, increasing rates of cc were measured in samples showing prolonged TTL, although not being statistically significant. Interestingly, delayed TTL directly correlated to downstream ac. Most importantly, CRAC, subsuming proficient apoptosis signaling showed a direct positive correlation to TTL.

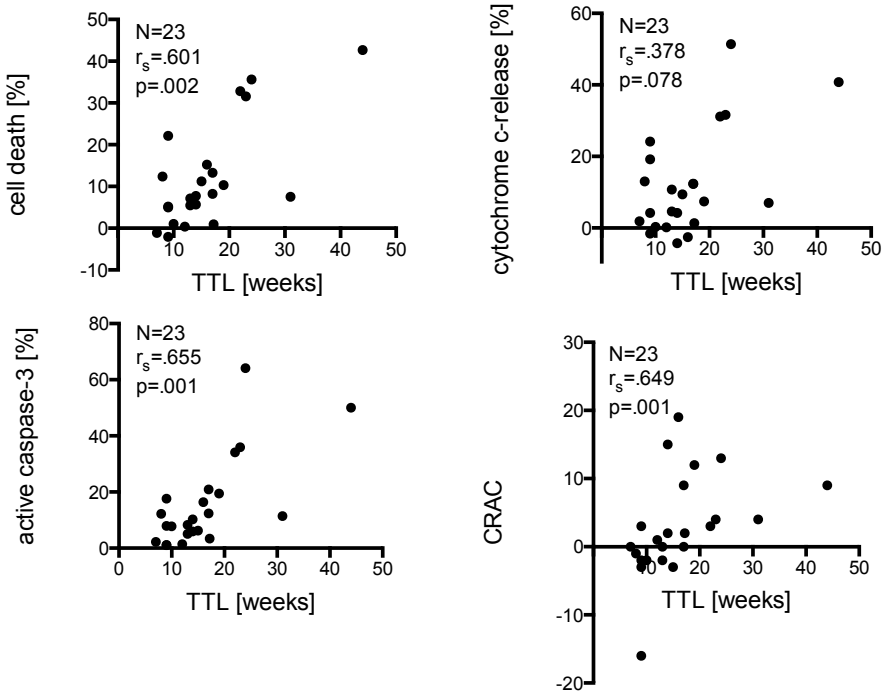

Figure 6. Correlation of apoptosis signaling parameters and TTL
Apoptosis signaling parameters were correlated to time to leukemia (TTL). Cell death, active caspase-3 and CRAC (cytochrome c-related activation of caspase-3) were significantly correlated to TTL. A trend was found towards correlation of cytochrome c-release and TTL. Spearman correlation, r_s is Spearman's rho, p significance.

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3.7 Deficient apoptosis signaling in poor prognosic subgroups

Concomitant release of cytochrome c (cc) from mitochondria to the cytosol and activation of caspase-3 (ac) is indicative of intact apoptosomal function and proficient apoptosis signaling as previously shown in primary leukemia cells by our research group [92, 93]. In this study, we simultaneously quantified percentages of cc and ac of patient derived xenograft ALL cells obtained from leukemia bearing mice. In order to estimate apoptosis proficiency in different subgroups analyzed,

cc and ac were correlated to each other, a significant correlation being indicative for apoptosis proficiency and absence indicating apoptosis deficiency in the subgroup. Proficient apoptosis signaling was exclusively found in *ex vivo* experiments derived from xenografts from patients, who were in remission on day 15 of chemotherapy, which is a prognostic factor for good patient outcome [103, 138, 156], not in patients with residual leukemic infiltration of the bone marrow on day 15. Moreover, proficient apoptosis signaling was only present in samples from patients, stratified as non-high risk according to the ALL-BFM criteria, which is predictive for favorable prognosis [26] and only in samples from non-relapsed patients. Additionally, apoptosis proficiency was observed in TTL^{long}-samples in contrast to TTL^{short}-samples, suggesting an association between apoptosis deficiency and prolonged NOD/SCID engraftment. Further, it was exclusively present in samples with high expression of *DAPK1*, pointing towards enhanced apoptosis signaling in this subgroup (Table 3).

Table 3. Correlation of cc and ac in distinct prognostic subgroups

	n	r_s	p
remission on day 15			
yes	7	0.893*	.007
no	7	0.643	.119
ALL-BFM risk group			
non-HR	19	0.793*	< .001
HR	4	-0.400	.600
relapse			
no	16	0.759*	.001
yes	5	0.600	.285
time to leukemia (TTL)			
TTL^{long}	16	0.750*	.001
TTL^{short}	7	0.750	.052
***DAPK1*, transcript expression**			
high	11	0.791*	.004
low	12	0.517	.085

Cytochrome c release (cc) and active caspase-3 (ac). High indicates expression above median. Low indicates below median. Spearman correlation, r_s indicates Spearman's rho, *P* significance. *Correlation is significant.

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3.8 NOD/SCID engraftment and patient outcome according to CRAC

Analyzing time to clinical manifestation of leukemia after transplantation in the recipient animals, we found accelerated engraftment of samples showing apoptosis deficiency in the CRAC-assay, compared to samples showing apoptosis proficiency (Figure 7A). Additionally, we analyzed the clinical outcome of

corresponding patients according to apoptosis signaling parameters in the CRAC-assay. Interestingly, we found superior relapse free survival of patients with intact apoptosis signaling (Figure 7B).

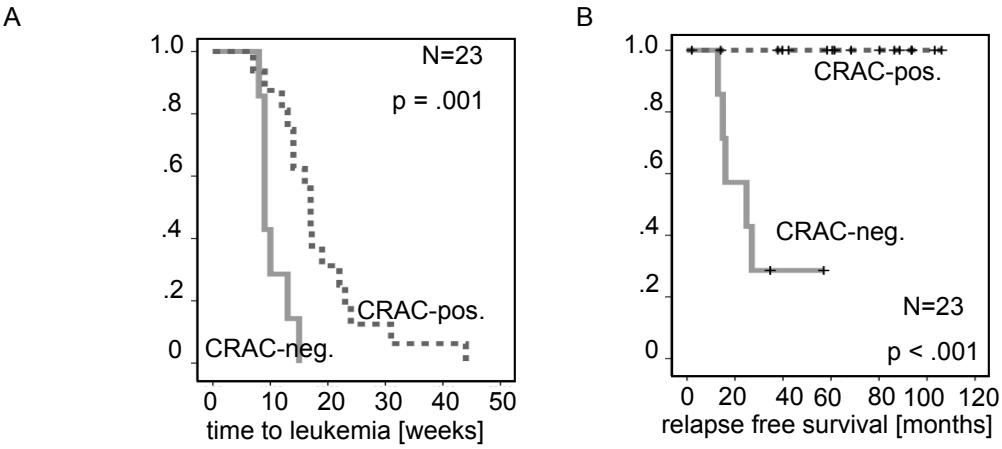

Figure 7. NOD/SCID engraftment and patient outcome according to CRAC
(A) Time to leukemia manifestation according to CRAC. CRAC indicates cytochrome c-related activation of caspase-3, CRAC-pos. intact apoptosis signaling, CRAC-neg. apoptosis deficiency.
(B) Relapse-free survival of the corresponding patients according to CRAC in the xenograft samples. Kaplan-Meier analysis. N=23. p was calculated by log rank test.

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3.9 Reverse phase protein array (RPPA) of ALL samples

In the second part, we analyzed protein expression levels of numerous molecules in a series of 16 patient-derived leukemia xenograft samples with distinct engraftment phenotypes in the NOD/SCID mouse model, aiming to identify differentially regulated proteins in these subgroups. In this part of the study, we exclusively analyzed samples, which were negative for the fusion genes *ETV6-RUNX1*, *BCR/ABL* and rearrangements of *MLL,* in order to reduce the heterogeneity of the samples. Characteristics of this patient cohort and derived xenograft samples are shown in Table 4. Altogether, 16 samples were analyzed, of which 14 also have been investigated in the first part of this study.

The time after transplantation of human ALL onto the recipients until onset of disease in the animals was determined and samples were classified as TTL^{short} and TTL^{long}, as described before [95]. Nine samples showed prolonged NOD/SCID engraftment. In contrast, seven samples led to rapid NOD/SCID engraftment (Figure 8A). Also in this cohort analyzed, the corresponding patients showing a TTL^{long} phenotype in the recipient animals showed a superior outcome compared to patients with a TTL^{short} phenotype (Figure 8B).

