Tampere University

ARTTURI MÄKINEN

Novel Biomarkers of Pediatric B-cell Acute Lymphoblastic Leukemia

Tampere University Dissertations 629

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ACADEMIC DISSERTATION
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Tampere, May 2022
Artturi Mäkinen

ABSTRACT

B-cell acute lymphoblastic leukemia (B-ALL) is the most common pediatric malignancy. It is characterized by the fast proliferation of clonal white blood cells in the bone marrow and blood. Occasionally, B-ALL may infiltrate lymph nodes and other sites outside of bone marrow, such as the CNS. Although the prognosis has improved dramatically during the past decades through intensification of the treatment and monitoring of response to treatments, relapse of the disease is still a major cause of morbidity and mortality. This prevalent hardship forces the continuous re-evaluation of disease classification and investigation for novel treatments.

The pathogenesis of B-ALL includes the aberrant expression of different transcription factors that lead to uncontrolled proliferation and derailed migration of leukemic populations. This dissertation focused on two transcription factors, namely neurodevelopmental and mantle cell lymphoma-associated transcription factor SOX11 and the germinal center-originated lymphoma-associated proto-oncogene BCL6, along with the oncofetal post-transcriptional modifier protein IGF2BP3. We utilized pre-existing gene expression datasets, collected a population-based immunohistochemistry sample cohort, and correlated the findings to treatment response and patient survival.

We found BCL6 was associated with the *TCF3-PBX1* subtype and several novel subtypes, such as, *MEF2D* and components of the pre-BCR signaling pathway. At mRNA level, higher *BCL6* levels indicated better prognosis. SOX11 was expressed particularly in *ETV6-RUNX1* and *TCF3-PBX1* subtypes of B-ALL, and high expression of SOX11 protein was associated with a favorable outcome. Low level of *SOX11* promoter methylation was likely behind the high expression and high methylation levels could be overcome by treatment with demethylating agent decitabine. Finally, IGF2BP3 was discovered to be widely expressed in B-ALL at protein and mRNA levels and associated with a proliferative cell phenotype. High levels of *IGFBP3* mRNA were associated with better treatment response in high-risk B-ALL cases.

In summary, we found several potential novel biomarkers for B-ALL. Although the results suggest prognostic utility in patient care, they require further validation in

prospective cohorts and with current treatment protocols before application in clinics.

TIIVISTELMÄ

B-soluinen akuutti lymfoblastileukemia on yleisin lapsilla esiintyvä syöpä. Tälle klonaaliselle taudille on ominaista syöpäsolujen nopea jakaantuminen luuytimessä ja veressä. Ajoittain tautia voi esiintyä esimerkiksi imusolmukkeissa tai keskushermostossa. Vaikka taudin ennuste on parantunut merkittävästi viimeisten vuosikymmenten aikana taudin intensiivisemmän hoidon ja tarkemman hoitovasteseurannan avulla, aiheuttaa taudin uusiutuminen edelleen sairastavuutta ja kuolleisuutta. Tämän vuoksi tautia pyritään edelleen luokittelemaan tarkemmin, jotta hoitoa osataan kohdentaa paremmin.

Taudin syntyyn liittyy olennaisella tavalla geenien ilmentymistä säätelevien transkriptiotekijöiden ilmentyminen normaalista poikkeavalla tavalla leukemiasoluissa, mikä voi johtaa leukemiasolujen kasvuun ja hallitsemattomaan jakaantumiseen. Tässä väitöskirjassa keskityttiin kahteen transkriptiotekijään: SOX11-nimiseen transkriptiotekijään, joka kytkeytyy hermoston kehitykseen sekä manttelisolulymfoomaan sekä itukeskuslähtöisissä lymfoomissa ilmentyvään BCL6-proto-onkogeeniin. Lisäksi tutkittiin RNA:ta sitovan IGF2BP3:n roolia leukemiassa. Tutkimuksissa hyödynnettiin olemassa olevia geenieksпressiotiedostoja sekä tutkimusta varten kerättyä väestöpohjaista immunohistokemian näytesarjaa. Ilmentymistuloksia verrattiin potilaiden kliinisiin tietoihin.

BCL6:n ilmentyminen oli korkein *TCF3-PBX1*- sekä mm. uudessa *MEF2D*-alaryhmässä, ja se nivoutui myös pre-BCR-signalointireitin osien ilmentymiseen. Korkeampi mRNA-ekspressiotaso yhdistyi suotuisampaan ennusteeseen lapsipotilailla. Havaitsimme SOX11-ekspression yhteyden *ETV6-RUNX1*- ja *TCF3-PBX1*-alaryhmiin sekä suotuisampaan ennusteeseen B-ALL-potilailla. Lisäksi havaitsimme, että *SOX11*-geenin promoottorialueen korkea metylaatio liittyi geenin ilmentymisen säätelyyn, ja sitä voitiin muokata metylaatiota poistavalla lääkeaineella, desitabiinilla. IGF2BP3 ilmentyi laajalti eri B-ALL-alaryhmissä, ja korkeampi ilmentyminen mRNA-tasolla liittyi parempaan hoitovasteeseen korkean riskin potilailla.

Väitöskirjatutkimuksessa tunnistettiin useita ennusteellisia biomarkkereita akuuttiin lymfoblastileukemiaan, ja löydökset viittaavat niiden mahdolliseen hyötyyn potilaiden ennustetta arvioitaessa. Tulokset tulisi kuitenkin jatkossa vahvistaa

prospektiivisessa koeasetelmassa ja nykyisissä hoitoprotokollissa ennen niiden mahdollista kliinistä käyttöä.

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ABBREVIATIONS

muHC	immunoglobulin heavy constant mu
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
B-ALL	B-cell acute lymphoblastic leukemia
BCL6	BCL6 transcription repressor
BM	bone marrow
CD19	CD19 molecule
CDK6	cyclin dependent kinase 6
CHL	classic Hodgkin lymphoma
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CNS	central nervous system
DAB	3,3'-Diaminobenzidine
DBD	DNA-binding domain
DC	dendritic cell
DLBCL	diffuse large B-cell lymphoma
DN	double negative
DNA	deoxyribonucleic acid
DP	double positive
DUX4	double homeobox 4
EBF1	EBF transcription factor 1
EDTA	ethylenediaminetetraacetic acid
EFS	event-free survival
EOI	end of induction
ERG	ETS transcription factor ERG
ETS	erythroblast transformation-specific family
ETV6	ETS variant transcription factor 6
FC	flow cytometry
FFPE	formalin-fixed, paraffin-embedded
FL	follicular lymphoma

GC	germinal center
HSC	hematopoietic stem cell
IGF2BP3	insulin like growth factor 2 mRNA binding protein 3
IHC	Immunohistochemistry
IKZF1	IKAROS family zinc finger 1
IL7R	interleukin-7 receptor
Ki-67	marker of proliferation Ki-67
KMT2A	lysine methyltransferase 2A
LMPP	lymphoid-primed multipotent progenitor
MCL	mantle cell lymphoma
MEF2D	myocyte enhancer factor 2D
MK	megakaryocyte
MPP	multipotent progenitor
MRD	minimal residual disease
mRNA	messenger ribonucleic acid
MYC	MYC proto-oncogene
NOS	not otherwise specified
NOTCH	notch receptor
NUTM1	NUT midline carcinoma family member 1
OS	overall survival
PAX5	paired box 5
PBX1	PBX homeobox 1
Ph	Philadelphia chromosome
Ph-like	Philadelphia chromosome-like
Pre-BCR	pre-B-cell receptor
pSTAT5	phosphorylated signal transducer and activator of transcription 5
PU.1	transcription factor PU.1
RNA-seq	ribonucleic acid sequencing
RT-qPCR	quantitative reverse transcription polymerase chain reaction
RUNX1	RUNX family transcription factor 1
siRNA	small interfering ribonucleic acid
SOX11	SRY-box transcription factor 11
T-ALL	T-cell acute lymphoblastic leukemia
TCF3	transcription factor 3
TF	transcription factor

TMA	tissue microarray
TP53	tumor protein P53
WBC	white blood cell
WGS	whole genome sequencing
WHO	World Health Organization
WTS	whole-tissue section
XBP1	X-box binding protein 1
ZAP70	zeta chain of T cell receptor associated protein kinase 70

ORIGINAL PUBLICATIONS

Publication I **Mäkinen, A.**, Nikkilä, A., Mehtonen, J., Teppo, S., Oksa, L., Nordlund, J., Rounioja, S., Pohjolainen, V., Laukkanen, S., Heinäniemi, M., Paavonen, T., & Lohi, O. (2021). Expression of BCL6 in paediatric B-cell acute lymphoblastic leukaemia and association with prognosis. *Pathology, 53*(7), 875-882. doi: 10.1016/j.pathol.2021.02.013.

Publication II Grönroos, T.,* **Mäkinen, A.,*** Laukkanen, S., Mehtonen, J., Nikkilä, A., Oksa, L., Rounioja, S., Marincevic-Zuniga, Y., Nordlund, J., Pohjolainen, V., Paavonen, T., Heinäniemi, M., & Lohi, O. (2020). Clinicopathological features and prognostic value of SOX11 in childhood acute lymphoblastic leukemia. *Scientific Reports, 10*(1), 2043. doi: 10.1038/s41598-020-58970-z.

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AUTHOR'S CONTRIBUTION

Studies I and III: The author designed the studies together with supervisors. The author conducted the literature search and collected the clinical data and archived samples for the studies. The author organized and performed the laboratory work and was responsible for the data analysis and interpretation with the aid of supervisors and coauthors. The author wrote the first drafts and was the primary author for the papers with the help of supervisors and other authors. The author submitted the manuscripts to the journals and prepared the final version of the papers.

Study II: The author planned in the designing of the study, performed data collection, and participated in data interpretation. The author was responsible for generation of figure panels, literature search and writing as an equal contribution with the first author with the aid of supervisors and coauthors.

1 INTRODUCTION

Although cancer in children is rare, B-cell acute lymphoblastic leukemia (B-ALL) is the most common pediatric malignancy. B-ALL is a neoplasm of the hematopoietic tissue originating from the bone marrow (BM). The uncontrolled proliferation of clonal and undifferentiated precursor lymphoid blast cells causes B-ALL. The prognosis has significantly improved over the last decades. However, significant morbidity and mortality associated with B-ALL still remain, even with the current risk-adapted treatment protocols (Malard & Mohty, 2020; Toft et al., 2018). Therefore, better tools for disease classification and novel targets for precision therapy are needed to improve the prognosis and lessen the therapy burden.

Age and white blood cell count (WBC) at diagnosis, genetics, and minimal residual disease (MRD) at the end of induction (EOI) therapy are the main prognostic markers in ALL. While genetic subtypes of B-ALL are essential for risk stratification, MRD is the most accurate predictive marker for relapse. The Philadelphia chromosome (Ph) defining the *BCR-ABL1* subtype and a distinct gene expression profile in the Ph-like subtype serve as examples of disease subtypes where predictive biomarkers guide targeted tyrosine kinase inhibitor therapy.

Here, we sought to find new biomarkers to understand the pathogenesis of B-ALL and to aid in the risk classification of patients. To this end, we collected a retrospective patient cohort of pediatric B-ALL patients with archived BM biopsy samples used for immunohistochemistry (IHC) analyses. Expression at the protein level was supplemented with expression data at mRNA level, where we utilized several large gene expression datasets. Finally, identified novel biomarkers (BCL6, SOX11, and IGF2BP3) were correlated with clinical and prognostic parameters. In summary, several novel biomarkers with possible prognostic value in B-ALL were identified and characterized.

2 REVIEW OF THE LITERATURE

2.1 Hematopoiesis

Hematopoiesis is a process where all blood cells, including red blood cells, platelets, and WBCs, are formed. Disruptions in hematopoiesis lead to anemia, bleeding, and infection (Birbrair & Frenette, 2016; Crane, Jeffery, & Morrison, 2017; Cumano & Godin, 2007). Generation of blood cells occurs in different locations and multiple waves during the lifespan of an individual. The first wave of hematopoiesis is called the "primitive wave" that occurs in the yolk sac during early embryonic life from the second gestational weeks through week 8. The main function of this ephemeral primitive phase is the production of red blood cells for oxygenation of tissues in the fast-growing embryos (Cumano & Godin, 2007; Zhu et al., 2020). The primitive phase is followed by the transient "definitive wave" of adult-type hematopoiesis, which begins in the aorta-gonad-mesonephros at gestational day 24 (Tavian, Hallais, & Péault, 1999) and is later evident in the fetal liver, thymus, and spleen in perivascular niches (Birbrair & Frenette, 2016). At 14 weeks of gestation, the definitive hematopoiesis is moving into the fetal BM and, after birth, BM is the exclusive site of hematopoiesis under normal circumstances (Charbord, Tavian, Humeau, Péault, & Peault, 1996). Extramedullary hematopoiesis (EMH) can occur under hematopoietic stress and most commonly appears in the spleen or liver, but almost any tissue can be the site of EMH (Crane et al., 2017; Orkin & Zon, 2008).

Classical model of hematopoiesis was based on strict hierarchical structures with separate compartments for each cell type and differentiation stage (Ceredig, Rolink, & Brown, 2009; Liggett & Sankaran, 2020). Recent studies have revealed more plasticity in cell differentiation pathways and new models have been suggested wherein cells acquire cell-type specific features gradually (Ceredig et al., 2009; Fischer et al., 2020; Liggett & Sankaran, 2020; Paul et al., 2015; Perié, Duffy, Kok, de Boer, & Schumacher, 2015). The classical model of the genesis of blood cells is illustrated in Figure 1.

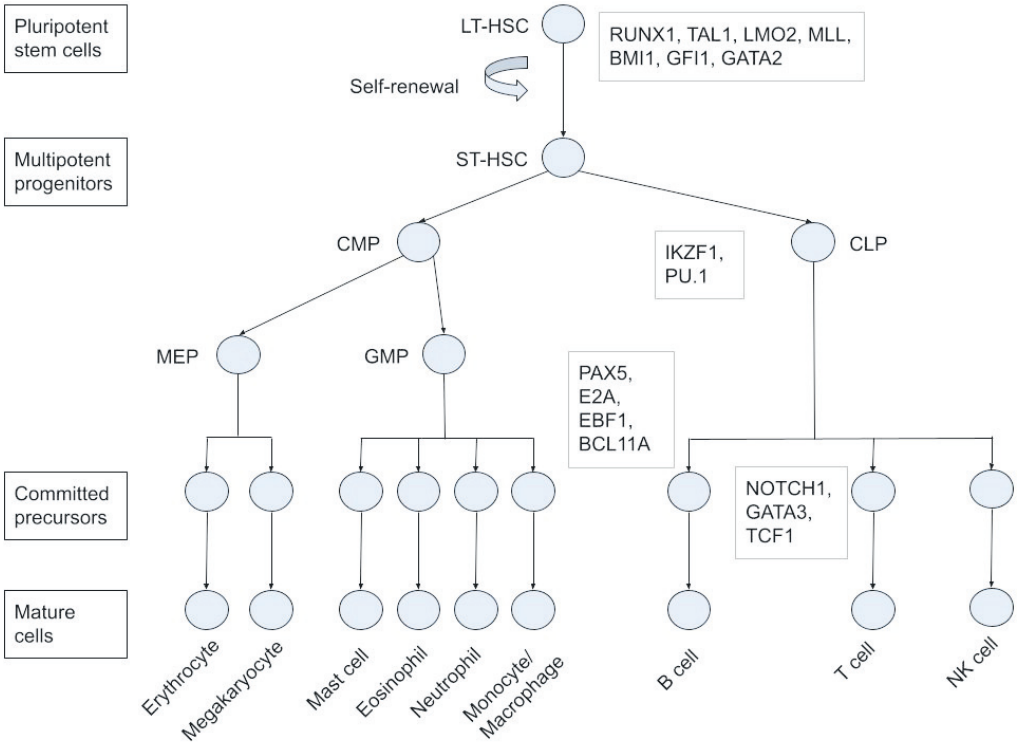

Figure 1. The classical model of hematopoiesis. Central transcription factors are indicated in the boxes. LT-HSC: Long-term hematopoietic stem cell; ST-HSC: short-term hematopoietic stem cell; CMP: common myeloid progenitor; CLP: common lymphoid progenitor; MEP: megakaryocyte/erythroid progenitor; GMP: granulocyte/macrophage progenitor. Adapted from: Orkin & Zon, 2008; Ceredig, Rolink, & Brown, 2009.

2.1.1 Hematopoietic stem cells

Hematopoietic stem cells (HSC) are pluripotent cells that give rise to all cells of blood lineage ((Sawai et al., 2016). HSCs were first detected over 90 years ago in the aorta of a developing pig and chick (Sabin, 1917) and were among the first tissue-specific stem cells identified (Till & McCulloch, 1961). HSCs arise from the hemogenic endothelium during embryonic development (Lancrin et al., 2009) and can be categorized based on their self-renewal properties, lifespan, and differentiation abilities into long-term and short-term HSCs (Birbrair & Frenette, 2016). These cells circulate in the blood stream in small numbers but mostly reside in the BM in adults (Ceredig et al., 2009). As many blood cells are short-lived, HSCs and active hematopoiesis are necessary to sustain life throughout an individual's life

(Orkin & Zon, 2008). Self-renewing LT-HSCs are primarily dormant and divide at low frequency (ca. 1/40 weeks) (Catlin, Busque, Gale, Guttorp, & Abkowitz, 2011), thus reducing replication-induced physiological deoxyribonucleic acid (DNA) damage. Clonal tracking of transplanted HSCs suggests that most of blood production is derived from ST-HSCs (Biasco et al., 2016).

2.1.2 Lymphopoiesis

The immune system consists of multiple cell types that ensure normal immune defense. Lymphopoiesis refers to the generation of T-lymphocytes, B-lymphocytes, and natural killer (NK) cells. These cells are essential components of the innate (NK cells) and adaptive immune system (B and T-lymphocytes) (Abel, Yang, Thakar, & Malarkannan, 2018; Cooper, 2015). Lymphopoiesis begins in the primary lymphoid organs (BM, thymus) and continues in secondary lymphoid tissues (spleen, lymph nodes, mucosa-associated lymphoid tissues). Lymphatic cells interact with each other and the different cells of the innate immune system to elicit immune responses against infective agents, neoplasms, and the individual itself in the setting of autoimmune diseases.

2.1.2.1 Development and function of B-cells

The antibody-mediated humoral response was discovered in 1890, but the B- and T-cell lymphocytes and their immunological role were deciphered later in the 1960s (Cooper, 2015). B-cells are involved in the humoral immunity of the adaptive immune system by the generation, processing, and secretion of antibodies, with each B-cell expressing a unique recombination of immunoglobulin genes that recognize a distinct epitope on an antigen.

B-cell development is guided by lineage-specific transcription factors (TFs) and further modified by interactions with stromal and immune cells. The stage of development can be identified by the expression of surface markers and degree of plasticity in differentiation. The multipotency in differentiation decreases from lymphoid-primed multipotent progenitors, all-lymphoid progenitors, and B-cell-biased lymphoid progenitors (1–3) into fully committed pro-B cells that can be recognized by expression of CD19 antigen and CXCR4 and dependency of interleukin-7 receptor (IL7R)-signaling (Fischer et al., 2020).

These pro-B cells undergo immunoglobulin heavy chain (IgH) gene rearrangement mediated by RAG1 and RAG2 enzymes. If IgH rearrangement is successful, a pre-B-cell receptor consisting of two IgH molecules and surrogate light chain proteins (VPREB1, lambda5) is expressed on the cell surface. Unsuccessful rearrangement leads to apoptosis. Oligomerization of the pre-B-cell receptor (pre-BCR) complex emits proliferation signals partially, simultaneously with IL7R-signaling and survival signals at the large pre-B cell phase. Pre-BCR signaling suppresses IgH rearrangement and leads to cell cycle exit and immunoglobulin light chain (IgL) rearrangement at the small pre-B cell phase (Buchner, Swaminathan, Chen, & Müschen, 2015; Eswaran et al., 2015; Rickert, 2013). Also, murine models suggest that CXCR4-signaling might seize proliferation and drive light chain rearrangement and survival of small pre-B cells (K. C. McLean & Mandal, 2020).

Through successful IgL rearrangement, a B-cell receptor is formed and an immature B-cell stage is reached. These cells differentiate into mature naïve B-cells that express immunoglobulin D and egress from the BM into peripheral circulation and lymphoid tissues. After antigen stimulus, in the germinal centers (GCs) of lymphoid follicles, B-cells can undergo somatic hypermutation and class-switch of immunoglobulin genes and may turn into memory B-cells and long-lived plasma cells. Alternatively, B-cells can develop into short-lived plasma cells in the interfollicular areas and eventually into memory B-cells in the marginal zones of lymphoid follicles (Fischer et al., 2020). The stages of B-cell differentiation, key signaling pathways, and TFs are presented in Figure 2.

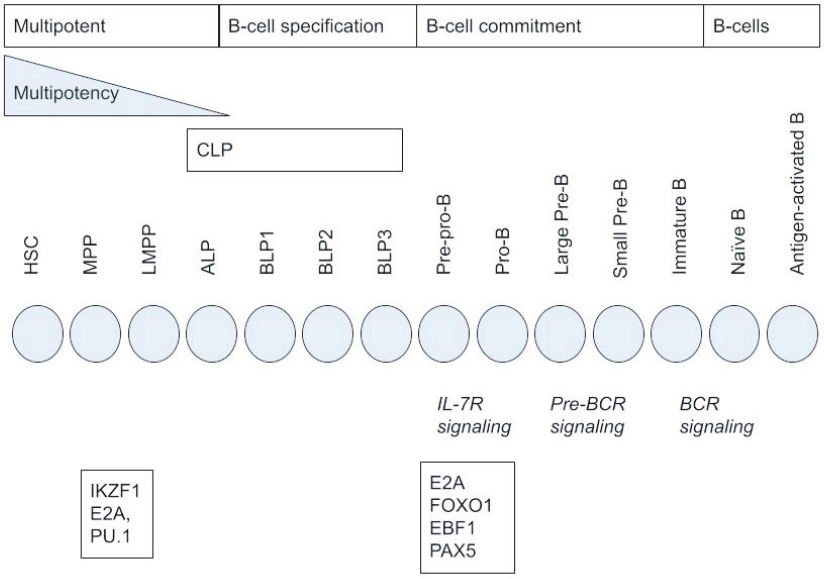

Figure 2. A schematic model of B-cell development along with key transcription factors indicated in the boxes. ALP: all-lymphoid progenitor; BCR: B-cell receptor; BLP: B-cell biased lymphoid progenitor; CLP: common lymphoid progenitor; HSC: hematopoietic stem cell; LMPP: lymphoid-primed multipotent progenitor; MPP: multipotent progenitor. Adapted from: Buchner et al., 2015, Fischer et al., 2020.

2.1.2.2 Development and function of T-cells

T-cells are effectors in the adaptive and innate immune system by detecting antigens, regulating inflammatory reactions, and actively killing cells. T-cells are populated across different organs (Kumar, Connors, & Farber, 2018). The development of T-cells starts early in the fetus, during 9–12 weeks of gestation, and humans are born with an immunocompetent T-cell complement. As other hematopoietic cells mature in the BM, BM-derived T-cell progenitors arrive at the corticomedullary junction of the thymus and undergo lineage commitment and further maturation in the thymus; therefore, the process is called thymopoiesis. Antigen-independent differentiation proceeds in the subcapsular cortex from prothymocytes through CD4-/CD8-double negative (DN1–4) cells. The T-cell receptor (TCR) γ, δ, and β gene rearrangement in DN2 and DN3 stages leads to pre-TCR expression and DN4 stage (Krueger, Ziętara, & Łyszkiewicz, 2017; Park et al., 2020; Taghon, Yui, Pant, Diamond, & Rothenberg, 2006). This beta-selection leads to αβ-T-cell lineage commitment. DN4

thymocytes proliferate and begin co-expressing CD4 and CD8, becoming double-positive (DP) thymocytes, which go through TCRα recombination, and αβ-TCR interacts with cortical thymic epithelial cells in positive selection process. Finally, positively selected T-cells express either CD4 or CD8, becoming single positive (SP) cells. SPs migrate to the thymic medulla for negative selection, where self-reactivity leads to apoptosis. SP cells that pass negative selection as naïve T-cells leave into peripheral lymphoid tissues for further maturation. At antigen encounter, CD4+ T-cells develop through blast forms into different subtypes, T-helper 1 (Th1), T-helper 2 (Th2), regulatory T-cell (Treg), T-helper 17 (Th17), T-helper 9 (Th9), T-helper 22 (Th22), and follicular helper T-cells (Tfh). CD8+-cells become memory T-cells and effector T-cells. The activity of thymopoiesis declines during adulthood (Krueger et al., 2017; Kumar et al., 2018).

2.1.2.3 Development and function of natural killer cells

Natural killer (NK) cells are cytotoxic and cytokine-producing lymphocytes and are part of the innate immune responses against viral infections and malignancies (Cheent & Khakoo, 2009; Spits, Lanier, & Phillips, 1995). Natural killer cells belong to a group of innate lymphoid cells (ILC), including ILCs 1–3 (Spits et al., 2013). These cells develop from common lymphoid progenitors, but early thymocytes and B-cell biased lymphoid progenitors (BLPs) can also differentiate into NK cells (Ceredig et al., 2009; Fischer et al., 2020; Galy, Travis, Cen, & Chen, 1995). Maturation of NK cells occurs in the BM and secondary lymphoid tissues, such as tonsils, spleen, and lymph nodes. Mature NK cells express CD56 and NKp46 but lack CD3 expression. As NK cells lack germline-rearranged and antigen-specific receptors, NK cells express combinations of activating and inhibiting receptors eliciting diverse immune reactions. NK cells secrete cytokines and chemokines to recruit and modulate other immune cells (Abel et al., 2018). Memory-like functions are present in NK cells to enhance immune reactions in reactivation (Romee et al., 2012).

2.1.3 Myelopoiesis, erythropoiesis and thrombopoiesis

The myelopoiesis gives rise to neutrophilic, eosinophilic, basophilic granulocytes, monocytes, macrophages, and dendritic cells (DCs). These myelopoietic cells are first-line effectors in infection, inflammation, cellular immunity, and tissue

homeostasis. During myelopoiesis, common myeloid progenitors (CMPs) give rise to granulocyte/macrophage progenitors, which differentiate into granulocytes, monocytes, macrophages, and DCs. New myelopoietic differentiation stages and routes have been identified (Dress, Liu, & Ginhoux, 2020; Orkin & Zon, 2008). The megakaryopoiesis gives rise to polyploid giant cells called megakaryocytes (MKs). MKs remodel BM and shed platelets through cytoplasmic fragmentation. Platelets contribute to hemostasis and wound healing and regulate inflammation, angiogenesis, and immune responses. In classical models, CMPs give rise to megakaryocyte/erythroid progenitors (MEPs), which, through committed progenitors, differentiate into MKs, but in adults, MKs may differentiate from multipotent progenitors (Geddis, 2010; Notta et al., 2016; Orkin & Zon, 2008). Megakaryocyte/erythroid progenitors give rise to erythroid cells that develop into erythrocytes. Erythropoiesis produces red blood cells that transport the respiratory gases, namely oxygen and carbon dioxide.

2.1.4 Bone marrow microenvironment

A specific microenvironment is required to form blood cells in the BM. The stroma of BM regulates the renewal, proliferation, differentiation, and trafficking of HSCs and progenitors. The stroma in BM includes various cells such as mesenchymal cells, pericytes, osteocytes, adipocytes, endothelial cells, and nerve cells. Stromal extracellular matrix (ECM) gives anchorage for hematopoietic and stromal cells. ECM is composed of fibrous proteins, glycoproteins, and proteoglycans, such as collagens, fibronectin, laminin, and tenascin (Birbrair & Frenette, 2016; Crane et al., 2017; Orkin & Zon, 2008). The bone-forming osteoblasts affect hematopoiesis by producing growth factors and cytokines (i.e., G-CSF, IL-7, CXCL12), which contribute to the proliferation and development of B-cell progenitors (Birbrair & Frenette, 2016; Crane et al., 2017). The BM has a rich vasculature but it lacks lymphatic drainage. As arterioles are located near endosteal spaces, sinusoids are situated throughout the BM and permit the passage of mature blood cells into the circulation (Birbrair & Frenette, 2016; Crane et al., 2017). Endothelial cells facilitate self-renewal, regeneration, and maintenance of HSCs through the expression of stem cell factor (SCF), glycoprotein 130, and CXCL12 (Ding, Saunders, Enikolopov, & Morrison, 2012; Greenbaum et al., 2013; Yao, Yokota, Xia, Kincade, & McEver, 2005). The arteriolar endothelial cells drive HSC quiescence, and permeable sinusoidal endothelial cells cause HSC and progenitor activation and cell trafficking

(Itkin et al., 2016). Pericytes are located near endothelial cells and regulate HSCs through CXCL12 and SCF (Méndez-Ferrer et al., 2010; Pinho et al., 2013). These perivascular MSCs are forming partially overlapping subgroups. CXCL12-abundant cells (CARs) maintain HSCs and are in part dependent on TF Forkhead box C1 (FoxC1) (Omatsu, Seike, Sugiyama, Kume, & Nagasawa, 2014). Also, PDGFRα+Sca-1+ (PαS) cells and leptin receptor-expressing cells have been described as necessary for HSC maintenance. The arteriolar pericytes express glycoprotein NG2 and drive HSC quiescence (Kunisaki et al., 2013). Nerves follow arterioles to serve smooth muscle cells or mix with hematopoietic cells. Nonmyelinated Schwann cells surround the sympathetic and sensory nerves in the BM. Schwann cells secrete transforming growth factor beta (TFGβ), which drives HSC dormancy and catecholamines from sympathetic nerves influence HSC migration (Fitch et al., 2012; Yamazaki et al., 2011). The BM fat differs from brown and white fat through smaller cellular size and gene expression profile. Although BM adipocytes produce cytokines, fatty acids, and hormones, the effect on HSCs in normal BM homeostasis and disease is still obscure (Birbrair & Frenette, 2016; Crane et al., 2017).

Hematopoietic cells also modulate and regulate the BM microenvironment. Macrophages residing in the BM induce HSC retention through integrin VLA and osteopontin M. Also, macrophages maintain erythropoiesis in erythroblastic islands. Osteoclasts resorb bone allowing bone renewal and clear space for hematopoiesis. By releasing calcium ions, osteoclasts may contribute to the homing of HSCs into the endosteal niche (Birbrair & Frenette, 2016; Crane et al., 2017). Megakaryocytes have a dichotomous effect on HSCs. In steady-state conditions, megakaryocytes secrete TGFB, CXCL4, and thrombopoietin to drive HSC quiescence, and in stress, they secrete fibroblast growth factor 1 (FGF-1) to activate HSCs (Bruns et al., 2014; Zhao et al., 2014). Lymphocytes are dispersed throughout the BM. As natural killer cells have a negative effect on HSC differentiation, CD4+ T cells are required for the differentiation of myeloid cells. Regulatory CD4+ T cells suppress colony formation and myeloid differentiation of HSCs. FoxP3+ regulatory CD4+ T cells co-localize with HSCs into the endosteal region and possibly protect HSCs from immune attacks (Birbrair & Frenette, 2016).

2.2 Acute leukemia

Acute leukemias are a heterogeneous group of hematological neoplasms that originate from the BM, and they are the most common pediatric malignancy forming approximately 30% of cancer diagnoses among children (Linabery & Ross, 2008; Puumala, Ross, Aplenc, & Spector, 2013). The first morphological and clinical descriptions of leukemias as white or milky blood were reported in the 19th century (Kampen, 2012). In 1976, the French-American-British (FAB) classification was created. It divided lymphoblastic leukemias into three and myeloid leukemias into six groups by morphology and cytochemical properties (Bennett et al., 1976). This classification has evolved through the WHO classification of 1997 and continues over the current WHO classification (Swerdlow et al., 2017). ALL and AML are further categorized by clinical features, morphology, immunophenotype, and genetic abnormalities (Puumala et al., 2013). In addition, some leukemias are of ambiguous lineage, undifferentiated, mixed-phenotype, or NK lymphoblastic leukemia (Swerdlow et al., 2017; Weinberg et al., 2021; Wolach & Stone, 2015). The prevalence of acute leukemia patients is increasing. Also, morbidity among long-time survivors is a relevant problem (Phillips et al., 2015).

Symptoms and clinical findings of acute leukemia are associated with the packing of BM with blast cells and dysfunction of normal hematopoiesis. The shortage of healthy blood cells causes anemia, bleeding or bruising, and infections. The enlargement of the spleen and liver is frequent along with lymphadenopathy, and pain in the bones and joints is common (Clarke et al., 2016).

2.2.1 B-cell acute lymphoblastic leukemia

B-ALL is a malignant clonal proliferation of early B-lineage cells and is composed of small to medium-sized blast cells originating from the BM and with a general involvement of blood. Moreover, B-ALL can also infiltrate extramedullary sites such as the lymph nodes, the central nervous system (CNS), or, rarely, the skin. BM-involvement of over 25% of leukemic blasts is used in many treatment protocols as a criteria for B-ALL diagnosis (Malard & Mohty, 2020; Swerdlow et al., 2017).

2.2.1.1 Epidemiology

Although B-ALL is a rare disease, it is the most common malignancy in pediatric patients. The age-standardized incidence of leukemia in children between 1–14 years in Europe between 2000–2010 was 50 per million person-years (Steliarova-Foucher et al., 2017). Age-specific incidence is highest among children aged 1–4 years and lowest among adults aged between 25 and 45 years. Sixty percent of ALL cases are diagnosed before 20 years of age (Siegel, Miller, Fuchs, & Jemal, 2021). Before the 1950s, all patients succumbed to the disease, whereas the 5-year survival rates in most developed countries is currently around 90% (Pui et al., 2015). Treatment results in most risk groups of ALL have improved during the last four decades, but infants (< 1 year) have the lowest survival rates (Bonaventure et al., 2017; Pulte, Gondos, & Brenner, 2009; Teachey, Hunger, & Loh, 2021). Relapse occurs in less than 10% of pediatric ALL patients (Toft et al., 2018). There is a need for better treatments, especially in high-risk genetic groups of ALL, in patients with suboptimal responses to treatments and in a proportion of adult patients (Malard & Mohty, 2020).

2.2.1.2 Genetics and risk factors of ALL

There are many characteristic features in cancer development, but genetic alterations define the development of malignancies (Fouad & Aanei, 2017; Hanahan & Weinberg, 2000, 2011).

Prenatal initiation of leukemogenesis has been observed in many studies. For example, *ETV6-RUNX1* fusion can be found in B-cells of cord blood samples in as much as 1–5% of healthy newborns. Most of these children will not develop B-ALL, which indicates required secondary genetic alterations (Gale et al., 1997; M. F. Greaves, Maia, Wiemels, & Ford, 2003; Hein et al., 2019; Maia et al., 2003; Mori et al., 2002; Schäfer et al., 2018). Environmental factors as causative factors have been investigated and ionizing radiation has been shown as a causal factor, but the role of non-ionizing radiation (e.g., electromagnetic fields), has not been verified (Ahlbom et al., 2000; Bartley, Metayer, Selvin, Ducore, & Buffler, 2010; Eden, 2010; Hsu et al., 2013). Lack of infections in early childhood and abnormal immune response to infective agents in pre-leukemic clones have been associated with ALL (M. Greaves, 2018). Exposure to benzene and pesticides has been associated with an elevated ALL risk (Carlos-Wallace, Zhang, Smith, Rader, & Steinmaus, 2016; Ward et al., 2009).

Genetic susceptibility contributes partially to leukemogenesis. Down syndrome increases risk of ALL 20-fold (Buitenkamp et al., 2014; Hasle, 2001). Other syndromes increase the risk of ALL, for example, Fanconi anemia, Ataxia telangiectasia, Bloom syndrome, Li-Fraumeni syndrome, Nijmegen breakage syndrome, constitutional mismatch repair deficiency syndrome, neurofibromatosis (type 1), and Noonan syndrome (Saida, 2017). Also, syndromes involving hematopoiesis-associated TFs include *IKZF1*-, *PAX5*-, and *ETV6*-associated leukemia predispositions (Churchman et al., 2018; Noetzli et al., 2015; Shah et al., 2013). In addition to syndromes, some germline variants add to ALL susceptibility (e.g., *ARID5B*, *CEBPE*, *CDKN2A*, *CDKN2B*, *PIP4K2A*) (Prasad et al., 2010; Xu et al., 2012, 2013, 2015).

2.2.1.3 Subtypes of B-ALL

B-ALL can be classified into many different subtypes. Categories are based on, for example, aneuploidy or genetic translocations that generate chimeric genes disrupting protein expression of hematopoietic TFs, cytokine receptors, epigenetic modifiers, and tyrosine kinases. Primary genetic alterations are rarely sufficient for the onset of overt leukemia but require additional genetic and epigenetic lesions (Malard & Mohty, 2020; Pui, Nichols, & Yang, 2019; Swerdlow et al., 2017). Over 90% of pediatric B-ALL can be grouped into a specific prognostically distinct subtype (Fischer et al., 2020).

High hyperdiploidy (HeH) is characterized by a gain of at least five entire chromosomes (or DNA index over 1.16), occurs in 25–30% of pediatric cases, and is associated with favorable prognosis. HeH is less common in adolescents and adults with heterogeneity in the prognostic associations of different trisomies (Chilton et al., 2014). In addition to extra copies of chromosomes (typically X, 4, 6, 10, 14, 17, 18, and 21) that drive the leukemogenesis, HeH leukemia cells have secondary mutations in genes of histone modifiers (*CREBBPE*, *WHSC1*) or the RTK-RAS signaling pathway-associated genes (*KRAS*, *NRAS*, *PTPN11*) (Jerchel et al., 2018; J. F. Li et al., 2018; Paulsson et al., 2015).

Balanced *ETV6-RUNX1*-translocation is more frequent in children than in adolescents or adults (ca. 20–30% in pediatric patients) and is associated with favorable prognosis. The translocation fuses two hematological TFs, namely ETV6 and RUNX1. The *ETV6-RUNX1*-translocation occurs in utero in HSCs or early progenitors and is a driving event in leukemogenesis, but overt leukemia requires

secondary hits. Secondary genetic alterations are driven by aberrant RAG recombination and infrequent point mutations. Deletions and other copy number aberrations of genes involved in B-cell development (e.g., *CDKN2A, PAX5, BTG1, TBLXR1, RAG1, RAG2*, and *wild type ETV6*) are common and stall the early B-cell development (Bhojwani et al., 2012; Enshaei et al., 2013; Papaemmanuil et al., 2014; Romana, Mauchauffe, et al., 1995).

