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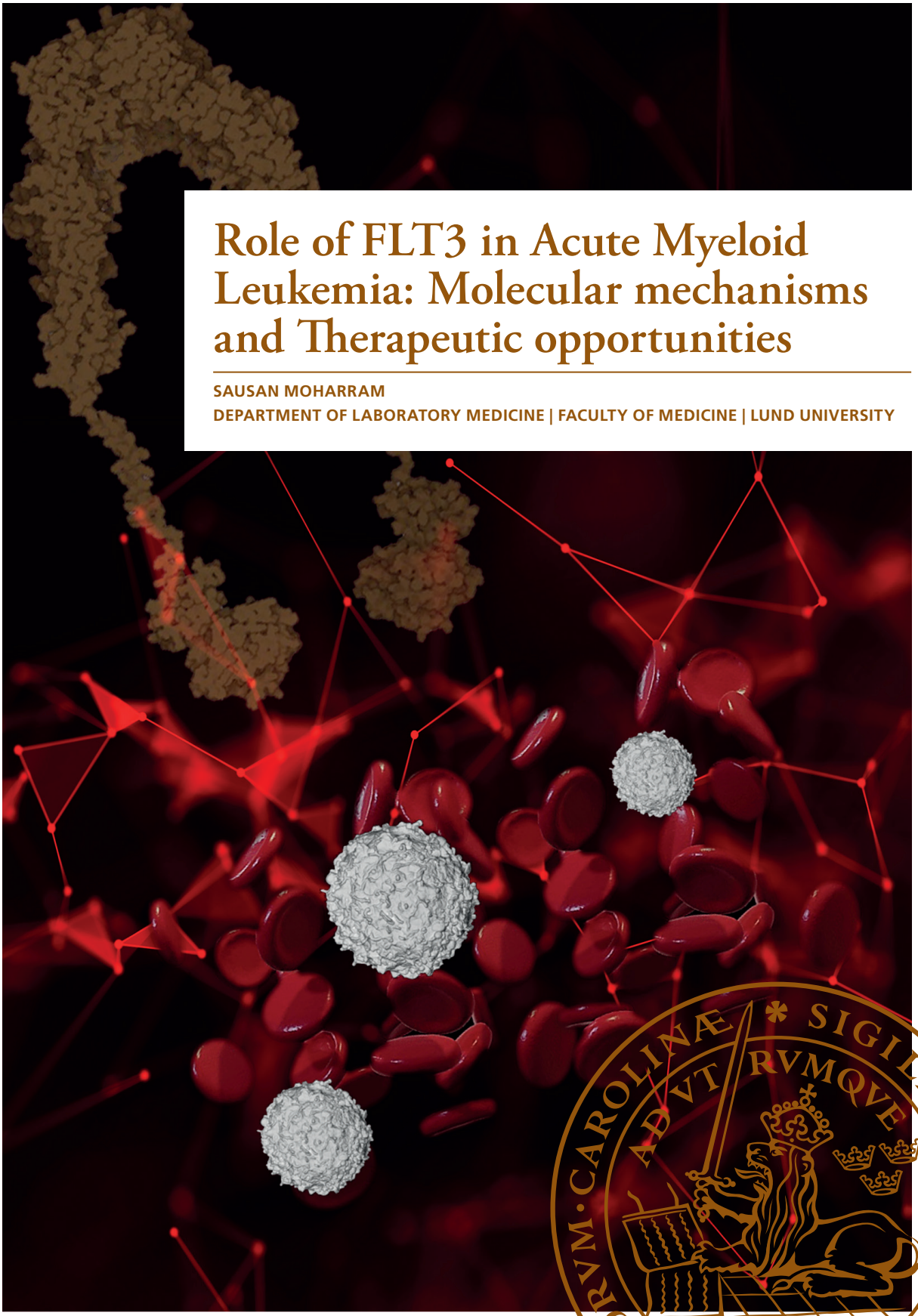

Role of FLT3 in Acute Myeloid Leukemia: Molecular mechanisms and Therapeutic opportunities