Table 4. Characteristics of ALL samples analyzed by RPPA

		N	%
total number		16	*100*
patient characteristics			
gender	female	7	*44*
	male	9	*56*
age at diagnosis	1-9 years	8	*50*
	>9 years	8	*50*
immunophenotype	pro-B ALL	0	*0*
	c-ALL	10	*63*
	pre-B ALL	6	*38*
initial white blood cell count (WBC)	≤ 100,000/µl	14	*88*
	> 100,000/µl	2	*12*
prednisone response (PR)[1]	good	16	*100*
day 15, blast cell persistence in bone marrow (BM)	≤ 5%	6	*38*
	> 5%	4	*25*
	not analyzed	6	*38*
minimal residual disease (MRD)[2]	MRD-SR	5	*31*
	MRD-IR	7	*44*
	MRD-HR	0	*0*
	not analyzed	4	*25*
BFM risk groups	SR	7	*44*
	IR	9	*56*
	HR	0	*0*
xenograft characteristics			
time to leukemia (TTL)	long	9	*56*
	short	7	*44*
NOD/SCID passage used for RPPA	P 0	1	*6*
	P 1	5	*31*
	P 2	5	*31*
	P 3	1	*6*
	P 4	2	*13*
	P 5	2	*13*

ALL indicates acute lymphoblastic leukemia. RPPA indicates reverse phase protein array. TTL indicates time to leukemia; SR, standard risk; IR, intermediate risk; HR, high risk.
[1] prednisone response is good if blast cell count in PB is lower than 1000/µl at day 8
[2] MRD-SR if MRD is negative at day 33 (sensitivity 10^{-4}); MRD-HR if at least 10^{-3} at day 78 and MRD-IR all others

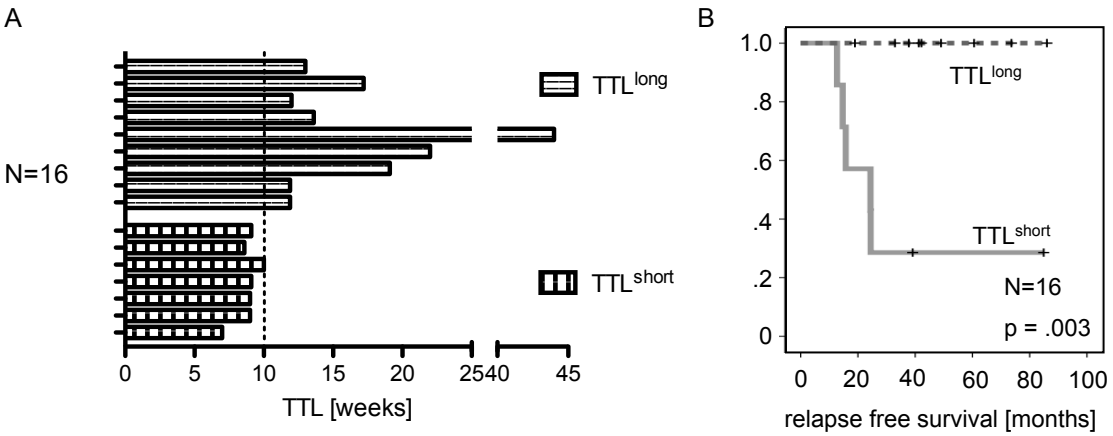


Figure 8. TTL and patient outcome
(A) The time after transplantation of leukemia cells onto the recipients until clinical manifestation of leukemia was determined. TTL indicates time to leukemia. TTL^{long} indicates prolonged engraftment within a time period of more than 10 weeks. TTL^{short} indicates accelerated NOD/SCID engraftment within the first 10 weeks.
(B) Relapse-free survival of the patients according to TTL. Kaplan-Meier analysis. p was calculated by log rank test.

The protein expression levels of leukemia samples of both engraftment phenotypes were analyzed by reverse phase protein array (RPPA), enabling quantitative detection of protein expression data in all samples simultaneously at the same conditions. Altogether, 51 different target molecules, which have been described to be involved in the regulation of different important pathways such as apoptosis, proliferation and cell cycle, were investigated; a list of all antibodies used in this study is shown in Table A1 in the appendix. We performed protein extraction and transferred all samples onto nitrocellulose-coated slides (Figure 9A). Each single slide, carrying protein of all selected samples located at different spots in different dilutions, was stained with a specific antibody directed to phosphorylated or non-phosphorylated proteins of interest. Thereafter, each slide was incubated with a biotinylated secondary antibody. Finally, slides were stained with diaminobenzidine (DAB), visualizing the signal intensity of a sample for a specific protein. An example of a stained slide is shown in Figure 9B.

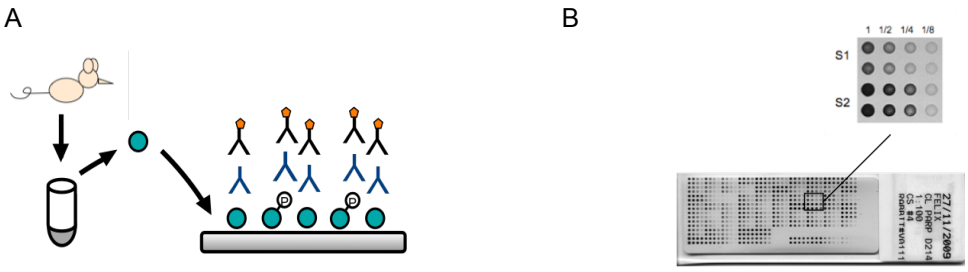


Figure 9. Reverse phase protein array (RPPA) strategy
(A) Leukemia cells were isolated from xenografted human ALL samples of short time to leukemia (TTL^{short}) or TTL^{long} phenotype. Protein extraction was performed. Samples were immobilized onto nitrocellulose-coated glass slides. Staining with primary antibodies and biotinylated secondary antibodies was done.
(B) Example of a stained RPPA slide. Each sample was printed as duplicate and at four different dilutions (from undiluted to 1:8). S1 indicates first sample, S2 second sample.

The expression data was normalized and protein levels were quantified and compared between the TTL subgroups. Differential protein expression with a fold change of 1.5 and a q-value (adjusted p-value) of 0.05 and below was further evaluated. According to these criteria, comparison of the protein expression data identified four proteins to be differentially expressed: CYCLIN B, beta-CATENIN and ANNEXIN I were over-expressed in TTL^{short} leukemia samples while decreased phosphorylated PKC alpha was detected (Table 5). Expression values of all molecules are summarized in supplementary Table A2.

Table 5. Differentially expressed proteins identified by reverse phase protein array

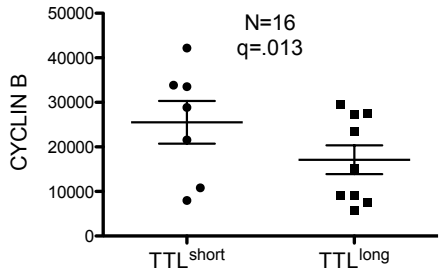
	CYCLIN B			beta-CATENIN			ANNEXIN I			p-PKCα S657		
	mean	**SEM**	***q***	**mean**	**SEM**	***q***	**mean**	**SEM**	***q***	**mean**	**SEM**	***q***
TTL			*.013*			*.006*			*.033*			*8.5e-12*
long	2.6e4	4.8e3		1.9e4	3.7e3		2.5e4	1.0e4		8.3e4	1.0e4	
short	1.7e4	3.2e3		1.1e4	1.0e3		1.4e4	4.3e3		1.4e5	1.5e4	

TTL indicates time to leukemia. Shrinkage t-test, q < .05, fold change ≥ 1.5. SEM indicates standard error of the mean; q FDR adjusted p-value. N=16.

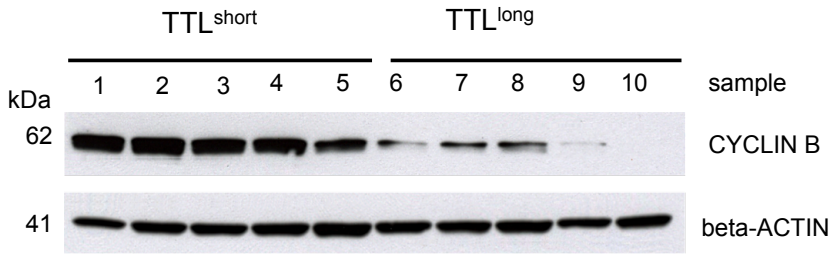
3.10 Up-regulation of CYCLIN B in TTL^{short} leukemia samples

Analysis of the protein expression data obtained from reverse phase protein array (RPPA) identified CYCLIN B to be expressed at higher levels in TTL^{short} leukemia samples, compared to TTL^{long} (Figure 10A). RPPA results were evaluated by western blot analyses using ten exemplary samples from which protein lysates were available in the cohort analyzed by RPPA, thereby enabling the technical validation of this screening method. The results obtained from RPPA were confirmed by western blot analysis, showing an up-regulation of CYCLIN B in the TTL^{short} group (Figure 10B), which was confirmed by densitometric quantification from the western blot results (Figure 10C).