Ph-like ALL has a similar gene expression profile with *BCR-ABL1* ALL but lacks the *BCR-ABL1* fusion gene (M. L. Den Boer et al., 2009; Harvey et al., 2010). Ph-like ALL consists of a heterogeneous group of leukemias that carry distinct gene fusions involving tyrosine kinases and cytokine receptors such as *CRLF2* and *JAK2*, with common deletions in *IKZF1*, *TCF3*, *EBF1*, *PAX5,* and *VPREB1*. There are numerous different fusion partners involved. The fusion proteins drive cytokine-independent proliferation in B-cell precursors (Roberts et al., 2014). The incidence of Ph-like ALL is 10% among childhood ALL and is associated with poor prognosis. For example, in Children's Oncology Group's randomized controlled trial for HR-ALL, 5-year event-free survival (EFS) was 63% in Ph-like ALL vs. 86% in non-Ph-like cases. Still, this prognosis might be improved with tyrosine kinase inhibitors targeting *ABL1* and *JAK2* (Loh et al., 2013).

DUX4-rearranged ALL is associated with deregulation or deletion of *ERG* (Zhang et al., 2016). The gene fusion with *IGH* and *ERG* is common, leading to overexpression of truncated DUX4. As wild-type DUX4 is pro-apoptotic, the C-terminus of *DUX4* is disrupted in the translocation leading to oncogenic activity (Yasuda et al., 2016). DUX4- rearrangement is the driving event with secondary hits such as *ERG* deletion generated by aberrant RAG activity (Clappier et al., 2014) and mutations in genes such as *ZEB2* (Lilljebjörn et al., 2016; Y. F. Liu et al., 2016; Zhang et al., 2016). This subtype composes 5–10% of childhood ALL and has good prognosis even with coexistent *IKZF1* deletion (Zhang et al., 2016). Aberrant CD2 positive immunophenotype is prevalent although not specific (Clappier et al., 2014).

The *TCF3-PBX1*-subtype harbors a balanced or unbalanced translocation between two TFs, *TCF3* (formerly known as *E2A*) at 19p13 and *PBX1* at 1q23 (Carroll et al., 1984; Crist et al., 1990; Felice et al., 2011; S. Hunger et al., 1991; Paulsson, Horvat, Fioretos, Mitelman, & Johansson, 2005). *PHF6* and *PAX5* mutations have been identified as driver mutations (Ueno et al., 2020). *TCF3-PBX1*-translocation is present in 5% of pediatric cases and associated with favorable prognosis under current treatment protocols (Felice et al., 2011; S. P. Hunger, 1996; Kager et al., 2007). Secondary mutation, such as loss of *PAX5*, is common, and activating mutations in JAK/STAT signaling pathways are possible. As the direct

oncogenic mechanism of *TCF3-PBX1* is elusive, it seems that TCF3-PBX1 enhances B-cell progenitor renewal. Mutations in *PBX1*, *PAX5*, *CTCF*, and *SETD2*; amplification of *AKT3*; and deletion of *CDKN2A/B* are often encountered (Duque-Afonso et al., 2015; Zhou et al., 2021).

ZNF384-rearrangement is present in 5% of pediatric ALL. *ZNF384* usually has a regulator of chromatin modulation gene as a fusion partner. ZNF384 regulates the promoter of extracellular matrix genes. Expression of myeloid markers CD13 and CD33, reduction of cell cycle regulation, and DNA repair with activation of JAK-STAT signaling is typical (Gocho et al., 2015; Y. F. Liu et al., 2016; McClure et al., 2018). ZNF384 subtype is sometimes diagnosed as a mixed phenotype B-cell/myeloid acute leukemia suggesting a possible earlier stage of the cell of origin. The cell of origin might be a pre-VDJ-recombination pro-B cell precursor cell, at least in *TCF3-ZNF384* cases (Bueno et al., 2019). The prognosis has been investigated in small cohorts and reported as intermediate. Nevertheless, larger retrospective consortia data have shown lower relapse rates in the *EP300-ZNF384* subtype (Hirabayashi et al., 2021).

MEF2D (Myocyte enhanced factor 2D) is rearranged in 4% of pediatric ALL (Y. F. Liu et al., 2016). Immunophenotype includes negativity for CD10 and expression of CD38 and cytoplasmic muHC (Y. F. Liu et al., 2016; Ohki et al., 2019). The fusion of *MEF2D* and *DAZAP1* was revealed in a cell line with t(1;19), which did not involve *TCF3* and *PBX1* (Yuki et al., 2004). There are several fusion partners, and *BCL9* is the most common of these. *MEF2D* subtype shares gene expression profile with the *TCF3-PBX1* subtype and is associated with pre-BCR signaling with increased expression of *HDAC9* and decreased expression of *MEF2C* (Gocho et al., 2015; Y. F. Liu et al., 2016). Furthermore, this subtype is associated with a poor prognosis and older age (Y. F. Liu et al., 2016; Ohki et al., 2019).

B-ALL with rearrangement of *KMT2A* (*KMT2A*-re) (previously known as *MLL,* mixed lineage leukemia) with over 80 partner genes, is most frequent in infants (0–1 years) and adults resulting from mostly unbalanced and complex translocations. The cell of origin is a pre-VDJ progenitor (Agraz-Doblas et al., 2019; Pieters et al., 2007). *KMT2A*-re of leukemia harbors few non-silent additional mutations. Primarily these are found in kinase PI3K-RAS signaling pathway (e.g., *KRAS* and *NRAS*) (Agraz-Doblas et al., 2019). Infant ALL is one of the least mutated cancers. Most of these missense mutations are expressed with harmful effects on protein function. Mutations are more frequent in older patients. This *KMT2A*-re ALL has poor prognosis and more efficient treatment protocols are required (Andersson et al., 2015; Forgione, McClure, Eadie, Yeung, & White, 2019; Tomizawa et al., 2020).

BCR-ABL1 translocation is present in 2–5% of pediatric patients and 25% of adult cases (Ribeiro et al., 1987). Although this *BCR-ABL1* subtype has been associated with poor prognosis, treatment with tyrosine kinase inhibitors has improved prognosis (Jeha et al., 2021; Van Der Veer et al., 2014). *IKZF1* mutations are typically found in over 80% of the *BCR-ABL1* subtype and are associated with an inadequate treatment response (Mullighan et al., 2008; Slayton et al., 2018; Van Der Veer et al., 2014). Mutations in the kinase domain of *BCR-ABL1* can lead to treatment resistance (Jones et al., 2008; Soverini et al., 2021).

ALL cases with less than 44 chromosomes belong to the hypodiploid subtype, which is rare (< 1%) in pediatric B-ALL. Near haploid (23–29 chromosomes) subclass of hypodiploid ALL is very rare. Low hypodiploid (32–39 chromosomes) ALL is more common and harbors alterations in *TP53*, *IKZF2, CDKN2A,* and *RB1* and is frequently associated with a germline mutation in the *TP53* gene (Holmfeldt et al., 2013; Safavi et al., 2012). Low hypodiploid ALL is associated with poor prognosis (McNeer et al., 2019; Mehta et al., 2015; Pui, Rebora, et al., 2019).

The intrachromosomal amplification of chromosome 21 (iAMP21) usually includes amplification of *RUNX1* (three or more extra copies) and B-ALL-associated deletions of *PAX5*, *CDKN2A,* and *IKZF1* (Harewood et al., 2003; C. J. Harrison et al., 2005; Rand et al., 2011). *RUNX1* itself is not mutated. The iAMP21 is present in 2% of pediatric ALL, and it is seen in other subtypes such as *ETV6-RUNX1* and *BCR-ABL1*-like (Harrison et al., 2014; Zaliova et al., 2019). This group is seen more often in older children (> 10 years). The possible mechanism behind this aberration is the breakage-fusion-bridge cycle (Robinson, Harrison, Moorman, Chudoba, & Strefford, 2007). iAMP21 subtype is associated with poor prognosis in standard risk therapy, and the patients benefit from high-risk treatment protocols (Harrison et al., 2014; Heerema et al., 2013; Moorman et al., 2007, 2013).

ETV6-RUNX1-like ALL is found in 3% of B-ALL cases. It is characterized by a lack of *ETV6-RUNX1* fusion and coexisting lesions in *ETV6*, *IKZF1,* and *ARPP22* with a similar gene expression profile to that of the *ETV6-RUNX1* subtype (Gu et al., 2019; Lilljebjörn et al., 2016; Zaliova et al., 2019). Also, *ETV6-RUNX1*-like cases share *RAG1* gene expression signature and CD27+/CD44-immunophenotype with *ETV6-RUNX1* (Chen et al., 2021; Zaliova et al., 2017). The prognostic effect of *ETV6-RUNX1*-like GEP is still somewhat unclear. As intermediate or favorable treatment response are previously described, Jeha et al. reported worse outcomes in this subtype (Jeha et al., 2021; Lilljebjörn et al., 2016; Zaliova et al., 2019).

TCF3-HLF is a rare subtype of pediatric ALL associated with poor prognosis, but it is present in less than 1% of ALL cases. As HLF is not usually expressed in

lymphoid cells, TCF3-HLF fusion is an exception (S. P. Hunger, Ohyashiki, Toyama, & Cleary, 1992; Inukai et al., 2007). Intragenic deletions of *PAX5* and *VPREB1* are observed in this subtype. Genetic aberrations of *TCF3* upstream of *PAX5* are found in B-cell progenitors with arrested maturation at the pro- to the pre-B stage (Fischer et al., 2015).

IGH locus is involved in translocations with several fusion partners (e.g., *CRLF2*, *ID4*, *CEBP* family, *BCL2*) and is associated with poor prognosis and older patient groups. *IGH* translocations may occur as genetic primary or secondary events and occur with other recurrent genetic aberrations (Russell et al., 2014). B-ALL with t(5;14)(q31;q32) IGH-*IL3* is a rare entity included in the WHO classification. Eosinophilia is associated with this disease due to the constitutional overexpression of interleukin-3 (IL3) (Grimaldi & Meeker, 1989). *IKZF1* deletions are countered. The prognosis of this disease is considered intermediate, and pediatric cases have been observed with IGH-*IL3* B-ALL (Fournier et al., 2019).

PAX5 haploinsufficiency promotes leukemogenesis, but it requires sequential alterations that disrupt lymphoid development (Dang et al., 2015). Also, cases with PAX5p.Pro80Arg acquire secondary alterations such as deletions of the wild-type allele (Gu et al., 2019). *PAX5* gene has genetic alterations in 31% of B-cell lineage ALL (Mullighan et al., 2007), *PAX5*-translocation is present in 2–3% of patients (*PAX5*alt), and loss of *PAX5* function accelerates the development of B-ALL (Nebral et al., 2009). *PAX5*-translocations are uncommon with concomitant recurrent translocations, but PAX5 deletions are common among other subtypes (Coyaud et al., 2010).

NUTM1-rearranged (*NUTM1*) B-ALL is a novel subtype and is involved with multiple partner genes such as *ACIN1* with a possibly favorable outcome (J. M. Boer et al., 2021; Gu et al., 2019). *ZNF384*-like and *KMT2A*-like B-ALL have gene expression profiles similar to *ZNF384* and *KMT2A*-re subtypes but lack defining rearrangements. *CRLF2*-non Ph-like subtype has *CRLF2* rearrangement without the Ph-like gene expression profile (Gu et al., 2019). *IKZF1*^{plus} has *IKZF1* deletion with *PAR1*, *PAX5*, *CDKN2A*, *CDKN2B* without *ERG* deletion. This prognostic profile comprises 6–14% of B-ALL in childhood and is associated with poor prognosis. *IKZF1*^{plus} is enriched in the *BCR-ABL1*-like subtype (Stanulla et al., 2018; Zaliova et al., 2019).

The other subtype of B-ALL (also called B-ALL, nos) includes cases without known recurrent genetic aberrations. The different genetic subtypes and proportions in children are presented in Figure 3.

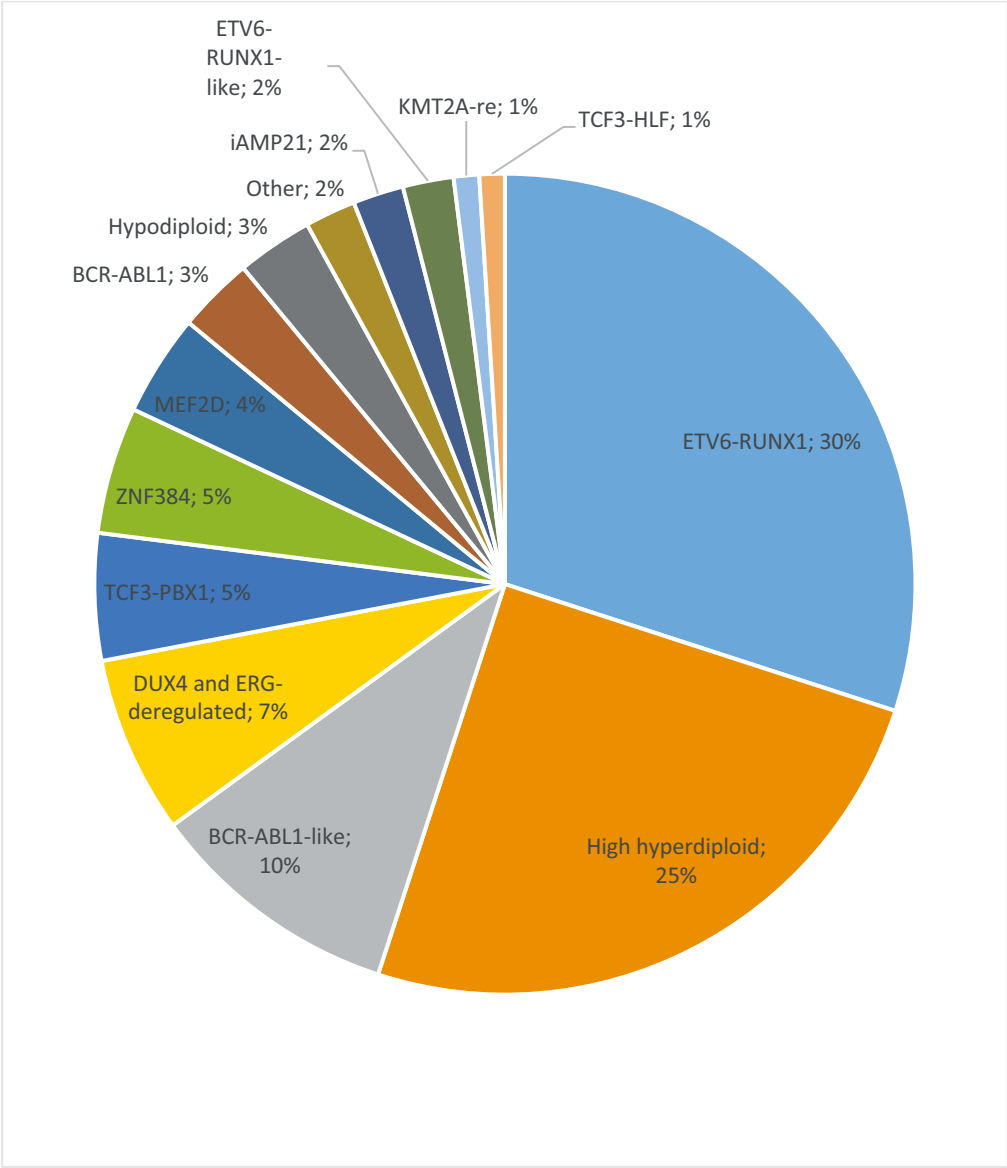

Figure 3. B-ALL subtypes and percentages from children between 1–14 years. Modified from: (Malard & Mohty, 2020; Pui, Nichols, et al., 2019). KMT2A-re, KMT2A-rearranged.

2.2.1.4 Cooperative mutations

Cooperative mutations that are not considered founding events in leukemogenesis frequently occur in ALL. These mutated genes can be involved in the factors

associated with lymphoid development (*PAX5*, *IKZF1*, *EBF1*), tumor suppression (*CDKN2A*, *CDKN2B*, *RB1*, *PTEN*), cytokine receptor signaling (*CRLF2*, *IL7R*, *FLT3*), RAS-signaling (*NRAS*, *KRAS*, *PTPN11*), transcriptional regulation (*ETV6*, *ERG*, *TBL1XR1*, *CREBBP*) and epigenetic modification (*CREBBP*, *EP300*, *SETD2*, *NDS2*(*WHSC1*)). Also, noncoding genetic alterations influence leukemogenesis (Mullighan et al., 2007). Different fusion transcripts can drive the same pathways in leukemogenesis, such as pre-BCR signaling being driven by *TCF3-PBX1* and *MEF2D* translocations and the resulting fusion proteins (Geng et al., 2015; Tsuzuki et al., 2020).

2.2.1.5 Genetics of relapse

Compared to other tumors such as solid tumors in adults, the mutational burden in B-ALL is generally low. Still, in relapsed B-ALL, mutations are increased compared to the leukemic cells at diagnosis (Dobson et al., 2020). Initial modifications can prevail, and additional mutations can be generated. Some mutations prevalent at relapse can be found in a minor population at diagnosis. In part, these mutations can be therapy-induced (B. Li et al., 2020). Usual genetic alterations in relapsed ALL involve genes of chromatin modulation and epigenetic regulators. Mutations of *CREBBP*, a transcriptional coactivator, and histone acetyltransferase are found in 20% of relapsed B-ALL and in parts of primary samples. These mutations can increase glucocorticoid resistance (Inthal et al., 2012; Malinowska-Ozdowy et al., 2015; Mullighan et al., 2011; Waanders et al., 2020). Other common mutations include *NT5C2*, *WHSC1*, *TP53*, *USH2A*, *NRAS,* and *IKZF1*, with *PMS2* and *MSH6* (Ma et al., 2015; Oshima et al., 2016). These mutations hamper the function of, for example, tumor suppressors and components DNA repair and can lead to further drug resistance (Barz et al., 2020; Oshima et al., 2020).

2.2.1.6 Diagnostics and treatment

The diagnosis of ALL is based on the WHO classification (Swerdlow et al., 2017), integrating morphology, immunophenotyping, and genetics. The morphology assessment from blood and BM smears can determine blood and BM involvement. Immunophenotyping by flow cytometry (FC) reveals cancer cell lineage and useful aberrant markers for surveillance of residual disease (Béné et al., 2011). Conventional cytogenetics are performed and complemented with FISH and RT-PCR, for

example, to reveal cryptic genetic alterations. FC is also used to detect aneuploidy. Novel gene sequencing methods may replace many applied methods, but the availability and affordability of these novel methods must be enhanced (Malard & Mohty, 2020).

Prognostication of ALL requires the assessment of multiple attributes. Classic clinical risk factors include age, WBC count, CNS involvement, and immunophenotype, but some of these features are associated with other risk factors such as recurrent genetic abnormalities (Malard & Mohty, 2020). For example, favorable genetic subtypes are more prevalent in children. *KMT2A*-rearrangement is more common in infants and high blood cell counts are associated with this same subtype (Andersson et al., 2015; Pieters et al., 2007). Minimal residual disease (MRD) determined by FC and molecular methods is an important prognostic factor (Abou Dalle, Jabbour, & Short, 2020; Bassan et al., 2009; Brüggemann et al., 2006; Jacquy et al., 1997; Raff et al., 2007; Ribera et al., 2014; Sutton et al., 2009; Van Dongen, Van Der Velden, Brüggemann, & Orfao, 2015).

Treatment results of B-ALL has dramatically improved during the last seven decades and 5-year survival now exceeds 90% in developed countries (Pui, Nichols, et al., 2019). Combination chemotherapy is the cornerstone of ALL treatment. Directed antibody therapies and CAR-T cell therapies are used in relapse settings. Agents acting on signaling pathways, such as tyrosine kinase inhibitors, are used in a minority of cases, particularly in Bcr-Abl1 cases. It is important to recognize patients at high risk; however, even patients at low risk might benefit from less intensive treatment protocols, possibly enabled by the new treatment strategies (Ahmad, Uddin, & Steinho, 2020; Malard & Mohty, 2020; Phillips et al., 2015; Pui, Nichols, et al., 2019). The effective treatment strategies have been investigated in large multi-center groups such as the Nordic Society of Pediatric Hematology and Oncology (NOPHO) group (Teachey et al., 2021).

Current treatment of ALL usually lasts for 2–3 years and consists of four phases: induction, consolidation, intensification, and maintenance. Preventive treatment of CNS disease is also given. Induction therapy eradicates the bulk disease, restores normal hematopoiesis (so-called remission). The consolidation and intensification phases deepen remission and are followed by lengthy per oral maintenance therapy phase. The CNS prophylaxis is performed through serial intrathecal chemotherapy with methotrexate singly or in combination with cytarabine and glucocorticoids. Cranial irradiation is preserved for patients with CNS relapse. Imatinib is also used in cases of *BCR-ABL1* subtype with improved results (Biondi et al., 2018; Malard &

Mohty, 2020; Toft et al., 2018). Allogenic transplantation of HSCs is used with high-risk patients and patients with persistent minimal residual disease.

2.2.2 T-cell acute lymphoblastic leukemia

T-cell acute lymphoblastic leukemia (T-ALL) is a malignancy formed by T-cell lineage lymphoblasts. T-ALL accounts for 15% of pediatric ALL cases and is more common in adolescents (Toft et al., 2018). Elevated WBC counts are typical and mediastinal masses with lymphadenopathy are common (Quist-Paulsen et al., 2020; Toft et al., 2018). Patients with T-ALL have inferior prognosis compared to B-ALL patients. The diagnostic strategy in T-ALL cases is similar to that of B-ALL. However, the clinical significance of genetic subtypes is not clear in T-ALL.

Genetic alterations in T-ALL are highly heterogeneous. T-ALL can be divided into subtypes based on thymic maturation or gene expression profiles (Yu Liu et al., 2017). The activation of NOTCH-signaling through mutations in *NOTCH1* is seen in over 50% of T-ALL cases and deletion of *CDKN2A* locus or loss of function mutations in *FBXW7* promote leukemogenesis are common (Jang et al., 2019; Weng et al., 2004). Activation of NOTCH-signaling and deletions in the *CDKN2A* locus cooperatively promote oncogenesis (Ferrando et al., 2002). Multiple translocations also occur in T-ALL. These translocations usually include T-cell-specific enhancers (T-cell receptor alpha, beta, or delta) and oncogenic TFs (TAL1, TLX1, TLX2) or, less commonly, deletions of tumor suppressors (*WT1*, *LEF1*, *ETV6*) (Van Vlierberghe & Ferrando, 2012). Genetic lesions also occasionally affect the genes involved in signal transduction (*PTEN* loss, *ABL1* translocation) and chromatin modeling (*AZH2, SUZ12*) (Ntziachristos et al., 2012; Palomero et al., 2007). Mutations in glucocorticoid receptor coding NR3C1 are also seen (Wandler et al., 2020). Epigenetic regulation of T-cell development also affects leukemogenesis (Maćkowska, Drobna-śledzińska, Witt, & Dawidowska, 2021).

2.2.3 Acute myeloid leukemia

Acute myeloid leukemia (AML) forms a minority of pediatric acute leukemias, comprising about 15–20% of cases (De Kouchkovsky & Abdul-Hay, 2016; Linabery & Ross, 2008; Puumala et al., 2013; Yamamoto & Goodman, 2008). Pediatric AMLs are a heterogeneous group of nonlymphoid, hematological malignancies and can be further classified based on genetic abnormalities, morphology, and

immunophenotype. The five-year survival for AML in children < 15 years old is 64% and varies significantly among different genetic subtypes (Hasle et al., 2012; Pui, Carroll, Meshinchi, & Arceci, 2011). For example, children with acute promyelocytic leukemia have better prognosis than children with leukemia harboring mutations such as *FLT3-ITD*, monosomy 7, del 5q, and poor disease response (Meshinchi et al., 2006). The genetic subtyping holds prognostic value, but the implication of this classification into clinical management requires establishment. There are generally accepted risk factors, such as Down syndrome and ionizing radiation in utero (Doll & Wakeford, 1997; Xavier, Ge, & Taub, 2009). Also, there is evidence of neonatal origin in AML (McHale et al., 2003). MRD is also used in risk-stratification of pediatric AML patients (Dix, Lo, Clark, & Abadir, 2020; Rubnitz et al., 2010).

2.3 Transcriptional regulation of hematopoiesis and leukemic cells

Hematopoiesis is a complex process, which is largely orchestrated by different TFs, cofactors, chromatin modelers, and noncoding RNAs (T. I. Lee & Young, 2013; Orkin & Zon, 2008). The multiple TFs involved in hematopoiesis drive hematopoietic cell fate toward one or several developmental routes, while the same TFs can also inhibit other routes (Ceredig et al., 2009; T. I. Lee & Young, 2013). Also, chromatin formation and DNA accessibility through nucleosomal formation and modification and DNA methylation changes during this highly regulated process. Genetic lesions affect the function and expression levels of TFs involving hematopoiesis. These modifications derail and halt physiological developmental processes and drive leukemogenesis by accelerating cell proliferation.

2.3.1 Transcription factors

TFs are proteins that bind to specific DNA sites and activate or repress gene expression. TFs form less than 5% of human protein-coding genes, but they make possible the development and maintenance of multiple different cell types and organs (Fulton et al., 2009; Lambert et al., 2018; Vaquerizas, Kummerfeld, Teichmann, & Luscombe, 2009; Yusuf et al., 2012). TFs control the development of cells, organs, and organisms; elicit responses to intercellular signals and environment; control cell cycle; and contribute to pathogenesis (Spitz & Furlong, 2012).

The modular structure of TFs allows sequence-specific binding of promoter and enhancer regions of DNA through DNA-binding domains (DBD) (Fulton et al., 2009; Latchman, 1997). The activation domains (ADs) allow binding of other proteins such as TFs. Signal sensing domains, for example, bind ligands, which can attach, for instance, ligands, which can activate other TFs. TFs are commonly classified based on the structure of their DBDs (Luscombe, Austin, Berman, & Thornton, 2000).

The expression and function of TFs are regulated by controlling their synthesis by themselves in autoregulatory loops or by other TFs. Their localization can be changed by ligand binding, and TFs can be activated, for example, by phosphorylation or interaction with other TFs. The accessibility of binding sites is altered by DNA methylation, histone modification, and occupation by different TFs. The availability of other cofactors and TFs also influences the function of TFs. TFs can bind to different sites such as promoters and regulatory elements or enhancer regions in the DNA through DNA-binding domains, singly, as dimers, or by recruiting cofactors. Additionally, accessory TFs that do not bind DNA are essential in, for example, histone modification adding to the spatiotemporal regulation of gene expression activity (Fulton et al., 2009; Spitz & Furlong, 2012).

2.3.2 Central hematopoietic transcription factors

Hematopoiesis is strictly orchestrated by the timely expression of multiple different TFs. The self-renewal of HSCs and other multistepped lineage-restricted differentiation into mature cell types involves dozens of TFs from different DNA-binding protein families. HSCs express TAL1, RUNX1, and LMO2, among other TFs. These TFs are redeployed at various stages of blood cell development, exhibiting dynamic expression patterns of key regulators. For example, TAL1 and LMO2 participate in a protein complex driving erythroid differentiation. In primitive hematopoiesis, master regulators GATA1 and PU.1 drive erythroid and myeloid differentiation, respectively, and counteract each other (Orkin & Zon, 2008; Wadman et al., 1997). Numerous TFs are more lineage-specific, such as EBF1 and PAX5 for B-cell development and Notch and GATA3 for T-cell development (Nutt, Morrison, Dörfler, Rolink, & Busslinger, 1998; Radtke et al., 1999; Ting, Olson, Barton, & Leiden, 1996). Further differentiation into plasma cells is repressed by PAX5 and driven by XBP1 (Reimold et al., 1996).

2.3.3 Aberrant transcriptional regulation in B-ALL

Transcriptional regulation in precursor cells leading to B-ALL can become aberrant through several genetic alterations. Aneuploidy such as hyperdiploidy causes widespread transcriptional dysregulation through chromosomal gains and lower-level expression of CTCF and cohesin, leading to the disarray of chromosomal architecture and misconfiguration of regulatory elements and promoter elements (Paulsson et al., 2015; Yang et al., 2019). The hematopoietic TFs can be involved in translocations such as *ETV6-RUNX1* or *TCF3-PBX1* or sequence mutations such as *PAX5* or *IKZF1*. These genetic lesions affecting TFs dysregulate physiological transcriptional programs of healthy developing B-cells and lead to developmental arrest and less controlled proliferation in leukemic cells (Dang et al., 2015). Further mutations and epigenetic dysregulation contribute and are mandatory for leukemia initiation.

2.3.3.1 Fusion transcription factors

Many B-ALL subtypes include recurrent translocations. These translocations involve common hematopoietic and B-cell lineage-associated TFs and other genes, such as cytokine receptors, epigenetic modulators, and tyrosine kinases. These translocations cause chimeric fusion-proteins, which lead to aberrant transcriptional programs in leukemic cells (Look, 1997; Pui, Nichols, et al., 2019).

ETV6-RUNX1 (t(12;21)(p13;q22)) is a common in-frame fusion in pediatric B-ALL and involves two hematopoietic TFs. The 5' region of *ETV6* fuses to almost the entire *RUNX1* locus (Romana, Poirel, et al., 1995; Zelent, Greaves, & Enver, 2004). The ETV6-RUNX1 chimeric protein lacks the ETS DBD but includes functional regions of RUNX1 (Berger, 1997; Golub, Barker, Stegmaier, & Gilliland, 1996). *ETV6-RUNX1* is evident in fetal blood spot samples. A popular theory of the two-hit model includes this neonatal first hit in HSC or pro-B cell and the development of a pre-leukemic clone. ETV6-RUNX1 acts as a transcriptional repressor of RUNX1 gene targets and drives self-renewal, cell survival, proliferation, and inhibition of apoptosis through, for example, driving EPOR, PI3K/AKT/mTOR, and JAK2/STAT5 signaling and increasing MDM2 and SMAD3, which leads to inhibition of P53 and TGF-beta (Sun, Chang, & Zhu, 2017).

Subclonal deletion of remaining *ETV6* gene is common, and the lack of normal ETV6 protein possibly enhances transforming effect as *ETV6-RUNX1* chimeric protein forms homodimers or heterodimers with ETV6. As ETV6-RUNX1 causes

developmental arrest and survival, while secondary genetic hits are required for overt leukemia (Cavé et al., 1997; Lopez et al., 1999; T. McLean et al., 1996; Mori et al., 2002; Morrow, Horton, Kioussis, Brady, & Williams, 2004; Romana et al., 1996). Extra copy of RUNX1 is also frequently observed (Stams et al., 2006).

Usual secondary hits, deletions, or mutations in *ETV6-RUNX1* subtype are found in lymphoid TFs (such as monoallelic deletion of *PAX5*), cell-cycle regulation and tumor suppression (*CDKN2A*, *BTG1*), regulation of apoptosis, transcriptional regulation and coactivation (*ETV6*), members of the immunoglobulin superfamily (*VPREB1*, *BTLA*), nuclear receptors (*TBL1XR1*, *NR3C2*), and epigenetic alterations such as demethylation (Busche et al., 2013; Fenrick et al., 2000). These secondary genetic hits are possibly caused by aberrant RAG recombinase activity (Mullighan et al., 2007; Papaemmanuil et al., 2014; Parker et al., 2008).

As ETV6 and RUNX1 are involved in earlier hematopoiesis, genes involved in *TCF3-PBX1* (t(1;19) (q23;p13.3)) fusion are vital for B-cell development (TCF3) and lymphoid development (PBX1) (Boisson et al., 2013; Carroll et al., 1984; Ficara et al., 2013; Sanyal et al., 2007). *TCF3-PBX1* is the most common cytogenetic fusion involving *TCF3* in B-ALL (Barber et al., 2007). *TCF3* encodes basic helix loop helix proteins E12 and E47 by alternative splicing. TCF3-PBX1 and ETV6-RUNX1 block B-cell differentiation and cause pooling of pre-B cell clones but cannot cause overt leukemia. Deletion/downregulation of *PAX5* and mutation of *TCF3* are common (Barber et al., 2007).

The TCF3-PBX1 fusion protein combines transactivation domains of TCF3 with the DNA binding homeodomain of PBX1 (Kamps, Murre, Sun, & Baltimore, 1990; Nourse et al., 1990). The heterozygosity of *TCF3* declines B-cell development through reducing immunoglobulin rearrangement and expression (Bain et al., 1994; Zhuang, Soriano, & Weintraub, 1994). The fusion protein causes aberrant transcriptional activation of *PBX1* targets (LeBrun & Cleary, 1994).

2.3.3.2 BCL6

BCL6 transcription repressor (BCL6) is a 95 kDa protein, a transcriptional repressor coded from a 24kb gene on chromosome 3q27 (B. H. Ye et al., 1993). The protein has an N-terminal BTB/POZ domain for protein–protein interactions, 3 PEST domains in the middle of the protein for protein activity and stability, and 6 C-terminal zinc fingers for DNA-binding (Wong & Privalsky, 1998). BCL6 belongs to the BTB/POZ/ZF TF family, and it acts through sequence-specific DNA-binding

and recruiting histone deacetylase complexes (HDACs) and corepressors. BCL6 acts as dimers (Lemercier et al., 2002).

BCL6 is an active component of the pre-BCR signaling, opposing IL7R/JAK-STAT-signaling pathway during early B-cell development (Buchner et al., 2015; Chan et al., 2020; Eswaran et al., 2015; Geng et al., 2015). In mature B-cell development, BCL6 transcription is promoted by IRF8 and engaging of B-cell receptors by antigens in naïve B cells. BCL6 is involved in the formation of GCs in lymphoid follicles of lymphoid tissue and partially regulates somatic hypermutation of immunoglobulin genes (Dent, Shaffer, Yu, Allman, & Staudt, 1997; Duy et al., 2010; Hatzi & Melnick, 2014; B. H. Ye et al., 1997). BCL6 represses its expression in an autoregulatory loop. Acetylation in the PEST domain leads to reduced recruiting of corepressors, and phosphorylation of BCL6 leads to degradation of the protein through the ubiquitin-proteasome pathway. BCL6 promotes tolerance toward DNA damage during somatic hypermutation and class-switch recombination in B-cells by, for example, repressing *TP53* (Bereshchenko, Gu, & Dalla-Favera, 2002; Phan & Dalla-Favera, 2004; Phan, Saito, Kitagawa, Means, & Dalla-Favera, 2007). After antigen stimulus from BCR leads to MAPK-pathway activation and phosphorylation of BCL6, it leads to further degradation (Niu, Ye, & Dalla-Favera, 1998). CD40-signaling through NF-KB-pathway leads to repression of BCL6 expression through IRF4 binding of multiple BCL6 promoter regions (Saito et al., 2007). BCL6 also regulates CD40-NFKB- and BCR-MAPK-signaling to prevent premature activation and differentiation of B-cells. Likewise, BCL6 represses PRDM1 and MUM1, which promote plasma cell differentiation (Basso et al., 2010; Tunyaplin et al., 2004) and anti-apoptotic molecules such as BCL2, adding to a safer GC reaction (Klein et al., 2003).

BCL6 is a crucial oncogene in diffuse large B-cell lymphoma (DLBCL). As high expression of *BCL6* is associated with the GC type DLBCL and, in some studies, with favorable prognosis, *BCL6* translocation is not linked with high *BCL6* expression or favorable prognosis (Alizadeh et al., 2000; Lossos et al., 2001). Also, transient expression of BCL6 in lymphomagenesis might cause genetic alterations that drive tumorigenesis in a "hit-and-run" manner (Green et al., 2014). BCL6 is strongly expressed in follicular lymphoma (FL) and Burkitt lymphoma (Bosga-Bouwer et al., 2006; Seegmiller et al., 2010). Subsequently, it is also a potential target for targeted treatment (Cardenas et al., 2017).

In B-ALL, evident nuclear BCL6 expression has been associated with the *TCF3-PBX1* subtype (Deucher, Qi, Yu, George, & Etzell, 2015; Geng et al., 2015). Furthermore, it has been identified in pre-BCR+ ALL, which is a new subtype of B-

ALL with tonic pre-BCR signaling with active SRC kinases and PI3K-AKT signaling and lack of pSTAT5 activity. Thus, BCL6 has been proposed as a diagnostic and a predictive biomarker for this subtype (Geng et al., 2015). Moreover, pre-BCR signaling and BCL6 expression have been reported in the novel *MEF2D* subtype of B-ALL (Tsuzuki et al., 2020) and *KMT2A*-re subtype, in which it has been proposed as a treatable target (Hurtz et al., 2019). The pre-BCR+ ALL could be targeted with tyrosine kinase inhibitors (Geng et al., 2015; Köhrer et al., 2016)

The prognostic effect of BCL6 at protein level has not been fully elucidated in population-based studies. Nonetheless, in high-risk B-ALL patients, high *BCL6* mRNA expression has been associated with an inferior outcome in several studies (Ge et al., 2017; Hurtz et al., 2019; Swaminathan et al., 2013).

2.3.3.3 SOX11

The SOX11 protein is an intronless TF from the group C SRY-related HMG-box (SOXC) TF family. The *SOX11* gene is located in locus 2p25.2 (Jay et al., 1995). This protein has two functional domains: the N-terminal DNA-binding domain and the C-terminal transactivation domain. The SOXC family includes SOX4 and SOX12 (Dy et al., 2008; Penzo-Méndez, 2010; Tsang, Oliemuller, & Howard, 2020). SOX11 cooperates with POU3F2/BRN2 or POU3F1/OCT6 when binding gene promoters in motif TTGT and enhances transcriptional activation (Badis et al., 2009; Kuhlbrodt et al., 1998). SOX11 also harbors pioneer TF capabilities (Dodonova, Zhu, Dienemann, Taipale, & Cramer, 2020). Cellular location of SOX11 is regulated post-translationally by phosphorylation (Balta et al., 2018).

