Identifying therapeutic targets by elucidating signaling pathways in pediatric lymphoid leukemias
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General introduction and Scope of the thesis
GENERAL INTRODUCTION

Normal and malignant hematopoiesis

The biological process of blood cell formation is called hematopoiesis. All types of blood cells originate from a small pool of pluripotent stem cells, known as hematopoietic stem cells (HSC). The maintenance, commitment, proliferation, and differentiation of HSCs is dependent on the bone marrow microenvironment. In the bone marrow, bone marrow stromal cells (including stromal fibroblasts, osteoblasts, endothelial cells, and adipocytes) support the production of blood cell maturation by producing growth factors, direct stromal cell-blood cell contact, and the production of the extracellular bone marrow matrix. In this bone marrow niche, HSCs are multipotent and have the capacity to differentiate towards all types of mature blood cells. Before differentiating into various mature blood cells, the committed HSC will first differentiate into lineage-specific progenitor cells; the common myeloid progenitor and the common lymphoid progenitor (Figure 1).2,3

Genetic alterations in hematopoietic stem and progenitor cells disturb the regulation of normal hematopoiesis leading to a clonal proliferation of hematopoietic progenitor cells in the bone marrow.4,5 Accumulation of subsequent genetic changes can lead to leukemia formation. Leukemia is characterized by an uncontrolled proliferation of immature blood cells, accompanied by a block in maturation and frequently escape from apoptosis, resulting in an excess of malignant cells.6

![Figure 1 Hematopoiesis](image-url)
Leukemias can be stratified according to the affected lineage into either lymphoid or myeloid. When the differentiation block occurs in the myeloid lineage the disease is called ‘myeloid leukemia’, whereas a blocked lymphoid lineage differentiation is called ‘lymphoid leukemia’. Leukemias can also be classified based on clinical presentation and disease development in ‘acute’ or ‘chronic’. In total we can classify four main categories: Acute Lymphoblastic Leukemia (ALL), Acute Myeloid leukemia (AML), Chronic Lymphoblastic Leukemia (CLL), and Chronic Myeloid Leukemia (CML). This thesis will focus on therapeutic targets in the context of pediatric ALL and a progressed phase of pediatric CML (lymphoid blast crisis CML).

**Acute Lymphoblastic Leukemia**

*Epidemiology and clinical presentation*

With approximately 25% of all pediatric malignancies diagnosed, ALL is the most common type of cancer among children (<15 years). The peak incidence occurs between two and five years of age with a male:female prevalence of 1.3:1.

The symptoms of ALL are mainly nonspecific e.g. fatigue, pale skin, fever, bleeding, hematomata, petechiae, bone pain, and lymphadenopathy. Symptoms occur by displacement of normal bone marrow cells by immature blasts, resulting in anemia, thrombocytopenia, and granulocytopenia or lymphocytopenia. Bone pain, most frequently affecting the long bones, is caused by leukemic involvement of the periosteum. Mediastinal mass, testicular enlargement, and focal neurological signs are rare.

*Pathobiology*

In general, ALL arises from various genetic, epigenetic, and environmental lesions in a lymphoid progenitor cell leading to an uncontrolled cell proliferation and stage-specific development arrest. Historically, ALL was classified based on morphology using the French-American-British (FAB) system incorporating information regarding the size, amount of cytoplasm, and prominence of nucleoli of tumor cells from the bone marrow. The World Health Organization (WHO) published a classification system for tumors and leukemias incorporating immunophenotypic, genetic, and clinical information to define a clinically and biologically relevant disease nomenclature. According to the immunophenotype, ALL is first classified into B-cell progenitor ALL (BCP-ALL) and T-cell ALL (T-ALL) and secondly subclassified according to cytogenetic and molecular genetic abnormalities. The complete WHO classification is shown in Table 1.