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Sausan Moharram

LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Abstract

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We identified SLAP2 and ABL2 as potent interaction partners of FLT3 through their SH2 domain. Our results show that SLAP2 suppresses FLT3 downstream signaling pathways including AKT, ERK, p38 and STAT5 and facilitates FLT3 degradation through enhancing ubiquitination while ABL2 expression does not alter FLT3 stability or ubiquitination but partially suppresses FLT3 downstream signaling through the PI3K/AKT pathway. In contrast to the case of many kinases, we have found that the activation loop of FLT3 is not essential for its activation. Rather, we found that phosphorylated activation loop Y842 serves as a binding site of SHP2, which is required for FLT3-induced activation of RAS/ERK pathway. Our results suggest that SLAP2 and ABL2 regulate FLT3 signaling and modulation of SLAP2 expression levels or targeting ABL2 could potentially synergize with FLT3 inhibitors to treat FLT3-ITD positive AML. Furthermore, Y842 is found to be critical for FLT3-mediated RAS/ERK signaling and cellular transformation.

Using a panel of kinase inhibitors, we found ALK inhibitor AZD3463 selectively inhibited the activation and downstream signaling of FLT3-ITD and did not affect the wild-type FLT3 (FLT3-WT). These findings are interesting from a therapeutic point of view since FLT3-WT is essential for normal hematopoiesis process. Moreover, we showed that AZD3463 effectively overcame the secondary resistance to sorafenib in FLT3-ITD positive AML cells. Thus, this suggests that AZD3463 is a promising inhibitor to target FLT3-ITD positive AML. In conclusion, this thesis explores the mechanisms of regulating FLT3 signaling and therapeutic targeting opportunities.

Key words: AML, FLT3, Signaling pathways, FLT3-ITD, Inhibitors resistance

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Sausan Moharram

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Constitutive signaling displayed by mutated FLT3

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سورة طـه: آية ١١٤

To my Parents

To my daughters; Sara and Sofia

In Ever-Loving Memories of my Grandpapa

Table of Contents

Abbreviations

ABL2	Abelson tyrosine-protein kinase 2
AKT	a serine/threonine kinase, Protein Kinase B
ALK	Anaplastic Lymphoma Kinase
ALL	Acute lymphocytic leukemia
AML	Acute Myeloid Leukemia
ANG1	Angiopoietin 1
ATP	Adenosine Triphosphate
BCL-2	B-cell Leukemia/Lymphoma 2
Bcl-xL	B-cell Lymphoma-extra large
BCR	Breakpoint Cluster Region Protein
BM	Bone Marrow
CBL	Casitas B-lineage Lymphoma
CEBPA	CCAAT/Enhancer Binding Protein Alpha
CLL	Chronic Lymphocytic Leukemia
CLP	Common Lymphoid Progenitor
CML	Chronic Myeloid Leukemia
CMP	Common Myeloid Progenitor
c-Myc	Cellular Myelocytomatosis oncogene
CR	Complete Remission
CRK	CT10 (chicken. tumor virus no. 10) Regulator of Kinase
CRKL	CRK Like Proto-Oncogene
CSF	Colony Stimulating Factor
CSK	C-terminal Src kinase
CXCL12	C-X-C motif chemokine 12
CXCR4	C-X-C chemokine receptor type 4
ERK	Extracellular Signal-regulated Kinase
FAB	French American British
FDA	Food and Drug Administration
FES	Feline sarcoma oncogene
FGFR1	Fibroblast Growth Factor Receptor 1
FL	FLT3 Ligand
FLT3	FMS-like Tyrosine Kinase 3
FOXO3a	Forkhead box O3a
GAB2	GRB2-associated-binding protein 2
GMP	Granulocyte Myeloid Progenitor
GRB2	Growth Factor Receptor Bound Protein 2
GSEA	Gene Set Enrichment Analysis
HER2	Human Epidermal growth Receptor 2
HPC	Hematopoietic Progenitor Cell
HSC	Hematopoietic Stem Cell
ITD	Internal Tandem Duplication