During embryogenesis, SOX11 is expressed in the CNS (Jay et al., 1995). In adult tissues, SOX11 expression is found in keratinocytes and squamous epithelium. Furthermore, Schwann cells express SOX11, but BM and brain do not express this protein (Ek, Dictor, Jerkeman, Jirström, & Borrebaeck, 2008). Knockdown models have shown the vital role of SOX11 for embryogenesis (Kato, Bhattaram, Penzo-Méndez, Gadi, & Lefebvre, 2015; Sock et al., 2004), and neurodevelopmental disorders are associated with *SOX11* mutations and deletions (Hempel et al., 2016).

In malignant neoplasms of epithelial origin, SOX11 is expressed in, for example, epithelial ovarian cancer with possible protective survival effect (Sernbo et al., 2011) and in basal-like breast cancer with a potential oncogenic role (Shepherd et al., 2016). The effects of *SOX11* upregulation on prognosis are mostly favorable in solid tumors (Grimm et al., 2020).

In hematological malignancies, SOX11 is a specific and well reproducible diagnostic biomarker for conventional mantle cell lymphoma (MCL) (Croci et al., 2020; Ek et al., 2008; Fernàndez et al., 2010; Mozos et al., 2009; Zeng et al., 2012). As SOX11 is useful in the diagnostics of mature B-cell malignancies, its utility is compromised among blastoid B-cell malignancies (M. T. Ye et al., 2021). SOX11 is an essential factor in the genesis of MCL (Beekman, Amador, & Campo, 2018). In a mouse model, overexpression of SOX11 in B-cells led to a MCL-like phenotype and activated B-cell receptor signaling (Kuo et al., 2018). The prognostic role of SOX11 expression is ambivalent in the literature. Its high expression has been associated with improved survival (Kuo et al., 2015; Nygren et al., 2012). SOX11 was associated with inferior prognosis in MCL in one study and the indolent MCL is considered SOX11 negative (Meggendorfer, Kern, Haferlach, Haferlach, & Schnittger, 2013; Navarro et al., 2012)). Moreover, the subcellular location of SOX11 into the cytoplasm has been associated with lower survival in one study (Wang et al., 2008). Interestingly, *TP53* mutations have been associated with low or negative *SOX11* levels (Federmann et al., 2020). Also, SOX11 expression has been associated with increased angiogenesis and T-cell infiltration in MCL (Annese et al., 2020). Recent studies have shown promising cytotoxicity with SOX11 inhibitors in MCL cell lines (Jatiani et al., 2021). In AML of adults, high SOX11 expression was associated with dismal prognosis (Tosic et al., 2018).

The effect of SOX11 on proliferation are ambivalent in the literature. Knockdown of *SOX11* by small interfering ribonucleic acid (siRNA) increased the proliferation in MCL cell lines (Gustavsson et al., 2010). Knockdown of HIG-2 led to decreased SOX11 expression and increased proliferation in MCL cell lines (Kuci, Nordström, Conrotto, & Ek, 2016). In pro-B-cell Ba/F3 cell models, SOX11 expression has decreased proliferation and increased cell aggregation (Lord et al., 2018). On the contrary, SOX11 has been shown to promote tumor growth in vivo (Vegliante et al., 2013). An increased proliferation has been associated with high SOX11 expression (Conrotto, Andréasson, Kuci, Borrebaeck, & Ek, 2011).

In lymphoblastic leukemias, nuclear SOX11 expression has been reported on the protein level in a small series (Dictor et al., 2009). Burkitt leukemias were also found to express SOX11 in this study. Also, in an anecdotal cohort of one case, SOX11 was positive in a B-cell lineage lymphoblastic disease (Mozos et al., 2009). Elevated *SOX11* mRNA expression was found in *ETV6-RUNX1* and *TCF3-PBX1* subtypes of B-ALL in one previous study (Vegliante et al., 2011). Two studies have reported *SOX11* expression in the *ETV6-RUNX1* subtype (Busche et al., 2013; Nordlund et al., 2012). The prognostic role of SOX11 expression in B-ALL is not fully elucidated.

2.3.3.4 IGF2BP3

Insulin-like growth factor II mRNA-binding protein 3 is a 69 kDa protein. The gene *IGF2BP3* is in the locus 7p15.3. This oncofetal protein is mainly found in the cytoplasm of cells (Monk et al., 2002; Mueller-Pillasch et al., 1999). IGF2BP3 belongs to the IGF2BP-family of three proteins, IGF2BP1–3. The amino acid sequence of these proteins shares 59–73% similarity (Mancarella & Scotlandi, 2020; Nielsen et al., 1999). As an RNA-binding protein (RBP), IGF2BP3 binds RNA molecules regulating mRNA localization and stability. In adult tissues, IGF2BP3 expression is evident only at low levels. In numerous malignancies, the expression is elevated (Burdelski et al., 2018; Mancarella & Scotlandi, 2020).

In gastrointestinal and urogenital malignancies, high IGF2BP3 expression is linked to a more aggressive phenotype (Burdelski et al., 2018; Mancarella & Scotlandi, 2020). In lymphoid malignancies, IGF2BP3 expression has been reported in malignancies of GC-origin origin (Findeis-Hosey & Xu, 2011; King, Pasha, Roullet, Zhang, & Bagg, 2009). In classic Hodgkin lymphoma (CHL), IGF2BP3 expression has been reported in the Reed–Sternberg cells adding to the differential diagnostic potential of immunophenotyping (Masoud, Ibrahiem, Tantawy, & Eldosoky, 2019; Sennekamp & Seelig, 2016; Tang et al., 2013).

Increased proliferation has been associated with higher IGF2BP3 expression on several malignancies of epithelial and hematolymphoid origin (Hartmann et al., 2012; Liao, Hu, Herrick, & Brewer, 2005; Mancarella & Scotlandi, 2020). IGF2BP3 also promotes cell survival during ionizing radiation in B-cells (Liao, Hu, & Brewer, 2011).

IGF2BP3 was weak or absent in HSCs and mature cells in a previous study. Different B-ALL subtypes were also investigated in this study, and *IGF2BP3* expression was highest in the *ETV6-RUNX1* and *KMT2A*-re subtypes (Stoskus et al., 2011). The previous literature supported this by including a small cohort of histologic lymphoblastic lymphoma cases (King et al., 2009; Natkunam et al., 2007). Furthermore, by siRNA and CRISPR-CAS9 silencing, two studies have shown that *IGF2BP3*-knockdown decreases proliferation and induces apoptosis in leukemia cell lines (Liao et al., 2005; Palanichamy et al., 2016). Yet, the role of IGF2BP3 in pediatric B-ALL remains elusive.

2.4 Histopathological diagnostic methods in hematological malignancies

The diagnostic workup in hematological malignancies such as leukemias is multimodal. It consists of clinical presentation, morphology, immunophenotyping, and genetics. For morphology, peripheral blood and BM aspirate smears are highly informative and adequate in many hematological malignancies. Histological evaluation of tissue biopsies from BM, lymph nodes, and other sites gives further information on topography and elements such as fibrosis (Swerdlow et al., 2017).

2.4.1 Trephine biopsy and morphology

When a hematological malignancy, such as a myeloid or lymphoid neoplasm, is suspected, histologic BM trephine biopsy is essential in the characterization of hematological neoplasms and the differential diagnostics with nonhematological malignancies and reactive conditions (Orazi, 2007). The histological specimen yields information on histologic topography, overall age-adjusted cellularity, and the proportions of maturation in hematopoietic cells. The biopsy also allows the evaluation of morphology, growth pattern and the molecular composition. Stromal cells of the BM parenchyma, and the structure of the cancellous bone can also be deciphered. In myelofibrosis and many myeloproliferative neoplasms, histological evaluation of fibrosis and architectural disruption is required. Especially in cases of "dry tap," trephine biopsy is essential as myelofibrosis or an infiltrating process such as a metastatic neoplasm hampers the assessment of an aspiration sample. In the routine diagnostic workup of B-ALL, histology is not often needed to set the diagnosis (Malard & Mohty, 2020). Histological images of reactive BM and B-ALL are shown in Figures 4 and 5.

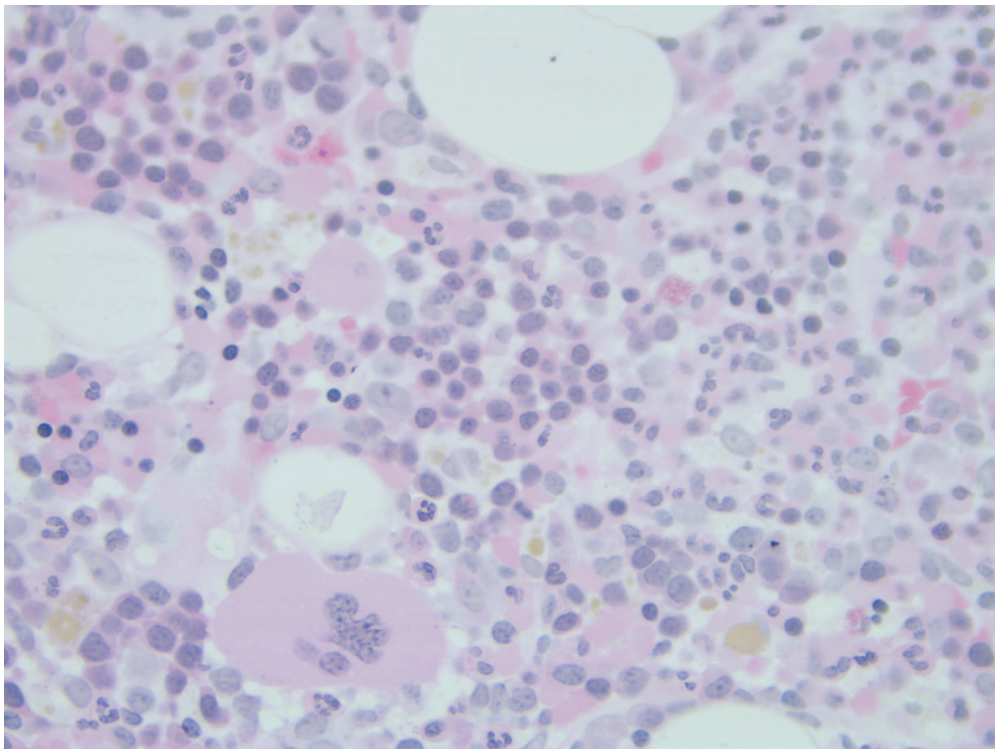

Figure 4. Hematoxylin and eosin-stained section of reactive bone marrow at 20x magnification. The tissue section is from a plastic-embedded bone marrow trephine biopsy sample.

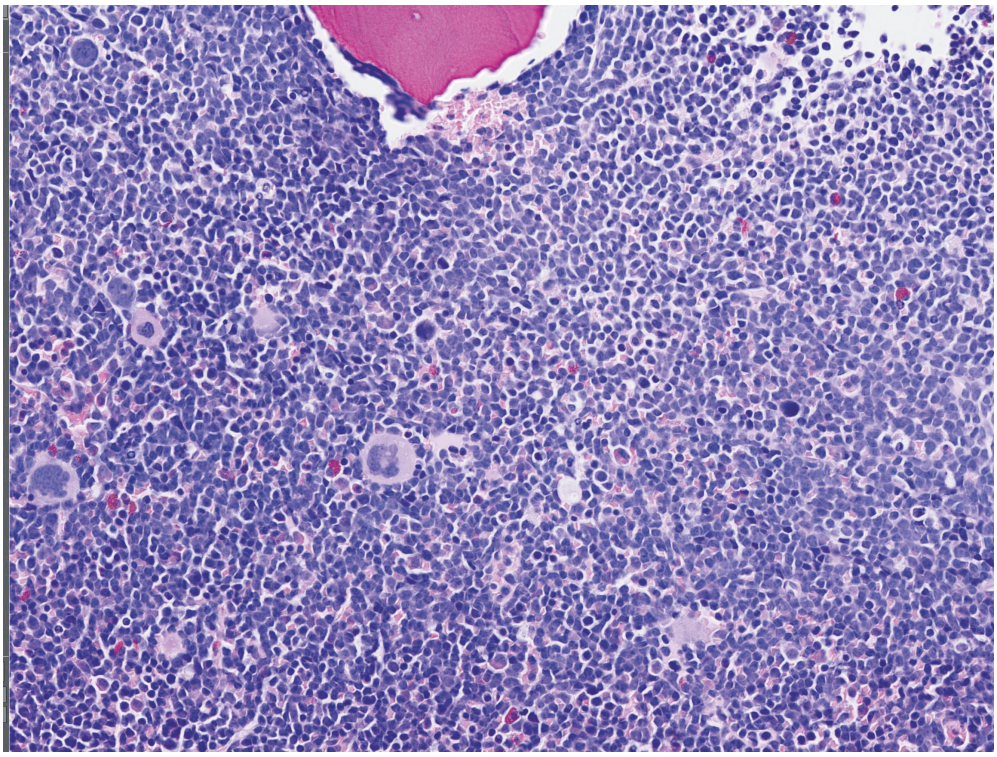

Figure 5. Hematoxylin and eosin-stained section of a bone marrow sample from a B-ALL case at 10x magnification. The tissue section is from a formalin-fixed, paraffin-embedded sample.

2.4.2 Immunohistochemistry

The preferred method for immunophenotyping of hematological malignancies such as leukemias is FC. In rare cases, IHC can aid as a substitute or complementary study, when for instance, a histological sample such as skin biopsy initiates the diagnostic buildup of B-ALL (Al Gwaiz & Bassioni, 2008; Cho-Vega, Medeiros, Prieto, & Vega, 2008). Investigation of B-ALL can be gained from IHC in finding biomarkers or when investigating the topography or microenvironment of the samples. As some proteins are challenging to assess by FC and as archived formalin-fixed, paraffin-embedded (FFPE) material is usually available, IHC can be executed also in a retrospective manner.

2.4.3 Tissue microarray

Tissue microarray (TMA) can be utilized as a high-throughput method for biomarker analysis from hundreds and even thousands of histologic samples simultaneously. This method has been used for several decades, and it has many advantages compared with WTSs (Battifora, 1986; Battifora & Mehta, 1990; Kononen et al., 1998). Areas of interest from the donor blocks are punched out and positioned into the recipient TMA block. Samples in TMA sections are more easily immunostained in single batches for a more consistent expression pattern. Also, the logistical usage of a single block vs. dozens of blocks is more efficient. As there is more dropout due to detachment or other technical insults with TMA, multiple replicates are usually created if there is sufficient material to conduct this. In diagnostic usage, TMAs can create control tissue blocks with multiple control tissues combined. As the diagnostic work-up of individual cases is rapid, TMAs require numerous cases from the same disease group to match the turnaround time of the pathology laboratory processes.

2.4.4 Digital pathology

Digitalization of clinical and experimental pathology began several decades ago. The method of remote tissue analysis, named telepathology, was invented in the 1960s and in the late 1990s saw the introduction of the virtual microscope (Ferreira et al., 1997; Weinstein, Bloom, & Rozek, 1987). In addition to interinstitutional and institutional consultations, the digital form of tissue sections with different stainings brings the vast possibilities of image analysis and neural-network-based automated analysis. Although automated analysis of cells, tissues, and IHC have been experimented with for decades (Bacus, Flowers, Press, Bacus, & McCarty, 1988; Hamilton et al., 2014), its routine use in diagnostics is limited. Most of the whole-slide image analysis and validation has concentrated on solid tumors. Identification and grading of different hematolymphoid malignancies from digitized tissue sections have also been investigated (Amin, Mori, & Itoh, 2019; Mohlman et al., 2020). In biomarker research, digital pathology and image analysis with whole-slide images is increasing and changing the biomarker analysis from qualitative toward more quantitative analysis (Bankhead et al., 2017). Furthermore, digital pathology brings speed and reproducibility into the study of IHC, which exhibits inter- and intra-observer variability even in expert laboratories, especially in heterogeneous tumor samples (Polley et al., 2013).

3 AIMS OF THE STUDY

B-ALL is the most common pediatric malignancy, and the relapse of the disease is a significant cause of mortality and morbidity. Novel prognostic biomarkers and druggable targets are needed to aid in risk stratification and to design more effective targeted treatments.

The aims of this study were as follows:

1. to investigate the expression and clinicopathological effects of the transcription factor BCL6 in pediatric B-ALL (I);

2. to explore the functional role and influences of the transcription factor SOX11 in pediatric B-ALL (II); and

3. to evaluate the prognostic effects and association between IGF2BP3 expression and proliferation in pediatric B-ALL (III).

4 SUBJECTS AND METHODS

4.1 Pediatric cohort of ALL patients

A retrospective cohort of pediatric leukemia patients with trephine biopsy specimens was collected for IHC assays. The cases were identified from the local cancer registry, POTTI. Patients were treated in the Tampere University Hospital during 1990 and 2017. The study cohort included patients under 18 years old who had B-ALL and T-ALL cases at the time of diagnosis. Patients with AML or acute leukemia of ambiguous lineage were excluded. The patients were treated with consecutive NOPHO ALL treatment protocols.

Clinical data were collected from the POTTI registry and manually curated from laboratory information management systems and electronic health care records. Survival status was confirmed from the patient records. Variables included age, cytogenetic subtype, karyotype, immunophenotype by FC, CNS disease, WBC count, blast count, MRD at the EOI/consolidation therapy, relapse, site of relapse, survival status, secondary malignant neoplasm, and the used treatment protocol.

4.2 Bone marrow trephine biopsy samples

Archived BM biopsies were chosen based on original pathology reports, and inadequate or technically unsatisfactory samples were excluded. The diagnostic BM biopsies were fixated in phosphate-buffered 10% formalin (pH 7.0) for one day. After that, BM biopsies were transferred into the formalin-including ethylenediaminetetraacetic acid (EDTA) solution for decalcification. The duration of the decalcification process was 3–4 days. The hard cartilage was cut from the samples, and the soft marrow was transferred into the tissue processor. In a few cases, the formalin-fixation was performed in +60 °C pre-heated formalin in a hot air oven. The decalcification process was conducted using 5% formic acid and a microwave oven after tissue-processing samples were embedded in paraffin for tissue sectioning and archiving.

4.3 Immunohistochemistry and imaging

4.3.1 Immunohistochemical stainings

BM biopsies from paraffin blocks were sectioned into four-micrometer thick whole-tissue sections (WTS) (I, II). For the TMA, representative areas 1.5 mm in diameter were punched from the donor tissue block into the recipient TMA block (III) for sectioning. WTS and TMA sections were transferred on glass slides. Ventana Benchmark Classic (I, II) and Ventana Benchmark Ultra (III) instruments were used for IHC. The Ultraview Universal DAB detection kit (I, II), Optiview DAB detection kit (III), and Ultraview Universal Alkaline Phosphatase Red detection kit were used for antibody detection, and slides were counterstained using hematoxylin. CD19 antibody was combined with Ki-67 for double-staining, and alkaline phosphatase and 3,3'-diaminobenzidine (DAB), respectively, were used for detection. The antibodies used for IHC and tissue samples used for control material are shown in Table 1.

Table 1. Antibodies and control material used for immunohistochemistry. BL: Burkitt lymphoma; IDC: invasive ductal carcinoma; MCL: mantle cell lymphoma; pSTAT5: pSTAT5-Y694; RTU: ready-to-use.

Target	Clone	Product number	Manufacturer	Dilution	Control tissue
SOX11	MRQ-58	382M	Cell-Marque	1:50	MCL
BCL6	LN22	PA0204	Leica	1:50	appendix, BL
pSTAT5	E208	ab32364	Abcam	1:50	IDC
muHC	polyclonal	A0425	Dako	1:5500	appendix, BL
ZAP70	YE291	ab32429	Abcam	1:100	tonsil
CD19	EP169	119-R18	Cell-Marque	RTU	appendix
Ki-67	30-9	790-4286	Ventana	RTU	appendix
IGF2BP3	69.1	M3626	Dako	1:100	appendix

4.3.2 Light microscopy

Immunohistochemically stained BM WTSs were evaluated by two pathologists independently and without clinical information. In cases of discrepancy, a third pathologist evaluated the sample to settle the grade. The staining pattern of leukemic blast cells was assessed using a light microscope with magnification up to 200x. The intensity of expression was graded semiquantitatively as negative, positive, or strong positive. "Negative" cases showed positivity in under 20% of the leukemic blast cells, while "positive" cases expressed antigen in 20–50% of the blasts. "Strong positive" cases had antigen expression of over 50% in the leukemic blast cells.

4.3.3 Digital pathology

Immunostained TMA section slides were scanned with Hamamatsu Nanozoomer XR with 40x magnification. QuPath (version 0.2.3) software was used for image analysis and immunostaining detection (Bankhead et al., 2017). Areas with leukemic cells were annotated and cell detection parameters were set by a pathologist. Inadequate and technically unfit samples were removed from the analysis. The results were confirmed by a pathologist by comparing the original image and detected cells.

4.4 Fluorescence in-situ hybridization (FISH)

Fluorescence in-situ hybridization (FISH) analysis was performed on either the frozen BM aspiration samples or the FFPE BM biopsy samples for cases that lacked cytogenetic information. DNA probes with fluorescent labels were hybridized with the processed samples. A total of 100–200 cells were analyzed for translocations. Probes that were used for the detection of translocations were Metasystems *E2A* Break Apart Probe 19p13 (Lot #18216), Metasystems XL *MLL* plus Break Apart Probe 11q23 (Lot #18451), Metasystems XL *BCR/ABL1* plus (Lot #19082), and Metasystems XLXL t(12;21) (Lot #19133).

4.5 Gene expression datasets

Hemap, a microarray gene expression dataset, is an interactive online resource that includes molecular phenotypes across hematological malignancies (Mehtonen et al., 2019; Pölönen et al., 2019). This dataset includes 6,832 patient samples from hematological malignancies with 1,304 B-ALL samples from different cytogenetic subtypes. Out of these B-ALL samples, 662 are from pediatric patients.

The publicly available PanALL study cohort dataset consists of RNA-sequencing (RNA-seq) data from 1,988 B-ALL patients, of which 1,234 are pediatric (Gu et al., 2019).

The publicly available TARGET dataset includes 155 pediatric high-risk B-ALL patients with clinical information and RNA-seq data (Yu Liu et al., 2017; Roberts et al., 2014).

RNA-seq data of a pediatric B-ALL cohort of 115 cases includes clinical, survival, and methylation data (Marincevic-Zuniga et al., 2017; Nordlund et al., 2013).

4.6 Cell lines and cell culture

The commercial cell lines used are shown in Table 2. These cell lines were cultured according to the instructions provided by the manufacturer. Mycoplasma was tested regularly, and short tandem repeats genotyping was used to authenticate the cell lines.

Table 2. Cell lines used in the studies.

Cell line	Disease	Genetics	Donor age
NALM-6	B-ALL	*ETV6*/*PDGFRB*, *DUX4*/*ERG*	19 years
REH	B-ALL	*ETV6-RUNX1*	15 years
697	B-ALL	*TCF3-PBX1*	12 years
RCH-ACV	B-ALL	*TCF3-PBX1*	8 years
KOPN-8	B-ALL	*KMT2A-ENL*	3 months
KASUMI-2	B-ALL	*TCF3-PBX1*	15 years
JURKAT	T-ALL	*TAL1*	14 years
MOLT-16	T-ALL	*TAL1*	5 years
P12-ICHIKAWA	T-ALL	*LYL1*/*LMO2*	7 years
HPB-ALL	T-ALL	*TLX3* subtype	14 years
CCRF-CEM	T-ALL	*NKX2-5* expression	3 years

4.6.1 Cell viability

Cell viability assay was performed on transfected RCH-ACV, 697, and REH cell lines. A total of 10,000 cells were counted per well on a 96-well plate. RCH-ACV and 697 cells were grown for 72 h and REH cells for 120 h. Alamar blue with 2 h of incubation was applied to measure the cell viability every 24 h. Tecan fluorometer Infinite 200 was used to measure fluorescence, and 4 technical replicates per sample were used to calculate the mean value.

4.6.2 Knockdown of gene expression

The knockdown of *SOX11* was performed by nucleofection of cell lines with gene sequence-specific siRNA, using 4D-nucleofector for transfections according to the manufacturer's instructions. Nonspecific siRNA acted as the control. One million cells were used for every nucleofection reaction. The success of knockdown levels was evaluated from the transfected cells using Western blot and RT-qPCR. The transfected cell lines and controls were used for cell viability assays and RNA-seq.

4.6.3 In vitro drug treatments

To inhibit methyltransferase, the cultured cell lines (KOPN-8, REH) were treated with decitabine, and 5-Aza-2'-deoxycytidine dissolved in dimethyl sulfoxide in different concentrations. Corticosteroid (prednisolone and dexamethasone) and chemotherapies (asparaginase and vincristine) were used in various concentrations for the in vitro drug treatments of knockdown and control cell lines (697, RCH-ACV, and REH). After treatment, the cell viability assay was measured, and RT-qPCR analysis was performed on the extracted RNA in hypomethylation assays.

4.7 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics (version 26) and RStudio (version 3.6.1). Mann–Whitney U test and Kruskal–Wallis U test were used for nonparametric variables, and Chi-squared test and Fisher's exact test were used for categorical variables. In analyzing EFS and overall survival (OS), the log-rank test was used to test statistical significance between groups. Events in the EFS analysis were death, relapse, resistant disease (MRD > 25% at the EOI/consolidation therapy), and secondary malignant neoplasm. All tests were 2-sided, and p-values under 0.05 were considered statistically significant. Cox proportional hazards model was fitted for survival data to estimate the hazard of different individual risk factors. The proportionality of various covariates in the models was tested. The expression levels of "metagenes" were formed from the mRNA expression data by calculating an arithmetic mean of gene expression in a given set of genes.

4.8 Ethical considerations

The Regional Ethics Committee approved the studies of the Expert Responsibility area of Tampere University Hospital (R16054, R13109, and R19060B). The permit to use the pediatric BM sample tissue for the studies was obtained from the National Supervisory Authority for Welfare and Health (Valvira, Dnro: 4243/06.01.03.01/2016 and V/3994112019). All cell lines used were purchased from commercial vendors. Gene expression datasets used were retrieved from publicly available sources.

5 RESULTS

5.1 The characteristics of BCL6-positive cases of pediatric B-ALL (I)

5.1.1 BCL6 protein is expressed in a small subset of pediatric B-ALL

The Hemap dataset is a web-based resource that contains data on the expression of thousands of genes across all hematological cancers, many cell lines, and healthy donors (Mehtonen et al., 2019; Pölönen et al., 2019). This dataset was employed to explore the expression of *BCL6* mRNA across hematological malignancies. The highest median expression of *BCL6* was found in FL, DLBCL, and Burkitt lymphoma. At the same time, B-ALL and T-ALL exhibited a wide range of expression with median expression at intermediate levels. When the median expression levels of *BCL6* were assessed among distinct cytogenetic subtypes of B-ALL, the *TCF3-PBX1* and *BCR-ABL1* subtypes showed the highest expression levels, and the *KMT2A*-re subtype showed the lowest levels (Kruskal–Wallis U test p-value < 0.001). The PanALL dataset, based on RNA-seq of 1,988 ALL cases (Gu et al. 2019), corroborated the findings and indicated high *BCL6* mRNA also in novel subtypes of ALL, namely the *MEF2D*, *NUTM1,* and *BCR-ABL1*-like subtypes.

The functional unit of TF is protein, and the correlation of expression between mRNA and protein abundance is circa 0.50, but it varies greatly depending on multiple biological and technical factors (Csárdi, Franks, Choi, Airoldi, & Drummond, 2015; Latonen et al., 2018; Yansheng Liu, Beyer, & Aebersold, 2016; Sjöstedt et al., 2020; Uhlen et al., 2019). Therefore, the expression of the BCL6 protein was explored by IHC in a population-based pediatric cohort of B-ALL. The BCL6 protein was expressed either moderately or strongly in 8 out of 117 cases (6.8%); 3 out of 4 *TCF3-PBX1* cases were positive, while none of the 5 KMT2A-re cases were positive for BCL6, in agreement with the mRNA data. The B-ALL sample with BCL6 expression is shown in Figure 6. A case summary of the IHC patient cohort and IHC results from the WTS cohort are shown in Tables 3 and 4.

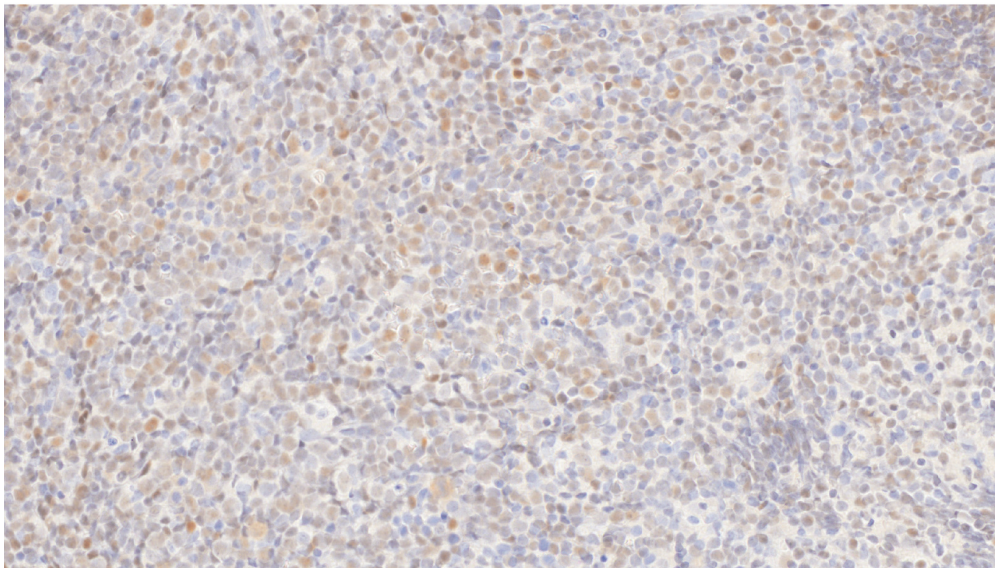

Figure 6. Light microscopic image of BCL6-stained B-ALL sample. Diffuse nuclear staining is observed with varying intensity in majority of the leukemic blast cells.

Table 3. Case summary for the immunohistochemistry cohort. CNS: central nervous system; EOI: end of induction; IQR: interquartile range; KMT2A-re, KMT2A-rearranged; Md: median; MRD: minimal residual disease; n: number; WBC: white blood cell; WHO: World Health Organization.

	Md	**IQR**
Age (years)	4.2	0.9–17.6
WBC count (10^9/L)	7.1	1-311
MRD (%) at EOI	0.02	0–44
		n
Deceased		9
Relapse		15
CNS disease		6
	Total	**117**
WHO subtype	*ETV6-RUNX1*	33
	Hyperdiploid	30
	KMT2A-re	5
	BCR-ABL1	2
	TCF3-PBX1	4
	Hypodiploid	1
	Other	42

Table 4. Results of immunohistochemistry stainings and positive cases in the WTS cohort (I,II). IHC: immunohistochemistry; KMT2A-re, KMT2A-rearranged; n: number; WTS: whole-tissue section; TMA: tissue microarray.

	BCL6	**pSTAT5**	**ZAP70**	**SOX11**	**Total n in subtype**
ETV6-RUNX1	0	31	32	26	33
Hyperdiploid	1	28	30	1	30
***KMT2A*-re**	0	3	4	0	5
BCR-ABL1	1	2	2	0	2
TCF3-PBX1	3	4	4	3	4
Hypodiploid	0	1	1	1	1
Other	3	37	37	7	42
Total n of IHC positive cases	8	106	110	38	117

5.1.2 BCL6 expression associates with markers of pre-BCR signaling and a more mature immunophenotype

BCL6 has been proposed as a biomarker for the new pre-BCR+ ALL subtype and a downstream effector of the active pre-BCR signaling cascade in B-ALL (Chan et al., 2020; Geng et al., 2015; Tsuzuki et al., 2020). Signaling through pre-BCR receptor and IL7R/JAK-STAT pathway were shown to be mutually exclusive (Geng et al., 2015).

Therefore, ZAP70, a marker for pre-BCR signaling, and phopho-STAT5-Y694 (pSTAT5), a marker for JAK/STAT5 signaling, were chosen as targets for further IHC. pSTAT5 was positive in 108 cases (92%), a high proportion in line with previous literature (Geng et al., 2015), while pSTAT5 and BCL6 were co-expressed in 7 cases (87.5% of the BCL6-positive cases). The ZAP70 protein was expressed in 110 cases (94%), with only 2 cases co-expressing BCL6 and ZAP70 (25%). A pre-BCR "metagene" described by Geng et al. (2015), which is composed of *IGLL1, IGLL3, VPREB1, VPREB3, IGHM, SYK, ZAP70* genes, showed a positive correlation between the expression of the highest 10th percentile of "metagene" and *BCL6* (Mann–Whitney U test p-value < 0.001). Previously, high BCL6 expression has been associated with a more mature CD34-negative immunophenotype and *TCF3-PBX1* subtype (Deucher et al., 2015). CD34, which is a marker of HSCs, was expressed in 4 out of 7 BCL6-positive cases (57%) and 96 out of 101 BCL6-negative cases (95%) by FC (Chi-squared test p-value = 0.001). These results mostly support the association between high BCL6 and pre-BCR expression, a more mature phenotype, and *TCF3-PBX1*.

Staining in control tissues was evaluated throughout the validation process of the antibodies. BCL6 and muHC were expressed in the GCs of lymphoid follicles in the appendix control. ZAP70 expression was seen in T-cells in the tonsil sample. The malignant epithelial cells in an invasive ductal breast carcinoma sample showed pSTAT5 expression.

5.1.3 High *BCL6* mRNA associates with favorable survival

Previously, high *BCL6* mRNA has been associated with a poor outcome in a high-risk cohort of B-ALL patients (Ge et al., 2017; Hurtz et al., 2019; Swaminathan et al., 2013). Here, the association of BCL6 expression with treatment outcome was explored in two datasets. The first cohort, which included 115 patients and had RNA-seq data on *BCL6* mRNA, showed better EFS in cases with a higher than the

median level of *BCL6* (log-rank test p-value = 0.03). In a multivariate Cox regression model, high *BCL6* mRNA showed lower hazard of relapse (HR 0.47; 95% CI: 0.21–1.04; p-value 0.06). In the IHC dataset, positivity for BCL6 protein did not show a statistically significant association with outcome in EFS or OS.

5.2 Prognostic value of SOX11 in children with B-ALL (II)

5.2.1 SOX11 associates with distinct subtypes of pediatric B-ALL

SOX11 is a well-established marker of MCL (Beekman et al., 2018). We assessed its expression in other hematological malignancies with a focus on pediatric B-ALL. By IHC, SOX11 expression was strong in 5 cases (4%), moderately positive in 33 cases (28%) and negative in 79 cases (68%). SOX11 expression was evident in the nuclei of the leukemic blasts with varying intensity. The background stromal tissue, hematopoietic cells, bone remodeling cells, and adipocytes were negative. A total of 26 out of 33 (79%) *ETV6-RUNX1* cases were positive, and 3 out of 4 (75%) *TCF3-PBX1* cases were positive. One SOX11-positive hypodiploid case was included in the cohort, and 1 out of 30 (3%) HeH cases was positive. In the "Other" subtype, 7 out of 42 (17%) cases were SOX11 positive. MCL control was positive in these analyses. B-cells or T-cells in the remission BM trephine biopsies did not show SOX11 expression. CD5 expression in FC was not associated with SOX11 expression. SOX11 IHC is shown in Figure 7.

In the Hemap dataset, AML and T-ALL showed minimal expression of *SOX11*. Overall, B-ALL cases displayed low levels of expression, while MCL and *ETV6-RUNX1* and *TCF3-PBX1* subtypes of B-ALL showed high levels of expression. Likewise, gene expression data from the PanALL study confirmed high expression of *SOX11* in the *ETV6-RUNX1* and *TCF3-PBX1* subtypes. The new subtypes *MEF2D*, *IKZF1*, *ETV6-RUNX1*-like, and *DUX4* showed high expression levels. The high expression levels of *SOX11* in *TCF3-PBX1* and *ETV6-RUNX1* subtypes were also observed in the Nordic RNA-seq cohort (Marincevic-Zuniga et al., 2017; Nordlund et al., 2013).

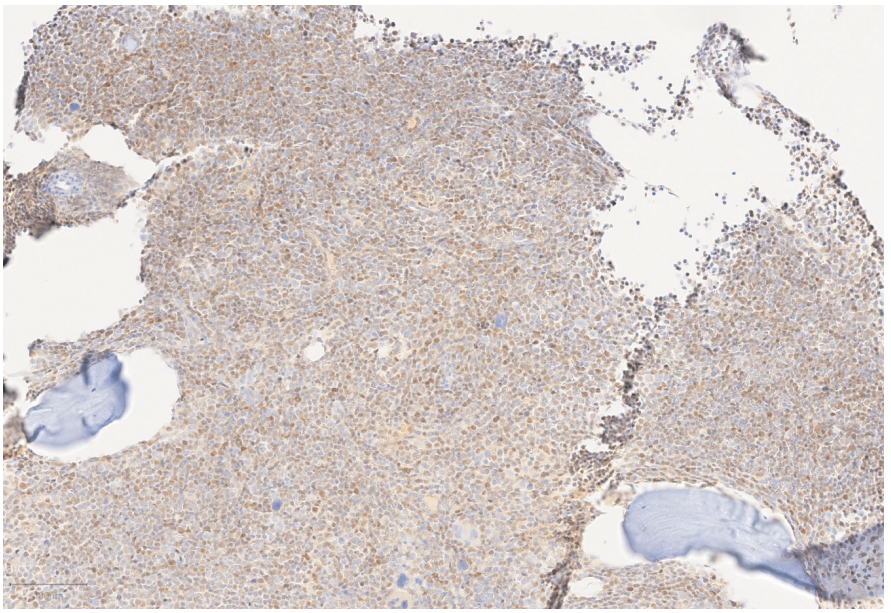

Figure 7. Light microscopic image of SOX11 immunostaining in a SOX11-positive B-ALL sample.