A minority of all ALL cases (<5%) is associated with inherited, predisposing genetic syndromes as for example Down’s syndrome. Cytogenetic and molecular genetic abnormalities are known in approximately 75% of the cases. In BCP-ALL these abnormalities include hyperdiploidy
(>50 chromosomes), hypodiploidy (<44 chromosomes), and the translocations t(12;21)(p13;q22) encoding ETV6-RUNX1, t(1;19)(q23;p13) encoding TCF3-PBX1, t(9;22)(q34;q11) encoding BCR-ABL1, and MLL rearrangements (Figure 2). Dysregulation of the T-cell receptor (TCR) genes TLX1, TLX3, HOXA, TAL1, TAL2, LMO1, LMO2, and LYL1 often occurs in T-ALL (Figure 2). However, these translocations alone do not induce leukemia in experimental mouse models and in 25% of the cases no chromosomal alteration are known, suggesting additional genetic alterations contributing to leukemogenesis. High-resolution microarray profiling revealed the presence of more genetic alterations involved in prognosis and development of leukemia and defined new leukemia subtypes (e.g. IKZF1, PAX5, and CDKN2A/B deletions in BCP-ALL, and CDKN2A deletions, activating NOTCH1 mutations, and PTEN loss in T-ALL). Similarly, gene-expression profiling revealed new ALL subtypes based on gene-expression signatures such as BCR-ABL1-like in BCP-ALL (also known as Philadelphia-like), a subtype of precursor BCP-ALL with a similar gene expression profile compared to Philadelphia-positive ALL, and early T-cell precursor (ETP) ALL in T-ALL.

**Table 1** WHO classification of acute lymphoblastic leukemias (World Health Organization 2008)

<table>
<thead>
<tr>
<th>Acute lymphoblastic leukemia</th>
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<tbody>
<tr>
<td>1. B lymphoblastic leukemia</td>
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<tr>
<td>B lymphoblastic leukemia, not otherwise specified</td>
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<tr>
<td>B lymphoblastic leukemia with recurrent genetic abnormalities</td>
</tr>
<tr>
<td>B lymphoblastic leukemia with t(9;22)(q34;q11.2); BCR-ABL1</td>
</tr>
<tr>
<td>B lymphoblastic leukemia with t(v;11q23); MLL-rearranged</td>
</tr>
<tr>
<td>B lymphoblastic leukemia with t(12;21)(p13;q22); TEL-AML1 (ETV6-RUNX1)</td>
</tr>
<tr>
<td>B lymphoblastic leukemia with hyperdiploidy</td>
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<tr>
<td>B lymphoblastic leukemia with hypodiploidy</td>
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<tr>
<td>B lymphoblastic leukemia with t(5;14)(q31;q32); IL3-IGH</td>
</tr>
<tr>
<td>B lymphoblastic leukemia with t(1;19)(q23;p13.3) E2A-PBX1 (TCF3-PBX1)</td>
</tr>
<tr>
<td>2. T lymphoblastic leukemia</td>
</tr>
</tbody>
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**Prognostic factors and prognosis**

Clinical and biological factors are important prognostic predictors of outcome. Age (infant or ≥ 10 years of age), leukocyte count (≥ 50x10^9/L), race (black and Hispanic), and male sex are unfavorable clinical prognostic factors. Unfavorable biological prognostic factors include T-ALL, hypodiploidy, MLL-translocations, and BCR-ABL fusion. Recently, IKZF1 deletions are identified as an unfavorable prognostic factor. The most relevant prognostic factor is
the early response to therapy e.g. minimal residual disease (MRD). The prognosis of patients with ≥1% leukemic cells after remission-induction therapy is almost as poor as those who fail to achieve complete clinical remission (≥5% leukemic cells).

Since the introduction of chemotherapy, and especially the introduction of combination chemotherapy, overall 5-year event-free and overall survival rates for ALL have increased dramatically. Current overall survival rates are approaching 90%. Nevertheless, despite intensive chemotherapy, still about 20% of ALL patients struggle with ALL relapse.