A



B



C

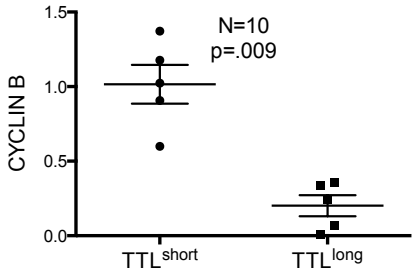


Figure 10. Protein expression of CYCLIN B in leukemia samples showing different engraftment properties in the xenograft mouse model

(A) Reverse phase protein array (RPPA) identified CYCLIN B protein being expressed at higher levels in samples showing short time to leukemia (TTL^{short}), compared to samples of the TTL^{long} group. Shrinkage t- test, q= adjusted p-value.

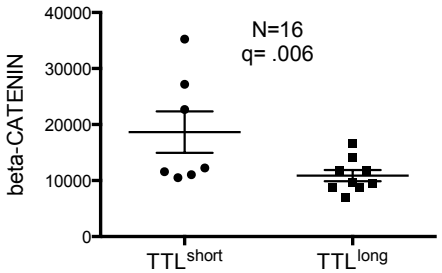
(B) Western blot analysis of CYCLIN B of TTL^{short} versus TTL^{long} leukemia samples.

(C) Densitometric quantification of CYCLIN B relative to beta-Actin. TTL^{short} versus TTL^{long}, U-test. p= significance. Data are presented as the means of all samples ± SEM (standard error of the mean).

3.13 Protein levels of beta-CATENIN

Analyzing results of the reverse phase protein arrays revealed the molecule beta-CATENIN to be expressed at higher levels in TTL^{short} samples in contrast to TTL^{long} (Figure 11A). However, the difference between the groups was not found in the validation by western blot (Figure 11B) and subsequent quantification (Figure 11C).

A



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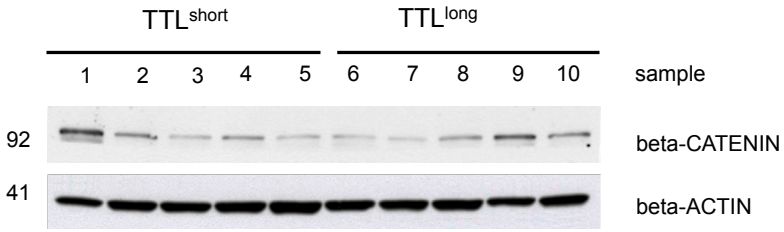


C

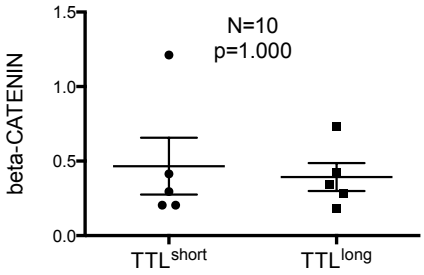


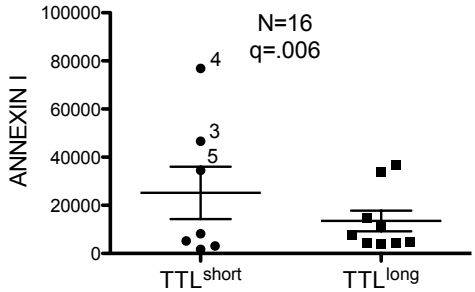
Figure 11. Protein expression of beta-CATENIN
(A) Protein expression of beta-CATENIN on reverse phase protein array. TTL indicates time to leukemia. Shrinkage t-test, q= adjusted p-value. p= significance.
(B) Western blot of beta-CATENIN.
(C) Densitometric quantification relative to beta-ACTIN was performed, which did not show a different expression of beta-CATENIN within the subgroups. U-test, p= significance. Data are presented as the means of all samples ± SEM (standard error of the mean).

3.14 Protein expression of ANNEXIN I in TTL^{short} versus TTL^{long}

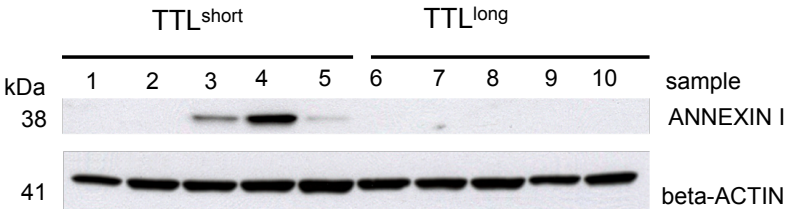
Comparing protein expression data of the reverse phase protein arrays identified ANNEXIN I to be expressed at higher levels in TTL^{short} leukemia samples (Figure

12A). Corresponding to the RPPA results, the western blot analysis also showed high expression of ANNEXIN I in two samples of the TTL^{short} group (sample # 3 and 4 in Figure 12), compared to low or absent expression in the other samples (Figure 12B). However, upon quantification and statistical analysis of ANNEXIN I protein expression analyzed by western blot, a significant difference between TTL^{short} and TTL^{long} leukemia samples was not observed (Figure 12C).

A



B



C

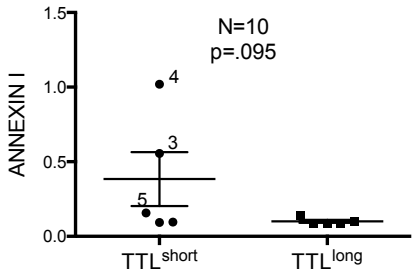
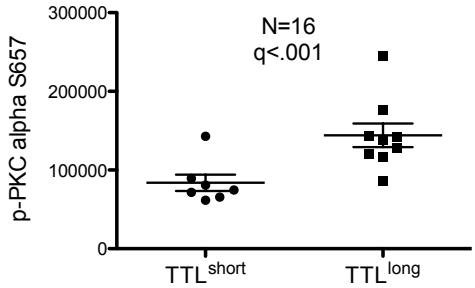


Figure 12. Protein expression ANNEXIN I
(A) Protein expression of ANNEXIN I analyzed by reverse phase protein array (RPPA). TTL indicates time to leukemia. Shrinkage t-test, q= adjusted p-value.
(B) Western blot analysis of Annexin I.
(C) Densitometric quantification of ANNEXIN I relative to beta-ACTIN. U-test, p= significance.
The numbers indicate the sample ID, showing high ANNEXIN I levels in the corresponding samples analyzed by RPPA and western blot. Data are presented as the means of all samples ± SEM (standard error of the mean).

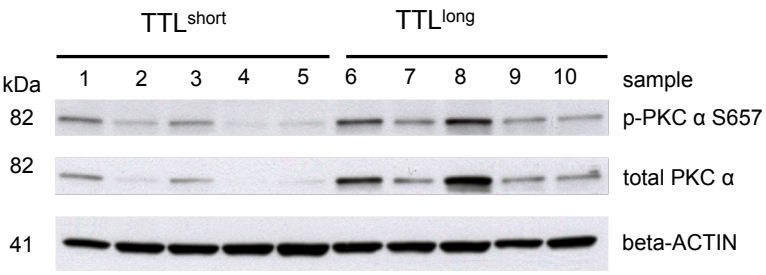
3.15 Down-regulation of PKC alpha protein levels in TTL^{short} samples

In contrast to the above described molecules, p-PKC alpha S657 (phosporylated serine 657) was down-regulated in poor prognostic TTL^{short} (Figure 13A). This result was also confirmed by western blot (Figure 13B), where we found lower levels of p-PKC alpha S657 in TTL^{short} leukemia samples upon quantification (Figure 13C). Additionally, western blot analysis of total PKC alpha was performed, since the total form was not included in the RPPA analyses. However, the total form of PKC alpha was down-regulated in the TTL^{short}-group as well, indicating that the difference of the phospho-signal is likely to be due to a reduced expression of total PKC alpha (Figure 13B,C).

A



B



C

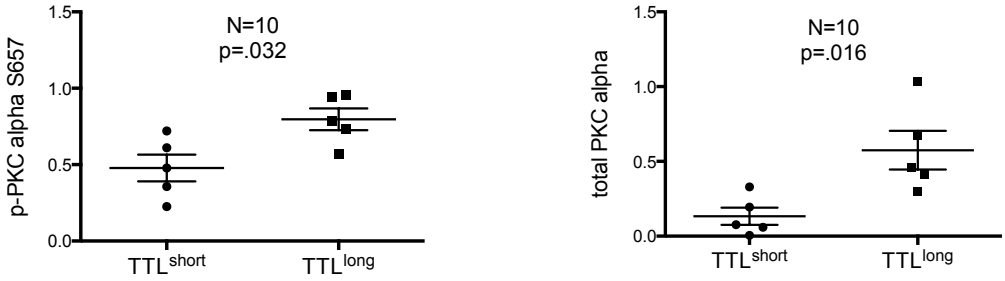


Figure 13. Distinct PKC alpha levels in xenografted leukemia samples
(A) The protein expression of p-PKC alpha S657 was analyzed by reverse phase protein array (RPPA), resulting in lower expressions in time to leukemia short (TTL^{short}) samples. Shrinkage t-test, q= adjusted p-value.
(B) Validation by western blot of PKC alpha in TTL^{short} versus TTL^{long}.
(C) Densitometric quantification of p-PKC alpha S657 and total PKC alpha relative to beta-Actin in TTL^{short} versus TTL^{long}; U-test, p= significance. Data are presented as the means of all samples ± SEM (standard error of the mean).