5.2.2 Hypomethylation of *SOX11* is accompanied with elevated expression

To better understand the biology behind altered expression of SOX11, we explored WGS or GRO-seq data of eight ALL cases but found no somatic mutations or aberrant enhancer activities that could explain the change in expression. Therefore, genome-wide CpG hypomethylation data were explored for the presence of an altered methylation pattern that could explain the modified expression of SOX11. Indeed, hypomethylation of *SOX11* was evident in the *SOX11* gene locus in the 23 identified CpG loci including the promoter region. When KOPN-8 or REH cells were treated with decitabine, an inhibitor of DNA methyltransferase, an increase in *SOX11* expression was seen in a concentration-dependent manner, indicating that methylation levels are associated with SOX11 expression.

5.2.3 Knockdown of SOX11 does not reduce cell viability or proliferation

Expression of SOX11 was silenced by siRNA in REH, RCH-ACV, and 697 cell lines, which exhibit robust expression of endogenous SOX11. Knockdown did not affect

cell viability or proliferation and did not lead to changes in response to corticosteroid or chemotherapy treatments. RNA-seq of wild-type and knockdown cell lines showed alterations in the expression of genes associated with cell migration, adhesion, and differentiation.

5.2.4 High expression of SOX11 associates with better survival

Association of SOX11 with clinical parameters and patient outcome has not been previously assessed in pediatric B-ALL. No association was found between SOX11 expression and WBC count or the presence of central nervous disease at diagnosis. No deaths occurred among patients with positive immunohistochemical staining for SOX11, leading to better OS (log-rank p-value = 0.039). EFS similarly showed a trend to favorable outcomes. In a Cox multivariate regression model with age, WBC count, cytogenetic subtype, and MRD at the EOI as covariates, a trend to lower hazard for survival was observed (HR 0.37; 95% CI: 0.10–1.43). Among the *ETV6-RUNX1* subtype, a protective survival effect was observed (log-rank p-value = 0.004). A favorable prognostic association was supported by data in the Nordic cohort of pediatric B-ALL, which showed a trend to lower hazard for events in cases with a higher-than-median expression of *SOX11*. OS between SOX11-positive and -negative cases is shown in Figure 8.

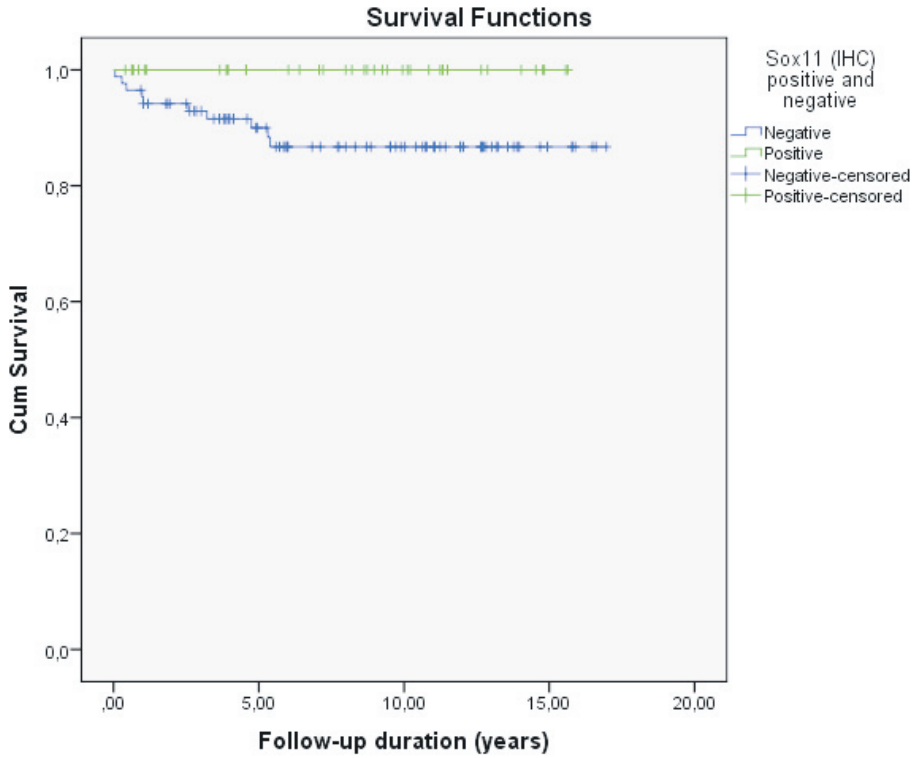

Figure 8. Overall survival among SOX11-positive and -negative cases. Log-rank p-value = 0.039. IHC: immunohistochemistry.

5.3 IGF2BP3 links to proliferation and outcome in pediatric B-ALL (III)

5.3.1 Wide-spread expression of IGF2BP3 in B-ALL

The elevated expression of IGF2BP3 has been associated with several malignancies (Mancarella & Scotlandi, 2020). IGF2BP3 IHC in the population-based TMA cohort of 83 cases showed protein expression in a cytoplasmic granular pattern in 74 cases (89%). Positive cell percentage in leukemic blasts ranged from 1 to 100%. The highest median expression levels were found in *ETV6-RUNX1*, and *KMT2A*-re,

and "Other" subtypes. The appendix control tissue for the IHC showed positive expression in GCs of lymphoid follicles.

In the Hemap dataset, Burkitt leukemia, DLBCL, FL, juvenile myelomonocytic leukemia, and *KMT2A*-re, *ETV6-RUNX1,* and HeH among B-ALL showed the highest expression of *IGF2BP3*. PanALL dataset validated findings in *ETV6-RUNX1* and *KMT2A*-re subtypes, showing strong expression in new B-ALL subtypes CRLF2, *ETV6-RUNX1*-like, iAMP21, *KMT2A*-like, and *NUTM1*. The results of IGF2BP3 IHC are shown in Table 5.

Table 5. Results of immunohistochemistry stainings and positive cases in the TMA cohort (III). IHC: immunohistochemistry; KMT2A-re, KMT2A-rearranged; n: number; WTS: whole-tissue section; TMA: tissue microarray.

	IGF2BP3	**Total** **n in subtype**
ETV6-RUNX1	17	20
Hyperdiploid	10	22
***KMT2A*-re**	4	4
BCR-ABL1	0	1
TCF3-PBX1	1	3
Hypodiploid	1	1
Other	21	32
Total n of IHC positive cases	54	83

5.3.2 IGF2BP3 associates with proliferation activity

IGF2BP3 is associated with the active proliferative phenotype in several malignancies (Mancarella & Scotlandi, 2020). To this end, a proliferative "metagene" signature (Giuliano, Lin, Smith, Palladino, & Sheltzer, 2018) was applied in the Hemap and PanALL datasets. Across all hematological malignancies, a high level of proliferation "metagene" was associated with higher-than-median expression of *IGF2BP3* (Mann–Whitney U test, p-value < 0.001). Likewise, a positive association was observed among B-ALL cases in the Hemap and PanALL datasets.

At the protein level, co-staining of CD19 and Ki-67 demonstrated higher expression of Ki-67, a marker for proliferation, among B-ALL cases with a higher-than-median percentage of IGF2BP3 positive blasts.

5.3.3 Improved outcome of high-risk B-ALL patients with elevated levels of *IGF2BP3*

In the TARGET cohort of 155 pediatric high-risk B-ALL patients, higher-than-median expression of *IGF2BP3* was associated with statistically significantly better EFS and OS (log-rank p-value = 0.0001). We composed a Cox regression model of proportional hazards to explore the association of various covariates with survival. The covariates of age and MRD at the EOI were fitted for the EFS model and, in addition, WBC count was fitted into the OS model. In these models, higher-than-median *IGF2BP3* showed a protective effect for events and death (HR 0.49; 95% CI: 0.34–0.71; p-value < 0.001 and HR 0.44; 95% CI: 0.28–0.68; p-value < 0.001, respectively). Finally, no statistically significant association between IGF2BP3 protein expression and survival or clinicopathological effects was found.

6 DISCUSSION

6.1 The discovery and validation of novel biomarkers for B-ALL

B-ALL is often driven by genetic or epigenetic alterations in TFs or other regulators of gene expression. However, a detailed understanding of dysregulation of transcription is still largely lacking. With this in mind, we decided to explore the gene expression landscape of B-ALL in the context of all hematological malignancies, leukemia cell lines, and healthy samples. This was made possible by the recently developed gene expression data resource called Hemap (Mehtonen et al., 2019; Pölönen et al., 2019), which includes gene expression data on 6,832 different patient samples from 36 hematological disease entities. This resource is based on gene expression data that has been generated by using a uniform microarray platform (Affymetrix HG U133 Plus 2.0) and includes manually curated clinical data. Hemap dataset consists of 1,689 lymphoblastic leukemias (1,304 B-ALL and 385 T-ALL), with approximately half of the B-ALL cases from children (662 children and 642 adults). This dataset was used as a starting point for this thesis project when searching for novel entity-specific and aberrantly expressed transcriptional regulators. Other gene expression datasets (PanALL and Nordic dataset) were used to validate the findings. Moreover, we used a WTS IHC cohort and developed an in-house TMA on pediatric ALL cases to explore the expression of the gene expression findings at the protein level. Overall, these studies provide holistic insights into the expression of the new biomarkers in B-ALL.

Three relevant biomarkers were discovered: BCL6, SOX11, and IGF2BP3. SOX11 is not expressed in normal lymphoid tissues, while BCL6 is transiently expressed early in B-cell development and later in maturing B-cells in GCs. Likewise, IGF2BP3 is also expressed in GCs (Ek et al., 2008; King et al., 2009; Swaminathan et al., 2013). These proteins act already as diagnostic biomarkers in MCL (SOX11), FL (BCL6), and CHL (IGF2BP3) (Cardenas et al., 2017; Dogan, Bagdi, Munson, & Isaacson, 2000; Lederer, Bley, Schleifer, & Hüttelmaier, 2014; W. Lee, Shin, Kim, & Kim, 2019; Tsang et al., 2020). The expression of these molecules has been identified previously in B-ALL and its different subtypes by using either high-risk mRNA cohorts or relatively small patient cohorts with protein data (Geng et al., 2015;

Palanichamy et al., 2016; Vegliante et al., 2011). We, therefore, explored their expression and role as potential biomarkers, particularly in pediatric B-ALL, by using both gene and protein expression datasets coupled with detailed and curated information on clinical parameters.

6.2 Association of BCL6 expression with pre-BCR signaling and clinical outcome

In line with previous studies, BCL6 was expressed in hematological malignancies of GC origin, some T cell malignancies, and myeloid lineage malignancies (Cardenas et al., 2017). In B-ALL, BCL6 expression was associated with the *TCF3-PBX1*, *BCR-ABL1*, and *MEF2D* subtypes, which was in line with the earlier literature (Deucher et al., 2015; Ge et al., 2017; Tsuzuki et al., 2020). In addition, BCL6 expression was found expressed in several new subtypes, such as *NUTM1* and *PAX5*alt. Association of high BCL6 expression with *KMT2A*-re was recently reported, but, in our data, BCL6 expression was low in this subgroup (Hurtz et al., 2019).

Geng et al. (2015) suggested BCL6 as a biomarker for a novel pre-BCR+ ALL subtype. We found a similar, more mature pre-BCR-associated phenotype for the BCL6 expressing B-ALL cases. Still, we could not confirm the reported clear-cut dichotomy between the pre-BCR negative (pSTAT5+) and pre-BCR+/BCL6 expressing cases. As mRNA levels of pre-BCR components are not direct evidence of the pre-BCR activity, we interrogated the protein samples for the activity of IL7R (pSTAT5) and pre-BCR signaling (ZAP70). This analysis showed that components of IL7R and pre-BCR signaling pathways were partially overlapping. This suggests that BCL6 alone seems to lack the required specificity to act as a diagnostic or predictive biomarker for pre-BCR+ ALL subtype and its targeted treatments. Similar findings have been observed by others in high-risk B-ALL patients, and, recently, BCL6 expression has been reported in several other subtypes, which are not considered as pre-BCR expressors (Chen et al., 2016; Ge et al., 2017; Hurtz et al., 2019). In the differential diagnosis of B-cell malignancies, BCL6 expression in a subset of cases must be taken into consideration, that is, the lymphoblastic transformation of follicular lymphoma (Slot et al., 2016). In addition to immunophenotyping, these cases also require genetic confirmation.

High *BCL6* mRNA expression has been previously associated with poor outcomes in high-risk patient groups of ALL and pre-BCR+ ALL cases (Ge et al., 2017; Geng et al., 2015; Hurtz et al., 2019; Swaminathan et al., 2013). As a novel

finding, we observed that high *BCL6* mRNA expression was associated with favorable patient outcomes in a cohort of pediatric B-ALL patients from all risk groups. This is in agreement with the findings by Chen et al. (2016), who reported that high expression of the components of the pre-BCR complex was associated with better prognosis in high-risk B-ALL patients. (Geng et al., 2015; Tsuzuki et al., 2020).

6.3 SOX11 expression, methylation status and favorable prognosis

As expected, SOX11 was strongly expressed in the MCL cases. Among B-ALL cases, expression of SOX11 focused on the *ETV6-RUNX1* and *TCF3-PBX1* subtypes of B-ALL, supporting previous literature (Busche et al., 2013; Dictor et al., 2009; Nordlund et al., 2012; Vegliante et al., 2011, 2013). As new findings, strong *SOX11* mRNA expression levels were detected in novel subtypes, such as *ETV6-RUNX1*-like and *DUX4*. Interestingly, SOX11 expression was strong in two B-ALL subtypes with slightly different developmental stages and immunophenotypes (*ETV6-RUNX1* and *TCF3-PBX1*). This could indicate different biology behind the expression variability or that the role of SOX11 in leukemogenesis is secondary. In MCL, PAX5 upregulates the expression of *SOX11* directly. PAX can also activate a super-enhancer, which promotes high *SOX11* expression through hypomethylation (Vilarrasa-Blasi et al., 2021). We were also the first to explore the expression of SOX11 at the protein level by using a population-based pediatric sample cohort. This verified that a significant proportion of pediatric B-ALL expresses SOX11.

We also investigated the biology behind the high expression of SOX11 and found that the methylation level of the *SOX11* gene, including the promoter region, affected its expression. Hypomethylation of *SOX11* was associated with higher *SOX11* expression. Accordingly, *SOX11* expression could be increased with a demethylating agent, decitabine, in KOPN-8 cells that had low basal expression level of *SOX11*.

The role of SOX11 in cell proliferation has remained unclear (Beekman et al., 2018). We did not observe marked effects on cell viability or proliferation by KD of *SOX11*. RNA-seq analysis comparing the wild type and SOX11 KD cells showed changes in genes involved in cell adhesion, migration, and differentiation. The possible role of SOX11 as an oncogene or as a tumor suppressor cannot be deciphered from our findings. An oncogenic role in MCL has been suggested

(Beekman et al., 2018), but might be context-dependent, as SOX11 has been described as a tumor suppressor as well in several malignancies (Tsang et al., 2020).

Several disease entities such as MCL and Burkitt's lymphoma can exhibit a blastoid morphology with the expression of SOX11 (Zeng et al., 2012). However, these diseases are rare in pediatric patients. Still, cyclin-D1 negative blastoid MCL cases especially present as a differential diagnostic challenge with B-ALL in adult patients with bulky disease, and the mutual SOX11 expression must be taken into account (Dictor et al., 2009; M. T. Ye et al., 2021). To compare the similarity between immunophenotypes of MCL and SOX11 positive B-ALL, we successfully tested CD5 expression by FC as a marker for SOX11 positivity. This showed no association between these markers.

TFs are considered challenging drug targets. In MCL cell lines, SOX11 inhibitors, which perturb DNA interactions of SOX11, have shown promising results (Jatiani et al., 2021). As SOX11 was positive in a substantial proportion of B-ALL cases, the effects of SOX11 inhibitors could be further explored in B-ALL.

Investigations with SOX11 as a prognostic biomarker in B-ALL have been lacking. In our studies, we found high SOX11 expression at mRNA and protein level associated with favorable patient outcomes, which could be utilized in risk-stratification of B-ALL patients. It would be essential to explore further the clinicopathological effects of SOX11 in prospective studies, which include novel subtypes. For example, the *ETV6-RUNX1*-like subtype, which showed high *SOX11* mRNA expression, has not yet been established with a clear prognostic role (Jeha et al., 2021; Lilljebjörn et al., 2016).

6.4 IGF2BP3 expression is associated with active cellular proliferation and survival in high-risk B-ALL

IGF2BP3 is expressed in multiple malignancies and, to a lesser extent, in normal adult tissues (Mancarella & Scotlandi, 2020). Expression of IGF2BP3 in hematological malignancies has been associated with B-cell lymphomas of the GC origin—a finding validated in our study. Also, in normal tissues, IGF2BP3 expression has been observed in GCs of lymphoid follicles.

Previous investigations on IGF2BP3 expression in B-ALL have shown high gene expression in the *ETV6-RUNX1* and *KMT2A*-re subtypes (Palanichamy et al., 2016; Stoskus et al., 2011). In addition to these two well-established subtypes, we observed that HeH cases and several novel subtypes showed high expression of *IGF2BP3*. As

fusion protein in the *KMT2A*-re subtype directly upregulates *IGF2BP3* mRNA expression, and as IGF2BP3 drives expression of the targets of this fusion protein, the leukemogenesis is driven in a feed-forward loop (Tran et al., 2021). Whether similar mechanisms are functional also in other leukemia subtypes remains to be researched further.

IGF2BP3 expression has been associated with cell proliferation in multiple cell types and different neoplasms (Mancarella & Scotlandi, 2020). High enforced *IGF2BP3* expression was associated with the increased proliferation of hematopoietic stem and progenitor cells in murine models (Palanichamy et al., 2016). We found an association between increased cell proliferation in B-ALL cases at both gene and protein level in patient samples. This is also seen in solid tumors and lymphomas (Guenther et al., 2008; Mancarella & Scotlandi, 2020; Palanichamy et al., 2016). The proliferative phenotype associated with IGF2BP3 expression fits well with the expression in GCs, which show high proliferation. One cellular mechanism leading to more increased proliferation is stabilizing of *MYC* and *CDK6* oncogenes by IGF2BP3 (Palanichamy et al., 2016). We found indirect and partial support for this at the gene expression level by showing that high *CDK6* expression was associated with high *IGF2BP3* expression. However, as IGF2BP3 binds hundreds of different RNA particles and other RBPs bind these transcripts, there are likely multiple mechanisms by which IGF2BP3 might promote proliferation and leukemogenesis.

We found common expression of IGF2BP3 in different B-ALL subtypes. Thus, the utility of IGF2BP3 as a diagnostic tool in this disease group or the differential diagnosis with other blastoid diseases is questionable. Several disease entities such as MCL can exhibit a blastoid morphology with the expression of IGF2BP3 (Hartmann et al., 2012).

Interestingly, the application of bromodomain and extraterminal domain inhibitors (BETi) has led to decreased IGF2BP3 expression, change in expression of its target genes, and inhibited tumor cell proliferation in ALL and Ewing sarcoma cell lines, which suggests that IGF2BP3 could be utilized as a predictive biomarker for targeted treatments (Mancarella et al., 2018; Palanichamy et al., 2016).

Investigations of the clinicopathological effects of IGF2BP3 expression in B-ALL have been lacking. In our studies, we found that high *IGF2BP3* mRNA expression was associated with favorable patient outcomes in high-risk patients. When considering the classical prognostic markers, response to treatment is a well-recognized prognostic factor in B-ALL (Berry et al., 2017). In our studies, we could find evidence that *IGF2BP3* mRNA expression is independent of MRD as a

prognostic marker, which might improve prognostication. The mechanism behind this favorable prognosis is unclear. The association with high proliferation might explain better treatment response. B-ALL cases with high proliferative features are more sensitive to induction treatment (Mehtonen et al., 2020). As we observed that *IGF2BP3* expression was evident in several novel B-ALL subtypes, such as *NUTM1* and *ETV6-RUNX1*-like, the prognostic role of this *IGF2BP3* could be further evaluated with prospective studies, including current diagnostic and treatment protocols with information on novel subtypes (J. M. Boer et al., 2021; Jeha et al., 2021).

6.5 Strengths and limitations of the studies

Previously, the expression of these biomarkers in B-ALL has been investigated by IHC in datasets including pediatric and adult cases with cohort sizes from 52 to 72 cases in studies on BCL6 and smaller samples with SOX11 and IGF2BP3 (Deucher et al., 2015; Dictor et al., 2009; Geng et al., 2015; Hurtz et al., 2019; King et al., 2009). Our IHC cohort was population-based and included a more significant number of pediatric B-ALL cases. The distribution of these cases was in line with the known proportions of subtypes in the WHO classification and covered most of the common subtypes(Swerdlow et al., 2017). Information of the novel subtypes such as *BCR-ABL1*-like was lacking, and these cases were included in the "Others" subtype. We excluded T-ALL and Burkitt leukemia cases from most of the analyses as there were only a very few cases from these disease groups. Protein data was analyzed together with large mRNA expression datasets of B-ALL cases, which also gave information on novel subtypes, such as *MEF2D* and *ETV6-RUNX1*-like.

We were able to correlate the expression findings with patient survival as two of our datasets had clinical information available. However, as they were retrospectively assessed, the validation with current treatment protocols should be done in the future. Variation between results in datasets was observed, and this may be explained by cohort sizes and proportions of different subtypes and risk groups. In IHC analyses, there were many missed cases due to technical reasons, which reduces the statistical strength of survival analysis with IGF2BP3 protein expression data. This could be overcome by using multiple replicates in the future, but limited sample material may become a problem (Limberger et al., 2017; Tzankov, Went, Zimpfer, & Dirnhofer, 2005).

As biomarkers should be objectively measurable, we used similar cut-offs with previous investigations for protein and mRNA levels in order to make our results more comparable (Geng et al., 2015). The IHC analysis was performed with tested antibodies, which are used in routine diagnostics, with automated platforms, and according to manufacturer's protocols. IHC requires scrutiny and supervision, and the validation of the used antibodies was performed with proper control materials and testing of different reagent and antibody dilutions for optimal performance.

One of the strengths of the IHC analysis is the topographical orientation of the biomarker expression when compared with clot samples (protein) or bulk expression (mRNA). From the IHC sections, it is possible to differentiate nonspecific background staining, which was evident, for example, in myeloid cells with BCL6 expression. Despite this, the effects of tissue degradation and processing, for example, decalcification on BM samples, could have affected our staining results (Fitzgibbons et al., 2014; Miquelestorena-Standley et al., 2020). Previous studies with BCL6 used mostly clot biopsies, which do not require decalcification (Deucher et al., 2015; Geng et al., 2015; Hurtz et al., 2019). Nevertheless, our cohort showed similar proportions of positive staining.

Antibody specificity is always a major concern. IGF2BP3 antibodies have cross-reactivity to IGF2BP paralogs and could complicate interpretation. However, a more specific antibody is not yet available (Lederer et al., 2014; Tschirdewahn et al., 2019). The antibody used to detect SOX11 has been described as specific (W. Lee et al., 2019; Nakashima et al., 2014). Antibodies against phosphoproteins are often more challenging to use as they are more prone to give varying results depending on sample handling and fixation protocols (Baker et al., 2005). The BM samples are usually quickly fixated, which might aid with this problem. We used pSTAT5 as a marker of active IL7R signaling. Phosphorylated ZAP70 antibody would better reflect the active pre-BCR signaling than the non-phosphorylated antibody. When studying the different signaling pathways in the future, multiple active molecules from pre-BCR and IL7R pathways should be interrogated. This may be challenging as the available material is scant and would require more prudent methods such as multiplexing or other proteomics methods.

The analysis of IHC was performed by conventional and digital methods. In conventional analysis, semiquantitative grading of protein expression is somewhat subjective, and inter- and intra-observer variability is a known challenge (Croci et al., 2020; Polley et al., 2013). To overcome this weakness, two pathologists analyzed the samples independently, and discrepant cases were settled by a third pathologist. As the conventional analysis of IHC gave graded semiquantitative results, digitized

images and image analysis software were used for additional accuracy and continuous values (Bankhead et al., 2017).

7 SUMMARY AND CONCLUSIONS

In summary, we investigated the expression and clinicopathological effects of two transcription factors and one post-transcriptional regulator in acute leukemia by utilizing microarray, RNA-sequencing, and immunohistochemistry cohorts and associated clinical findings.

Our primary findings are as follows:

1. BCL6 is associated with the *TCF3-PBX1* subtype of B-ALL and is expressed in novel subtypes such as *MEF2D*. High *BCL6* mRNA expression was associated with improved survival.

2. SOX11 is expressed in *ETV6-RUNX1* and *TCF3-PBX1* subtypes of B-ALL, and high SOX11 expression was associated with a favorable outcome.

3. IGF2BP3 expression is common in B-ALL, and elevated expression levels were associated with high proliferation activity. High *IGF2BP3* mRNA expression was associated with favorable survival in high-risk B-ALL.

In the future, large prospective cohorts are needed to evaluate the prognostic role of these biomarkers with current treatment protocols.

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PUBLICATIONS

PUBLICATION I

Expression of BCL6 in paediatric B-cell acute lymphoblastic leukaemia and association with prognosis

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HAEMATOLOGY

Expression of BCL6 in paediatric B-cell acute lymphoblastic leukaemia and association with prognosis

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Summary

B-cell lineage acute lymphoblastic leukaemia (B-ALL) is the most common paediatric malignancy. Transcription factor B-cell lymphoma 6 (BCL6) is essential to germinal centre formation and antibody affinity maturation and plays a major role in mature B-cell malignancies. More recently, it was shown to act as a critical downstream regulator in pre-BCR+ B-ALL. We investigated the expression of the BCL6 protein in a population-based cohort of paediatric B-ALL cases and detected moderate to strong positivity through immunohistochemistry in 7% of cases (8/117); however, only two of eight BCL6 cases (25%) co-expressed the ZAP70 protein. In light of these data, the subtype with active pre-BCR signalling constitutes a rare entity in paediatric B-ALL. In three independent larger cohorts with gene expression data, high *BCL6* mRNA levels were associated with the *TCF3-PBX1*, Ph-like, *NUTM1*, *MEF2D* and *PAX5*-alt subgroups and the 'metagene' signature for pre-BCR-associated genes. However, higher-than-median *BCL6* mRNA level alone was associated with favourable event free survival in the Nordic paediatric cohort, indicating that using BCL6 as a diagnostic marker requires careful design, and evaluation of protein level is needed alongside the genetic or transcriptomic data.

Key words: Acute lymphoblastic leukaemia; B-cell lineage; BCL6; immunohistochemistry; mRNA expression; paediatric.

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INTRODUCTION

Acute lymphoblastic leukaemia (ALL) is the most common malignancy in childhood. Although the long term survival has improved to 80–90%, several smaller subgroups have inferior outcomes.[1,2] Therapy response at the end-of-induction (EOI) measured by minimal residual disease (MRD) is the most important prognostic factor.[1–3] However, two major problems remain: first, relapses are still the major cause of mortality, and second, acute and long term side effects are experienced by many survivors due to toxicity in healthy tissues caused by chemotherapy.[1] Therefore, there is a need for new predictive biomarkers to better identify patients who either could be cured with less intensive treatments or could benefit from the novel targeted agents.[1,2]

B-cell lymphoma 6 (BCL6) is a zinc finger containing transcriptional repressor that is essential for germinal centre formation and plays an important role in the somatic hypermutation of immunoglobulin genes.[4–7] In diffuse large B-cell lymphoma, the BCL6 protein is a central oncoprotein.[8] BCL6 is highly expressed and is a potential drug target in follicular and Burkitt lymphoma.[8] In 2015, Geng *et al.* reported a new subgroup of ALL (pre-BCR+ ALL) with active pre-B-cell receptor (pre-BCR)-signalling marked by high expression of BCL6, and the authors suggested that it constitutes approximately 10–15% of paediatric and adult ALL cases.[9] Similar proportions of BCL6 expression have been reported by others.[10] The subgroup has been associated with the *TCF3-PBX1* subtype, and a CD34-negative immunophenotype.[9–11] Interestingly, pre-BCR+ leukaemia cells were found to be sensitive to tyrosine kinase inhibitors targeting the SYK, SRC, PIK3delta and BTK kinases,[9,12] which highlights the potential clinical utility of the novel subtype. Recently, Hurtz *et al.*[13] discovered that BCL6 could serve as a therapeutic target in *KMT2A*-rearranged leukaemia (*KMT2A*), while Tsuzuki *et al.*[14] identified BCL6-positive cases associated with the *MEF2D*-translocated ALL (*MEF2D*) and identified the SREBF1 protein in the regulatory network, thus serving as an additional potential drug target in this BCL6-positive entity. Finally, high *BCL6* mRNA has been associated with mutations of *PAX5* and *IKZF1* and inferior clinical outcomes in high-risk ALL.[13,15,16]

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The prevalence and clinical significance of BCL6 expression in childhood ALL is unclear, as studies with solely paediatric cases have not been conducted. Here, we studied a population based cohort of paediatric patients with bone marrow (BM) trephine biopsy samples, and utilised three distinct gene expression cohorts (the PanALL study, a Nordic cohort and a collection of studies in the Hemap resource) to investigate the expression of the BCL6 protein and mRNA in B-cell acute lymphoblastic leukaemia (B-ALL). Moreover, we explored the clinicopathological features and patient survival associated with BCL6 expression.

MATERIALS AND METHODS

Bone marrow biopsies and associated clinical data

We collected a retrospective patient cohort that included 117 paediatric precursor B-ALL patients who were treated at Tampere University Hospital between 1 January 2000 and 16 October 2017. Only cases under 18 years of age at diagnosis were included. Acute myeloid leukaemia, Burkitt leukaemia, T-cell lymphoblastic leukaemia, and all cases with either inadequate or plastic-embedded biopsy samples were excluded from the cohort. The primary diagnosis was based on the BM aspirate morphology, immunophenotyping and various cytogenetic and molecular genetic investigations. Patients were treated using successive Nordic Society for Pediatric Hematology and Oncology (NOPHO) ALL protocols. Associated clinical data included the following parameters: age, white blood cell count (WBC), blast count, immunophenotype by flow cytometry, cytogenetics and genetics information, involvement of the central nervous system at diagnosis, MRD at EOI, relapse data, secondary malignancies and death during the follow-up period. Subgroup allocation was performed according to the World Health Organization (WHO) 2017 classification criteria.[17] For cases lacking the genetic subtype information, fluorescence *in situ* hybridisation analysis was performed on either BM aspiration samples or formalin fixed and paraffin embedded (FFPE) samples.[18]

Immunohistochemical analysis of bone marrow biopsies

Decalcified (ethylenediaminetetraacetic acid) and FFPE histological BM trephine biopsy samples were collected from the pathology archives from the time of the primary diagnosis. Whole tissue sections 4 μm thick were stained with the primary antibodies against BCL6 (clone LN22, PA0204, mouse monoclonal, lot number 48794, dilution 1:50; Leica Biosystems, UK), pSTAT5-Y694 (clone E208, ab32364, lot number GR208043, dilution 1:50; Abcam, UK), ZAP70 (clone YE291, ab32429, rabbit monoclonal, lot number GR59787, dilution 1:100; Abcam) and muHC (clone A0425, rabbit polyclonal, lot number 00061133, dilution 1:5500; Dako, Denmark). Immunohistochemical staining was performed using the Ventana Benchmark Classic instrument (Ventana, USA). The Ultraview Universal DAB Detection kit (Ventana) and haematoxylin counterstain were used for antibody and cell detection, respectively. Appendix, tonsil, invasive ductal breast carcinoma and Burkitt lymphoma/leukaemia were used as control material. The immunohistochemically stained tissue sections were analysed independently by two pathologists using a light microscope. Positive staining in over 50% of the blast cell nuclei or cytoplasm were graded as strongly positive, 20–50% as moderately positive, and less than 20% as negative. Discrepant cases were resolved by a third pathologist.

Gene expression datasets

Three independent RNA expression datasets were used to analyse *BCL6* gene expression as previously described:[18] (1) the Hemap dataset, which is a microarray dataset of 36 haematological malignancies that includes 6832 samples, including 662 paediatric and 642 adult B-ALL cases collected from different original studies that represent both high risk cohorts and those that include also common good prognosis subtypes;[19,20] (2) the PanALL study dataset, an RNA-sequencing dataset (n=1988), which includes 1234 paediatric and 754 adult B-ALL cases from different patient cohorts and therapy risk groups;[21] (3) a Nordic dataset, which consists of RNA-sequencing data from 115 paediatric B-ALL cases that represent cases diagnosed between the years 1996 and 2010 from different therapy risk groups.[22] The pre-BCR 'metagene' signature was studied in the Hemap and PanALL datasets, and included *IGLL1, IGLL3, VPREB1, VPREB3, IGHM, SYK* and *ZAP70* genes, as described by Geng *et al.*[9]

Normalisation of the mRNA expression values has been described previously for the Hemap dataset[19,20] and the PanALL dataset.[21] Briefly, 'normalised expression values' in the Hemap microarray data[19,20] were normalised with RMA (robust multi-array analysis) probe summarisation algorithm and corrected for technical bias before log2-transformation. In the PanALL dataset,[21] 'normalised expression values' were corrected for batch effect.

Ethical considerations

The study was approved by the local ethical committee (Pirkanmaa Hospital District Ethical Committee, R16054 and R13109) and the National Supervisory Authority for Welfare and Health (Valvira, Dnro:4243/06.01.03.01/2016).

Survival and statistical analysis

Kaplan–Meier survival analysis was used to associate the biological findings with overall survival (OS) and event-free survival (EFS). The events that were factored into the EFS variable were death, relapse, resistant disease (MRD>25% at EOI) and secondary malignancy. Cox regression models were used to estimate hazard ratios (HRs) for EFS using the *BCL6* mRNA status (median expression as a cut-off), age, WBC, treatment group and cytogenetic subgroup as variables. Proportionality assumptions of the different time dependent variables were tested. The pre-BCR 'metagene' was formed as the arithmetic mean of the pre-BCR associated genes.

Statistical analysis was performed using SPSS Statistics (version 26; IBM, USA) and RStudio (version 3.6.1; RStudio, USA). The Mann–Whitney U and Kruskal–Wallis H tests were used to analyse continuous non-parametric variables, while the chi-squared test was applied on categorical variables and Spearman's rank order test for correlation. p values less than 0.05 were considered statistically significant, and all statistical tests were two-sided.

RESULTS

Clinicopathological features associated with BCL6 immunostaining

BCL6 is a transcriptional repressor that plays a major role in mature B-cell lymphoma.[8] Recently, BCL6 has been shown to contribute significantly to the pathogenesis of acute precursor B-cell acute leukaemia.[9,23] We investigated the expression of the BCL6 protein in childhood B-ALL by collecting trephine biopsy samples from 117 paediatric B-ALL cases. The cohort was population-based, and the main B-ALL subtypes were represented at expected proportions (Table 1).[17] During the follow-up period [median 8.3 years, interquartile range (IQR) 7.8 years], 15 relapses, nine deaths and two secondary malignancies were registered. Four patients had resistant disease with a blast count of over 25% at end induction.

Immunohistochemical staining of diagnostic biopsy samples was performed using an antibody against the BCL6 protein. As shown in Fig. 1, the BCL6 protein was expressed with varying intensity in the nuclei of 20–50% of lymphoblasts. Overall, BCL6-positive cells showed an evenly distributed staining pattern in the leukaemic BM microenvironment so that an approximately equal number of BCL6-positive cells were in close proximity of vasculature, bone trabeculae and other stromal cells (Fig. 1). Myeloid background staining was predominantly evident in the granulocytes.

We categorised BCL6 expression based on the staining intensity as either negative or positive (moderate or strong), and the cases were classified independently by two pathologists. With this categorisation, eight of the 117 (6.8%)

Table 1 Case summary for the patient cohort analysed by immunostaining

Age, years, median (range)	4.2 (0.9–17.6)
WBC × 10^9/L, median (range)	7.1 (1–311)
MRD EOI (%), median (range)	0.02 (0–44)
Deceased, *n* (%)	9 (7.7)
Relapsed, *n* (%)	15 (12.8)
CNS disease, *n* (%)	6 (5.1)
SMN, *n* (%)	2 (1.8)
WHO subgroup	
ETV6-RUNX1, *n* (%)	33 (28.2)
HeH, *n* (%)	30 (25.6)
KMT2A, *n* (%)	5 (4.3)
Ph, *n* (%)	2 (1.7)
TCF3-PBX1, *n* (%)	4 (3.4)
Hypodiploid, *n* (%)	1 (0.9)
Other, *n* (%)	42 (35.9)
Total, *n*	117

Age, age at diagnosis; CNS, central nervous system; HeH, high hyperdiploid; MRD EOI, minimal residual disease at the end of induction therapy; Ph, Philadelphia chromosome (*BCR-ABL1*); SMN, secondary malignant neoplasm; WBC, white blood cell count at diagnosis.

patients were positive for the BCL6 protein (Table 2). Three of four *TCF3-PBX1* cases were positive for BCL6 (chi-squared $p<0.001$), in agreement with previous work.[10] Statistical association with surface markers further confirmed a more differentiated phenotype: four of seven BCL6-positive cases (57%) were negative for the CD34 stem/progenitor cell marker expression when assessed by flow cytometry at the time of diagnosis, while the majority of BCL6 negative cases were CD34 positive (96/101, chi-squared $p=0.001$). No other surface markers showed a statistically significant association with the BCL6 protein (data not shown).

ZAP70 protein and pSTAT5 are commonly expressed in B-ALL

Expression of BCL6 has been suggested as a biomarker for the pre-BCR+ subgroup of ALL that has a concomitant high expression of components of the pre-BCR complex and downregulation of the interleukin-7 receptor pathway with downstream STAT5 activity.[9] We performed immunohistochemical staining of the phosphorylated form of STAT5-pY694 (pSTAT5) to assess its exclusivity with the BCL6 protein in B-ALL. The pSTAT5 was seen in 108 (92%) cases with staining in both the nuclei and cytoplasm of the leukaemia blasts (Fig. 1, Table 2). Of the eight BCL6-positive cases, seven were co-expressing pSTAT5 (Table 2). Immunostaining of the heavy chain component of the immunoglobulin receptor (muHC) was hampered by strong background staining of the serum. However, no association was found between the BCL6 protein level and cytoplasmic muHC expression, as assessed by flow cytometry (data not shown).