Figure 2 Frequencies of key subtypes of childhood ALL
Data were modified from Mullighan et al. Abnormalities that are exclusively seen in T-ALL are shown in blue.

**Chronic Myeloid Leukemia**

*Epidemiology and clinical presentation*

With an annual incidence of 0.6-1.2 per million children/year, accounting for approximately 2-3% of all pediatric leukemia cases, CML is very rare among children. With age, the incidence of CML increases with a median age at diagnosis of 65 years. The median age at presentation in children is 11 years. At initial presentation, 85% of pediatric CML patients is diagnosed in the chronic phase (CP). CML has historically been a triphasic disease. Without treatment, CML can rapidly progress to accelerated phase (AP) and blast crisis (BC), which in adults involves either myeloid (67%) or lymphoid (33%) transformation.

As with acute lymphoblastic leukemia, the most frequent symptoms of CML are nonspecific, including fatigue, night sweats, malaise and weight loss, left upper quadrant pain, and splenomegaly.
Pathobiology

CML is a clonal hematopoietic stem cell disorder characterized by the presence of the Philadelphia chromosome translocation t(9;22)(q34;q11), generating the BCR-ABL1 fusion gene which is giving rise to the constitutively active tyrosine kinase BCR-ABL. The initial chronic phase is characterized by a clonal expansion of the granulocytic cell lineage, even though theoretically all hematopoietic lineages can be produced from the CML stem cell.

The transition of CML chronic phase into blast crisis is not completely understood. Currently it is hypothesized that BCR-ABL activity cause genomic instability, resulting in the accumulation of genetic abnormalities activating oncogenic factors and/or mutations required for disease progression to blast phase. This phase is characterized by a block in cell differentiation of the myeloid or lymphoid lineage known as myeloid blast crisis (MyBC) or lymphoid blast crisis (LyBC), respectively.

Prognosis

With the introduction of tyrosine kinase inhibitors (TKIs), like imatinib (Gleevec®), CML has transformed from a fatal disease to a leukemia subtype with a favorable prognosis. Estimated progression-free survival rates are 98% among children with chronic phase CML at 36 months, which is comparable to the results achieved in adults. Unfortunately, when CML has progressed to blast crisis, treatment options are limited to only a few months.

Signal transduction pathways in cancer

The transformation from a normal cell to a malignant cell is a highly complex process and involves multiple genetic, epigenetic, and environmental events. Hanahan and Weinberg have defined specific characteristics of malignant cells, known as the hallmarks of cancer, which are: sustaining proliferative signaling, evading growth suppressors, avoiding immune destruction, enabling replicative immortality, tumor-promoting inflammation, activating invasion and metastasis, inducing angiogenesis, genome instability and mutation, resisting cell death, and deregulating cellular energetics. These characteristics are acquired through complex genetic changes including the activation of oncogenes by mutations, translocations, and amplifications resulting in enhanced cell signaling, which in turn leads to oncogenic transformation. Mutations in tumor suppressor genes, such as loss of function deletions, similarly contribute to oncogenesis. In addition to oncogenes and tumor suppressor genes, aberrations in proapoptotic and antiapoptotic genes as well as genes that control cell proliferation and differentiation contribute to cancer formation and metastasis.

Genetic alterations can lead to or coincide with epigenetic changes including loss of DNA methylation and alterations in the histone acetylation patterns. Many of the genetic and
epigenetic abnormalities which are at the basis of oncogenic transformation, maintenance, and tumor progression ultimately influence the activation of signal transduction pathways.\(^{37}\)

Signal transduction pathways are intracellular signaling cascades traditionally initiated by an extracellular stimulus e.g. binding of ligand by a transmembrane receptor that transfers the signal through a series of phosphorylation events until finally reaching regulatory molecules in the nucleus, such as transcription factors. Cellular signal transduction activity is controlled by protein phosphorylation. Therefore, the balance of kinase and phosphatase activity, responsible for protein phosphorylation and dephosphorylation, respectively, regulate many key aspects of intracellular processes. In the human body, 518 unique human protein kinases and 58 receptor tyrosine kinases have been identified.\(^{39,40}\) Malignant transformation of cells is highly dependent on the deregulation of protein kinases responsible for the activation of signal transduction pathways.\(^{41}\) In this regard, protein kinases are highly interesting targets to be investigated by pharmaceutical companies.