4. Discussion

4.1 NOD/SCIDhuALL xenotransplant mouse model

In the present study, we investigated molecular characteristics of childhood acute lymphoblastic leukemia samples in a xenograft mouse model with regard to the engraftment phenotype and to treatment response and relapse free survival of the corresponding patients. Although immortalized human cell lines have been useful to improve treatment options of leukemia patients, xenotransplantation of human leukemia cells is an important tool for studying biological characteristics of the primary disease [73, 89]. Previously, a xenotransplant mouse model using NOD/LtSz-scid/scid (NOD/SCID) mice was established in our research group [95]. Defects in innate and adaptive immunity of the NOD/SCID mice permit the engraftment of leukemic cells in the recipients without graft rejection [148]. Biologic features such as immunophenotype or patient-specific genetic aberrations of leukemia samples have been demonstrated to be stable upon serial transplantation onto consecutive xenograft passages [84, 142, 174].

In this study, primary ALL samples from patients with distinct clinical outcomes were characterized at a functional level in our NOD/SCIDhuALL xenotransplant mouse model. The time after transplantation until clinical manifestation of leukemia in the recipients, reflected by clinical signs including shaggy fur, loss of weight or reduced activity, was determined as TTL (time to leukemia) and samples were classified as TTL^{short} indicating accelerated engraftment within the first ten weeks or TTL^{long} representing prolonged engraftment of more than ten weeks. Previously, our group identified the TTL^{short}-phenotype to be characterized by a gene expression signature associated with survival pathways and to be indicative of poor patient outcome [95]. In line with these findings, inferior relapse free survival of patients showing the TTL^{short}-phenotype opposed to the TTL^{long}-group was also found in the cohort of this study.

Others have also reported engraftment properties of leukemia samples being associated with the clinical outcome of the corresponding patients. For instance, inferior survival of AML patients was demonstrated in patients showing NOD/SCID engraftment compared to cases that do not engraft [114]. Another group later also showed a correlation of patient prognosis with the extent of engraftment of blasts from pediatric ALL patients upon transplantation into NOD/SCID/IL2Rcγnull (NSG)

mice [174]. Furthermore, others observed faster engraftment of de novo high risk ALL samples showing positive MRD levels during consolidation therapy compared to standard risk ALL upon transplantation onto NSG mice [142].

However, the variety of leukemia xenograft models leads to difficulties in comparing results found in one model to the results of others [94]. For example, different research groups use distinct definitions of leukemia manifestation in the recipient animals. Additionally, the engraftment properties may be influenced by the transplantation procedure or by different levels of immunodeficiency.

4.2 Prognostic factors and outcome of leukemia patients

Despite stratification based on different prognostic factors, the majority of relapsed patients in the AIEOP-BFM ALL 2000 study was initially stratified into the non-high risk groups [26], indicating the need for additional approaches and alternative methods to predict patient outcome.

In this study, 25 primary patient-derived leukemia samples were analyzed in the xenotransplant mouse model. Overall, six of the patients suffered from relapse. Interestingly, none of these six patients was initially stratified as high risk. The incidence of gender, age and immunophenotype of the patients in this study was comparable to previously published patient populations [72, 90, 150]. According to several studies, white blood cell count and age at diagnosis are of prognostic value [62, 152]. However, in the current BFM trials these criteria are not used for risk stratification of the patients [26]. In line with this, we did not find a prognostic significance of age and WBC at diagnosis in the cohort of patients analyzed in this study. The cohort of samples analyzed on apoptosis signaling in this study also included four samples positive for *ETV6-RUNX1*, one sample positive for *BCR/ABL* and two samples with rearrangements of *MLL*. These are frequent genetic abnormalities in pediatric ALL, which are associated with patient outcome and characterized by distinct gene expression profiles [7, 131, 179]. Apoptosis signaling parameters were investigated in xenograft ALL samples irrespective of the presence of these fusion genes. However, samples carrying these alterations were excluded for analyzing protein levels by reverse phase protein arrays, since the aim was to identify differences in prognostic subgroups at protein level in a homogeneous cohort of leukemia samples.

4.3 Expression of apoptosis regulating molecules

Previously, our research group characterized the TTL-phenotypes by gene expression profiling, identifying a set of genes being up-regulated in one of these groups [95]. In this study, two genes were further evaluated since they have been reported to regulate apoptosis signaling. In line with our previous findings, the anti-apoptotic molecule *PDE4A* was up-regulated in TTL^{short} compared to TTL^{long} and pro-apoptotic *DAPK1* was expressed at higher levels in TTL^{long} than in TTL^{short} in this larger cohort of samples. In addition, we examined transcript and protein levels of other selected apoptosis regulating molecules: the anti-apoptotic BCL-2 family members BCL-2 and MCL-1 and the inhibitor of apoptosis proteins XIAP and LIVIN were analyzed.

PDE4a is a member of the cyclic nucleotide phosphodiesterases and it is involved in the down-regulation of the second messenger cAMP (cyclic adenosine monophosphate) [27]. Moreover, PDE4A has been described to have anti-apoptotic activity in leukemia cells by down-regulation of cAMP levels [68, 101]. Additionally, others have reported an increased sensitivity of leukemia cells to dexamethasone by inhibition of PDE4 with rolipram [96, 111]. In line with this, our group has previously found increased cell death rates upon *ex vivo* treatment of leukemia cells with rolipram [95]. Thus, inhibition of PDE4A might be a therapeutic tool in ALL, which can be further evaluated.

DAPK1 is a serine/ threonine kinase and it is a pro-apoptotic molecule [23, 24, 65]. In line with its pro-apoptotic function, we found reduced expression of *DAPK1* in TTL^{short} compared to TTL^{long} and an association of low *DAPK1* expression with deficient apoptosis signaling. The expression of *DAPK1* is described to be down-regulated by promoter methylation in different types of cancer. For example, promoter hypermethylation was found in head and neck cancer patients [137], advanced stage NSCLC (non-small cell lung cancer) patients [77] and urinary bladder cancer patients [64]. Moreover, promoter methylation and reduced *DAPK1* expression were found in chronic lymphocytic leukemia (CLL) patients compared to normal B cell samples [127]. Most importantly, treatment of *MLL*-rearranged leukemia cell lines with hypomethylating agents induced re-expression of genes including *DAPK1* [140]. Taken together, ongoing studies might help to evaluate the role of this molecule as prognostic marker and/ or therapeutic target in pediatric ALL.

The analysis of transcript and protein levels of the anti-apoptotic molecules BCL-2, MCL-1, XIAP and LIVIN did not reveal any significant differences within the different prognostic subgroups. Several other studies have also investigated the role of the expression of these apoptosis regulating molecules and their prognostic significance in distinct types of leukemia.

BCL-2 is a mitochondrial molecule that inhibits programmed cell death [57]. Despite its anti-apoptotic function, others similarly did not find an association of high BCL-2 protein levels with clinical outcome in childhood ALL [20, 58, 136]. The protein ratio of BCL-2 to BAX is known to be critical to determine survival or cell death [112]. In line with this, low BAX/BCL-2 protein ratios were reported to be associated with poor outcome in adults with AML [30], but did not show evidence for prognostic impact in a group of pediatric ALL patients on transcript levels [136].

MCL-1 is a member of the BCL-2 family and it is involved in the regulation of cell viability and apoptosis [80, 130]. In accordance with some previous findings in childhood ALL [58, 136], we did not find a differential expression on transcript or protein level in prognostic subgroups. Others have shown high levels of *MCL-1* expression to be associated with prednisone resistance in childhood ALL [59, 155] and with poor prognosis in adult CLL [115, 139].

XIAP is a suppressor of apoptosis by direct inhibition of effector caspases, such as caspase-3 [31, 160]. Whereas high expression levels were reported to be associated with unfavorable prognosis in childhood [158, 163] and adult [162, 164] AML, we did not find a significant difference on transcript or protein level in prognostic subgroups of pediatric ALL samples.

LIVIN is an inhibitor of apoptosis and directly inhibits downstream caspases such as caspase-3 [74]. In this study, *LIVIN* was only expressed in a few samples, while most samples lacked an expression and we did not find a statistically significant difference on transcript or protein level between the prognostic subgroups, which is in accordance with previously published data [178]. Interestingly, another report described the expression of *LIVIN* to be a favorable prognostic factor in childhood ALL, despite its anti-apoptotic activity [22]. However, in a further study of adult ALL and AML the gene expression of *LIVIN* was higher in patients with unfavorable prognostic factors [35].