ITK	Interleukin-2-inducible T-cell Kinase
JAK	Janus Kinase
JMD	Juxtamembrane Domain
KIT	KIT Proto-Oncogene Receptor Tyrosine Kinase
MAPK	Mitogen-activated Protein Kinase
MDS	Myelodysplastic Syndrome
MEK	Mitogen-activated protein kinase kinase
MEP	Megakaryocyte/Erythrocyte Progenitor
MPN	Myeloproliferative neoplasms
MPP	Multipotent Progenitor
mTOR	Mammalian Target of Rapamycin
NCK2	Non-catalytic region of tyrosine Kinase adaptor protein 2
NK cell	Natural Killer cell
NPM1	Nucleophosmin 1
OS	Overall Survival
PCK	Protein Kinase C
PDGFR	Platelet Derived Growth Factor Receptor
PI3K	Phosphoinositide 3 Kinase
Pim-1	Proviral integration site for Moloney murine leukemia virus-1
R/R	Relapsed/Refractory
RBC	Red Blood Cell
RTK	Receptor Tyrosine Kinase
RUNX1	Runt Related Transcription Factor 1
SCF	Stem Cell Factor
SH2	Src Homology 2
SHP2	Src Homology 2 containing Phosphatase 2
SLAP	Src-Like Adaptor Protein
SLAP2	Src-Like Adaptor Protein 2
SRC	Sarcoma
STAT5	Signal Transducer and Activator of Transcription 5
TGF-β	Transforming Growth Factor beta
TKD	Tyrosine Kinase Domain
TKI	Tyrosine Kinase Inhibitor
TPO	Thrombopoietin
VAV2	Vav guanine nucleotide exchange factor 2
VEGFR	Vascular Endothelial Growth Factor Receptor
WBC	White Blood Cell
WHO	World Health Organization
WT	Wild -Type

List of original papers

This thesis is based on the folowing papers:

I. **Sausan A. Moharram**, Rohit A. Chougule, Xianwei Su,Tianfeng Li, Jianmin Sun,1,Hui Zhao, Lars Rönnstrand, and Julhash U. Kazi. Src-like adaptor protein 2 (SLAP2) binds to and inhibits FLT3 signaling. *Oncotarget* 2016;7(36): 57770–82.

II. Julhash U. Kazi, Kaja Rupar, Alissa Marhäll, **Sausan A. Moharram**, Fatima Khanum, Kinjal Shah, Mohiuddin Gazi, Sachin Raj M. Nagaraj, Jianmin Sun, Rohit A. Chougule, Lars Rönnstrand. ABL2 suppresses FLT3-ITD-induced cell proliferation through negative regulation of AKT signaling. Oncotarget. 2017; 8(7): 12194–12202.

III. Julhash U. Kazi, Rohit A. Chougule, Tianfeng Li, Xianwei Su, **Sausan A. Moharram**, Kaja Rupar, Alissa Marhäll, Mohiuddin Gazi, Jianmin Sun, Hui Zhao, Lars Rönnstrand. Tyrosine 842 in the activation loop is required for full transformation by the oncogenic mutant FLT3-ITD. Cell Mol Life Sci. 2017; 74(14): 2679–2688.

IV. **Sausan A. Moharram**, Kinjal Shah, Fatima Khanum, Lars Rönnstrand, and Julhash U. Kazi. The ALK inhibitor AZD3463 effectively inhibits growth of sorafenib-resistant acute myeloid leukemia. Blood Cancer Journal 9:5.

Abstract

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Chapter 1

Hematopoiesis

Introduction

Hematopoiesis is the process by which different blood cellular components are generated. This process occurs throughout the embryonic development and during adulthood [1]. The hematopoietic stem cells (HSCs) are very specialized cells which are responsible to produce the functional mature blood cells through the entire life of vertebrates. In humans, the development of hematopoiesis undergoes two main distinct waves, primitive and definitive hematopoiesis. The primitive hematopoiesis occurs as early as the first few weeks of the embryo development in the yolk sac. This wave lacks lymphoid potential but provides the embryo with the essential blood cells namely erythrocytes, megakaryocytes, and macrophages required for tissue oxygenation, growth needs, and first innate defense system for the embryo [2, 3]. The definitive hematopoiesis occurs also at the yolk sac of the embryo where the first HSCs and progenitor cells are detected and subsequently migrate to the fetal liver and remain functional after birth as a source of hematopoiesis until they migrate and reside in the bone marrow (BM) [1, 4, 5]. Since HSCs are characterized by their ability of multi-potency and self-renewal, they can differentiate into all functional blood cells [6]. HSCs are rare and exist mainly in the BM in adult and only divide once every 145 days on average [7, 8]. Moreover, HSCs is differentiated into multipotent progenitor (MPP) which give raise to common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs). The CMPs can be further differentiated into Granulocyte myeloid progenitor (GMP) and Megakaryocyte/Erythrocyte progenitor (MEP) which are eventually differentiated into mature granulocytes, platelets, and erythrocytes while