The ZAP70 protein was expressed mostly in the cytoplasm and present in 110 of 117 (94%) B-ALL cases. Surprisingly, only two of eight BCL6-positive cases showed simultaneous immunostaining with the ZAP70 protein.

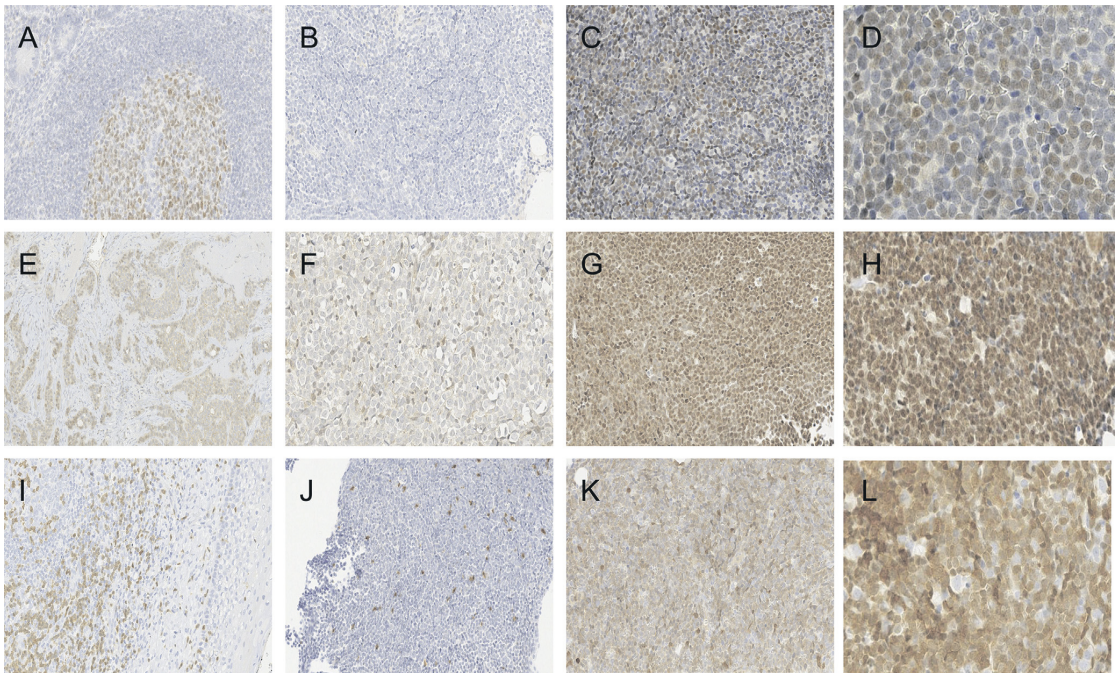

Fig. 1 Immunohistochemical staining of the diagnostic B-ALL trephine biopsy samples. (A–D) BCL6 staining in an appendix (positive control), a negative B-ALL case and two positive B-ALL cases, respectively. (E–H) Staining of the phosphorylated form of STAT5 (Y694) in an invasive ductal breast carcinoma (positive control), a negative B-ALL case and two positive B-ALL cases, respectively. (I–L) The ZAP70 protein immunostaining in the tonsil (positive control), a negative B-ALL case and two positive B-ALL cases, respectively.

Table 2 Expression of the BCL6, phospho-STAT5 (Y694) and ZAP70 proteins by immunohistochemistry in B-ALL WHO subgroups

	ETV6-RUNX1	HeH	*KMT2A*	*TCF3-PBX1*	Ph	Hypodiploid	Other	Total	%
BCL6									
Negative	33	29	5	1	1	1	39	109	
Positive	0	1	0	3	1	0	3	8	6.8
pSTAT5 (Y694)									
Negative	2	2	2	0	0	0	3	9	
Positive	31	28	3	4	2	1	39	108	92.3
ZAP70									
Negative	1	0	1	0	0	0	5	7	
Positive	32	30	4	4	2	1	37	110	94.0
Total	33	30	5	4	2	1	42	117	

HeH, high hyperdiploid; *KMT2A*, *KMT2A*-rearranged; Ph, Philadelphia chromosome (*BCR-ABL1*).

We evaluated co-expression of the mRNA for the pre-BCR genes (*IGLL1*, *IGLL3*, *VPREB1*, *VPREB3*, *IGHM*, *SYK* and *ZAP70*) and *BCL6* by discretising the gene expression to high versus low/non-expressed but did not find significant association between discrete expression groups or correlation between *BCL6* and the pre-BCR 'metagene' in the two different datasets[19–21] (Supplementary Fig. 1 and 2, Appendix A). We noted that *BCL6* mRNA did not show a clearly separable positive population in its signal distribution in any of the samples analysed. However, the pre-BCR 'metagene' expression was higher in the *TCF3-PBX1* and *MEF2D* subtypes (Mann–Whitney U-test $p<0.001$), and *BCL6* expression was higher in the cases with high pre-BCR 'metagene' expression (highest 10% quantile, Mann–Whitney U-test $p<0.001$).

Taken together, our results indicate that both on protein and mRNA level, BCL6 alone may not distinguish the patient group with concomitant pre-BCR signalling.

Expression of *BCL6* across haematological malignancies and B-ALL subtypes

BCL6 plays an essential role in B-cell maturation and has been associated with various haematological malignancies.[8] To obtain a comprehensive picture, we evaluated the expression of *BCL6* mRNA across 36 haematological disease entities, cancer cell lines and healthy cells utilising the Hemap dataset.[19,20] As shown in Fig. 2A, expression of *BCL6* mRNA was evident in B-cell malignancies, including acute leukaemia and lymphoma, and T-cell acute lymphoblastic leukaemia, while other entities, such as adult T-cell leukaemia/lymphoma, myeloid leukaemia and multiple myeloma, showed lower levels of expression. Among B-ALL, high hyperdiploid and *KMT2A* subtypes showed the weakest expression, whereas the *BCR-ABL1*, *TCF3-PBX1* and 'other' subtypes had the highest levels of expression (Kruskal–Wallis $p<0.001$) (Fig. 2B).

We retrieved two RNA-sequencing datasets that also included novel subtypes of B-ALL.[21,22] In addition to the well known subtypes (*TCF3-PBX1*, *ETV6-RUNX1*), several novel groups of ALL showed strong expression of the *BCL6* mRNA including the Ph-like, *MEF2D*, *NUTM1*-rearranged (*NUTM1*) and *PAX5*-altered (*PAX5*-alt) subgroups (Fig. 2C). Taken together, our analysis of mRNA expression captured the expected specificity of *BCL6* expression at disease and subtype levels, with the exception of MLL that showed weak expression in the microarray profiles.

High BCL6 mRNA is associated with favourable EFS in B-ALL

Previously, high expression of *BCL6* mRNA has been associated with inferior prognosis in high risk patient cohorts that included both children and adults.[9,13,15,16] To ascertain the prognostic value of expression of the *BCL6* mRNA in a paediatric setting, we evaluated EFS in the Nordic patient cohort by using the median expression of *BCL6* mRNA as a cut-off. In this setup, cases with higher-than-median expression of *BCL6* had better EFS (log-rank test $p=0.03$) (Fig. 3), but it did not translate into better OS (data not shown). In the univariate Cox regression model, higher-than-median *BCL6* mRNA was associated with a lower HR [0.46, 95% confidence interval (CI) 0.22–0.94, $p=0.03$]. In the multivariate model, higher-than-median *BCL6* mRNA showed a similar trend towards decreased HR (0.47, 95%CI 0.21–1.04, $p=0.06$) (Table 3).

We further evaluated the impact of the BCL6 protein expression to EFS and OS by using the dataset with immunohistochemistry data, but no statistically significant difference was noted between the BCL6-positive and -negative cases (Supplementary Fig. 3, Appendix A). Similarly, in the Cox univariate model, BCL6 expression did not show a statistically significant effect on OS or EFS. The association of BCL6 expression with diagnostic clinical findings (WBC and blast count, CNS disease), therapy response (MRD at EOI) and major events (relapse, death, secondary malignancy) did not reveal statistically significant findings. Similarly, positivity for either ZAP70 or pSTAT5 did not associate significantly with the patient survival (data not shown).

DISCUSSION

BCL6 is a transcriptional repressor that is necessary for germinal centre formation and antibody affinity maturation.[8] It has a well established role in several mature B-cell malignancies, and recent studies have implicated a role in B-ALL as well.[9,23] We report here that approximately 7% of childhood B-ALL cases were positive for the BCL6 protein. High *BCL6* mRNA levels were most common among the *TCF3-PBX1*, Ph-like, *NUTM1*, *MEF2D* and *PAX5*-alt subgroups, and higher-than-median expression of *BCL6* was associated with favourable EFS.

Gene expression has been widely studied as a tool to improve classification of disease entities.[21,24] However, mRNA expression does not correlate fully with protein expression,[25–28] and therefore we collected a retrospective

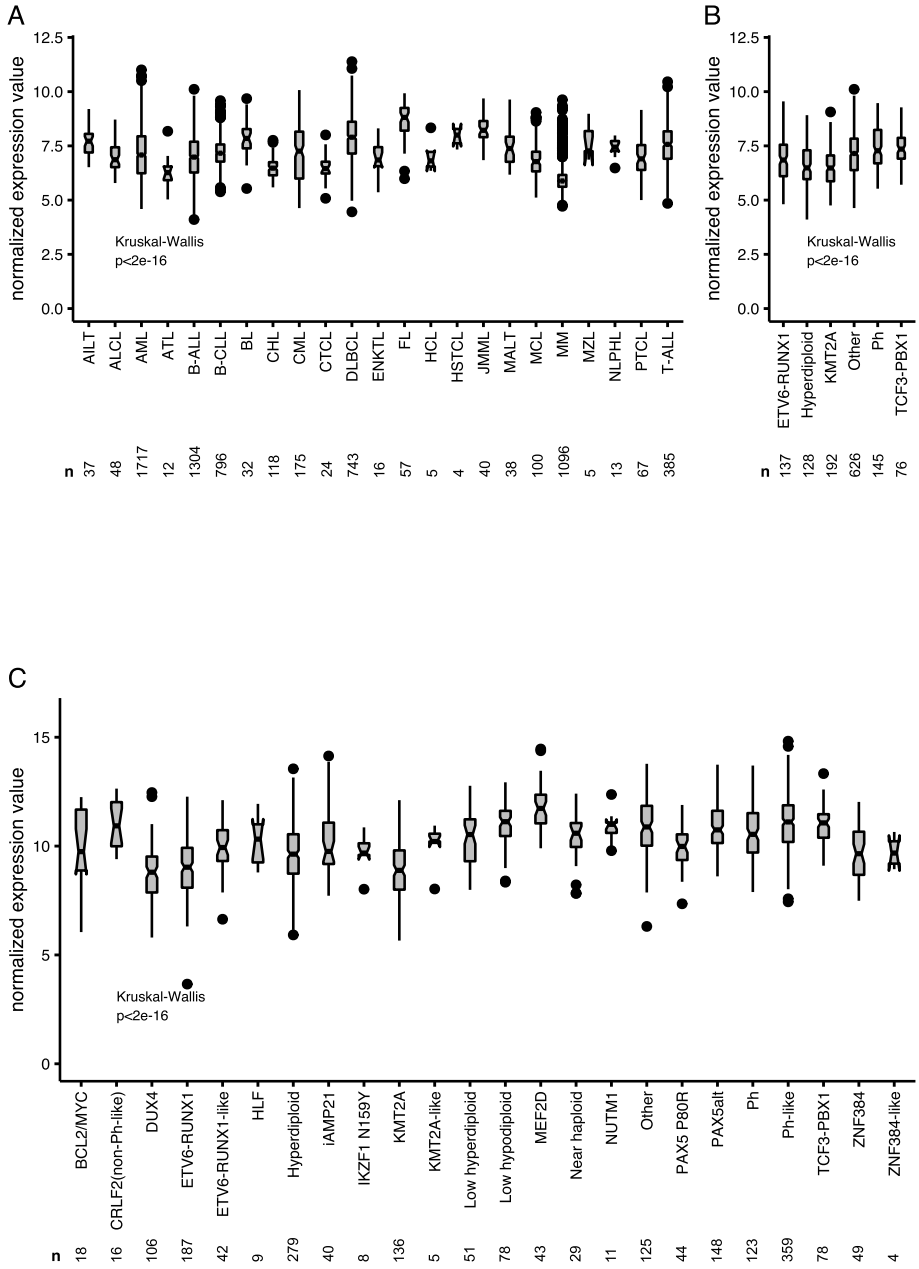

Fig. 2 Expression of *BCL6* mRNA in haematological malignancies and B-cell lineage acute lymphoblastic leukaemia (B-ALL). (A) Boxplot showing expression of the *BCL6* mRNA in different haematological malignancies in the Hemap dataset (n=6832).[19,20] (B) Boxplot showing expression of *BCL6* mRNA in the main subtypes of B-ALL in the Hemap dataset (n=1304). (C) Boxplot showing expression of the *BCL6* mRNA in different subtypes of B-ALL in the PanALL study dataset (n=1988).[21] AILT, angioimmunoblastic T-cell lymphoma; ALCL, anaplastic large cell lymphoma; AML, acute myeloid leukaemia; ATL, adult T-cell leukaemia; B-ALL, B-cell lineage acute lymphoblastic leukaemia; B-CLL, B-cell chronic lymphocytic leukaemia; BCL2/MYC, *BCL2/MYC* rearranged; BL, Burkitt lymphoma; CHL, classic Hodgkin lymphoma; CML, chronic myeloid leukaemia; CRLF2, CRLF2 (non-Ph-like); CTCL, cutaneous T-cell lymphoma; DLBCL, diffuse large B-cell lymphoma; DUX4, *DUX4*-rearranged; ENKTL, extranodal NK/T-cell lymphoma; FL, follicular lymphoma; HCL, hairy cell leukaemia; HLF, *TCF3/TCF4-HLF*; HSTCL, hepatosplenic T-cell lymphoma; iAMP21, intrachromosomal amplification of chromosome 21; IKZF1 N159Y, *IKZF1* missense alteration encoding p.Asn159Tyr; JMML, juvenile myelomonocytic leukaemia; KMT2A, *KMT2A* rearranged; MALT, extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue; MCL, mantle cell lymphoma; MEF2D, *MEF2D* rearranged; MM, multiple myeloma; MZL, marginal zone lymphoma; n, number of cases; NLPHL, nodular lymphocyte predominant Hodgkin lymphoma; NUTM1, *NUTM1* rearranged; PAX5alt, *PAX5* alterations; PAX5 P80R, PAX5 p.Pro80Arg (P80R) alteration; Ph, Philadelphia chromosome (*BCR-ABL1*); PTCL, peripheral T-cell lymphoma, not otherwise specified; T-ALL, T-cell lineage acute lymphoblastic leukaemia; ZNF384, *ZNF384* rearranged.

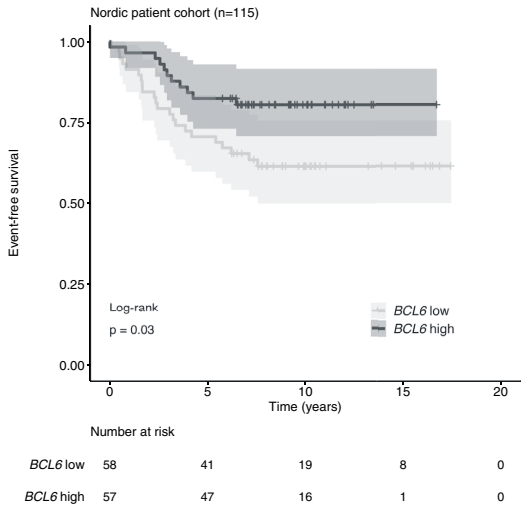

Fig. 3 Association of *BCL6* expression with the survival of patients. Kaplan–Meier survival curve and log-rank test *p* value for EFS analyses comparing EFS in cases with higher- or lower-than-median *BCL6* mRNA in the Nordic patient cohort with mRNA sequencing data (n=115).[22]

patient cohort that had archived trephine biopsy samples. Our cohort was population based and represented the most common subtypes of B-ALL according to the WHO 2017 classification.[17] We note that knowledge of Ph-like, IGH/*IL3*-rearranged B-ALL and iAMP21 B-ALL was lacking in our biopsy cohort, as novel subtypes have been identified only during the last few years. These cases were likely among the 'other' group of B-ALL in our cohort.

BCL6 protein expression was found in 7% of paediatric B-ALL cases, which is lower than the 12–17% previously reported.[9,10,13] Previous sample cohorts with BCL6 protein expression were composed of both paediatric and adult patients.[9,10,13] In addition, the published cohorts had fewer patients (52–72 patients) and used BM aspirate clot specimens, in contrast to the formalin fixed BM trephine biopsies in our study. In order to ensure the quality of the analysis, two pathologists independently evaluated the immunohistochemically stained samples, and the discrepant cases were solved by a third pathologist. The decalcification process or the used antibody, which was different from the one used in previous studies, might have affected the efficiency of the immunohistochemistry, thereby decreasing the number of positive cases in our cohort.[9,10,13,29] However, three Burkitt leukaemia/lymphoma cases, which were used as the positive controls, were all BCL6 positive as expected, suggesting that neither the antibody nor the sample processing were behind the low prevalence of BCL6 positivity.

High BCL6 has been suggested to represent the more differentiated precursor B-cell leukaemia than the progenitor-type B-cell leukaemia.[9] However, co-immunostaining of BCL6 with either the ZAP70 (pre-B-ALL) or phosphorylated STAT5 (pro-B-ALL) antibody did not show a clear dichotomy, despite the fact that the proportions of positivity to ZAP70, phosphorylated STAT5 and BCL6 were roughly in line with the previous literature.[9] It is possible that the sensitivity of the antibody-based detection of active signalling pathways by IHC is not as good as the multiparametric flow cytometry-based assay,[30] and could explain the low co-expression. On the other hand, mRNA expression of the pre-BCR+ 'metagene',[9,14] which was associated with the *TCF3-PBX1* and *MEF2D* subtypes in two different datasets, did not correlate with the *BCL6* expression.[14] However, *BCL6* mRNA expression was statistically significantly higher in the 10% of cases with highest pre-BCR 'metagene' expression. In addition, BCL6 protein expression did associate with the CD34-negative immunophenotype in line with the previous literature.[9,10] Combined, these results suggest that an association exists between pre-BCR-signalling and BCL6, but there is more heterogeneity in signalling pathways in BCL6-positive B-ALL than earlier reported. Thus, it is possible that other routes for activation of BCL6 exist, or the cell population is heterogeneous with respect to signalling pathway activity.

Table 3 Cox proportional hazards models for survival in the Nordic patient cohort (n=115)[22]

	n	Multivariate Cox regression			Univariate Cox regression		
		HR	95% CI	*p*	HR	95% CI	*p*
Age							
1 year	7	1.0			1.00		
1–10 years	80	0.38	0.08–1.94	0.25	0.30	0.10–0.87	**0.03**
>10 years	28	0.31	0.06–1.74	0.19	0.36	0.11–1.19	0.09
WBC							
$\leq 50 \times 10^9$/L	101	1.00			1.00		
$>50 \times 10^9$/L	14	0.63	0.16–2.54	0.52	1.88	0.78–4.56	0.16
WHO subgroup							
Other	55	1.00			1.00		
HeH	42	0.72	0.31–1.67	0.45	0.63	0.29–1.36	0.24
ETV6-RUNX1	18	0.89	0.28–2.82	0.84	0.58	0.20–1.71	0.32
Treatment							
Standard risk	32	1.00			1.00		
Other	83	1.85	0.70–4.84	0.21	1.94	0.80–4.70	0.14
BCL6 mRNA expression							
Low ($\leq$md)	58	1.00			1.00		
High (>md)	57	0.47	0.21–1.04	0.06	0.46	0.22–0.94	**0.03**
Total *n*	115						

CI, confidence interval; HeH, high hyperdiploid; HR, hazard ratio; md, median; WBC, white blood cell count.
Statistically significant *p* values <0.05 in bold.

To further evaluate the expression of *BCL6*, we retrieved three independent microarray or RNA-sequencing datasets. High levels of *BCL6* mRNA were associated with the *TCF3-PBX1*, Ph-like, *NUTM1,* and *PAX5*-alt subgroups. Also, expression of *BCL6* mRNA was prominent in the newly described *MEF2D* subtype, and fittingly, Tsuzuki *et al.* recently identified SREBF1 as a possible drug target in this subtype.[14] In contrast, we could not show an association with the *KMT2A* (formerly MLL) subtype, a finding recently reported by Hurtz *et al.*[13]

MuHC has been proposed as an additional biomarker for the pre-BCR+ ALL subgroup.[9] We tested muHC immunohistochemistry in the trephine biopsy samples, but strong background staining hampered the evaluation, suggesting that the accurate detection of muHC expression might be more suitable from clot samples and flow cytometry than the trephine biopsy specimens.

The association of BCL6 protein expression on survival has not been previously investigated in a paediatric B-ALL cohort. In our local cohort, we did not notice a statistically significant difference in survival based on BCL6 positivity, possibly due to rarity of its occurrence. In contrast, higher-than-median expression of *BCL6* mRNA was associated with favourable EFS in a Nordic patient cohort and showed a similar trend in the multivariate model. Restricting the analysis to the strongest expressors only (top 10%) did not change the result. Unfortunately, we lacked the mRNA data for the biopsy cohort in order to determine the mRNA threshold when the protein expression turns positive in biopsy samples (although the association is likely not linear). Our results do not indicate that BCL6 protein or mRNA levels alone would associate with an inferior prognosis as suggested by earlier literature.[9,13,15,16] These discrepancies may relate to the differences in the composition of the patient cohorts and the thresholding used. Instead, based on the results obtained, a combination of protein markers, or the pre-BCR 'metagene' may better stratify the patient group referred to as pre-BCR+ ALL.

In summary, BCL6 is associated with distinct diagnostic and novel subgroups of B-ALL. BCL6 expression is widely used as a diagnostic and classification tool in B- and T-cell lymphomas and Hodgkin lymphomas.[17] In B-ALL, its utility as a biomarker for pre-BCR+ warrants further studies with co-staining of additional markers and parallel transcriptome profiling in larger patient cohorts to distinguish patients that may benefit from its therapeutic targeting.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pathol.2021.02.013.

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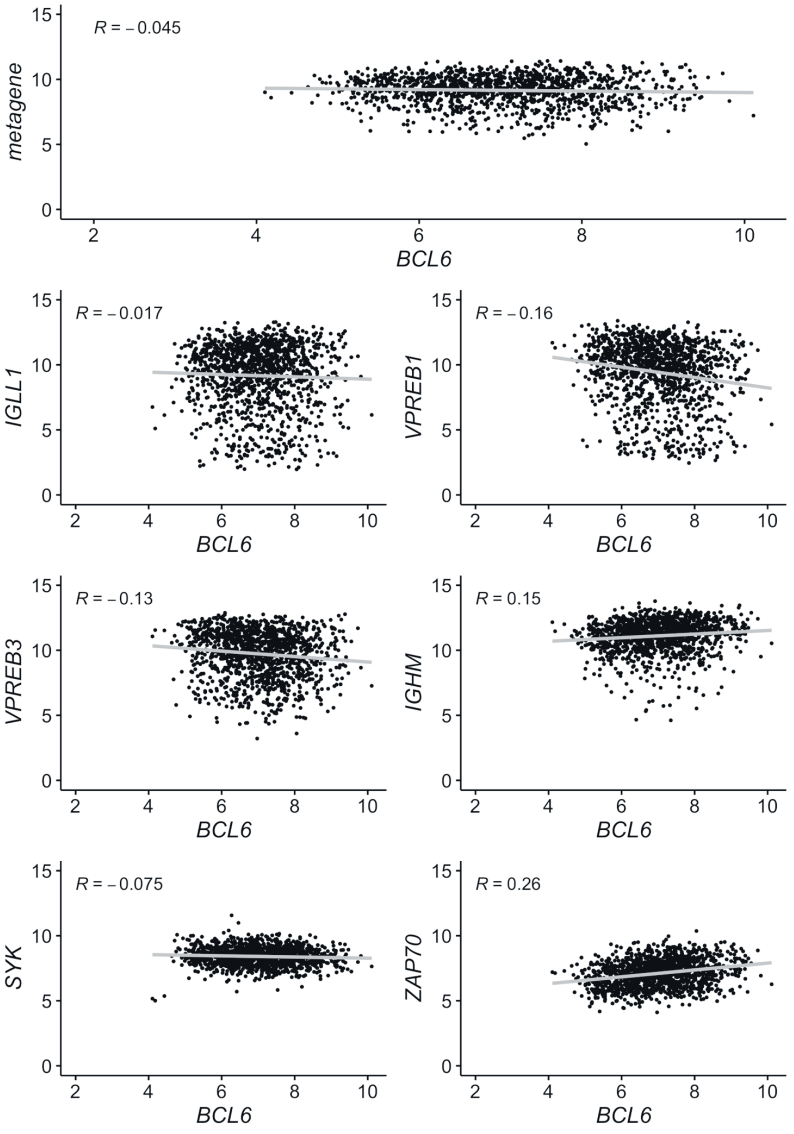
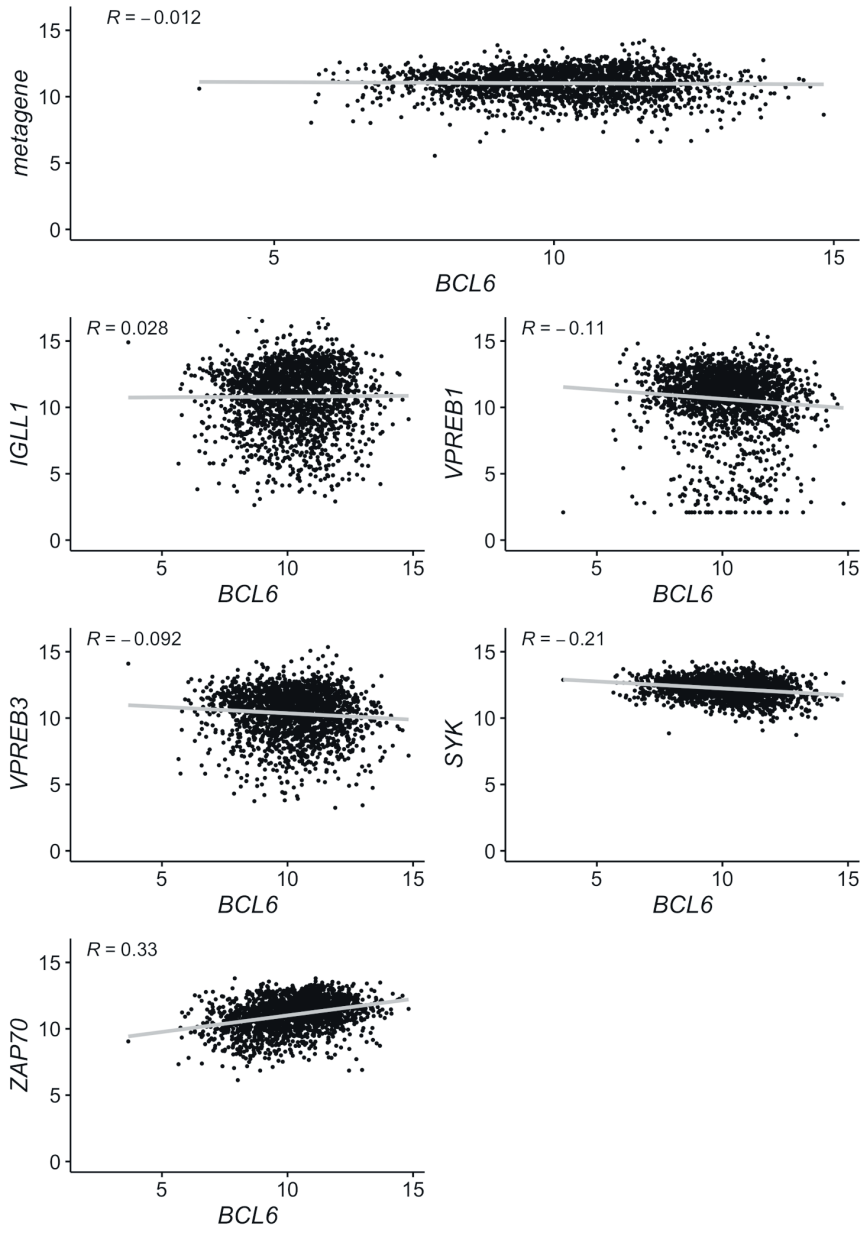
APPENDIX A: SUPPLEMENTARY DATA

Expression of BCL6 in paediatric B-cell acute lymphoblastic leukaemia and association with prognosis

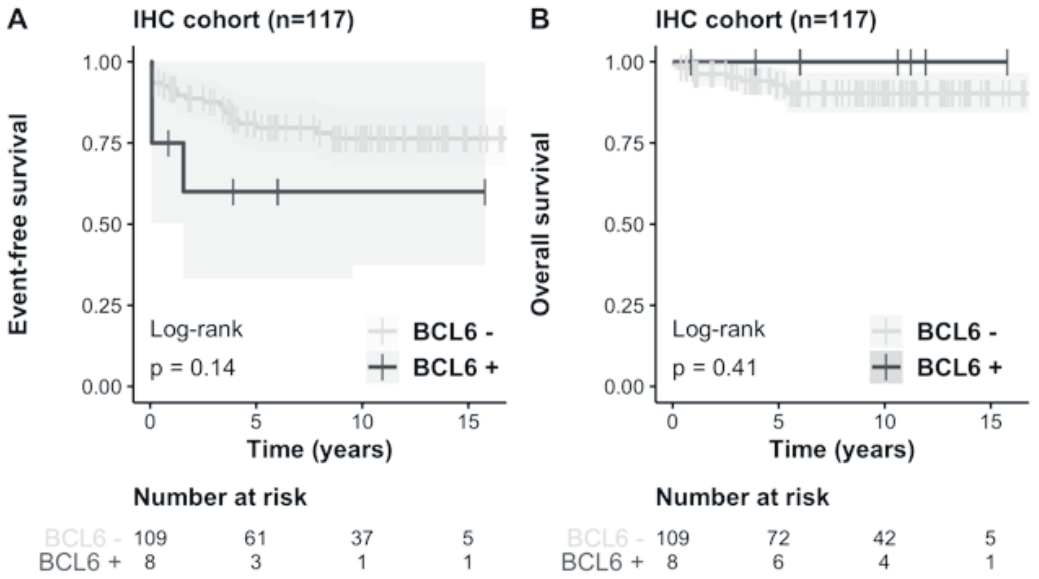
Supplementary Fig. 1 Scatterplots of Pre-BCR 'metagene' and individual pre-BCR 'metagene' components (*IGLL1, VPREB1, VPREB3, IGHM, SYK* and *ZAP70*) with *BCL6* expression in the Hemap[1,2] B-ALL cases (*n*=1304). Metagene, arithmetic mean of *IGLL1, VPREB1, VPREB3, IGHM, SYK* and *ZAP70*.



Supplementary Fig. 2 Scatterplots of pre-BCR 'metagene' and individual pre-BCR 'metagene' components (*IGLL1, VPREB1, VPREB3, SYK* and *ZAP70*) with *BCL6* expression in the PanALL study cohort[3] B-ALL cases (*n*=1988).



Supplementary Fig. 3 Association of BCL6 expression with the survival of patients. Kaplan–Meier survival curve and log-rank test *p* value for (A) EFS and (B) OS analyses comparing cases with negative and positive BCL6 immunohistochemistry in the paediatric IHC B-ALL cohort (*n*=117).



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2. Mehtonen J, Pölönen P, Häyrynen S, *et al*. Data-driven characterization of molecular phenotypes across heterogeneous sample collections. *Nucleic Acids Res* 2019; 47: 76.
3. Gu Z, Churchman M, Roberts K, *et al*. PAX5-driven subtypes of B-progenitor acute lymphoblastic leukemia. *Nat Genet* 2019; 51: 296–307.

PUBLICATION II

Clinicopathological features and prognostic value of SOX11 in childhood acute lymphoblastic leukemia

Grönroos T, Mäkinen A, Laukkanen S, Mehtonen J, Nikkilä A, Oksa L, Rounioja S, Marincevic-Zuniga Y, Nordlund J, Pohjolainen V, Paavonen T, Heinäniemi M, Lohi O.

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Clinicopathological features and prognostic value of SOX11 in childhood acute lymphoblastic leukemia

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Acute lymphoblastic leukemia is marked by aberrant transcriptional features that alter cell differentiation, self-renewal, and proliferative features. We sought to identify the transcription factors exhibiting altered and subtype-specific expression patterns in B-ALL and report here that SOX11, a developmental and neuronal transcription factor, is aberrantly expressed in the ETV6-RUNX1 and TCF3-PBX1 subtypes of acute B-cell leukemias. We show that a high expression of *SOX11* leads to alterations of gene expression that are typically associated with cell adhesion, migration, and differentiation. A high expression is associated with DNA hypomethylation at the *SOX11* locus and a favorable outcome. The results indicate that SOX11 expression marks a group of patients with good outcomes and thereby prompts further study of its use as a biomarker.

SOX11 is a transcription factor (TF) encoded by the *SOX11* gene located in chromosome 2p25[1]. It is a member of the *SoxC* (sex-determining region Y-related HMG box) group of genes and consists of two functional domains—the N-terminal DNA-binding and the C-terminal transactivation domains[2,3]. Other SoxC family members include SOX4 and SOX12. Of these, SOX4 is a crucial TF in B lymphopoiesis and is expressed in the B- and T-cell lineages[2,4,5]. SOX11 is normally expressed in the developing central nervous system of the embryo, in keratinocytes, and in some other epithelial tissues[1,6–8]. It is also expressed in ovarian and breast cancer, in which both tumor suppressor and oncogenic functions have been suggested[9,10]. A knockout mouse model revealed the vital role of *SOX11* during embryonic development, as *SOX11*-deficient mice died after birth likely from multiple heart defects, asplenia, and organ hypoplasia in the lungs, stomach, and pancreas[11]. *SOX11* deletions and mutations are associated with neurodevelopmental disorders[12].

Previous studies have shown that SOX11 mRNA and nuclear protein expression is a specific marker for conventional but not indolent mantle cell lymphoma (MCL)[8,13–15]. In MCL, SOX11 has been associated with either increased or reduced cell proliferation[16–23] and either good or bad prognosis[24–27]. In a cohort of 50 adult acute myeloid leukemia (AML) patients, a high *SOX11* expression was associated with FLT/ITD and NPM1 mutations and a shortened disease-free survival[28].

There is other evidence linking SOX11 with B-lineage malignancies. Dictor *et al.* (2009) reported that the nuclear expression of SOX11 was found in eight cases of B-lymphoblastic neoplasias[29]. Another study reported the strong nuclear expression of SOX11 in a single B-cell and five T-cell lymphoblastic lymphoma/leukemias[13]. Vegliante *et al.* (2011) demonstrated the increased expression of *SOX11* mRNA in ETV6-RUNX1 (E/R) and

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TCF3-rearranged B-cell precursor acute lymphoblastic leukemia (BCP-ALL)[30], whereas Nordlund *et al.* (2012) and Busche *et al.* (2013) observed the prominent expression of *SOX11* in the E/R subtype of ALL[31,32].

We investigated the expression of SOX11 across lymphoid malignancies, focusing on B-lymphoblastic leukemias. The function of SOX11 in leukemias and its clinical significance as a biomarker were further explored.

Materials and Methods

Microarray datasets. We used three independent datasets to study *SOX11* expression—a combined microarray dataset ("Hemap") retrieved from Gene Expression Omnibus (GEO)[33,34], the GEO series GSE47051[35], and the publicly available BCP-ALL data from the recent PanALL study[36]. The sample sizes for each dataset are shown in Supplementary Table 1.

Cell lines, cell culture, and drug treatments. NALM-6, REH, 697, RCH-ACV, KOPN-8, KASUMI-2, JURKAT, MOLT-16, P12-ICHIKAWA, HPB-ALL, and CCRF-CEM were cultured in RPMI Medium 1640 (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) with 2 mM L-glut, 100 U penicillin, 100 µg/ml streptomycin with 10% FBS (Gibco), and MOLT-4, PEER, and MHH-CALL3 with 20% FBS (Gibco) at 37 °C in 5% CO_2. An inducible *E/R* fusion in the NALM-6 cell line and a knockdown of *E/R* by a short hairpin RNA (shRNA) in the REH cell line have been previously described[37]. *E/R* expression was induced with 500 ng/ml doxycycline (Clontech). *E/R* expression changes were confirmed with RT-qPCR, with fusion gene-specific primers (Table S2). Mycoplasma tests were done regularly for the cell lines, and Eurofins Genomics (Ebersberg, Germany) services were used to authenticate the cell lines by STR genotyping. All cell lines used in this study were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).

For the methyltransferase inhibition experiments, the cultured cells were treated for 72 h with decitabine, 5-Aza-2′-deoxycytidine solved in DMSO (A3656, Sigma-Aldrich, St. Louis, MO, USA) at 0, 0.1, and 1 µM concentrations. The media were changed at a 24 h interval to compensate for decitabine instability under cell culture conditions. After the treatment cells were collected, RNA was extracted for RT-qPCR analyses. Corticosteroid and chemotherapy treatments were conducted with the indicated concentration ranges, and cell viabilities were measured after either 72 (697 and RCH-ACV) or 96 h (REH). The corticosteroids included prednisolone (P6004, Sigma-Aldrich) and dexamethasone (D8893, Sigma-Aldrich), and the chemotherapy agents used were asparaginase (A3809, Sigma-Aldrich) and vincristine (V8879, Sigma-Aldrich). The applied concentrations for each cell line are indicated in Table S3.

Quantitative real-time PCR. Total RNA was extracted using the PureLink™ RNA Mini Kit, and the On-Column PureLink® DNase Treatment Protocol was used for DNA removal (Ambion® by Life Technologies and Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA); 100–500 ng of the extracted RNA was used as a starting material for cDNA synthesis, which was performed with iScript (Bio-Rad, Hercules, CA, USA). RT-qPCR reactions were conducted according to the manufacturer's instructions with SsoFast EvaGreen® Supermix (Bio-Rad). The following program was performed with the Bio-Rad CFX96™ Real-Time System (Bio-Rad): initial denaturation at 96 °C for 30 s, 39 cycles of denaturation at 96 °C for 2 s, annealing at 60 °C for 5 s, and plate read. Independent experiments performed in triplicate were used as the starting material for the RT-qPCR measurements, and the relative $2^{-\Delta\Delta C}{}_{T}$ method was used for quantification[38]. The primer sequences used in RT-qPCR are listed in Table S2.