Taken together, these findings suggest that the expression level of those single anti-apoptotic molecules may not be sufficient to reflect the integrity of the

apoptosis pathway and its impact on the engraftment phenotype and the treatment response and outcome of corresponding patients.

4.4 Functional analysis of apoptosis signaling

In order to interpret the ability of apoptosis signaling of single leukemia xenograft samples we employed the functional CRAC-assay, which assesses the interplay between two key molecules of the apoptosis pathway, cytochrome c-release and activation of caspase-3, thereby subsuming many other upstream single molecules. We determined the parameter cytochrome c-related activation of caspase-3 (CRAC) as described before by our group [92]. Samples showing a TTL^{long}-phenotype in our mouse model presented higher levels of CRAC, indicating proficient apoptosis signaling, compared to TTL^{short}-samples. Additionnally, higher levels of active caspase-3 were found in TTL^{long}-, compared to the TTL^{short}-group. Consistently, others have shown a correlation of higher caspase-3 activity with better risk groups in patients with CML [128] and high levels of caspase-3 were reported to be associated with achievement of complete remission in adults with ALL [39]. Furthermore, others have shown a reduction of caspase-3 activation in relapsed childhood ALL samples, when compared to initial diagnosis [120]. In another study however, the expression of procaspase-3 in adult AML and ALL was not of prognostic relevance [159].

Interestingly, we found a positive correlation of ac, cd and CRAC to TTL, demonstrating a direct association of apoptosis signaling and engraftment characteristics of human leukemia cells. Most importantly, CRAC-negative leukemia samples showed accelerated NOD/SCID-engraftment, suggesting apoptosis deficiency to be critical for *in vivo* proliferation. Moreover, patients presenting deficient apoptosis signaling ($CRAC^{neg.}$) in our xenotransplant model showed inferior relapse free survival compared to the $CRAC^{pos.}$-group, supporting previous findings of our group from retrospective studies in primary ALL and AML showing superior outcome of patients presenting apoptosis proficiency [92, 93].

Overall, these results support the impact of proficient or deficient apoptosis signaling on the engraftment phenotype of leukemia cells in NOD/SCID mice and on treatment response and clinical outcome of the corresponding patients.

4.5 Reverse phase protein array (RPPA) strategy

In the second part of this study we employed reverse phase protein arrays to identify differentially expressed proteins in TTL^{short} versus TTL^{long} leukemia samples. This approach enables measuring overall protein levels or protein phosphorylation sites of a large number of molecules [166]. The use of RPPA has been shown to be a precise and highly reproducible method [87, 166]. Additionnally, it provides post-translational information on the protein phosphorylation status, which are not seen by gene expression analyses [37, 116]. Previously, several studies analyzed mRNA expressions and the corresponding protein levels and found discordant expressions, underlying the need for quantitative analyses of protein levels instead of estimation from mRNA transcripts [21, 45, 50]. Using the RPPA method might help identifying risk-associated proteins or potential therapeutic targets in cancer patients.

Others have already successfully applied RPPA analyses in different types of cancer. For instance, activation of the AKT/mTOR pathway has been identifed to be negatively associated with patient survival in childhood rhabdomyosarcoma [117]. In another study, activation of EGFR (epidermal growth factor receptor) and COX2 (cytochrome c oxidase subunit 2) signaling was found in metastatic in contrast to nonmetastatic colorectal cancer patients [118]. Additionnaly, RPPA studies of leukemia patient samples revealed important results that might be useful to predict patient prognosis or to optimize therapy of patient subgroups. For example, high levels of phosphorylated FOXO3A (forkhead box O3) have been associated with adverse prognosis in adult AML [79]. Moreover, protein expression signatures identified by RPPA have been associated with outcome in adult AML [78] and CML [126]. Furthermore, AMPK (AMP-activated protein kinase) was identified by RPPA to be hyperactivated in poor prognostic leukemia patients with *MLL* gene rearrangements [2] and pharmacological inhibition of AMPK enhanced apoptosis in *MLL*-rearranged leukemia cells [3].

In our study, protein levels of 51 key signaling molecules were analyzed using the RPPA method. Protein expression levels were quantified and compared between the different prognostic subgroups.

4.6 Differentially expressed proteins in TTL$^{short/\ long}$

We analyzed 16 xenografted pediatric ALL samples being negative for the tested fusion genes *BCR/ABL*, *ETV6-RUNX1* and rearranged *MLL*. Analyzing the results from the reverse phase protein arrays revealed three proteins to be expressed at higher levels in TTL^{short} and one protein expressed at lower levels in TTL^{short} compared to TTL^{long} leukemia samples.

CYCLIN B protein expression was up-regulated in TTL^{short}, suggesting an association of NOD/SCID engraftment and cell cycle progression. CYCLIN B is a positive regulator of the cell cycle and it is important for the G2 – M phase transition [119, 171]. The inhibition of CYCLIN B1 might be important for controlling malignant transformation [63]. Previous reports, using immunohistochemistry or RPPA propose high CYCLIN B1 expression to be associated with adverse outcome in different types of breast cancer [1, 5]. Moreover, high protein expression was associated with poor prognosis in esophageal squamous cell carcinoma [108, 161], gastric carcinoma [13] and non-small cell lung cancer [154]. High CYCLIN B expression might reflect the overall aggressiveness of the cancer cells and further studies in larger cohorts of samples will possibly further characterize its role in tumor development and as potential prognostic marker.

The RPPA analysis showed beta-CATENIN to be expressed at higher levels in TTL^{short}, though the difference was not confirmed in the validation by western blot. Beta-CATENIN is involved in WNT-signaling and down-regulated by the tumor suppressor APC (adenomatous polyposis coli) [107]. Interestingly, activating mutations have been described in prostate cancer [170], medulloblastoma [183] and hepatocellular carcinoma [100]. Furthermore, high beta-CATENIN activity was demonstrated as poor prognostic marker in breast cancer [86].

ANNEXIN I is involved in inflammatory pathways, cell proliferation and carcinogenesis [85]. Gene and protein expression of ANNEXIN I are up-regulated in hairy cell leukemia [40]. Over-expression on transcript level has been reported in poor prognostic ALL samples with *MLL* translocations [10]. In our study, analyzing a cohort of ALL samples without fusion genes, ANNEXIN I was only expressed in a few samples, leading to a significant up-regulation in TTL^{short} in the RPPA analysis. However, the analysis by western blot did not reveal a significant difference between TTL^{short} and TTL^{long}.

Protein kinase C (PKC) is a Ca^{2+}-dependent kinase and involved in different cellular functions such as proliferation, differentiation and apoptosis [19, 97, 110]. The activation of PKC alpha is strictly regulated [109]. Three different phosphorylation sites have been identified to be crucial for its activation: T497 (activation loop site), T838 (autophosphorylation site) and S657 (hydrophobic C-terminal site) [113]. The phosphorylation on serine 657 in the hydrophobic region is important for its full activation and contributes to a phosphatase-resistant state [18]. In our study, we investigated protein levels of total PKC alpha and p-PKC alpha S657. The protein analyses by RPPA and western blot in this study revealed a reduced activation of PKC alpha (phosporylated serine 657, p-PKC alpha S657) in TTL^{short} leukemia samples. Furthermore, the total form of PKC alpha was down-regulated in TTL^{short}, as well. In contrast to these findings, others have shown PKC alpha to increase cell cycle progression in glioma cells [17] and to promote cell proliferation, migration and invasion in human hepatocellular carcinoma [175]. Additionally, PKC alpha is described to suppress apoptosis and to mediate chemoresistance in ALL cells through BCL-2 phosphorylation [70, 134]. On the other hand, PKC alpha overexpression induced a less aggressive biological behavior of breast cancer cells [91] and activation of PKC is described to promote apoptosis in hepatocytes [71], thymocytes [146] and a human T cell leukemia cell line [133]. Most importantly, low levels of p-PKC alpha S657 were recently identified to be associated with a high probability of relapse in pediatric T-ALL patients [98]. Moreover, the authors of this study analyzed PKC alpha transcript levels in a large cohort of childhood T-ALL patients of the AIEOP study group, identifying an increased cumulative incidence of relapse in patients with PKC alpha down-regulation. Particularly, low PKC alpha expression identified patients with very poor outcome within the MRD-stratified high risk group.

Taken together, the role of PKC alpha in childhood ALL remains ambiguous. Further evaluation of the molecule in a larger cohort of patients, functional assays by PKC alpha overexpression or treatment with specific PKC alpha enhancing drugs will possibly better define its function as prognostic marker or potential therapeutic target.

In conclusion, this study found an association of deficient apoptosis signaling of leukemia cells with accelerated NOD/SCID engraftment (TTL^{short}) and poor

outcome of corresponding patients. Moreover, reverse phase protein array (RPPA) and western blot analyses identified high protein levels of CYCLIN B and low levels of PKC alpha in TTL^{short} compared to TTL^{long} samples. Succeeding studies are needed to further investigate to the role of these molecules as potential prognostic markers or therapeutic targets.