**Signal transduction for the identification of druggable targets**

Over the past decade, molecular profiling of the genome, epigenome, transcriptome, and proteome has markedly increased our knowledge of the molecular background underlying cancer development. As a consequence, treatment strategies have changed from a “one size fits all” principle to a more personalized approach.\(^{42}\) The dictionary of cancer terms from the National Cancer Institute (NCI) defines personalized medicine as “A form of medicine that uses information about a person’s genes, proteins, and environment to prevent, diagnose, and treat disease” (www.cancer.gov/dictionary). A breakthrough for targeted therapy was the introduction of the tyrosine kinase inhibitor imatinib (Gleevec ®) for the treatment of CML. Imatinib blocks the ATP binding site on the constitutively activated protein kinase BCR-ABL1 and thereby inhibits the phosphorylation of downstream substrates.\(^{43}\) To date, 29 small molecule inhibitors have received US Food and Drug Administration (FDA) approval for cancer indications. Currently, targeted therapy is also used in clinical practice for patients with non-small cell lung cancer (NSCLC) harboring ALK-rearrangements, EGFR-mutated NSCLC, KIT-mutated gastrointestinal stroma tumors, and melanoma harboring BRAF V600 mutations.\(^{44-47}\)

From the clinical point of view, in pediatric lymphoid leukemias, kinase inhibitors only have a revolutionary impact to the treatment of Philadelphia-positive (Ph\(^+\)) ALL and CML.\(^{48}\) However, genomic profiling of high risk BCP-ALL identified several subgroups sensitive to kinase inhibitors.\(^{49,50}\) Genomic profiling of the BCR-ABL1-like ALL subtype identified rearrangements involving EBF1 and PDGFRB giving rise to the constitutively active PDGFRB kinase which is sensitive to FDA-approved ABL1 inhibitors as imatinib and dasatinib.\(^{50-52}\) Additionally, characterization of the signaling consequences of CRLF2-rearrangements in ALL showed increased basal levels of pSTAT5 and pJAK2 which can be targeted with JAK inhibitors.\(^{53}\) In addition to CRLF2-rearranged ALL, JAK inhibitors are interesting for the treatment of human
leukemic cells with JAK2 rearrangements as well as erythropoietin receptor (EPOR) rearranged ALL.\textsuperscript{49}

In T-ALL the rationale for the use of kinase inhibitors is less intensively studied compared to BCP-ALL. Genomic profiling of early T cell precursor (ETP) ALL, an aggressive subtype of T-ALL, showed activating mutations in genes regulating cytokine receptor, RAS, and JAK/STAT signaling in more than 60\% of the cases, thus suggesting the use of targeted therapy for this specific T-ALL subtype.\textsuperscript{54,55} Unfortunately, intensive genome profiling has not yet resulted in the widely implementation of kinase inhibitors for the treatment of ALL and CML-LyBC.

**SCOPE OF THE THESIS**

Although the survival of pediatric leukemias, in particular acute lymphoblastic leukemia, has improved remarkably over the last decades, ALL relapse remains a leading cause of cancer death among children and the survival of lymphoid blast crisis CML is limited in terms of months.\textsuperscript{23,34} Major improvements in outcome for ALL are the result of improved scheduling and dosing of combination chemotherapies. However, these improvements in clinical outcome have reached a plateau, mostly due to chemotherapy related toxicity.\textsuperscript{56} Furthermore, treatment options for lymphoid blast crisis CML are limited due to tyrosine kinase inhibitor resistance.\textsuperscript{34} Therefore, new treatment options are needed to cure all children with lymphoid leukemia.