Western blot. Protein extraction was performed using M-PER reagent (Thermo Fisher Scientific) according to the manufacturer's instructions to lyse the cells, and protein concentrations were measured with *DC* Protein Assay (Bio-Rad); 15–20 µg of a protein sample was loaded into the precast 10% Mini-PROTEAN® TGX Stain-Free™ Gels (Bio-Rad). After the electrophoresis run, Trans-Blot® Turbo™ Pack (Bio-Rad) was used to transfer the proteins from the gel to the nitrocellulose membrane. Transfer was done with the Trans-Blot® Turbo™ Transfer system according to the manufacturer's instructions (Bio-Rad). A prestained protein ladder, PageRuler Plus (#26619, Thermo Fischer Scientific), was used as a protein size marker. We utilized antibodies against SOX11 (1:1,000) (HPA000536, Sigma-Aldrich, Lot # BB107024) and Histone H3 (1:75,000) (ab4729, Abcam, Cambridge, UK, Lot # GR167613-1). Horseradish peroxidase conjugated anti-rabbit (1:2,000) (P0217, Lot # 00069121) was used as a secondary antibody (Agilent Technologies, Santa Clara, CA, USA). Chemiluminescence reaction by Amersham ECL reagent was detected with ChemiDoc™ XRS+using Image Lab™ Software (Bio-Rad).

Gene silencing with nucleofection. Knockdowns for suspension cells were performed using 4D-Nucleofector™ (Lonza, Basel, Switzerland) for transfections. *SOX11* knockdown was done using gene sequence-specific small interfering RNA (siRNA) (Sigma-Aldrich), and a non-specific siRNA was used as a control (Table S4). Before nucleofection, 20 µM stock solutions were diluted in Resuspension Buffer (SR30005, OriGene, Rockville, MD, USA) so that the final concentration per reaction was 300 nM. One million cells were used for each nucleofection reaction. Nucleofection reactions were conducted in proper solutions and programs according to the manufacturer's instructions in single nucleocuvettes (Table S5). Then, the cells were transferred to 12-well plates with prewarmed fresh media. The transfected cells were used in cell viability assays and RNA sequencing (RNA-seq). Western blot and RT-qPCR were used to assess knockdown levels.

RNA sequencing of cell lines. *SOX11*-specific and control siRNAs were used in the nucleofection for the 697, RCH-ACV, and REH cell lines to create *SOX11* knockdown and control samples. After 48 h of nucleofection, three million cells per sample were collected for the total RNA extraction. RNA extraction was performed with the PureLink™ RNA Mini Kit, and the On-Column PureLink® DNase Treatment Protocol was used to avoid contamination by genomic DNA (Ambion® by Life Technologies and Invitrogen). Protein samples were also

collected, and the *SOX11* knockdown level was verified by both RT-qPCR and Western blotting. Three independent biological replicates were collected for each transfected cell line, and each sample had 25 ng of RNA in 40 µl. Library preparation and RNA-seq (GSE123943) were performed in the Finnish Functional Genomics Centre (Turku, Finland). See more details in Supplementary Information.

The quality of the raw sequencing reads was ensured with FastQC (v0.10.1). Based on the FastQC results, reads were trimmed and their quality was filtered using the FASTX Toolkit (0.0.14). The reads were mapped to the human reference genome version hg19 using STAR aligner software (2.5.3a modified); reads aligning to more than two locations were discarded[39]. The alignment file was turned into tag directories, and read counts were calculated using the HOMER tool kit (v4.8).

Differential gene expression was analyzed using the quasi-likelihood F-test from edgeR, an R package[40]. Differentially expressed genes from all cell lines were filtered using the adjusted p-value < 0.05 (Benjamini–Hochberg method) as a cut-off, and the resulting gene lists from three different cell lines were compared by drawing a Venn diagram with an interactive Venn online tool (http://www.interactivenn.net/). Heatmaps, presenting all biological replicates, were drawn by using the z-scores of reads per kilobase of transcript, the per million mapped reads (RPKM) normalized count matrix, and the ComplexHeatmap R-package[41]. Gene set enrichment was analyzed by Gene Set Enrichment Analysis (GSEA) 3.0 software using logFC ranked lists of genes from differential gene expression analysis, and the results presented had an adjusted p-value < 0.02 (Benjamini–Hochberg method)[42,43]. Gene ontology (GO) term enrichment was studied with two approaches—with GSEA software using f-statistics ranked lists of genes and with the GOrilla online tool using unranked target and background lists[44,45]. The target lists were created with a threshold of adjusted p-value < 0.05 and logFC > 0.5 or < -0.5.

RNA sequencing and methylation analysis of the patient samples. Previous transcriptome sequencing of pediatric ALL cohort by Marincevic-Zuniga *et al.*[46] included 116 BCP-ALL cases, of which 115 cases were used to assess the SOX11 mRNA expression level in this study. DNA methylation data (GSE49031) were available for 112 of the 115 BCP-ALL cases[35]. See more details in Supplementary Information.

Cell viability and proliferation assays. Fresh media were replaced on the transfected cells after 24 h of transfection. For the cell viability assay, the cells were counted, and 10,000 cells per well were used in a 96-well plate. The cells were allowed to grow up to 72 (697 and RCH-ACV) and 120 h (REH), and cell viability was measured every 24 h with 10 µl of Alamar Blue reagent per well with a 2 h incubation before fluorescence measurement with excitation of 560 nm and emission of 590 nm using the Tecan fluorometer Infinite 200 (Tecan, Männedorf, Switzerland). For each time point, we used four technical replicates per sample to calculate the mean.

Cell proliferation assays were performed by counting the cells every 24 h after the transfection up to 96 (697 and RCH-ACV) and 120 h (REH).

Clinical data and the patient samples. The data on pediatric ALL cases below 18 years of age and treated in the Tampere University Hospital were retrieved from the clinical registry from years 1990 to 2017. Essential clinical information, such as age, leukocyte count, gender, relapse, death, central nervous system leukemia, immunophenotype based on flow cytometry, and clinical genetic information, was collected. The study obtained permission from the local ethical committee, and informed consent was sought, as needed (Pirkanmaa Hospital District Ethical Committee, R16054 and R13109). The use of old biopsy samples was approved by the National Supervisory Authority for Welfare and Heath (Valvira), and the samples were handled in accordance with relevant guidelines and regulations.

A total of 126 representative diagnostic formalin-fixed and paraffin-embedded (FFPE) decalcified bone marrow trephine biopsy samples were collected from the pathology department archives on the basis of the primary sample reports. For a proportion of cases, remission and relapse samples were also retrieved. Plastic-embedded and inadequate samples were excluded. The cases were classified based on the WHO 2017 Classification of Tumors and Haematopoietic and Lymphoid Tissues[47].

Immunohistochemistry. Four micron-thick whole tissue sections were used for immunohistochemistry. All cases were stained with anti-SOX11 antibody (clone MRQ-58, Cell Marque, Sigma-Aldrich, Lot # 1331005 A and 1430213 C) using Ventana Benchmark Classic at a dilution of 1:50. FFPE human MCL was used as a positive control material, and remission bone marrow samples were used as negative controls.

Staining intensity was graded, and cases with less than 20% of positivity of leukemic blast cell nuclei were interpreted as negative. Cases with immunohistochemical positivity ranging from 20% to 50% in the blast cell nuclei were graded positive, and cases with immunohistochemical nuclear positivity of over 50% were considered strongly positive. SOX11 expression was independently analyzed with a light microscope by two experienced pathologists without knowledge of the clinical data. Cases with discrepant scores were further analyzed by a third pathologist.

Fluorescence *in situ* hybridization. For cases lacking the genetic subtype information, fluorescence *in situ* hybridization analysis was performed on either the bone marrow aspiration samples or the FFPE samples. The following probes were used: Metasystems E2A Break Apart Probe 19p13 (Lot # 18216), Metasystems XL MLL plus Break Apart Probe 11q23 (Lot # 18451), Metasystems XL BCR/ABL1 plus (Lot # 19082), and Metasystems XL t(12;21) (Lot # 19133).

Flow cytometry analysis. For flow cytometry analysis, the cells were permeabilized using Fix&Perm reagent according to the manufacturer's instructions (GAS003, Invitrogen). The cells were then stained with Alexa Fluor 647® conjugated rabbit anti-human SOX11 antibody [EPR8191 (2)] (ab198540, Abcam), while CD3-APC antibody (345767) (Becton Dickinson, Franklin Lakes, NJ, USA) served as a negative control. The data were

acquired with the Beckman Coulter Navios cytometer (Beckman Coulter, Brea, CA, USA) using Red laser (638 nm) and 660/20 bandpass filter. Data analysis was conducted using Kaluza software (Beckman Coulter).

Statistical analysis. IBM SPSS Statistics (v. 22) and R (v. 3.40) were used for the statistical analysis. Kaplan–Meier survival analysis was performed, and Log-rank test was used, with a p-value < 0.05 considered statistically significant. Event-free survival (EFS) was analyzed using death, relapse, resistant disease (blast count >25% at the end of induction), and secondary malignancy as events. Cox proportional hazards models were fitted to estimate the effects of potential risk factors on survival. Chi-squared test was performed on SOX11 expression and clinicopathological prognostic variables. Kruskal–Wallis H and Mann–Whitney U tests were used to evaluate the differential expression of *SOX11* in distinct leukemia subgroups (Table S6). All performed statistical tests were two tailed, and no corrections for multiple testing were used.

Results

SOX11 is overexpressed in acute lymphoblastic leukemias. Derailed differentiation and abnormal proliferation of B-cells are thought to underlie the genesis of BCP-ALL. As TFs are key cell differentiation drivers, we sought to identify aberrantly expressed TFs in BCP-ALL. We utilized a large, curated dataset of microarray-based gene expression profiles retrieved from GEO[34]. This dataset comprises a total of 9,544 hematological gene expression profiles, including 4,418 leukemias, 428 healthy controls, and 862 cell lines. Among the genes with altered expression, TF *SOX11* showed prominent expression in MCL and in the E/R and T/P subtypes of BCP-ALL (Fig. 1a). Compared with healthy hematopoietic cells, *SOX11* had a 4.7- and 4-fold higher expression in the E/R and T/P subtypes, respectively, with an expression comparable to that in MCL (Fig. 1a). A similar subtype-specific expression of *SOX11* was replicated in two additional ALL gene expression datasets (Fig. 1a). Interestingly, the PanALL study revealed that *SOX11* expression is also elevated in novel subtypes, such as E/R-like, IKZF1 N159Y (*IKZF1* missense alteration encoding p.Asn159Tyr), MEF2D rearrangement, and DUX4 rearrangement.

As SOX11 belongs to the SoxC family of TFs, we investigated the expression of the two other members of this family. *SOX4*, which has been reported to affect survival, progression, and proliferation in BCP-ALL[48], was highly expressed in BCP-ALL and T-ALL, but it showed no subtype-specific expression pattern. The expression of *SOX12* did not vary markedly between the studied subtypes (Fig. S1a).

A subtype-specific expression pattern was also present in BCP-ALL cell lines, as REH cells, representing the E/R subtype, and RCH-ACV and 697 cells, representing the T/P subtype, showed an increased expression of *SOX11* by RT-qPCR (Fig. 1b). Neither knockdown of *E/R* in REH cells nor their overexpression in NALM-6 cells had any effect on *SOX11* expression levels (Fig. S2a).

Immunohistochemical analysis of SOX11 expression in ALL. In order to confirm the SOX11 expression at the protein level and relate it to clinical features, we collected a retrospective cohort of 119 B-ALL cases with available bone marrow biopsies and associated clinical data (Table 1). We performed immunohistochemical staining of the biopsy samples by using a SOX11-specific antibody. Staining intensity was graded from 0 to 2, with 0 marking negative, 1 positive, and 2 strongly positive samples. Eighty out of 119 primary B-ALL bone marrow samples stained negative for SOX11, whereas 34 cases were positive and 5 were strongly positive (Fig. 2a and Table 2). A total of 29/39 (74.4%) of the positively staining B-ALL cases represented either the E/R or T/P subtype. A statistically significant association was observed with the E/R subtype (p-value < 0.001) but not with the T/P subtype (likely because of the low number of cases: $N = 4$, 3 positive cases). Cases with either *KMT2A* rearrangement or the Philadelphia chromosome (*BCR-ABL1*-translocation) did not express the SOX11 protein, and the majority of hyperdiploid cases were also SOX11 negative. Additionally, seven T-ALL cases included in the whole ALL patient cohort were all negative.

SOX11 expression is associated with a favorable outcome. We next sought to evaluate the clinical significance of SOX11 expression in B-ALL ($N = 119$). The overall survival (OS) was better in the SOX11-positive group, and no deaths occurred among SOX11-positive cases ($p = 0.039$) (Fig. 2b). The EFS and relapse-free survival (RFS) adjusted for the competing event (death) showed similar trends but did not reach statistical significance (Fig. 2b). SOX11 positivity was not associated with good early therapy response, as measured by a minimal residual disease below 0.1% at the end of induction therapy (OR = 0.54, 95% CI 0.22, 1.28, $p = 0.17$). In multivariate analysis of EFS with covariates (age, WBC, MRD and subtypes), a positive immunohistochemical staining for SOX11 protein showed a favorable trend (Table 3).

When SOX11 immunostaining positivity was analyzed separately within the E/R subtype, SOX11-positive cases had a better OS (Log-rank test $p = 0.004$), but EFS did not show a statistically significant difference with a hazard ratio of 0.67 (95% CI 0.07, 6.43).

We replicated the survival findings in another dataset. Transcriptomic data from a patient cohort comprising 115 BCP-ALL cases were analyzed for *SOX11* expression and patients' survival status[46]. Figure 3a shows that a high *SOX11* mRNA expression was associated with a favorable trend in EFS analysis.

***SOX11* overexpression is associated with DNA hypomethylation.** We next investigated the biology behind the increased *SOX11* expression in leukemia. Neither primary transcription (GRO-seq, $N = 8$) nor whole-genome sequencing (WGS, $N = 8$) of the *SOX11* gene in BCP-ALL cases revealed any aberrant enhancer activity or somatic mutations, respectively, in the *SOX11* gene or nearby regions (data not shown), prompting us to look for other mechanisms. In MCL, hypomethylation (partly) drives the increased *SOX11* expression[30]. We utilized the above-mentioned BCP-ALL patient cohort with readily available genome-wide CpG methylation data[35,46]. Altogether, 23 CpG sites were located within the *SOX11* gene locus, and a strong pattern of DNA hypomethylation was seen in patients with a high *SOX11* mRNA expression (Fig. 3b,c). Nevertheless,

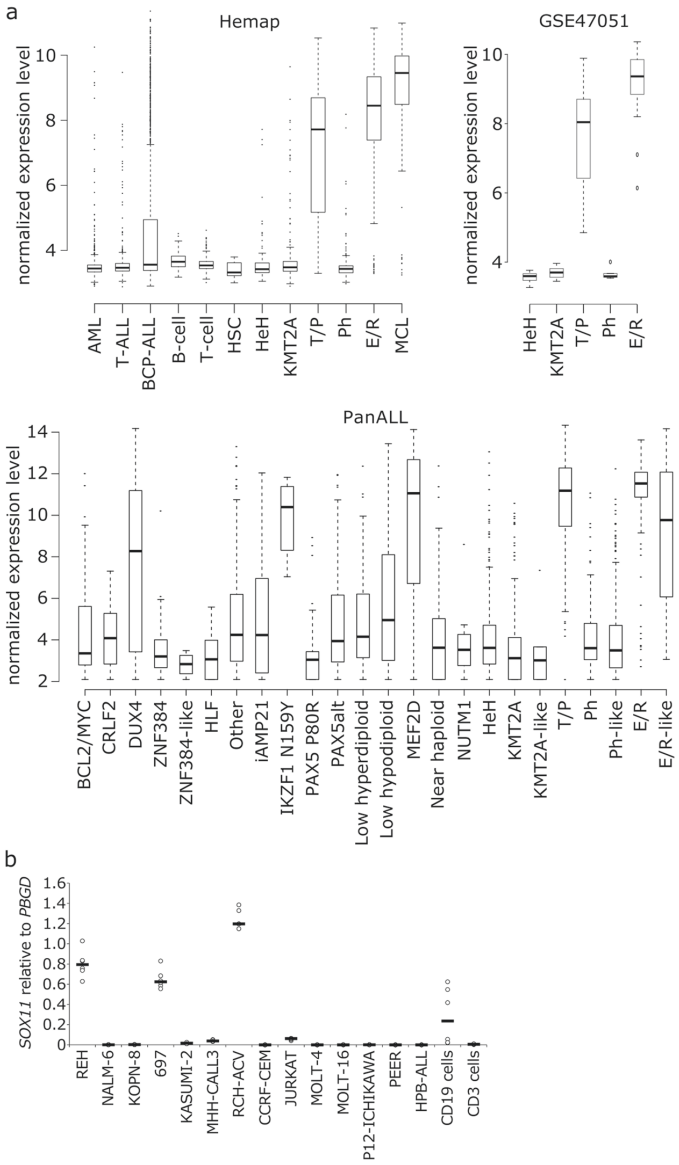

Figure 1. *SOX11* expression in hematological malignancies. **(a)** Expression boxplots of *SOX11* in healthy cells, leukemias, and mantle cell lymphoma. Data sources: Hemap microarray dataset[34], GSE47051[35], and the PanALL study[36]. See Supplementary Information for more details. **(b)** *SOX11* expression in ALL cell lines and healthy B- and T-cells, as measured by RT-qPCR (N = 2, black lines indicate the median). ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; BCL2/MYC, BCL2/MYC rearranged; BCP-ALL, B-cell precursor ALL; CRLF2, CRLF2 (non-Ph-like); DUX4, DUX4 rearranged; E/R, ETV6-RUNX1; HeH, high hyperdiploid; HLF, TCF3/TCF4-HLF; HSC, hematopoietic stem cell; iAMP21, intrachromosomal amplification of chromosome 21; IKZF1 N159Y, IKZF1 missense alteration encoding p.Asn159Tyr; KMT2A, KMT2A rearranged; MCL, mantle cell lymphoma; MEF2D, MEF2D rearranged; NUTM1, NUTM1 rearranged; PAX5alt, PAX5 alterations; PAX5 P80R, PAX5 p.Pro80Arg (P80R) alteration; Ph, Philadelphia chromosome; T/P, TCF3-PBX1; ZNF384, ZNF384 rearranged. Reproduced with permission[66].

	WHO Subtype								
	Burkitt	NOS	Ph	KMT2A	ETV6-RUNX1	Hyper-diploid	Hypo-diploid	TCF3-PBX1	Total/Combined
N of cases	2	42	2	5	33	30	1	4	119
Age, Md (min/max)	12.7	8.1	10.1	1.3	4	3.7	4.1	7.4	4.3 (0.9/17.6)
WBC count (10^9/l), Md (min/max)	18.9	6.7	156.3	109.7	7	7.1	1.9	43.4	7.1 (1/311)
Deceased	2	6	1	0	1	1	0	0	9
Relapse	0	7	1	0	3	3	0	1	15
CNS disease	0	2	1	0	1	2	0	0	6
Resistant disease	0	3	0	0	1	0	0	0	4
MRD (%) at EOI, Md (min/max)	0	0.05	1.75	0	0.06	0	0	0	0.02 (0/44)
Follow-up (years), Md (min/max)	7.5	9.7	8.4	4	7.1	9.2	11.5	7.6	8.2 (0.1/17)

Table 1. Summary of clinical characteristics of B-ALL patients. CNS, central nervous system; EOI, end of induction therapy; KMT2A, KMT2A rearrangement; Max, maximum; Md, median; Min, minimum; MRD, minimal residual disease; NOS, not otherwise specified; Ph, Philadelphia chromosome; WBC, white blood cell.

	WHO Subtype								
SOX11 IHC	Burkitt	NOS	Ph	KMT2A	ETV6-RUNX1	Hyper-diploid	Hypo-diploid	TCF3-PBX1	All cases
Negative	1	35	2	5	7	29	0	1	80
Positive	1	6	0	0	24	0	1	2	34
Strong positive	0	1	0	0	2	1	0	1	5
Total	2	42	2	5	33	30	1	4	119

Table 2. Summary of SOX11 protein expression by immunohistochemical staining in B-ALL. IHC, immunohistochemistry; KMT2A, KMT2A rearrangement; NOS, not otherwise specified; Ph, Philadelphia chromosome.

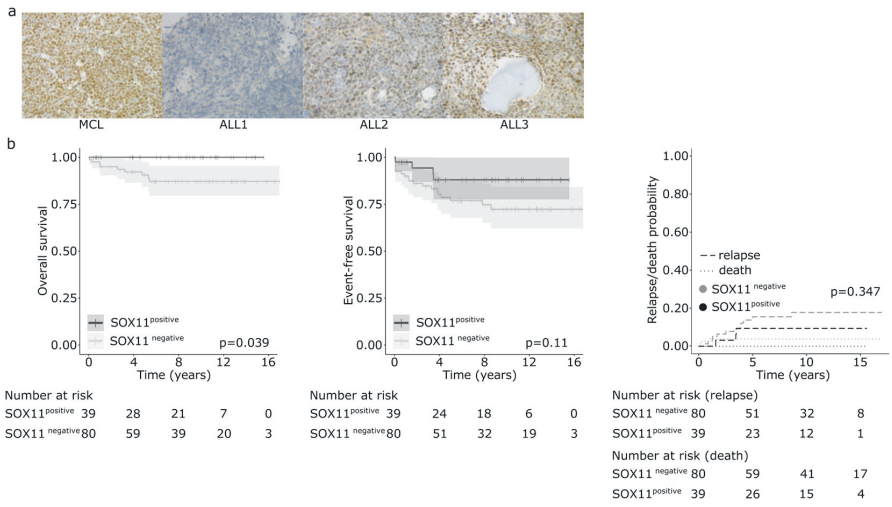

Figure 2. SOX11 protein expression and impact on the outcome in BCP-ALL. **(a)** Expression of SOX11 protein by immunohistochemistry. Mantle cell lymphoma (MCL), a strongly positive case for SOX11 (400×); ALL1, a negative B-ALL case (400×); ALL2, a positive B-ALL case (400×); ALL3, a strongly positive B-ALL case (400×). **(b)** Kaplan–Meier survival curves and Log-rank p-values for OS, EFS, and RFS in the SOX11-positive (high) and -negative (low) groups. Reproduced with permission[66].

DNA hypomethylation of the CpG sites at the *SOX11* locus was not associated with a better EFS (Fig. 3a, right panel). We also tested whether a methyltransferase inhibitor, decitabine, could reverse *SOX11* expression in leukemia cell lines. After 72 h of decitabine treatment, a marked increase in *SOX11* expression was observed in a concentration-dependent manner in KOPN-8 and REH cells (Figs. 3d and S2b).

	N	MULTIVARIATE			UNIVARIATE		
		HR	95% CI	*p-value*	HR	95% CI	*p-value*
Age (years)							
≥1 and ≤10	93	1.00*			1.00*		
<1 and >10	26	0.87	0.31–2.38	0.79	1.27	0.50–3.23	0.61
WBC count (10^9/l)							
<50	101	1.00*			1.00*		
≥50	18	0.55	0.16–1.92	0.35	0.90	0.27–3.04	0.87
Subtype							
Other B-ALL	56	1.00*			1.00*		
High hyperdiploidy	30	0.27	0.07–1.00	0.05	0.45	0.15–1.36	0.16
ETV6-RUNX1	33	0.72	0.18–2.83	0.64	0.50	0.17–1.51	0.22
SOX11 expression							
negative	80	1.00*			1.00*		
positive	39	0.37	0.10–1.43	0.15	0.43	0.15–1.26	0.12
MRD at EOI							
<0.1%	78	1.00*			1.00*		
≥0.1%	37	1.70	0.68–4.24	0.26	2.18	0.94–5.06	0.07

Table 3. Multivariate and univariate analyses of the event-free survival based on expression level of SOX11 protein in pediatric B-ALL. Cox proportional hazards regression calculated for known risk factors. SOX11 expression was treated as a binary variable. 95% CI, 95% confidence interval; B-ALL, B-cell acute lymphoblastic leukemia; HR, hazard ratio; WBC, white blood cell. *Marks reference groups of each categorical variable.

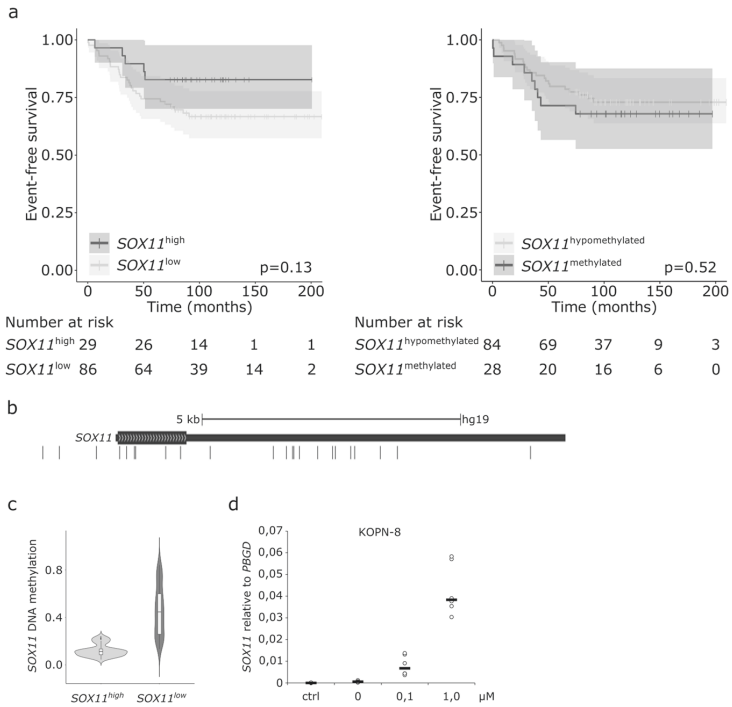

Figure 3. Survival analysis and methylation status of cases with either a high or low *SOX11* mRNA expression. **(a)** Kaplan–Meier survival curves and p-values of Log-rank test for EFS in patients with a low or high expression of *SOX11* and in patients with a low or high methylation of the *SOX11* gene locus. **(b)** CpG sites at the *SOX11* locus in chromosome 2[35]. **(c)** DNA methylation at the *SOX11* gene locus among patients with either a low or high expression of *SOX11*. **(d)** Effect of decitabine treatment on *SOX11* mRNA expression in the KOPN-8 cell line. Reproduced with permission[66].

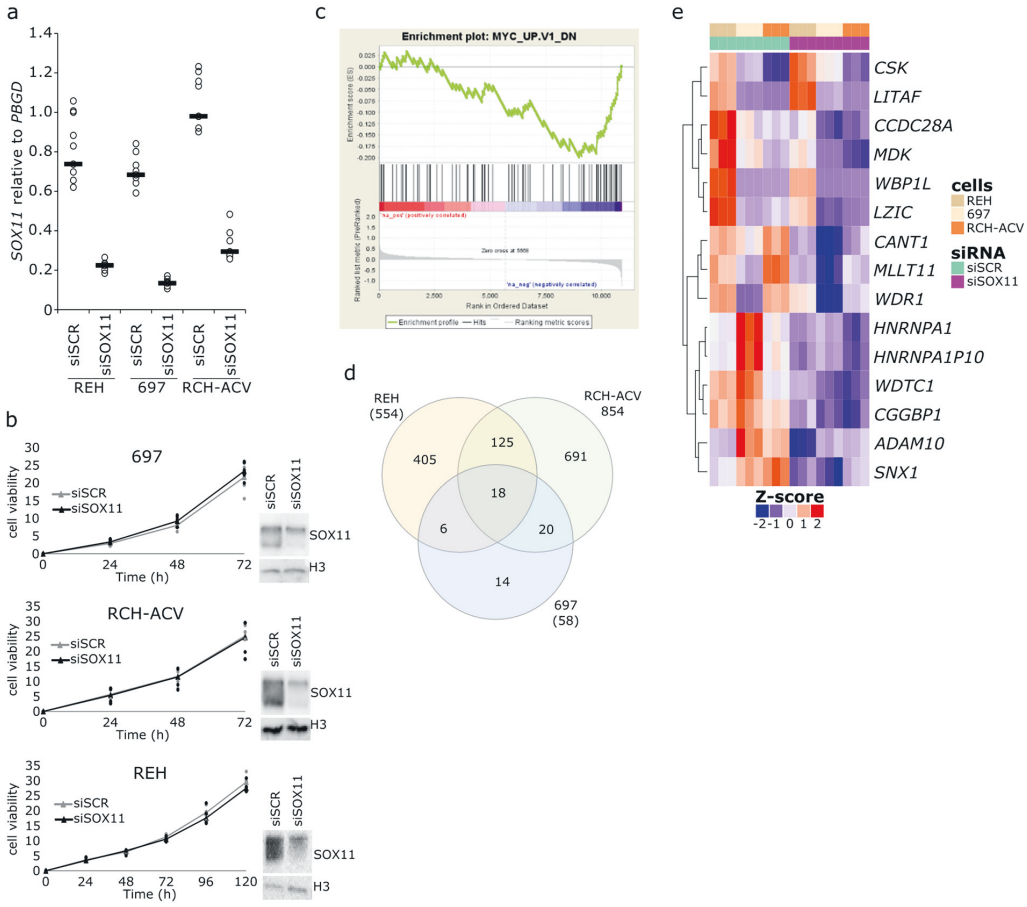

Figure 4. Cell viability and transcriptomic changes after knockdown of *SOX11*. **(a)** Expression level of *SOX11* after knockdown, as analyzed by RT-qPCR (N = 3, black lines indicate the median). **(b)** Knockdown of *SOX11* caused no evident changes in cell viability. Curves are drawn from the biological replicates using the median value at given time points. 697 and RCH-ACV cells represent the T/P subtype, and REH cells represent the E/R subtype. *SOX11* knockdown was confirmed by Western blotting, and cell viability assessments were conducted with the AlamarBlue assay. Measured absorbance intensities are reported as $x10^3$ (697 N = 6; RCH-ACV and REH N = 4). Western blot gel figures are cropped per cell line from the original blot figures shown in Fig. S3. **(c)** *SOX11* knockdown in 697 cells caused downregulation of genes that are known to be altered after MYC upregulation. **(d)** Venn diagram of differentially expressed genes in the REH, 697, and RCH-ACV cell lines after knockdown of *SOX11* (adjusted p-value < 0.05). **(e)** Heatmap of 15 concordantly differentially expressed genes in all three cell lines after knockdown of *SOX11*. Reproduced with permission[66].

Knockdown of *SOX11* alters gene sets related to cell development, motility, and drug response pathways. We next silenced *SOX11* expression in three cell lines that overexpress *SOX11* (REH, RCH-ACV, and 697) by using siRNA oligos. Figure 4a,b show that *SOX11* expression was decreased to 20–40% at both the mRNA and protein levels compared with scrambled siRNA-transfected cells. Cell viability and proliferation assays did not demonstrate any significant changes (Figs. 4b and S4), and *SOX11* knockdown did not have any impact on sensitivity to known leukemia drugs, such as dexamethasone, prednisolone, vincristine, and asparaginase (Fig. S5).

As SOX11 is a TF and it regulates gene expression, we measured changes in gene expression after *SOX11* knockdown by using RNA-seq (GSE123943). Three biological replicates were used for each cell line (REH, RCH-ACV, and 697), and the data were analyzed using the R package EdgeR. In GO annotations, many of the altered terms were related to cell migration, adhesion, and differentiation (Fig. S6 and Supplementary Dataset). On the other hand, GSEA implied the altered expression of MYC and EF2 target genes (Figs. 4c and S6). *SOX11* knockdown did not have a significant effect on the expression of other SoxC family members, such as *SOX4* and *SOX12* (Fig. S7).

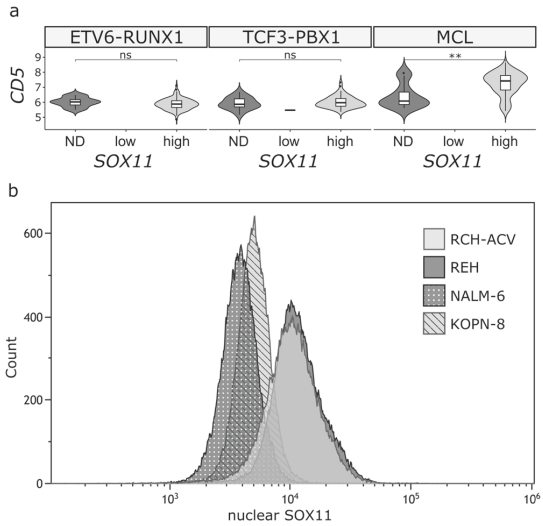

Figure 5. Detection of SOX11-positive cells by flow cytometry in BCP-ALL. **(a)** Correlation of *CD5* expression with *SOX11* mRNA expression in the E/R and T/P subtypes of B-ALL and MCL[34]. *SOX11* expression was categorized into three groups, not detected (ND), low and not detected (ND). **(b)** Flow cytometry analysis of nuclear SOX11 expression in cell lines with either a low (NALM-6, KOPN-8) or high (REH, RCH-ACV) expression of SOX11. ND, not detected, MCL, mantle cell lymphoma. Reproduced with permission[66].

The Venn diagram in Fig. 4d shows that 18 genes were differentially expressed in all of the three cell lines after *SOX11* knockdown (with an adjusted p-value < 0.05). Of these genes, 15 were concordantly down-regulated in all three cell lines and are shown in the heatmap (Fig. 4e, Supplementary Dataset). *SOX11* knockdown led to the downregulation of the *WD Repeat Domain 1* (*WDR1*) gene, which is involved in the remodeling of the actin cytoskeleton, regulation of cell migration, motility of neutrophils, and maturation of megakaryocytes[49,50]. Another gene down-regulated by *SOX11* knockdown is *Midkine* (*MDK*), a secreted growth factor that promotes cell migration and growth and is associated with an adverse prognosis in ALL possibly via increased drug resistance[51,52]. *WW Domain Binding Protein 1 Like* (*WBP1L*), also known as *OPAL1* (outcome predictor for acute leukemia 1), which was recently identified as a direct target of ETV6 in ALL[53], was down-regulated by *SOX11* silencing. Previous reports on *WBP1L*'s association with a favorable prognosis have since been refuted[54]. However, *WBP1L* expression had a 2.8-fold increase in the E/R subtype[55], which coincides with the overexpression of *SOX11* in the same subtype and may suggest co-regulation. *MLLT11* is involved in lymphoid regulation and is a known partner gene in rare leukemia translocations[56,57]. Similarly, *Coiled-coil domain containing 28 A* (*CCDC28A*) is a fusion partner to NUP98 in AML[58]. *CANT1* is a calcium-dependent nucleotidase involved in pyrimidine metabolism whose regulation by SOX11 could be related with drug metabolism (cytarabine) and therapy response in ALL[59]. The *lipopolysaccharide-induced TNF factor* (*LITAF*) has been suggested to sensitize leukemia cells to chemotherapeutic drugs, especially in cells with a lower expression of *LITAF*[60]. Both *LITAF* and *Sorting nexin 1* (*SNX1*) are involved in endosomal trafficking and regulation of cell-surface receptor signaling[61,62]. Taken together, *SOX11* knockdown leads to alterations in genes and cellular processes related to leukemia cell motility, adhesion, differentiation, and drug response.

SOX11 protein can be detected by flow cytometry. Finally, we searched for flow cytometry markers that could serve as surrogates of SOX11 positivity. In MCL, the surface expression of CD5 is correlated with SOX11 positivity[22], but in our immunohistochemical staining and microarray dataset[34], no positive correlation was observed in leukemias (Fig. 5a). Alternative surrogate markers were searched for among the routinely studied cell surface proteins, but none were associated with SOX11 positivity (data not shown). Therefore, we explored the suitability of SOX11 antibodies to discriminate SOX11-positive cases by flow cytometry. Figure 5b shows that the intensity of SOX11 antibody staining could readily separate the high expressors (REH and RCH-ACV) from the low expressors (NALM-6 and KOPN-8) in leukemia cell lines, suggesting that SOX11 antibodies could possibly be used as a biomarker in the future.

Discussion

BCP-ALL is caused by a relatively small number of genetic mutations that impede normal B-cell differentiation, entail self-renewal capacity, and increase proliferative activity. This is evident in transcriptional programs that govern leukemic B-cells. We report here that SOX11, a developmental and neuronal TF[6], is overexpressed in the E/R and T/P subtypes of BCP-ALL and also in novel E/R-like, IKZF1 N159Y, MEF2D rearrangement, and DUX4 rearrangement subtypes. A high expression is associated with DNA hypomethylation and a favorable clinical

outcome. The results suggest that a SOX11-associated transcriptional program is related with a less-aggressive disease and indicates that SOX11 warrants further study as a biomarker for low-risk ALL patients.

We observed the high expression of *SOX11* in the E/R and T/P subtypes of BCP-ALL in three separate gene expression datasets at the mRNA level[34–36] and confirmed this association at the protein level by immunohistochemistry, confirming previously published data[8,13,29,30] and adding further evidence at the protein level. We showed that the expression may be regulated epigenetically, that is, by hypomethylation of DNA at the *SOX11* locus, similar to what was earlier reported for MCL and five cases of BCP-ALL by Vegliante *et al.*[30]. We did not have material available for epigenetics studies, such as histone modifications, as these could also have a role in *SOX11* regulation[30]. Direct manipulation of the *E/R* fusion did not have any impact on *SOX11* expression, suggesting that regulation is indirect.

An interesting finding relates to the clinical significance of SOX11 expression in BCP-ALL, as we observed a better OS in the SOX11-positive cases. A similar trend was also seen in EFS, RFS, and early therapy response. This finding was supported (similar trend) in another dataset with transcriptome expression profiles[46]. In the subgroup analysis of E/R cases, SOX11 positivity retained its prognostic significance, suggesting that (a high) SOX11 expression could possibly be utilized as a biomarker for cases with a very good prognosis. It is noteworthy that our immunohistochemical staining series spans almost two decades, and patients have been treated using several distinct NOPHO ALL chemotherapy protocols[63,64]. As the most recent protocols have conferred the best survival results [63, trying to replicate these findings in the most recent protocols is necessary in the future.