5. Summary

In this thesis, molecular features of childhood acute lymphoblastic leukemia (ALL) samples were addressed in order to characterize potential prognostic markers associated with NOD/SCID/huALL (non-obese diabetic/severe combined immunodeficiency/human acute lymphoblastic leukemia) engraftment phenotypes.
Apoptosis signaling, which is known to be important for treatment response and outcome of leukemia patients was investigated. In accordance with previously published findings of our research group, we found an up-regulation of the anti-apoptotic molecule *PDE4A* (phosphodiesterase 4A) and down-regulation of pro-apoptotic *DAPK1 (*death-associated protein kinase 1) in leukemia samples with accelerated NOD/SCID engraftment (time to leukemia short, TTL^{short}) in a larger cohort of samples. We did not detect significant differences on transcript and protein levels of BCL-2 (B-cell lymphoma 2), MCL-1 (myeloid cell leukemia sequence 1), XIAP (X-linked inhibitor of apoptosis protein) and LIVIN (also known as BIRC7, baculoviral IAP repeat containing 7). In order to investigate apoptosis signaling at a functional level, two key steps of the apoptosis pathway, cytochrome c release (cc) and caspase-3 activation (ac) were analyzed after onset of leukemia in NOD/SCID/huALL xenograft samples. Apoptosis proficiency was associated with good treatment response, favorable patient outcome, prolonged time to leukemia and high *DAPK1* expression. Furthermore, this study demonstrated a correlation of prolonged NOD/SCID engraftment of leukemia cells to parameters reflecting proficient apoptosis signaling, namely caspase-3 activation, cell death and CRAC (cytochrome c-related activation of caspase-3). Moreover, superior relapse free survival was found in the corresponding patients showing intact apoptosis signaling.
In order to identify further differentially expressed molecules in TTL^{short} or TTL^{long}, a reverse phase protein array (RPPA) and subsequent western blot analyses were performed. Comparison of the protein levels between the two groups revealed CYCLIN B to be over-expressed in TTL^{short}. We also found beta-CATENIN and ANNEXIN I to be expressed at higher levels in TTL^{short} by RPPA analysis. However, validation of protein levels of these two molecules by western blot analyses did not reveal significant differences between the subgropus. Further, RPPA analysis identified p-PKC alpha S657 (phosho-protein kinase C) to be

down-regulated in TTL^{short}, however, subsequent western blot analyses revealed both reduced p-PKC alpha and total PKC-alpha. These findings in conjunction with other studies have implications for further characterization of CYCLIN B and PKC alpha in leukemia patients.

6. References

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6. Appendix

Table A1. Primary antibodies used for reverse phase protein array

Antibodies to phospho-proteins	**Catalog #**	**MW (kDa)**	**Company**	**Host**
4E-BP1 (S65)	9451	15-20	CellSig	Rabbit
Akt (S473)	9271	60	CellSig	Rabbit
AMPKalpha (T172)	2531	62	CellSig	Rabbit
AMPKbeta1 (S108)	4181	38	CellSig	Rabbit
ATF-2 (T71)	9221	70	CellSig	Rabbit
Bcl-2 (S70)	2827	28	CellSig	Rabbit
B-Raf (S445)	2696	95	CellSig	Rabbit
Caspase-6, cleaved (D162)	9671	18	CellSig	Rabbit
Caspase-7, cleaved (D198)	9491	20	CellSig	Rabbit
Caspase-9, cleaved (D315)	9505	35	CellSig	Rabbit
ERK 1/2, p44/42 (T202/Y204)	9101	42, 44	CellSig	Rabbit
FAK (Y397) (18)	611806	125	BD	Mouse
IRS-1 (S612)	2386	180	CellSig	Rabbit
Jak1 (Y1022/1023)	3331	130	CellSig	Rabbit
c-Jun S63 II	9261	48	CellSig	Rabbit
c-Jun S73	9164	48	CellSig	Rabbit
MARCKS (S152/156)	2741	80, 87	CellSig	Rabbit
mTOR (S2448)	2971	289	CellSig	Rabbit
NF-kappaB p65 (S536)	3031	65	CellSig	Rabbit
Cleaved NOTCH1 Val1744	2421	110	CellSig	Rabbit
p38 MAP Kinase (T180/Y182)	9211	40	CellSig	Rabbit
p70 S6 Kinase (T389)	9205	70, 85	CellSig	Rabbit
p90RSK (S380)	9341	90	CellSig	Rabbit
PARP, cleaved (D214)	9541	89	CellSig	Rabbit
PDK1 (S241)	3061	63	CellSig	Rabbit
PKA C (T197)	4781	42	CellSig	Rabbit
PKC alpha (S657)	06-822	82	Upstate	Rabbit
PKC delta (T505)	9374	78	CellSig	Rabbit
PRAS40 (T246)	44-1100	40	BioSource	Rabbit
PTEN (S380)	9551	54	CellSig	Rabbit
S6 Ribosomal Protein (S235/236) (2F9)	4856	32	CellSig	R mAb
SAPK/JNK (T183/Y185)	9251	46, 54	CellSig	Rabbit
SHIP1 (Y1020)	3941	145	CellSig	Rabbit
Stat1 (Y701)	9171	84, 91	CellSig	Rabbit
Stat3 (S727)	9134	79, 86	CellSig	Rabbit
Antibodies to total proteins	**Catalog #**	**MW (kDa)**	**Company**	**Host**
Annexin I	610066	38	BD	Mouse
Annexin II	610068	36	BD	Mouse

Bak	06-536	30	Upstate	Rabbit
Bax	2772	20	CellSig	Rabbit
Bcl-2	2876	28	CellSig	Rabbit
Bcl-xL	2762	30	CellSig	Rabbit
Bim	2933	12, 15, 13	CellSig	Rabbit
Catenina, Beta	610153	92	BD	Mouse
Cyclin A clone BF683	05-374	58	Upstate	Mouse
Cyclin B	610220	62	BD	Mouse
Cyclin D1 (G124-326)	554180	36	BD	Mouse
Cyclin E (HE12)	554182	50	BD	Mouse
Kip1/p27	610241	27	BD	Mouse
eNOS 49G3	9586	140	CellSig	Rabbit
Smac/Diablo	2954	21	CellSig	Mouse
XIAP	610716	57	BD	Mouse

Primary antibodies are directed to phosphorylated or total proteins. MW indicates molecular weight.

Table A2. Raw data of protein expression in RPPA analysis

sample (TTL)	bcl2 s70	cl casp6	cl parp d214	bak
125 p1 (long)	16764,18822	23623,55756	101722,0866	100710,008
068 p1 (long)	34891,55278	34891,55278	40134,85274	78433,03307
112 p2 (long)	61697,56432	62317,64942	65512,75612	113550,2039
132 p2 (short)	7824,382923	18807,29316	37797,56501	115844,0127
015 p4 (long)	7354,001589	16203,7802	66836,16844	97733,51031
093 p2 (short)	50513,70294	25848,29312	84965,48392	109097,8409
002 p5 (short)	16122,96167	25848,29312	37421,46439	103777,0566
076 p3 (long)	14868,50284	21439,69893	89321,68929	105873,4397
120 p1 (short)	14647,13464	22247,84055	82454,31776	105873,4397
018 p5 (short)	20537,34685	24100,79611	87553,04476	86681,85756
116 p1 (long)	27173,56863	30946,03477	60475,90069	111301,7083
139 p0 (long)	13200,36706	26370,47535	104820,0574	73865,41781
118 p1 (long)	18033,74837	30946,03477	38177,44559	131926,4648
006 p4 (short)	26108,07876	28001,11952	66836,16844	102744,4341
089 p2 (short)	15787,91127	28282,54192	108012,2745	169396,934
121 p2 (long)	33860,36059	22697,26548	43044,94478	90219,40784
sample (TTL)	bax	bcl 2	bcl xl	cl casp7
125 p1 (long)	78433,03307	52052,06032	33860,36059	18788,48767
068 p1 (long)	109097,8409	52052,06032	40945,61024	21057,24423
112 p2 (long)	77652,59358	47572,03929	43914,48857	19990,24889
132 p2 (short)	72402,7514	45706,6711	40538,18534	18657,42807
015 p4 (long)	95798,30465	54176,34313	43477,56362	19614,01861
093 p2 (short)	82454,31776	68186,37982	52575,20425	18269,70907
002 p5 (short)	80821,65296	64860,87846	47572,03929	17483,27613
076 p3 (long)	83283,01649	68871,68009	48533,03416	19360,6956
120 p1 (short)	48050,11141	58104,60892	30031,44924	9036,255979
018 p5 (short)	78433,03307	47572,03929	45706,6711	17137,07623
116 p1 (long)	79221,24074	54720,83696	55826,29674	17154,22881
139 p0 (long)	61697,56432	59874,14172	44355,84667	15138,55379
118 p1 (long)	87553,04476	70969,11606	39735,49519	18453,32692
006 p4 (short)	88432,98772	47098,67881	45251,91493	16514,5963
089 p2 (short)	86681,85756	44801,64059	49513,48916	18106,02257
121 p2 (long)	137310,4808	41357,12993	34544,36812	10582,95745
sample (TTL)	cl casp9	stat3 y727	pdk1 s241	pras40 t246
125 p1 (long)	24343,01871	49020,81049	54720,83696	36680,48794
068 p1 (long)	22247,84055	80821,65296	52575,20425	57526,44523
112 p2 (long)	22247,84055	82454,31776	54176,34313	43044,94478
132 p2 (short)	18269,70907	41772,78556	28566,79273	25084,3673
015 p4 (long)	20170,96963	40538,18534	33860,36059	28566,79273
093 p2 (short)	24100,79611	70969,11606	46166,04135	45706,6711
002 p5 (short)	20640,29309	48050,11141	28001,11952	29143,86577
076 p3 (long)	20994,16171	64215,48724	29436,77323	30333,24834
120 p1 (short)	12823,05028	19633,65037	4359,009425	11487,33011
018 p5 (short)	21439,69893	60475,90069	29436,77323	28566,79273
116 p1 (long)	22697,26548	66171,18541	30333,24834	29143,86577
139 p0 (long)	18087,93554	58104,60892	18807,29316	24100,79611
118 p1 (long)	21590,30267	65512,75612	31257,05474	33523,4367
006 p4 (short)	19574,83261	56954,0345	21568,735	26635,48372
089 p2 (short)	20332,99218	62943,96661	15475,29736	20910,35886
121 p2 (long)	14271,21692	52575,20425	8982,196111	11407,19993