Since genomic, epigenomic, and environmental alterations affect components of signaling pathways that lead to critical cellular processes such as proliferation and differentiation, the aim of this thesis is to identify therapeutic targets by elucidating signal transduction pathways in pediatric lymphoid leukemias.

We are currently facing significant limitations in implementing newly designed kinase inhibitors in the clinic for the treatment of pediatric malignancies. As many hallmarks of cancer are known to be regulated by intracellular protein signaling networks, we suggest to focus on these networks to improve the implementation of kinase inhibitors. Chapter 2 will provide a short overview of currently used strategies for the implementation of kinase inhibitors as well as reasons why kinase inhibitors has unfortunately not yet resulted in the widespread use for the treatment of pediatric cancer. Furthermore, we will propose a personalized medicine strategy, combining kinomics, proteomics, and drug screen approaches attempting to bridge the gap between pediatric cancers and the use of kinase inhibitors.

ALL arises after the occurrence of several known and unknown abnormalities.\textsuperscript{9} Previously we have shown that kinome profiling is an elegant approach to generate insight into active signal transduction pathways and is able to identify new potential druggable targets for future treatment
strategies.\textsuperscript{57-59} In Chapter 3 we used kinome profiling to study active signal transduction pathways in pediatric ALL and investigated known and unknown signaling proteins relevant to ALL and of potential interest for future targeted therapy approaches.

Subsequently, our kinase activity profiles showed a significantly elevated phosphorylation of a peptide derived from cyclic (c)-AMP response element binding protein\_Y134/S133 (CREB\_Y134/S133) compared to normal bone marrow.\textsuperscript{58} The applicability of CREB as a potential druggable target in ALL is described in Chapter 4. We studied the effects of a loss of CREB function on ALL cell survival and gene expression profiles using shRNA mediated knockdown of CREB and a CREB inhibitor. Moreover, we correlated CREB expression in primary ALL patients to outcome.

Genome wide approaches in ALL have increased the list of risk predictors and added \textit{IKZF1} deletions as an unfavorable prognostic factor.\textsuperscript{19,21,22} Although in adults \textit{IKZF1} deletions are most frequently identified in Philadelphia chromosome positive (Ph\textsuperscript{+}) ALL, in pediatric BCP-ALL 80\% of the \textit{IKZF1} deletions are found in a Philadelphia negative background. Effects of \textit{IKZF1} deletions on signaling pathways in this Philadelphia negative group is largely unknown. In Chapter 5 we studied the effect of \textit{IKZF1} deletions on active signal transduction pathways using kinome profiling and western blot analysis.

Since treatment options for CML are limited once the disease has progressed to lymphoid blast crisis, early recognition of patients at risk of disease progression to CML-LyBC is desirable. In Chapter 6 we used Multiplex Ligation-dependent Probe Amplification (MLPA) to determine whether B-cell lymphoid leukemia-specific copy number alterations (CNAs), including \textit{IKZF1}, \textit{PAX5}, and \textit{CDKN2A} deletions, could be detected in CML-CP and used to predict disease progression to CML-LyBC.

The activation of signaling pathways, independent of BCR-ABL1 activity, might be involved in disease progression to CML-LyBC and CML-LyBC cell survival. Therefore, inhibition of signaling hubs of these pathways might be of potential interest as new targets for the treatment of lymphoid blast crisis CML. In Chapter 7 we have studied the role of Akt, STAT3, and FAK for the survival of CML-LyBC cells to identify new potential therapeutic targets.

Finally, Chapter 8 will summarize the results of the studies described in this thesis and the results will be discussed in a broader perspective. In Chapter 9 a summary of the thesis in Dutch is given.
REFERENCES