To aid in the screening of SOX11 positivity at diagnosis, we also successfully tested a flow cytometry-based assay in cell lines. SOX11 inclusion into the flow cytometry panel would be convenient compared with immunohistochemical staining of bone marrow biopsies, which is slow and not routinely done in all treatment centers.

SOX11 knockdown did not markedly influence cell viability or proliferation, nor did it affect chemotherapy sensitivity. In MCL, conflicting reports have been made about the effect of knockdown or *SOX11* overexpression on cell proliferation and tumor growth[16–23]. As cell viability measurement is a relatively insensitive assay, we performed transcriptional profiling of *SOX11* knockdown cells by using RNA-seq and noticed changes in the genes associated with cell migration, adhesion, oxidative phosphorylation, hypoxia, glycolysis, and differentiation, which could explain the association of SOX11 with favorable clinical outcomes. Notably, the changes observed were mostly mild to moderate. There were only few overlapping genes with previous profiling studies in both pro-B-cells[18] and MCL cells[16,17,20,21,65]. For example, we did not see marked changes in the expression of either *PAX5*, as seen in MCL[21], or *Id1* and *Tal1* in pro-B-cells[18]. Interestingly, *MDK*, which is involved in cell migration and growth, was downregulated by *SOX11* knockdown in leukemia cell lines here and in an MCL cell line Z138[16].

In conclusion, the association of SOX11 expression with a favorable prognosis invites further studies to confirm its prognostic value and applicability as a part of the diagnostic workup.

Data availability

The datasets generated and analyzed in the current study are available in the GEO repository, GSE47051 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE47051), GSE123943 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE123943), and GSE49031 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE49031), or are included in the article or supplementary files. RNA-seq data of the patient samples[46] are not publicly available, as the patient/parent consent does not cover depositing data into repositories; however, they are available from the authors upon reasonable request.

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Author contributions

T.G., A.M.: Study design, data collection, data interpretation, figure designing, literature search, and writing (equal contribution as first authors). S.L.: Data analysis, data interpretation, figure designing, literature search, and writing. J.M.: Data collection, data analysis, figure designing, and writing. A.N.: Data collection, data analysis, data interpretation, figure designing, and writing. L.O.: Data analysis, data interpretation, figure designing, and writing. S.R.: Data collection, data analysis, data interpretation, figure designing, and writing. Y.M-Z., J.N.: Data collection, data analysis, and data interpretation. V.P.: Data collection and data interpretation. T.P.: Data analysis. M.H.: Study design, data interpretation, and writing. O.L.: Study design, data interpretation, literature search, and writing. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Clinicopathological features and prognostic value of SOX11 in childhood acute lymphoblastic leukemia

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Supplementary Table 1. Dataset sample sizes

Group	Hemap	GSE47051	PanALL
T-ALL	385	30	0
HeH	151	18	279
Low hyperdiploid	0	0	51
KMT2A	301	5	136
KMT2A-like	0	0	5
TCF3-PBX1	81	6	78
ETV6-RUNX1	171	16	187
ETV6-RUNX1-like	0	0	42
Ph	137	5	123
Ph-like	0	0	359
B-cell	75	0	0
HSC	21	0	0
T-cell	247	0	0
BCP-ALL	1304	0	0
AML	1713	0	0
MCL	100	0	0
BCL2/MYC	0	0	18
CRLF2	0	0	16
DUX4	0	0	106
HLF	0	0	9
iAMP21	0	0	40
IKZF1 N159Y	0	0	8
Low hypodiploid	0	0	78
MEF2D	0	0	43
Near haploid	0	0	29
NUTM1	0	0	11
Other	0	0	125
PAX5 P80R	0	0	44
PAX5alt	0	0	148
ZNF384	0	0	49
ZNF384-like	0	0	4

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; BCL2/MYC, BCL2/MYC rearranged; BCP-ALL, B-cell precursor ALL; CRLF2, CRLF2 (non-Ph-like); DUX4, DUX4 rearranged; HeH, high hyperdiploid; HLF, TCF3/TCF4-HLF; HSC, hematopoietic stem cell; iAMP21, intrachromosomal amplification of chromosome 21; IKZF1 N159Y, IKZF1 missense alteration encoding p.Asn159Tyr; KMT2A, KMT2A rearranged; MCL, mantle cell lymphoma; MEF2D, MEF2D rearranged; NUTM1, NUTM1 rearranged; PAX5alt, PAX5 alterations; PAX5 P80R, PAX5 p.Pro80Arg (P80R) alteration; Ph, Philadelphia chromosome; T-ALL, T-cell ALL; ZNF384, ZNF384 rearranged.

Supplementary Table 2. RT-qPCR primer sequences

Primer	Sequence 5'→ 3'
SOX11 forward	CGGTCAAGTGCGTGTTTCTG
SOX11 reverse	CACTTTGGCGACGTTGTAGC
PBGD forward	CGCATCTGGAGTTCAGGAGTA
PBGD reverse	CCAGGATGATGGCACTGA
SOX4 forward	GGCACATCAAGCGACCCATG
SOX4 reverse	CGTTTGCCCAGCCGCTTGG
SOX12 forward	CGAGGACGACGATGAAGACG
SOX12 reverse	GGACCATCCTCCACAGCTC
ETV6-RUNX1 forward	TGCACCCTCTGATCCTGAAC
ETV6-RUNX1 reverse	AACGCCTCGCTCATCTTGC

Supplementary Table 3. Used corticosteroid and chemotherapy concentrations

Cell line	Prednisolone (µM)	Dexamethasone (µM)	Asparaginase (U/ml)	Vincristine (nM)
697	0, 0.01, 0.1, 1	0, 0.01, 0.1, 1	0, 0.001, 0.01, 0,1	0, 0.1, 0.25, 0.5, 1
RCH-ACV	0, 0.01, 0.1, 1	0, 0.01, 0.1, 1	0, 0.001, 0.01, 0.1, 0.5	0, 0.1, 0.25, 0.5, 1, 2
REH	0, 100, 500, 1000	0, 10, 50, 100	0, 0.001, 0.01, 0.1	0, 0.1, 0.25, 0.5, 1

Supplementary Table 4. siRNA sequences

siRNA	Primers 5' → 3'	Reference
siSCR sense	GUUGCGUAGCGUACGUCGCAA	
siSCR antisense	UUGCGACGUACGCUACGCAAC	
siSOX11 sense	GAUAAGAUGUCGUGACGCA	[1]
siSOX11 antisense	UGCGUCACGACAUCUUAUC	[1]

Supplementary Table 5. Transfection protocols

Cell line	Solution	Reaction
697	SG	CA-137
RCH-ACV	SG	CM-137
REH	SF	CA-137

Supplementary Table 6. Kruskal-Wallis H test and Mann-Whitney U test was used to evaluate differential expression of *SOX11* in distinct leukemia subgroups

	Mann-Whitney U test (p-value)		
Groups	**Hemap**	**GSE47051**	**PanALL**
TCF3-PBX1 vs rest	9.09×10^{-20}	8.63×10^{-2}	1.69×10^{-27}
ETV6-RUNX1 vs rest	3.04×10^{-52}	1.81×10^{-9}	2.09×10^{-78}
	Kruskal-Wallis H test (p-value)		
	3.12×10^{-104}	3.16×10^{-7}	5.57×10^{-142}

Library preparation and RNA sequencing

The quality of the total RNA samples was ensured with Advanced Analytical Fragment Analyzer. Library preparation was done according to Illumina TruSeq® Stranded mRNA Sample Preparation Guide (part # 15031047) (Illumina, San Diego, CA, USA). The high quality of the libraries was confirmed with Advanced Analytical Fragment Analyzer and the concentrations of the libraries were quantified with Qubit® Fluorometric Quantitation (Life Technologies). Good quality libraries were exclusively selected and sequenced.

The samples were sequenced with Illumina HiSeq 2500 instrument using Truseq v2 Rapid sequencing chemistry. The samples were normalized and pooled for the automated onboard cluster preparation in HiSeq 2500. The 18 libraries were pooled into one pool and run in 2 lanes. Single-read sequencing with 1 x 50 bp read length was used, followed by 8 + 8 bp dual index run. The base calling was performed using Illumina's standard bcl2fastq2 software, automatic adapter trimming was used.

RNA sequencing and methylation analysis of patient samples

Briefly, strand-specific RNA-seq libraries were prepared from rRNA-depleted RNA using the ScriptSeq V2 Kit and paired-end sequenced to a minimum of 50 bp using a Hiseq2000/2500 or MiSeq instrument (Illumina), producing on average, 46 million read pairs per sample. Sequence reads were aligned to the human 1000 Genomes build 37 (GRCh37) using Tophat 2 (2.0.4) and gene expression levels were normalized to fragments per kilobase per million mapped reads (FPKM) using Cufflinks version 2.2.0.

Briefly, DNA methylation levels were measured using the Infinium HumanMethylation 450k BeadChip assay (Illumina). Intensity signals from the 450k array were converted to methylation beta values (ranging from 0-1) followed by probe filtering and normalization using Peak Based Correction as previously described [2].

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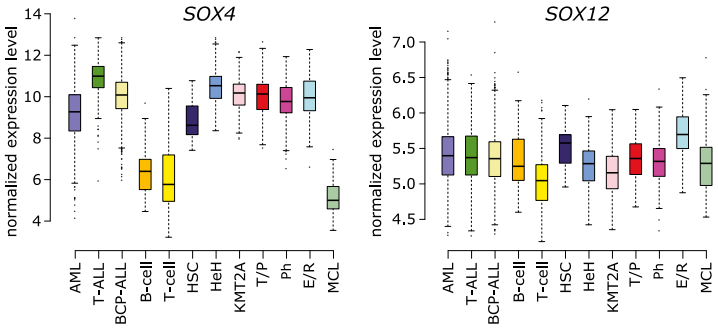

Supplementary Figure 1. *SOX4* and *SOX12* expressions. Expression boxplots of *SOX4* and *SOX12* in healthy cells, leukemias and mantle cell lymphomas. Data source: Combined microarray dataset [1,2]. ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; Ph, Philadelphia chromosome; BCP -ALL, B-cell precursor ALL; E/R, ETV6-RUNX1 subtype; HeH, high hyperdiploid subtype; HSC, hematopoietic stem cell; KMT2A, KMT2A rearrangement subtype; MCL, mantle cell lymphoma; T/P TCF3-PBX1 subtype.

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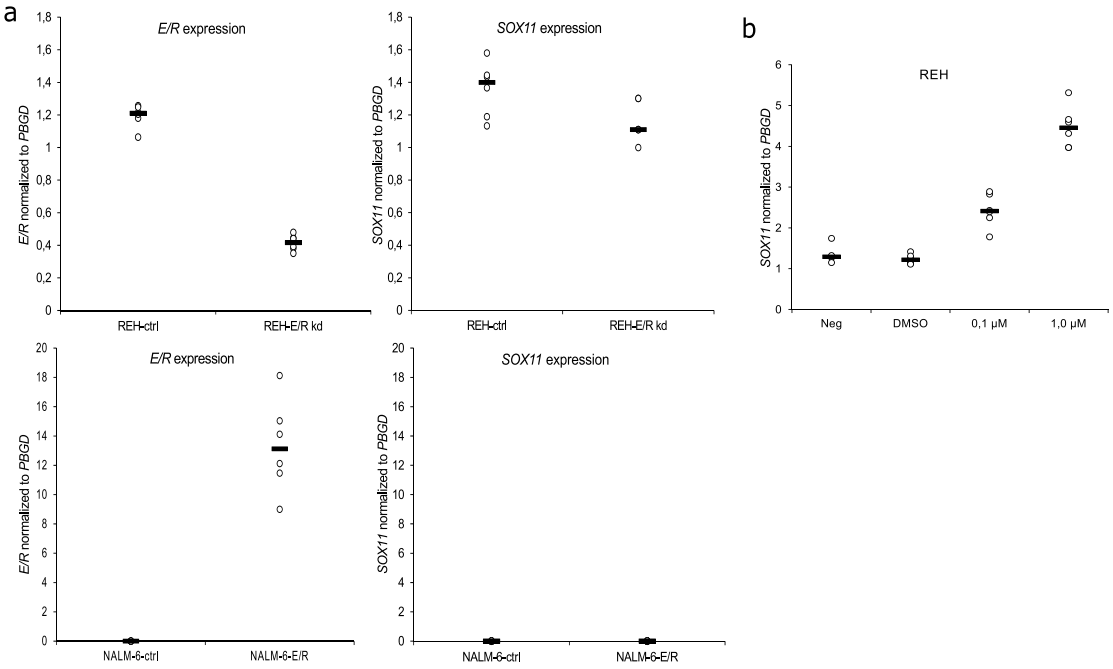
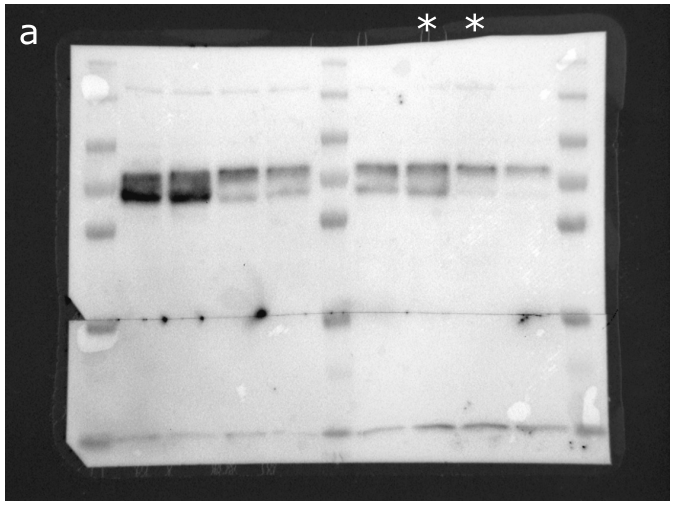
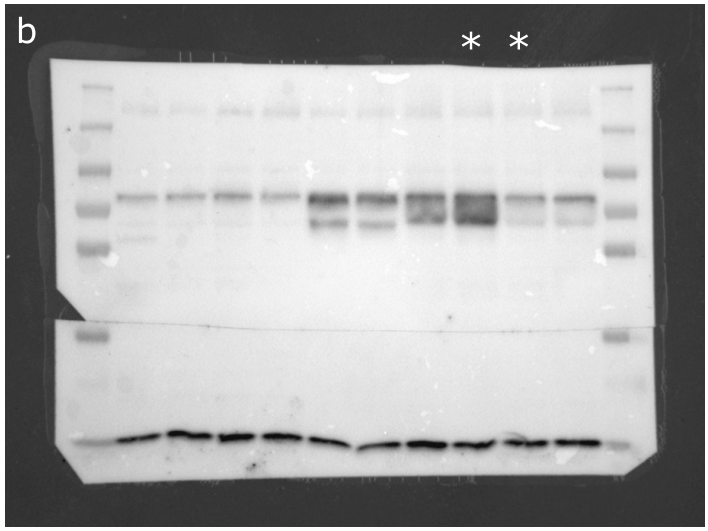
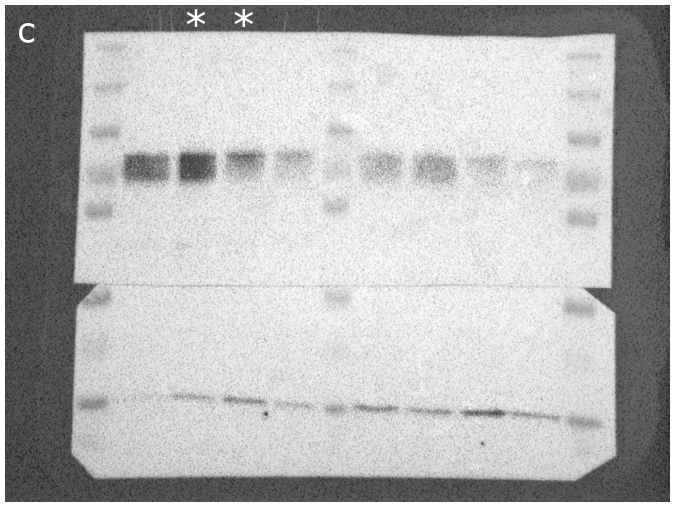


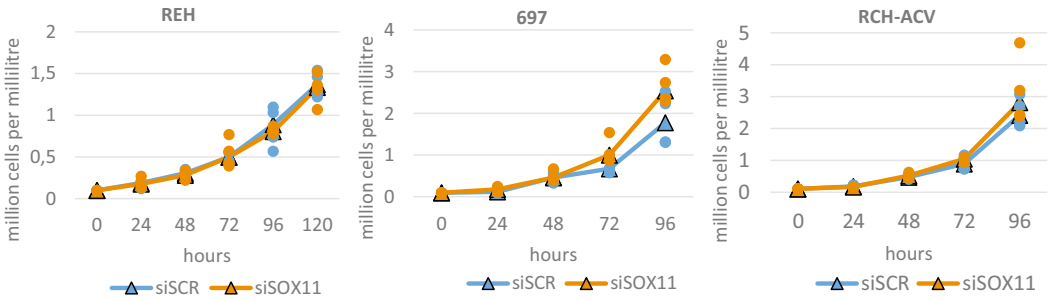
Supplementary Figure 2. *SOX11* expression. a. Changes in *E/R* expression has no marked change on *SOX11* expression (N=2). **b.** Effects of decitabine treatment on *SOX11* expression in REH cell line (N=2).



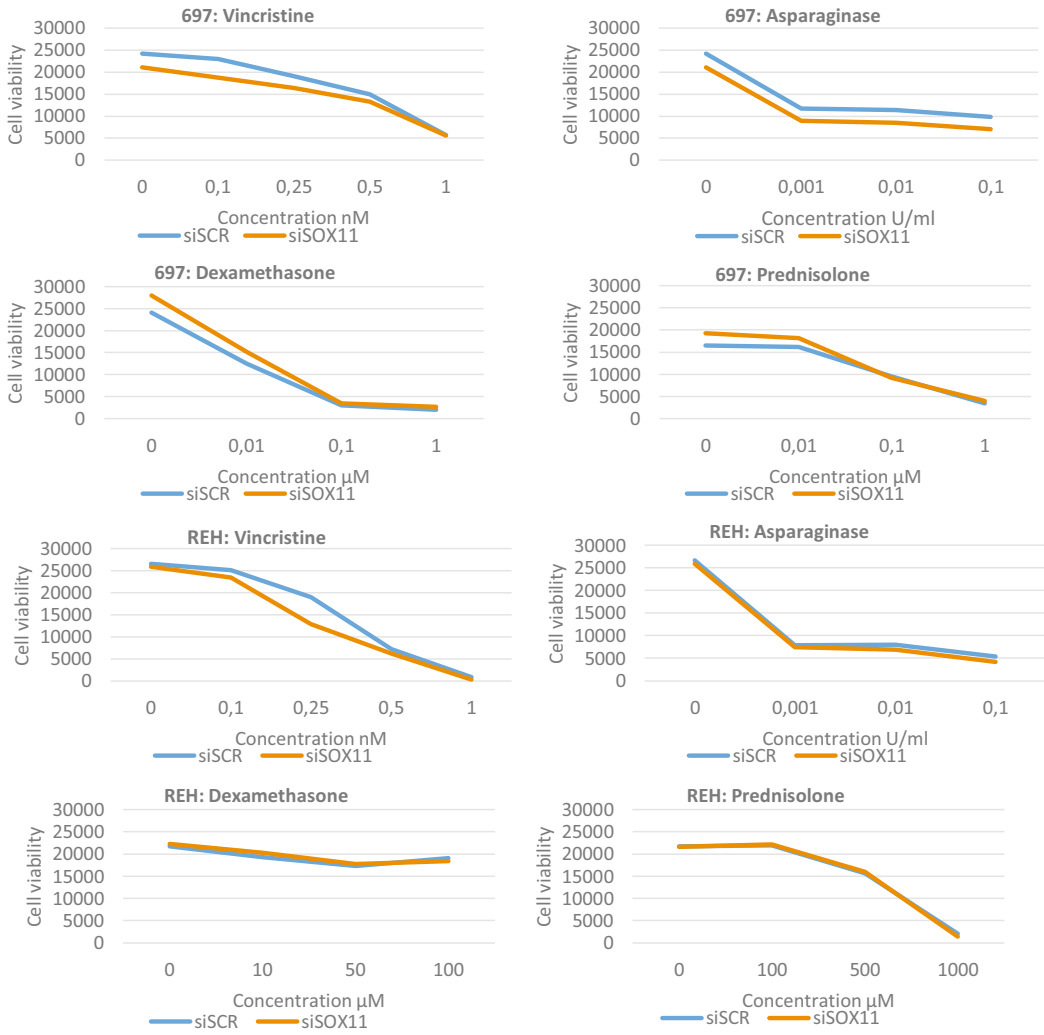




Supplementary Figure 3. Original Western Blots for Figure 4b. a. 697 **b.** RCH-ACV **c.** REH.
*) marks cropped lines for the figure 4 B western blots.

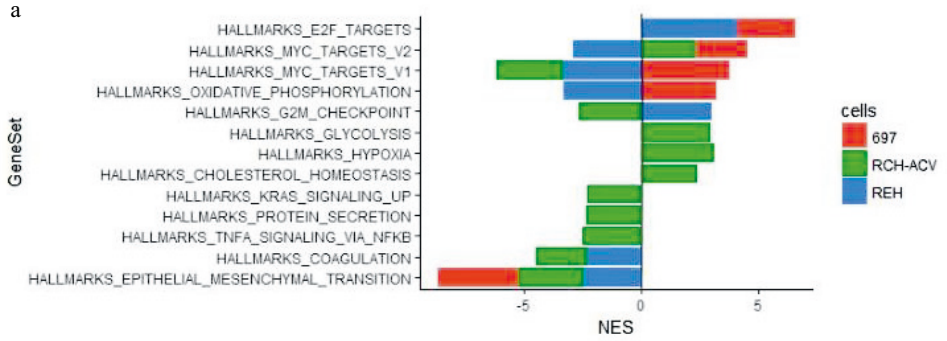


Supplementary Figure 4. Proliferation assay in REH, 697 and RCH-ACV cell lines after *SOX11* knockdown. Proliferation was assayed by counting the cells every 24 hours after the siRNA (siSOX11 = *SOX11* siRNA, siSCR = control siRNA) transfection. *SOX11* knockdown was confirmed with RT-qPCR and N=4.



Supplementary Figure 5. Chemotherapy agent and corticosteroid tretments on REH and 697 cell lines. Cell viabilities after siRNA transfection, *SOX11* siRNA (siSOX11) and control siRNA (siSCR) followed by drug treatments (697: 72 hours, REH: 96 hours; N=2). *SOX11* knockdown was confirmed with RT-qPCR and cell viability assessments were carried out with Alamar Blue assay.

a



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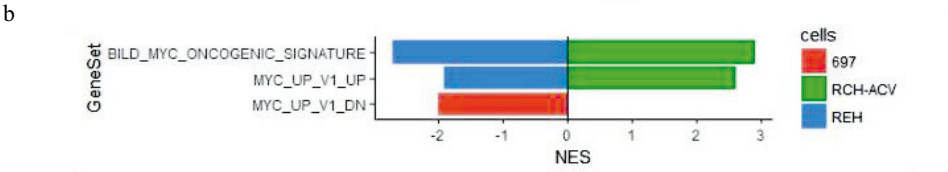

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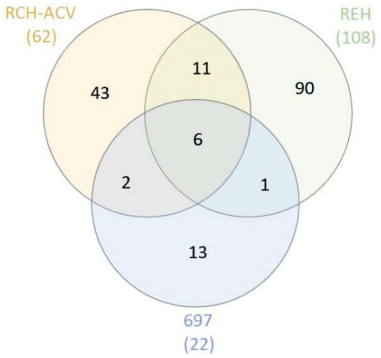


ALL (6)

- GO_HOMOPHILIC_CELL_ADHESION_VIA_PLASMA_MEMBRANE_ADHESION_MOLECULES
- GO_BIOLOGICAL_ADHESION
- GO_CELL_CELL_ADHESION_VIA_PLASMA_MEMBRANE_ADHESION_MOLECULES
- GO_CELL_CELL_ADHESION
- GO_TAXIS
- GO_POSITIVE_REGULATION_OF_CELL_DIFFERENTIATION

RCH-ACV and REH (11)

- GO_REGULATION_OF_MAPK_CASCADE
- GO_NEURON_DIFFERENTIATION
- GO_CELL_MOTILITY
- GO_CELL_MORPHOGENESIS_INVOLVED_IN_DIFFERENTIATION
- GO_POSITIVE_REGULATION_OF_IMMUNE_SYSTEM_PROCESS
- GO_NEURON_PROJECTION_DEVELOPMENT
- GO_IMMUNE_RESPONSE
- GO_REGULATION_OF_MAP_KINASE_ACTIVITY
- GO_MESENCHYMAL_CELL_DIFFERENTIATION
- GO_ACTIN_FILAMENT_BASED_PROCESS
- GO_REGULATION_OF_CELL_ACTIVATION

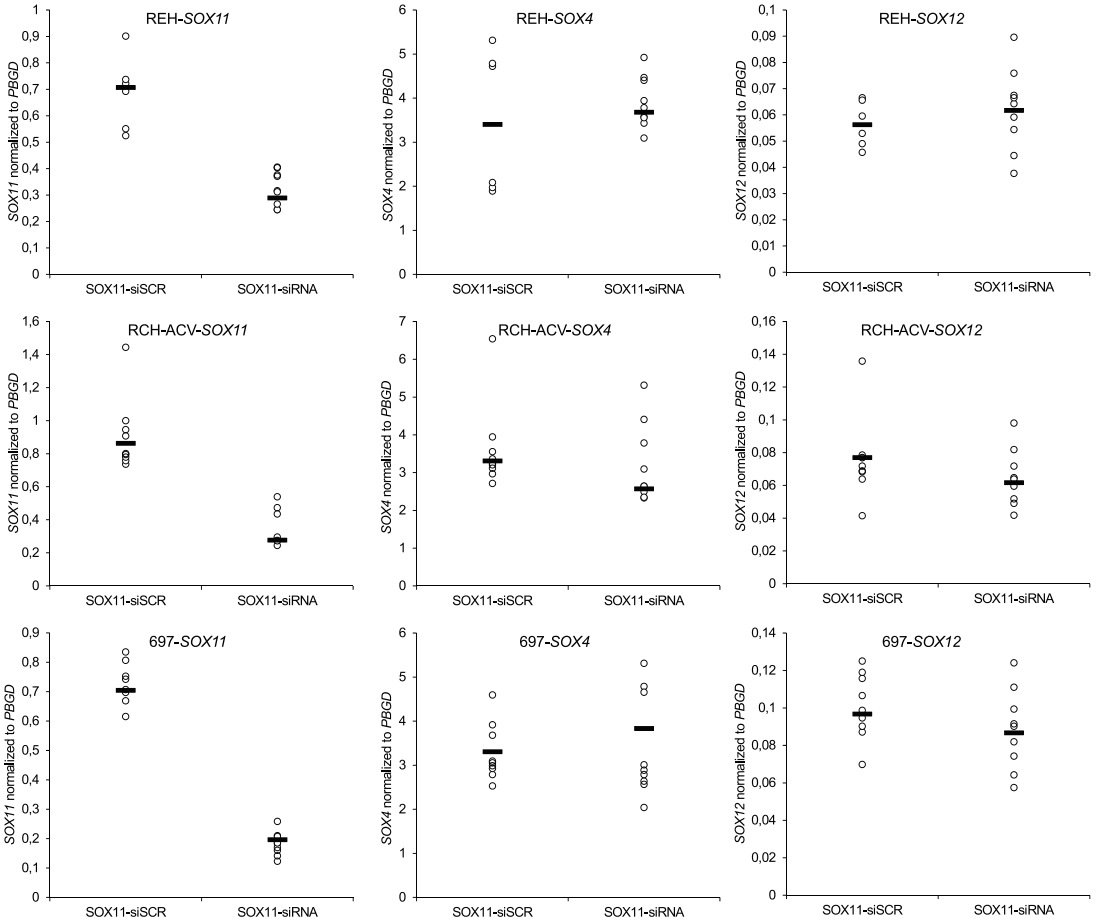
RCH-ACV and 697 (2)

- GO_REGULATION_OF_ERK1_AND_ERK2_CASCADE
- GO_NEUROLOGICAL_SYSTEM_PROCESS

REH and 697 (1)

- GO_REGULATION_OF_LEUKOCYTE_PROLIFERATION

Supplementary Figure 6. Enrichment of gene sets in 697, RCH-ACV and REH. a. Enrichment of hallmark gene sets retrieved from molecular signature database (MSigDB) in REH, RCH-ACV and 697 cell lines. Adjusted p-value of enrichment for all gene sets < 0.01. **b.** Enrichment of founder gene sets of "MYC targets V2" gene set in REH, RCH-ACV and 697 cell lines. Adjusted p-value of enrichment for all gene sets < 0.02. **c.** Venn diagram of significantly enriched GO terms in *SOX11* siRNA treated REH, RCH-ACV and 697 cell lines and list of GO terms overlapping between cell lines.



Supplementary Figure 7. SOX4 and SOX12 expressions after SOX11 knockdown. *SOX11* knockdown does not affect *SOX4* or *SOX12* expression levels in studied cell lines (N=3).

PUBLICATION III

IGF2BP3 Associates with Proliferative Phenotype and Prognostic Features in B-Cell Acute Lymphoblastic Leukemia

Mäkinen A, Nikkilä A, Haapaniemi T, Oksa L, Mehtonen J, Vänskä M, Heinäniemi M, Paavonen T, Lohi O.

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Article

IGF2BP3 Associates with Proliferative Phenotype and Prognostic Features in B-Cell Acute Lymphoblastic Leukemia

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Simple Summary: Although the prognosis of acute lymphoblastic leukemia (ALL) has improved significantly during the past decades, ALL remains a major cause of pediatric cancer mortality, and more accurate risk-stratification is required. We investigated *IGF2BP3*, which has previously been associated with aggressive cancers, and found high and subtype-specific expression of *IGF2BP3* in B-cell ALL, that was associated with good outcome in high-risk patients. Results suggest that *IGF2BP3* could be useful to improve stratification and prognosis of B-ALL.

Abstract: The oncofetal protein insulin-like growth factor 2 mRNA-binding protein 3 (*IGF2BP3*) belongs to a family of RNA-binding proteins involved in localization, stability, and translational regulation of target RNAs. *IGF2BP3* is used as a diagnostic and prognostic marker in several malignancies. Although the prognosis of pediatric B-cell acute lymphoblastic leukemia (B-ALL) has improved, a subgroup of patients exhibits high-risk features and suffer from disease recurrence. We sought to identify additional biomarkers to improve diagnostics, and we assessed expression of *IGF2BP3* in a population-based pediatric cohort of B-ALL using a tissue microarray platform. The majority of pediatric B-ALL cases were positive for *IGF2BP3* immunohistochemistry and were associated with an increased proliferative phenotype and activated STAT5 signaling pathway. Two large gene expression data sets were probed for the expression of *IGF2BP3*—the highest levels were seen among the B-cell lymphomas of a germinal center origin and well-established (KMT2A-rearranged and ETV6-RUNX1) and novel subtypes of B-ALL (e.g., NUTM1 and ETV6-RUNX1-like). A high mRNA for *IGF2BP3* was associated with a proliferative "metagene" signature and a high expression of *CDK6* in B-ALL. A low expression portended inferior survival in a high-risk cohort of pediatric B-ALL. Overall, our results show that *IGF2BP3* shows subtype-specificity in expression and provides prognostic utility in high-risk B-ALL.

Keywords: insulin-like growth factor 2 mRNA-binding protein 3 (*IGF2BP3*); mRNA; pediatric B-cell acute lymphoblastic leukemia; prognosis; proliferation; protein

1. Introduction

Pediatric B-cell acute lymphoblastic leukemia (B-ALL) is the most common malignancy in childhood. Despite the significantly improved prognosis, a subgroup of patients with either a poor therapy response or high-risk features still often experience a relapse. Better diagnostic tools are needed to enhance treatment stratification and prognosis, and to avoid overtreatment and adverse long-term side-effects [1–3].

Insulin-like growth factor II mRNA-binding protein 3 (*IGF2BP3*), also known as the *IGF2BP3* protein, is a 69 kDa protein that localizes mostly to the cytoplasm [4,5]. This oncofetal RNA-binding protein is a member of the IGF2BP-family, which also includes IGF2BP1 and IGF2BP2 proteins, and shares 59–73% similarity with the amino acid sequence with *IGF2BP3* [6,7]. *IGF2BP3* binds RNA molecules and acts as a regulator of mRNA localization and stability [7,8]. It is expressed only at a low level in most adult tissues, whereas in multiple human malignancies, it is overexpressed [7,8].

Mutations of *IGF2BP3* are rare, but the expression is dysregulated at epigenetic, transcriptional, and post-transcriptional levels. At a cellular level, *IGF2BP3* drives miRNA biogenesis; intercepts the cytoplasmic export of mRNA; and regulates mRNA stability, degradation, and transportation [7]. In gastrointestinal and urogenital malignancies, *IGF2BP3* is highly expressed, and is associated with cell adhesion, tumor invasion, metastasis, and inferior outcomes [7–9]. *IGF2BP3* exhibits a strong expression in lymphoid malignancies such as B cell lymphomas of a germinal center origin [10,11]. It is expressed in Reed–Sternberg cells and can be used as a supplementary diagnostic marker in Hodgkin's lymphoma [12–14]. Increased expression of *IGF2BP3* is associated with proliferative features in many solid tumors, mantle cell lymphoma, and chronic myeloid leukemia blast crisis [7,15,16], and promotes cell survival during ionizing radiation in B-cells [17].

Stoskus et al. [18] explored the expression of IGF2BP family members in hematopoietic tissues and ALL by using isoform-specific RT-qPCR. In healthy stem or mature hematopoietic cells, the expression of *IGF2BP3* was either weak or absent in contrast to *IGF2BP2*. The analysis of different mature cell populations demonstrated that only CD19+ B-cells expressed detectable levels of *IGF2BP3*, in line with previous literature [10,18,19]. Among B-ALL, the strongest expression was evident in ETV6-RUNX1 and KMT2A-rearranged subtypes. Liao et al. [16] and Palanichamy et al. [20] showed that siRNA or CRISPR-Cas9-mediated the knockdown of *IGF2BP3* reduced proliferation and increased apoptosis in several cell lines (K562, RS4;11, and NALM6).

While a growing body of data supports biological significance and prognostic utility of *IGF2BP3* in different epithelial and soft tissue tumors, to date, there are only two studies that have explored its expression in lymphoid leukemias [18,20], and no studies that have assessed expression at the protein level. Hence, we investigated the expression of *IGF2BP3* across hematological malignancies and in a trephine biopsy sample cohort of pediatric B-ALL and correlated its expression with cell proliferative features and patient survival.

2. Materials and Methods

2.1. Patient Cohort for Tissue Microarray and Immunohistochemistry

The formalin-fixed and paraffin-embedded bone marrow trephine biopsy samples of the pediatric B-ALL patients were collected into a tissue microarray (TMA) with 1.5 mm punches (see also [21]), and 4-micrometer TMA sections were used for immunohistochemistry. An appendix was used as a control material for the *IGF2BP3* and CD19/Ki-67 immunostainings. Immunohistochemistry was performed using the Ventana Benchmark Ultra instrument. BCL6 and pSTAT5 (Y694) immunohistochemistry was performed on whole tissue sections using Ventana Benchmark Classic [22]. The following antibodies were used: *IGF2BP3* (lot: 11085707, clone: 69.1, manufacturer: Dako, Santa Clara, CA, USA, id: M3626, dilution: 1:100, species: mouse monoclonal, Ig class: IgG2a, kappa), CD19 (lot: 000085227, clone: EP169, manufacturer: Cell Marque, Rocklin, CA, USA, id: 119R-18, dilution: ready-to-use, species: rabbit monoclonal, Ig class: IgG), Ki-67 (lot: F30644, clone: 30-9, manufacturer: Ventana, Tucson, AZ, USA, id: 790-4286, dilution: ready-to-use, species:

rabbit monoclonal, Ig class: IgG), BCL6 (lot: 48794, clone: LN22, id: PA0204, species: mouse monoclonal, manufacturer: Leica Biosystems, Newcastle, UK, dilution: 1:50), and pSTAT5 (Y694) (lot: GR208043, clone: E208, id: ab32364, manufacturer: Abcam, Cambridge, UK, dilution: 1:50). For the *IGF2BP3* and Ki-67 stainings, we used the OptiView DAB detection kit; for the CD19 stainings, the UltraView Universal Alkaline Phosphatase Red detection kit; and for BCL6 and pSTAT5 (Y694), the Ultraview Universal DAB detection kit. All of the slides were counterstained using hematoxylin. The expression of BCL6 and pSTAT5 was semiquantitatively graded as negative when antigen was expressed in under 20% of leukemic blasts, and positive when expressed in over 20%. Clinical data and the flow cytometry data (e.g., CD34 expression) were retrieved from patient hospital records gathered as described previously [21]. The flow cytometry results were graded as either negative or positive.

2.2. Image Analysis

Slides were scanned with Hamamatsu Nanozoomer XR using 40× magnification. QuPath software (version 0.2.3) [23] was used to detect cytoplasmic *IGF2BP3* positivity in TMA-sections from annotated areas with leukemic cells. A pathologist manually set detection parameters and thresholds using the cytoplasmic staining of *IGF2BP3* in germinal center cells as a reference, and the nuclear staining in germinal centers and proliferating epithelium as a reference for Ki-67 staining. The stain vectors and intensity thresholds for the cell and antibody detection were adjusted according to the instructions of the QuPath software in visual control. Inadequate samples were removed from the analysis. Areas with artifacts caused by compression or folding of the tissue were disregarded by setting the proper threshold values for background intensity. With the *IGF2BP3* and CD19/Ki-67-double-stained slides, stain vectors were adjusted for hematoxylin, 3,3′-diaminobenzidine (DAB), and alkaline phosphatase (AP) staining using a representative region of interest. Hematoxylin-stained cells were detected using the cell detection function in the QuPath, while the nuclear DAB of Ki-67-positive cells were recognized from the CD19-positive (AP) areas. Single intensity thresholds for *IGF2BP3*, CD19, and Ki-67 were used to assess the proportion of positive cells.