sample (TTL)	pten s380	s6ribprot s235 236	ship1 y1020	atf2 t71
125 p1 (long)	30638,10957	42616,63067	31257,05474	36315,50267
068 p1 (long)	39735,49519	42192,57845	45706,6711	41357,12993
112 p2 (long)	50011,07175	34891,55278	48050,11141	46630,02845
132 p2 (short)	21807,29381	24100,79611	29732,62452	30031,44924
015 p4 (long)	20272,07935	26635,48372	32859,61314	31888,47332
093 p2 (short)	34544,36812	32208,96518	34891,55278	33523,4367
002 p5 (short)	22697,26548	24834,76799	30031,44924	30031,44924
076 p3 (long)	20455,34796	24834,76799	34891,55278	32532,67811
120 p1 (short)	9759,533394	11181,31712	12556,57517	12938,9887
018 p5 (short)	23155,79126	27446,67381	33523,4367	30638,10957
116 p1 (long)	23860,98372	21547,16834	37421,46439	31888,47332
139 p0 (long)	15787,91127	14691,14583	22471,44021	23155,79126
118 p1 (long)	25848,29312	23860,98372	30031,44924	32859,61314
006 p4 (short)	19283,3947	20723,0136	32208,96518	29436,77323
089 p2 (short)	14987,91923	14559,52129	22247,84055	23155,79126
121 p2 (long)	11350,30498	9946,737866	18287,99531	22247,84055
sample (TTL)	braf s445	cjun s63	cjun s73	akt s473
125 p1 (long)	29436,77323	46166,04135	43914,48857	76114,93436
068 p1 (long)	39340,1114	60475,90069	55270,80316	73865,41781
112 p2 (long)	28853,90037	56954,0345	52575,20425	70969,11606
132 p2 (short)	29732,62452	28566,79273	31888,47332	63576,57854
015 p4 (long)	38561,14413	45706,6711	43044,94478	52052,06032
093 p2 (short)	28853,90037	43914,48857	43477,56362	72402,7514
002 p5 (short)	26370,47535	32208,96518	35596,39125	54720,83696
076 p3 (long)	35242,22678	39735,49519	39735,49519	52575,20425
120 p1 (short)	7540,17334	18124,14498	22247,84055	64215,48724
018 p5 (short)	30946,03477	38177,44559	38561,14413	50513,70294
116 p1 (long)	32208,96518	42616,63067	38561,14413	50513,70294
139 p0 (long)	23155,79126	24587,65229	25084,3673	65512,75612
118 p1 (long)	29436,77323	41357,12993	34891,55278	40538,18534
006 p4 (short)	24343,01871	28566,79273	27173,56863	60475,90069
089 p2 (short)	22471,44021	26108,07876	24587,65229	53637,31838
121 p2 (long)	16514,5963	17274,72914	21141,63576	55826,29674
sample (TTL)	eNOS	P38 MAPKT180 Y182	JAK1 Y1022-1023	Cyclin A
125 p1 (long)	47572,03929	73865,41781	51021,38577	22026,46579
068 p1 (long)	38561,14413	44355,84667	11896,50364	14457,96096
112 p2 (long)	31888,47332	46630,02845	12913,12614	16697,25432
132 p2 (short)	34200,63809	80821,65296	17783,03606	14044,69735
015 p4 (long)	39340,1114	38177,44559	12746,34788	10270,18653
093 p2 (short)	40945,61024	62317,64942	14838,78355	16139,09922
002 p5 (short)	35954,14916	47572,03929	16383,01609	19990,24889
076 p3 (long)	40538,18534	52575,20425	18434,87537	14574,08008
120 p1 (short)	20496,31617	22697,26548	6399,658695	9414,436787
018 p5 (short)	34544,36812	37421,46439	15382,71559	20312,66114
116 p1 (long)	42616,63067	50011,07175	18416,45982	15962,54709
139 p0 (long)	35596,39125	52575,20425	15552,86939	14515,90441
118 p1 (long)	34544,36812	32859,61314	13807,95276	15962,54709
006 p4 (short)	33189,86534	64215,48724	11884,61962	15382,71559
089 p2 (short)	37421,46439	71682,38394	15693,47425	17747,50811
121 p2 (long)	32208,96518	30031,44924	11896,50364	17378,68153

sample (TTL)	Cyclin B	Cyclin E	Cyclin D1	ann1
125 p1 (long)	9191,187324	3633,675517	34200,63809	36680,48794
068 p1 (long)	9009,185497	14200,0373	25591,09244	14853,63576
112 p2 (long)	15199,22487	8316,527034	29732,62452	7684,799377
132 p2 (short)	7958,536069	707,6855084	25336,47518	5192,654476
015 p4 (long)	7457,681223	1222,924262	27722,49738	11103,32154
093 p2 (short)	33860,36059	8821,966101	29732,62452	76879,91976
002 p5 (short)	42192,57845	5029,117314	30638,10957	8217,324422
076 p3 (long)	23388,51619	2237,243531	29732,62452	3869,960888
120 p1 (short)	10807,54853	3255,171683	20847,71637	3090,228303
018 p5 (short)	33523,4367	1471,444939	31571,17048	1670,703382
116 p1 (long)	27173,56863	11147,83117	31888,47332	4564,205067
139 p0 (long)	27446,67381	13480,5047	27722,49738	33860,36059
118 p1 (long)	5779,079555	9027,22059	29436,77323	4591,675
006 p4 (short)	28853,90037	5313,46832	29436,77323	46630,02845
089 p2 (short)	21568,735	17188,56911	34544,36812	34544,36812
121 p2 (long)	29436,77323	8725,459048	31257,05474	4363,368218
sample (TTL)	beta catenina	kip1 p27	smac diablo	fak y397
125 p1 (long)	11884,61962	29436,77323	65512,75612	5486,246794
068 p1 (long)	8866,187405	31888,47332	47098,67881	22247,84055
112 p2 (long)	9423,859744	23155,79126	49020,81049	15214,43785
132 p2 (short)	22697,26548	41772,78556	35954,14916	4807,823122
015 p4 (long)	11742,85547	36680,48794	43914,48857	6614,370644
093 p2 (short)	12258,80284	33189,86534	50513,70294	18160,42691
002 p5 (short)	11059,00017	37797,56501	42192,57845	12708,16284
076 p3 (long)	7044,480326	27446,67381	31257,05474	7030,406464
120 p1 (short)	27173,56863	12431,64417	10604,14301	7762,034672
018 p5 (short)	35242,22678	14429,06226	37797,56501	7420,485034
116 p1 (long)	14086,89836	22697,26548	48533,03416	10016,60899
139 p0 (long)	8777,965357	26635,48372	42616,63067	18939,40549
118 p1 (long)	9585,436758	31888,47332	43914,48857	19399,45293
006 p4 (short)	11579,60165	33189,86534	40134,85274	15490,77189
089 p2 (short)	10530,17352	39735,49519	51021,38577	14002,62276
121 p2 (long)	16647,24917	28566,79273	44355,84667	15756,36929
sample (TTL)	xiap	Marcks152	PKA c t197	PKC delta t505
125 p1 (long)	48533,03416	50513,70294	112420,3348	194852,9217
068 p1 (long)	74607,79468	36315,50267	125492,3113	117008,2912
112 p2 (long)	65512,75612	21547,16834	121783,4855	123007,458
132 p2 (short)	38177,44559	26108,07876	101722,0866	96761,02349
015 p4 (long)	52575,20425	25591,09244	130613,7447	129314,2099
093 p2 (short)	56954,0345	19438,28784	144350,5672	121783,4855
002 p5 (short)	45706,6711	17102,8388	144350,5672	110194,2126
076 p3 (long)	36680,48794	22925,38204	125492,3113	126753,559
120 p1 (short)	15168,859	58104,60892	159531,9576	176310,1473
018 p5 (short)	43044,94478	18882,66752	123007,458	121783,4855
116 p1 (long)	46630,02845	31257,05474	118184,2711	120571,692
139 p0 (long)	40945,61024	66836,16844	99707,90382	102744,4341
118 p1 (long)	54720,83696	8742,926161	133252,3784	117008,2912
006 p4 (short)	35596,39125	29732,62452	101722,0866	114691,3193
089 p2 (short)	47098,67881	20952,21839	98715,77101	120571,692
121 p2 (long)	70262,94547	19712,33654	112420,3348	110194,2126

sample (TTL)	AMPK beta s108	BIM	mTOR S2448	4EBP1 S65
125 p1 (long)	72402,7514	17000,5364	37049,14145	176310,1473
068 p1 (long)	98715,77101	4420,464471	114691,3193	125492,3113
112 p2 (long)	58688,52741	16680,57457	111301,7083	118184,2711
132 p2 (short)	62317,64942	4337,268278	134591,6178	80017,44576
015 p4 (long)	100710,008	10311,34656	131926,4648	114691,3193
093 p2 (short)	76879,91976	25084,3673	135944,1875	128027,4828
002 p5 (short)	94845,07388	6476,917767	144350,5672	100710,008
076 p3 (long)	126753,559	60475,90069	190994,4879	102744,4341
120 p1 (short)	58104,60892	15475,29736	93901,32814	80017,44576
018 p5 (short)	105873,4397	66171,18541	169396,934	129314,2099
116 p1 (long)	144350,5672	27722,49738	196811,0835	124243,6135
139 p0 (long)	80017,44576	23155,79126	190994,4879	103777,0566
118 p1 (long)	52052,06032	7273,553192	178082,1345	61083,70757
006 p4 (short)	91126,14882	12456,53053	187212,6365	96761,02349
089 p2 (short)	92042,00291	2713,515043	250196,104	94845,07388
121 p2 (long)	82454,31776	24587,65229	126753,559	91126,14882
sample (TTL)	P70s6k T389	AMPKa T172	PKCa S657	erk1 2 t202 y204
125 p1 (long)	84965,48392	114691,3193	176310,1473	39735,49519
068 p1 (long)	44801,64059	108012,2745	141492,1697	49513,48916
112 p2 (long)	62317,64942	99707,90382	142914,2224	40945,61024
132 p2 (short)	30031,44924	73130,42788	142914,2224	30333,24834
015 p4 (long)	34891,55278	111301,7083	128027,4828	40134,85274
093 p2 (short)	52575,20425	105873,4397	74607,79468	37421,46439
002 p5 (short)	29436,77323	80821,65296	61697,56432	30031,44924
076 p3 (long)	33523,4367	98715,77101	120571,692	33189,86534
120 p1 (short)	20434,91407	86681,85756	80821,65296	16848,22099
018 p5 (short)	16647,24917	92966,97303	89321,68929	33189,86534
116 p1 (long)	47098,67881	100710,008	138690,5058	33189,86534
139 p0 (long)	40538,18534	90219,40784	245241,7769	21698,52699
118 p1 (long)	33523,4367	90219,40784	117008,2912	32208,96518
006 p4 (short)	31888,47332	89321,68929	71682,38394	26635,48372
089 p2 (short)	54176,34313	95798,30465	65512,75612	20434,91407
121 p2 (long)	38948,66184	76114,93436	86681,85756	14928,09163
sample (TTL)	irs1 s612	p90 rsk s380	sapk jnk t183 y185	nfkb p65053
125 p1 (long)	59874,14172	48050,11141	38948,66184	28001,11952
068 p1 (long)	64215,48724	80017,44576	59278,37048	25848,29312
112 p2 (long)	39735,49519	81633,94276	31888,47332	21461,15803
132 p2 (short)	62943,96661	79221,24074	40945,61024	25591,09244
015 p4 (long)	64860,87846	41357,12993	44801,64059	31257,05474
093 p2 (short)	63576,57854	84120,04398	51534,17101	30333,24834
002 p5 (short)	70262,94547	38177,44559	51534,17101	43044,94478
076 p3 (long)	69563,80156	60475,90069	56387,31949	33860,36059
120 p1 (short)	67507,89855	33523,4367	54176,34313	21120,51629
018 p5 (short)	84120,04398	40134,85274	43044,94478	27173,56863
116 p1 (long)	72402,7514	79221,24074	47572,03929	28282,54192
139 p0 (long)	76879,91976	46166,04135	53103,606	25084,3673
118 p1 (long)	58104,60892	35954,14916	36315,50267	18788,48767
006 p4 (short)	66171,18541	64215,48724	45251,91493	26108,07876
089 p2 (short)	66836,16844	66836,16844	73130,42788	35954,14916
121 p2 (long)	58104,60892	62943,96661	35242,22678	23860,98372

sample (TTL)	cl notch val1744	stat1 y701	ann2	
125 p1 (long)	45251,91493	40134,85274	11778,12876	
068 p1 (long)	41357,12993	36680,48794	2565,734072	
112 p2 (long)	38948,66184	30333,24834	10456,72002	
132 p2 (short)	34544,36812	25848,29312	1288,198198	
015 p4 (long)	51021,38577	43914,48857	1818,923355	
093 p2 (short)	33860,36059	30031,44924	12271,07274	
002 p5 (short)	39735,49519	31257,05474	7903,021087	
076 p3 (long)	40538,18534	39340,1114	12295,63766	
120 p1 (short)	4614,691395	9877,35413	1853,813403	
018 p5 (short)	35242,22678	30333,24834	7442,781845	
116 p1 (long)	35596,39125	34891,55278	6768,26256	
139 p0 (long)	19930,36284	22697,26548	10785,9566	
118 p1 (long)	28853,90037	31571,17048	10959,91764	
006 p4 (short)	28853,90037	26903,18094	14943,03323	
089 p2 (short)	25084,3673	21894,71221	15568,42149	
121 p2 (long)	16317,60409	17396,07594	5480,7663	

Raw protein expression data was obtained by reverse phase protein array (RPPA) analysis. TTL indicates time to leukemia.

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8. Curriculum vitae

Lebenslauf aus Gründen des Datenschutzes entfernt.

Publication

Queudeville, M.*, Seyfried, F.*, Eckhoff, S. M., Trentin, L., Ulrich, S., Schirmer, M., Debatin, K. M., and Meyer, L. H.: Rapid engraftment of human ALL in NOD/SCID mice involves deficient apoptosis signaling. Cell Death Dis *3*: e364 (2012)

*equal contribution

Congress participations

Seyfried, F., Accordi, B., Queudeville, M., Eckhoff, S.M., Milani, G., Galla, L., Giordan, M., Kraus, J.M., Basso, G., Kestler, H.A., te Kronnie, G., Debatin, K.-M., Meyer, L.H.: High Risk Acute Lymphoblastic Leukemia with Rapid NOD/SCID Engraftment Is Characterized by High Protein Expression of CYCLIN B, Beta-CATENIN, ANNEXIN I and Decreased PKC Alpha Activation. 53rd ASH Annual Meeting. Poster presentation. San Diego (2011)

Seyfried, F., Accordi, B., Queudeville, M., Eckhoff, S.M., Milani, G., Galla, L., Giordan, M., Kraus, J.M., Basso, G., Kestler, H.A., te Kronnie, G., Debatin, K.-M., Meyer, L.H.: Reverse Phase Protein Array (RPPA) of High Risk ALL. Annual Meeting of the Kind-Philipp Foundation for Leukemia Research. Oral presentation. Wilsede (2012)

Seyfried, F., Queudeville, M., Eckhoff, S. M., Debatin, K.-M., Meyer, L. H.: Intact apoptosis signaling in pediatric ALL is associated with patient outcome, low expression of anti-apoptotic molecules and long NOD/SCID engraftment. Annual Meeting of the Kind-Philipp Foundation for Leukemia Research. Oral presentation. Wilsede (2010)

Seyfried, F., Queudeville, M., Eckhoff, S.M., Debatin, K.-M., Meyer, L.H.: Proficient apoptosis signaling in pediatric ALL is associated with survival, down-regulation of anti-apoptotic molecules and prolonged NOD/SCID engraftment. ACUTE LEUKEMIAS XIII. Poster presentation. Munich (2011)