2.3. Microarray and RNA-Sequencing Data Sets

Hemap is a microarray gene expression data set that includes 6832 cancer samples and 1304 B-ALL samples (662 pediatric and 642 adult cases) [24,25]. The RNA-sequencing data set from the PanALL study cohort includes 1988 B-ALL cases (1234 pediatric and 754 adult cases) [26]. For the survival analyses, the TARGET data set, which includes 155 cases of pediatric high-risk B-ALL patients, was retrieved along with the following clinical information: events (relapse, induction failure, death, and second malignancy), survival, age, leukocyte count, minimal residual disease (MRD) at the end of induction (EOI), and the cytogenetic subtype [27,28].

2.4. Statistical Analysis

The statistical analysis was conducted using IBM SPSS Statistics (version 26) and RStudio (version 3.6.1). The Mann–Whitney U test, Kruskal–Wallis U test, chi-squared test, Fisher's exact, and log-rank test were used to test the significance of the differences between groups. All tests were two-sided, and p-values under 0.05 were considered statistically significant. The ComplexHeatmap package in R was used to create heatmaps [29]. Cox proportional hazards models were fitted for survival data in order to estimate the hazard of individual risk factors.

3. Results

3.1. IGF2BP3 Protein Is Widely Expressed in Pediatric B-ALL

The *IGF2BP3* protein has shown diagnostic and prognostic utility in different malignancies [7]. To assess the expression of the *IGF2BP3* protein in B-ALL, we employed a

population-based pediatric cohort of 83 B-ALL cases, and immunostained the diagnostic bone marrow trephine biopsies embedded in a tissue microarray (TMA) with an antibody against *IGF2BP3*. The case summary for the TMA samples is shown in Table 1. The appendix was used as a positive control, and it was stained positively in the germinal centers of the lymphoid follicles, as expected (Figure 1A) [10]. Positivity (>1%) to *IGF2BP3* was detected in 74 out of 83 patients (89%; Figure 1B–D), while the proportion of positively stained leukemia cells ranged from 1 to 100% (median 34%). *IGF2BP3* exhibited a granular staining pattern and was localized mostly to the cytoplasm. Negative *IGF2BP3* staining was found in 9 out of 83 B-ALL cases (Figure 1E). No expression of *IGF2BP3* was found in the remission bone marrow specimens.

Table 1. Case summary of the tissue microarray (TMA) cohort.

Clinical Parameter	Median (IQR)
Age (years)	4.3 (2.7–9.7)
WBC (x 10E9/l)	6.3 (2.7–29.2)
MRD (%), EOI	0.01 (0.00–0.14)
	***n* (%)**
CNS disease	5 (6.0)
Total	83
WHO Subtype	
Other	32 (38.6)
BCR-ABL1	1 (1.2)
KMT2A-re	4 (4.8)
ETV6-RUNX1	20 (24.1)
Hyperdiploid	22 (26.5)
Hypodiploid	1 (1.2)
TCF3-PBX1	3 (3.6)

EOI—end of induction; IQR—interquartile range; KMT2A-re—KMT2A-rearranged; MRD—minimal residual disease; WBC—white blood cell count; WHO—World Health Organization.

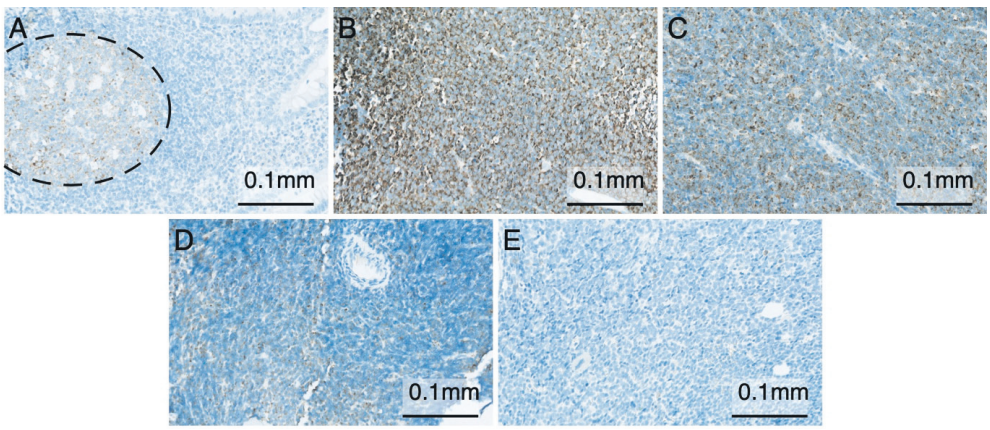

Figure 1. Immunohistochemistry of insulin-like growth factor II mRNA-binding protein 3 (*IGF2BP3*). (**A**) Appendix showing positivity (brown color) to *IGF2BP3* in the germinal center (dashed circle; 200× magnification). (**B**) Strongly *IGF2BP3*-positive bone marrow trephine biopsy of a B-cell acute lymphoblastic leukemia (B-ALL) patient (200× magnification). (**C**) Pediatric B-ALL case with a heterogeneous pattern of *IGF2BP3* expression (200× magnification). (**D**) Weakly *IGF2BP3*-positive B-ALL case with only singular positive cells visible (200× magnification). (**E**) *IGF2BP3*-negative B-ALL case (200× magnification).

We classified the cases into distinct subtypes according to the WHO 2017 Classification of B-ALL [30]. Expression of *IGF2BP3* protein was highest in the ETV6-RUNX1, "Other", KMT2A-rearranged, and hypodiploid subtypes (Figure 2A). The difference was

statistically significant between ETV6-RUNX1 and other subtypes (Mann Whitney U Test *p*-value = 0.04). The expression of *IGF2BP3* protein did not correlate with white blood cell count (WBC), MRD at the end of induction (EOI), CNS disease, or expression of specific cell surface markers.

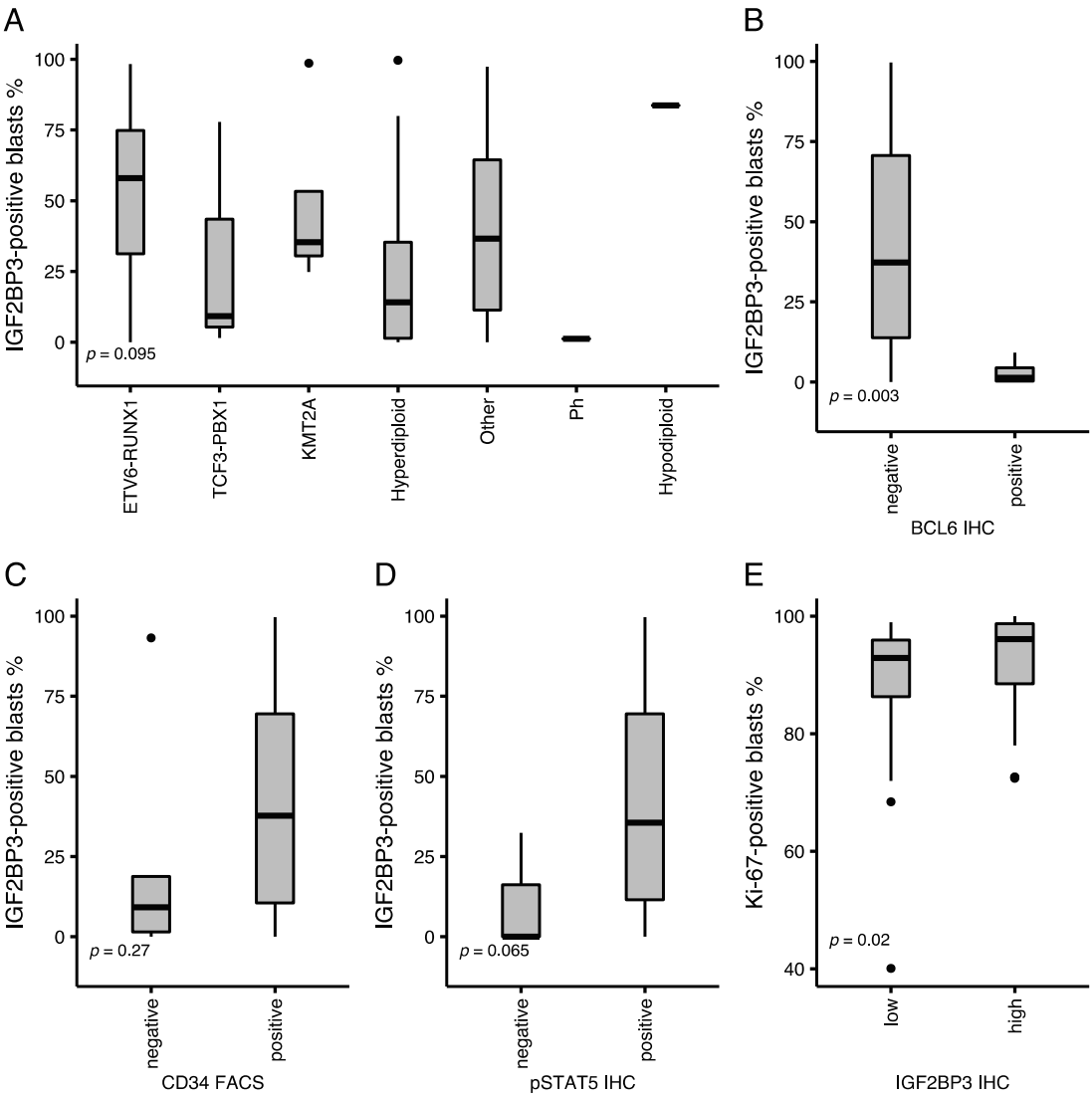

Figure 2. Immunophenotype of a B-cell acute lymphoblastic leukemia (B-ALL) tissue microarray cohort. (**A**) Expression of *IGF2BP3* according to the WHO classification of B-ALL. (**B**) Positivity to *IGF2BP3* among cases with either a negative or positive expression of the BCL6 protein. (**C**) Positivity to *IGF2BP3* in cases with negative or positive CD34. (**D**) Positivity to *IGF2BP3* in cases with a negative or positive pSTAT5 (Y694). (**E**) Expression of Ki-67 among cases with either a low or high *IGF2BP3* (median as a cut-off). Dots depict outliers. *p*-values of (**B**–**E**) Mann–Whitney U test and (**A**) Kruskal–Wallis test are shown.

IGF2BP3 is normally expressed in germinal centers, where the BCL6 protein is active and associated with germinal center-type B-cell lymphomas [10]. We recently showed that

the BCL6 protein is also expressed in a fraction of precursor B-ALL [22]. BCL6-positivity, CD34-negativity, and pSTAT5-negativity have been associated with a novel pre-B-cell receptor signaling subtype of B-ALL [31]. Hence, we tested the association between *IGF2BP3* and BCL6 proteins and discovered that the *IGF2BP3* protein was significantly lower among the BCL6-positive cases (Mann–Whitney U test; p-value = 0.003). Likewise, the mRNA expression of the pre-BCR "metagene" (see below), which is associated with BCL6-positivity [31], exhibited a significantly lower expression among the highest 10th percentile of the *IGF2BP3* expressing patients in the PanALL and Hemap data sets (Mann–Whitney U; p-value < 0.001). On the contrary, cases that exhibited phosphorylated STAT5 (pY694) protein or showed a high expression of the stem cell marker CD34 evidenced a higher-than-median level of the *IGF2BP3* protein (Figure 2B–D).

3.2. Expression of Ki-67 Is Associated with High IGF2BP3 Protein Expression

A high expression of *IGF2BP3* has been associated with proliferative phenotype in malignancies such as mantle cell lymphoma [7,15]. We assessed whether it is associated with cell proliferation in B-ALL by co-staining the trephine biopsy specimens with CD19, a marker of blast cells, and Ki-67, a well-established marker of cell proliferation [32,33]. Overall, the expression of Ki-67 was strong in proliferating cells of germinal centers and the epithelium of appendix (Figure S1A), and in CD19-positive cells of B-ALL samples (Figure S1B,C; proportion of positive cells, median 95%, interquartile range (IQR) 87–98%). A higher-than-median level of *IGF2BP3* was significantly associated with the expression of Ki-67 (Mann–Whitney U test; p-value = 0.02; Figure 2E).

3.3. Expression of IGF2BP3 in Hematological Malignancies and B-ALL

IGF2BP3 is associated with various malignancies of a B-cell origin, and particularly with germinal center lymphomas [10,11]. To get a comprehensive picture across hematological tumors, we assessed *IGF2BP3* mRNA levels in 6832 hematological cancers that included 24 different disease entities [24,25]. The median expression of the *IGF2BP3* mRNA was the highest in B-ALL, Burkitt lymphoma, diffuse large B-cell lymphoma, follicular lymphoma, mantle cell lymphoma, and juvenile myelomonocytic leukemia, while the lowest median expressions were observed in hairy cell leukemia, hepatosplenic T-cell lymphoma, and adult T-cell leukemia (Figure 3A). The *IGFBP3* mRNA was present in all subtypes of B-ALL, with the highest expression in the KMT2A-rearranged and ETV6-RUNX1 subtypes and the lowest in the TCF3-PBX1 and BCR-ABL1 subtypes (Figure 3B). Analysis of the PanALL data set [26], which comprises 1988 B-ALL cases, validated the findings, and also revealed a strong expression in novel subtypes such as NUTM1-rearranged, PAX5-altered, ETV6-RUNX1-like, BCL2/MYC, and CRLF2 (Figure 3C).

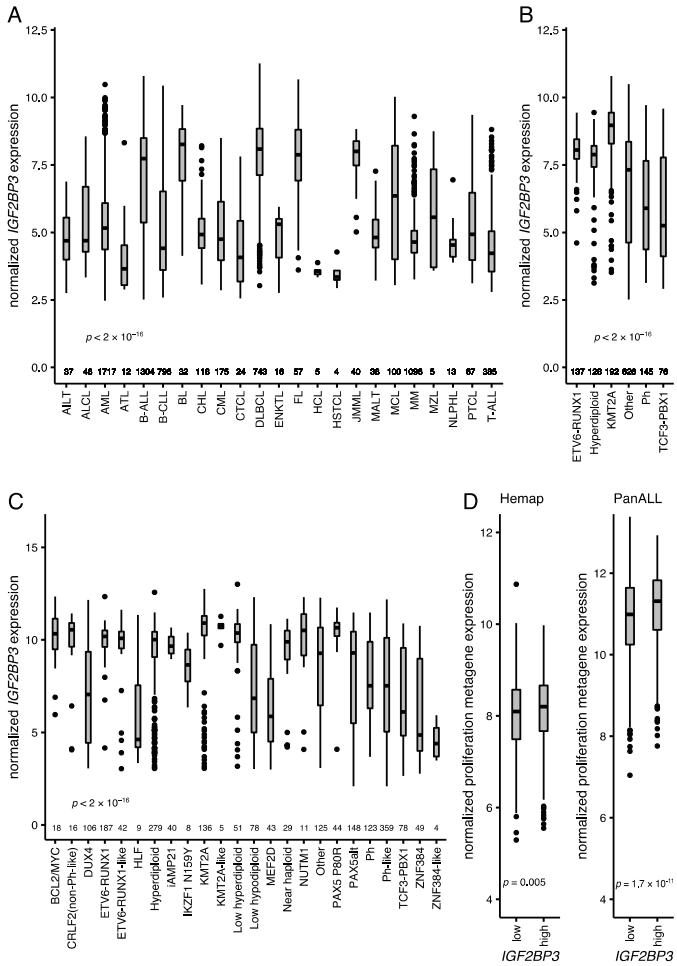

Figure 3. Expression of *IGF2BP3* across different hematological malignancies and subtypes of B-ALL. (**A**) *IGF2BP3* expression in the Hemap data set in different hematological malignancies (n = 6832) [24,25]. (**B**) *IGF2BP3* expression in different cytogenetic subtypes of B-ALL (n = 1304) in the Hemap data set [24,25]. (**C**) *IGF2BP3* expression in different B-ALL subtypes of B-ALL in the PanALL study cohort (n = 1988) [26]. (**D**) Proliferation-associated "metagene" [33] expression in B-ALL in the Hemap and PanALL data sets (median as a cut-off for the *IGF2BP3* expression groups). AILT—angioimmunoblastic T-cell lymphoma; ALCL—anaplastic large cell lymphoma; AML—acute myeloid leukemia; ATL—adult T-cell leukemia; B-ALL—B-cell lineage acute lymphoblastic leukemia; B-CLL—B-cell chronic lymphocytic leukemia; BCL2/MYC—*BCL2/MYC*-rearranged; BL—Burkitt lymphoma; CHL—classic Hodgkin lymphoma; CML—chronic myeloid leukemia; CRLF2—*CRLF2* (non-Ph-like); CTCL—cutaneous T-cell lymphoma; DLBCL—diffuse large B-cell lymphoma; DUX4—*DUX4*-rearranged; ENKTL—extranodal NK/T-cell lymphoma; FL—follicular lymphoma; HCL—hairy cell leukemia; HLF—*TCF3/TCF4-HLF*; HSTCL—hepatosplenic T-cell lymphoma; iAMP21—intrachromosomal amplification of chromosome 21; IKZF1 N159Y—*IKZF1* missense alteration encoding p.Asn159Tyr; JMML—juvenile myelomonocytic leukemia; KMT2A—KMT2A-rearranged; MALT—extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue; MCL—mantle cell lymphoma; MEF2D—*MEF2D*-rearranged; MM—multiple myeloma; MZL—marginal zone lymphoma; n—number of cases; NLPHL—nodular lymphocyte predominant Hodgkin lymphoma; NUTM1—*NUTM1*-rearranged; PAX5alt—PAX5 alterations; PAX5 P80R—PAX5 p.Pro80Arg (P80R) alteration; Ph—Philadelphia chromosome (*BCR-ABL1*); PTCL—peripheral T-cell lymphoma, not otherwise specified; T-ALL—T-cell lineage acute lymphoblastic leukemia; ZNF384—*ZNF384*-rearranged. Dots depict outliers. p-values of (**D**) Mann–Whitney U test and (**A**–**C**) Kruskal–Wallis test are shown.

3.4. Proliferative "Metagene" Signature in B-ALL

Recently, Giuliano et al. (2018) [33] described a proliferative "metagene" (*MKI67*, *PCNA*, *CCNB1*, *MCM2*, and *TOP2A*) that is correlated with cell proliferation. Supporting our earlier observations, the *IGF2BP3* mRNA was significantly associated with the proliferative "metagene" when assessed across all hematological malignancies (Mann–Whitney U test; p-value < 0.001). When the analysis was restricted to the B-ALL cases, the "metagene" signature and the *MKI67* mRNA showed elevated levels and were significantly associated with a higher-than-median expression of *IGF2BP3* (Mann–Whitney U test; p-value = 0.005 and p-value = 0.04, respectively; Figure 3D). A similar analysis in the PanALL data set replicated the findings: the *IGF2BP3* mRNA was significantly associated with the high *MKI67* mRNA (Mann–Whitney U test, p-value < 0.001) and the proliferation-associated "metagene" with a discretized expression of *IGF2BP3* (Mann–Whitney U test, p-value < 0.001; Figure 3D; see also heatmap in Figure S2A–C).

CDK6 and MYC oncoproteins have been reported as targets of the *IGF2BP3* protein [20]. In the PanALL data set, *CDK6* was higher and *MYC* was lower among cases with a higher-than-median *IGF2BP3* mRNA (Mann–Whitney U test p-value < 0.001, Figure S3A,B). In the Hemap data set, *CDK6* was strongly expressed among cases with a higher-than-median *IGF2BP3* mRNA, whereas the expression of *MYC* did not differ (Figure S3C,D).

3.5. High IGF2BP3 mRNA Associates with Favorable Survival in High-Risk B-ALL

The prognostic value of *IGF2BP3* mRNA was evaluated in the TARGET data set that included high-risk pediatric B-ALL cases [27,28]. Higher-than-median *IGF2BP3* mRNA showed a statistically significant association with favorable event-free (EFS) and overall survival (OS; Figure 4A,B). In a multivariate analysis that included age, white blood cell count (WBC), and minimal residual disease (MRD) at the EOI as covariates, higher-than-median *IGF2BP3* mRNA exhibited a decreased hazard ratio for events (HR 0.46, 95% CI 0.31–0.68) and death (HR 0.50, 95% CI 0.31–0.81; Table 2).

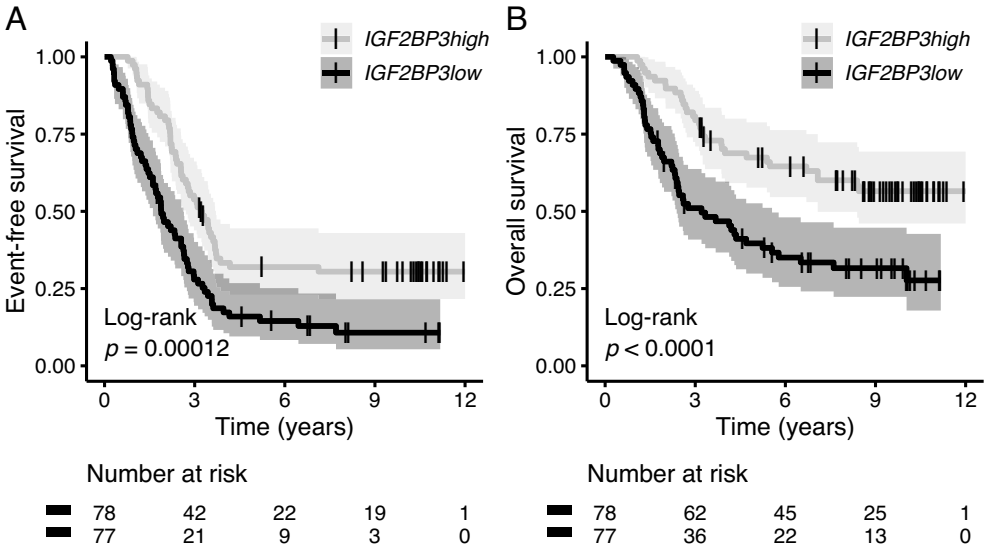

Figure 4. Association of *IGF2BP3* expression at an mRNA level on patient survival. Kaplan–Meier survival analysis for (**A**) event-free survival and (**B**) overall survival in the high-risk B-ALL TARGET cohort (n = 155) [27,28]. Statistical significance was tested using the log-rank test, while the median expression of *IGF2BP3* was used as a cut-off for the two different patient groups.

Table 2. Cox proportional hazards model for survival in the high-risk B-ALL TARGET cohort (n = 155) [27,28].

Event-Free Survival							
		Univariate			Multivariate		
		HR	95% CI	*p*	HR	95% CI	*p*
IGF2BP3 mRNA	≤median	1			1		
	>median	0.49	0.34–0.71	<0.001	0.46	0.31–0.68	<0.001
Age		1.01	0.97–1.05	0.69	0.97	0.93–1.01	0.19
MRD at the EOI		1.02	0.98–1.07	0.39	1.01	0.97–1.06	0.58
Overall survival							
		Univariate			Multivariate		
		HR	95% CI	*p*	HR	95% CI	*p*
IGF2BP3 mRNA	≤median	1			1		
	>median	0.44	0.28–0.68	<0.001	0.5	0.31–0.81	0.006
Age		1.06	1.01–1.11	0.01	1.03	0.98–1.08	0.24
WBC		1	1.00–1.00	0.58	1	0.99–1.00	0.68
MRD at the EOI		1.02	0.97–1.08	0.47	0.99	0.94–1.05	0.79

CI—confidence interval; EOI—end of induction therapy; HR—hazards ratio; MRD—minimal residual disease; WBC—white blood cell count at diagnosis.

In the population-based TMA cohort, positivity to *IGF2BP3* protein did not associate with patient survival (data not shown).

4. Discussion

IGF2BP3 is an oncofetal protein that is normally expressed in fetal/embryonic tissues, but is often aberrantly re-expressed in malignant tumors. In solid tumors, its expression is associated with increased proliferation and inferior outcomes. We report here that *IGF2BP3* is widely expressed in pediatric B-ALL, and shows a granular staining pattern fitting to the cytoplasmic ribonucleoprotein (RNP) complexes. Its expression is associated with proliferative features and a high level of *CDK6*, while a low expression confers inferior outcomes in a cohort of high-risk B-ALL patients.

Earlier gene expression studies have associated *IGF2BP3* with the ETV6-RUNX1 and KMT2A-rearranged subtypes of B-ALL [18,20]. This aligns well with our results, which were based on large gene expression microarray (Hemap) and RNA-sequencing data sets (PanALL). As novel findings, we report here a strong expression of *IGF2BP3* in new subtypes of B-ALL, such as ETV6-RUNX1-like, KMT2A-like, and NUTM1. Moreover, we extended analyses across the whole spectrum of hematological malignancies covering 24 diseases: B cell lymphomas were the strongest expressors, followed by juvenile myelomonocytic leukemia and mantle cell lymphoma, while most diseases of T-cell origin showed a lower expression.

At the protein level, our data are unique. We developed a population-based TMA platform that included 83 pediatric B-ALL cases. Almost 90% of the cases showed moderate or strong positivity to *IGF2BP3*, often in conjunction with a marker of activated JAK-STAT signaling (pSTAT5A-Y694). Fittingly, the expression of the BCL6 protein, which is not exhibited simultaneously with the activated JAK-STAT5 pathway [34], was absent among *IGF2BP3*-positive cases. The intracellular staining pattern of *IGF2BP3* was granular, possibly referring to the localization of *IGF2BP3* to cytoplasmic RNP complexes, where it exerts its function on the target mRNAs. One limitation of the immunohistochemistry results is the possibility of the cross-reactivity of the used *IGF2BP3* antibody (and other commercial *IGF2BP3* antibodies) with the paralogs of IGF2BP family [7,35,36].

By using the TMA platform, we performed co-stainings with a proliferative marker for Ki-67 and CD19, and showed that the *IGF2BP3* protein is associated with active cellular proliferation. This is not a surprise, as B-ALL is an aggressive malignancy with a high proliferative capacity. At the mRNA level, the proliferation-associated "metagene" signature was higher in patients with a strong expression of *IGF2BP3*. Although the absolute differences were small, the results fit well with earlier data in mantle cell lymphoma

and solid tumors, where the expression of *IGF2BP3* was similarly associated with proliferation [7,15]. Likewise, a study by Palanichamy et al. [20] showed that the exogenous expression of *IGF2BP3* increased the proliferation of bone marrow progenitor cells and provided them with a competitive survival advantage, and that *IGF2BP3* was essential for the survival of several B-ALL cell lines. Overall, *IGF2BP3* seems to play an active role in the proliferative capacity of B cell blasts, a feature that could possibly be utilized for diagnostic or therapeutic purposes.

We correlated the expression of *IGF2BP3* with the patient outcome in a high-risk pediatric B-ALL cohort, in which a higher-than-median *IGF2BP3* mRNA level was associated with improved survival. However, no association with outcome was evident for the *IGF2BP3* protein in our population-based biopsy cohort, possibly because of its relatively small size or low number of events. A recent single cell analysis of early therapy response in B-ALL showed that patients with high proliferative features are more sensitive to induction chemotherapy, and that therapy-resistant clones are more likely among the quiescent cell populations [37]. Hence, *IGF2BP3* expression is associated with cell populations that are actively dividing and more are likely to be killed by chemotherapy, which is reflected in the overall outcome of patients. We note that the prognostic effect was evident only in the high-risk cohort, and therefore in the future, outcome data need further exploration in larger population-based data sets.

5. Conclusions

In conclusion, we found that a high expression of *IGF2BP3* is associated with a proliferative phenotype in pediatric B-ALL at mRNA and protein levels, and portends a favorable survival high-risk B-ALL. Our results show that the subtype-specific expression of *IGF2BP3* provides diagnostic and prognostic utility in B-ALL.

Supplementary Materials: The following are available online at https://www.mdpi.com/2072-6694/13/7/1505/s1, Figure S1: Immunohistochemical co-staining of CD19 and Ki-67 in B-ALL, Figure S2: Heatmap illustration of expression of proliferation-associated genes (*MKI67*, *PCNA*, *CCNB1*, *MCM2*, and *TOP2A*) in cases with either a high or low expression of *IGF2BP3*, Figure S3: Boxplots showing expression of *CDK6* and *MYC* in cases with either a low or high expression of *IGF2BP3* in B-ALL.

Author Contributions: Conceptualization, O.L. and A.M.; data curation, A.M., A.N., J.M. and M.H.; formal analysis, A.M., A.N. and O.L.; funding acquisition, A.M., M.H. and O.L.; investigation, A.M.; methodology, A.M., T.H., T.P. and O.L.; project administration, O.L.; resources, O.L.; software, A.N.; supervision, M.V., M.H., T.P. and O.L.; validation, A.N.; visualization, A.M., L.O. and A.N.; writing—original draft, A.M. and O.L.; writing—review and editing, A.M. and O.L. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The results published here are in whole or part based upon data generated by the Therapeutically Applicable Research to Generate Effective Treatments (https://ocg.cancer.gov/programs/target) initiative, phs000463. The data used for this analysis are available at https://portal.gdc.cancer.gov/projects (accessed on 27 August 2020).

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IGF2BP3 Associates with Proliferative Phenotype and Prognostic Features in B-Cell Acute Lymphoblastic Leukemia

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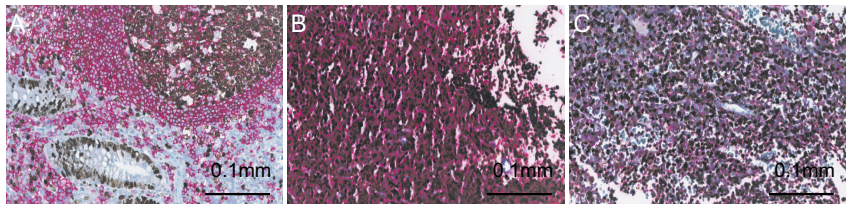

Figure S1. Immunohistochemical co-staining of CD19 and Ki-67 in B-ALL. (**A**) CD19 (red) and Ki-67 (brown) in the appendix show a high expression of Ki-67 in the germinal center (upper right) and crypt epithelium (lower left; 200X magnification). (**B**) Co-staining of CD19 and Ki-67 in the B-ALL case with a high proportion of blasts showing positivity to nuclear Ki-67 staining. (**C**) B-ALL case showing partial expression of the nuclear Ki-67 protein (200X magnification).

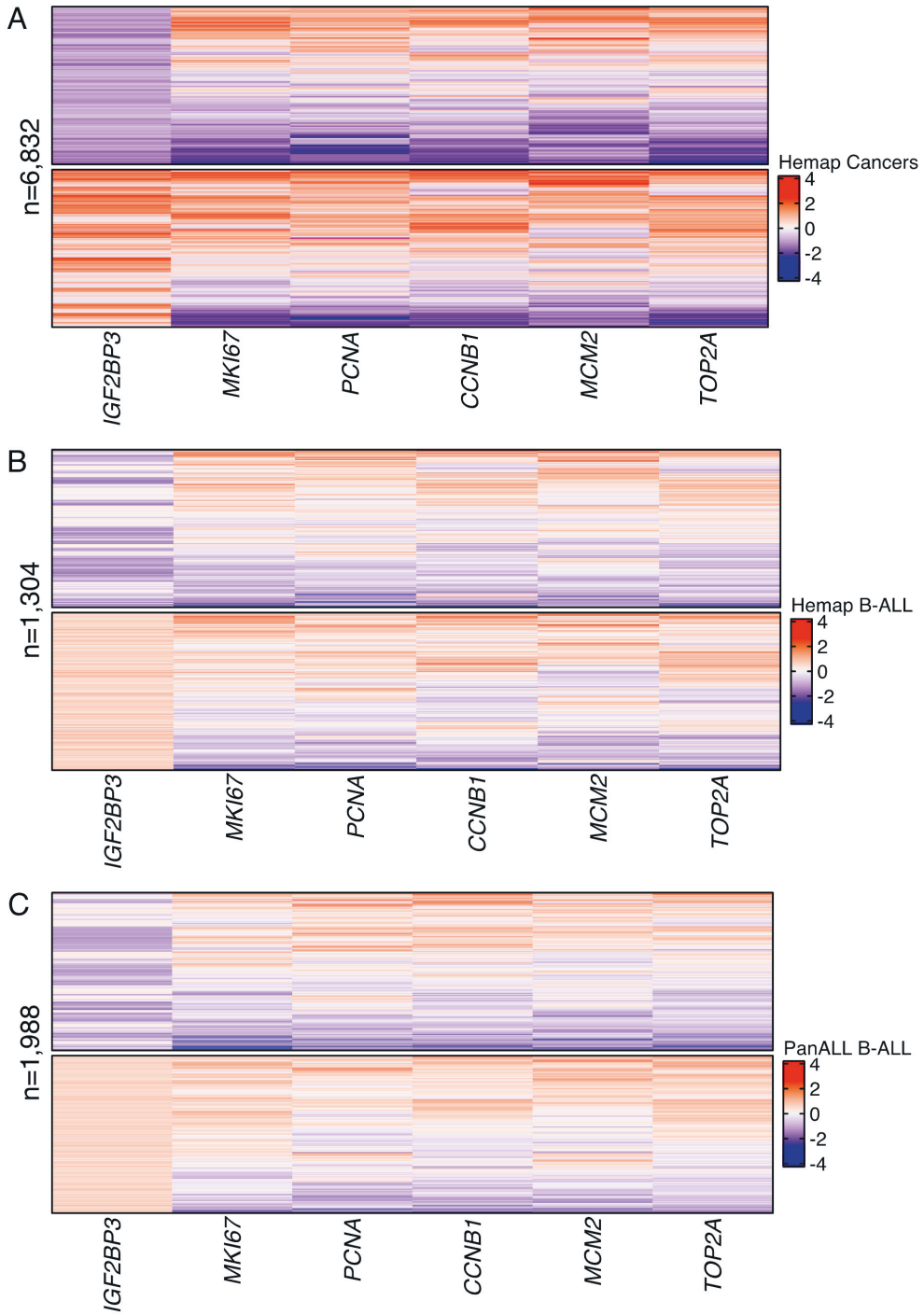

Figure S2. Heatmap illustration of the expression of proliferation-associated genes (*MKI67*, *PCNA*, *CCNB1*, *MCM2*, and *TOP2A*) in cases with either a high or low expression of *IGF2BP3* [29]. (**A**) Heatmap illustrations using (**A**) all hematological cancers or (**B**) B-ALL from the Hemap data set [24,25], and (**C**) B-ALL cases from the PanALL data set [26].

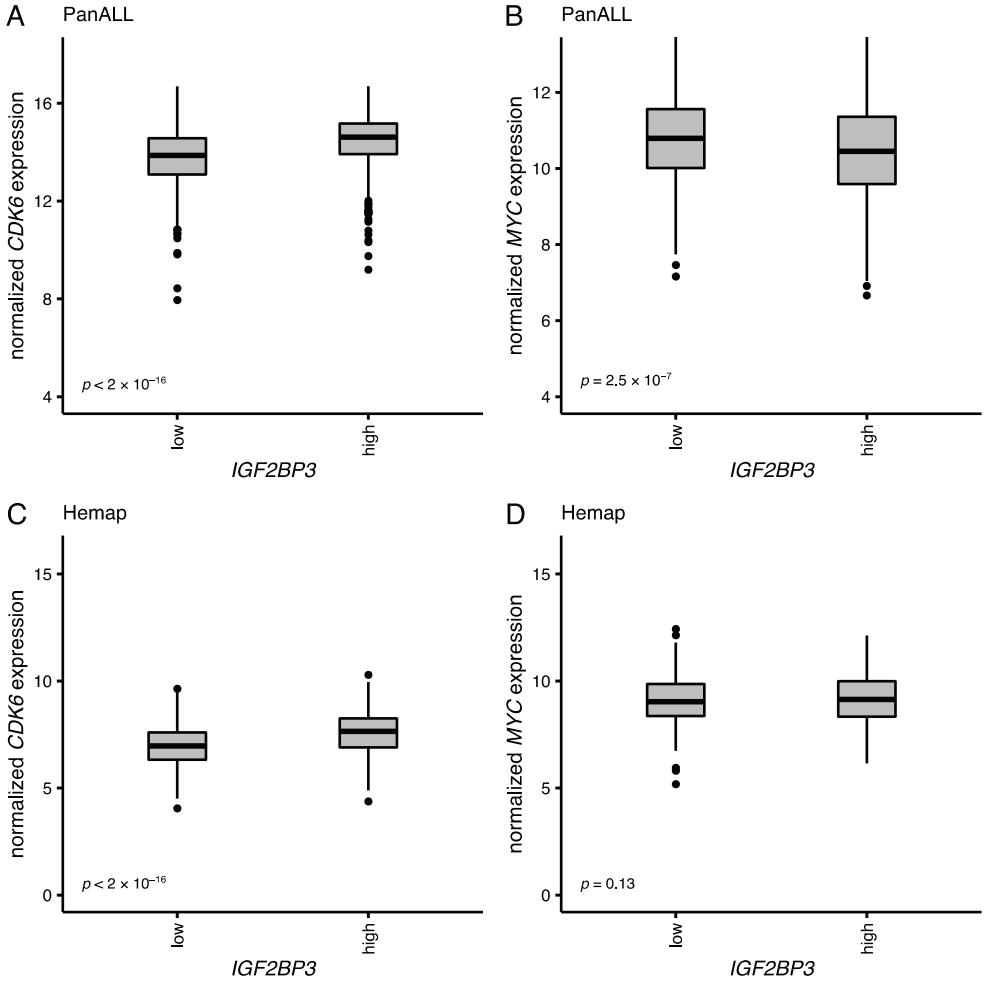

Figure S3. Boxplots showing expression of *CDK6* and *MYC* in cases with either a low or high expression of *IGF2BP3* in B-ALL for both of the data sets: (**A,B**) PanALL data set and (**C,D**) Hemap data set [24–26]. Dots represent outliers. The *p*-values of the Mann–Whitney U tests between groups are shown.