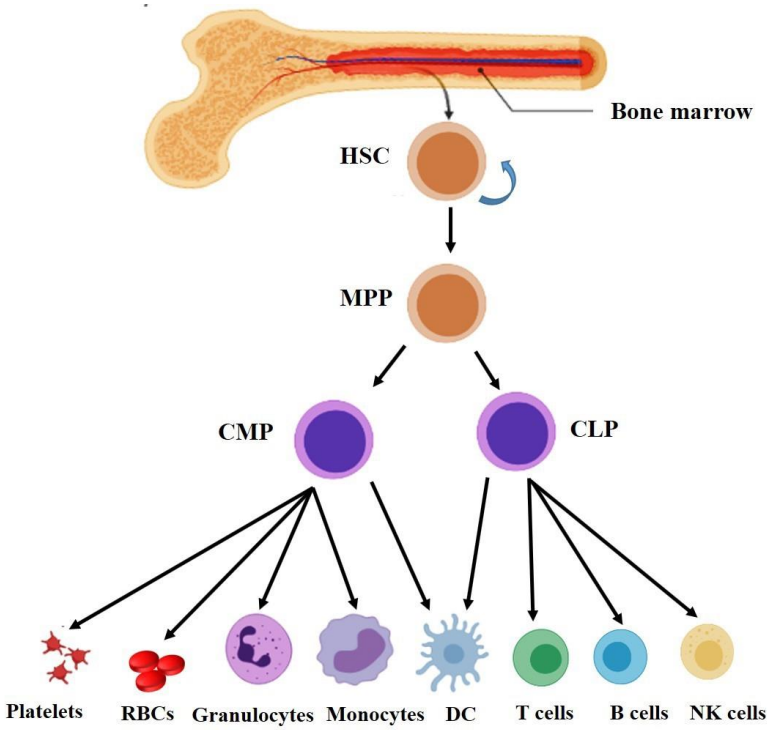

Figure 1. Schematic overview of hematopoiesis hierarchy in adult bone marrow. HSC: hematopoietic stem cell which has the ability for self-renewal and differentiation into all mature blood cells, MPP: multipotent progenitor cell, CMP: common myeloid progenitor cell, CLP: common lymphoid progenitor cell, RBCs: red blood cells, DC: dendritic cells, NK: natural killer cells.

CLPs are eventually differentiated into mature lymphocytes that form the innate and adaptive immunity (**Figure 1**).

Hematopoietic niche

HSC presents in few numbers in the BM which have the ability for extensive self-renewal and differentiation into different hematopoietic lineages. Therefore, HSCs maintenance and expansion are highly regulated to ensure sufficient production of blood cells under steady and stressful conditions. This balance is strictly controlled by intrinsic and extrinsic factors. The intrinsic factors include transcriptional regulation factors and epigenetic modifications within each individual HSCs. For example, the transcription factor FOXO3a plays a role in modulating the proliferative capacity of HSCs

[9]. Extrinsic factors include growth factors and cytokines such as stem cell factor (SCF), thrombopoietin (TPO), colony-stimulating factors (CSFs), transforming growth factor β (TGFβ), C-X-C motif chemokine 12 (CXCL12), and angiopoietin 1 (ANG1). These extrinsic factors are supplied by the BM microenvironment, also called BM niche. The concept of niche was introduced in 1978 by Schofield referring to a specific BM microenvironment that preserves the HSCs self-renewal capacity and contains different cells such as osteoblasts, mesenchymal stem cells, fibroblasts, and endothelial cells [10]. These cells collectively play a central role in HSCs protection from acquiring damage such as mutations. The BM niches provide limited nutrients and low oxygen which are important for HSCs maintenance and therefore prevent them from excessive proliferation.

Stem cell factor, SCF, also known as KIT ligand, is a growth factor which produced by fibroblasts and endothelial cells [11]. SCF binds KIT (CD117), its receptor, and stimulates the development and differentiation of the HSCs [12]. It has been shown that mutation or deletion of SCF or KIT during embryogenesis results in perinatal death of mice due to severe macrocytic anemia [13]. Besides SCF, CSFs play an important role in promoting growth and differentiation of hematopoietic progenitor cells as well as enhancing the function of the mature blood cells, especially macrophages and granulocytes cells [14, 15].

Cytokines and chemokines play an important role in regulating HSCs. For example, thrombopoietin (TPO) is a cytokine that is involved in HSCs maintenance as well as regulating megakaryocyte and platelet production [16]. TGF-β is family of cytokines which are implicated in the regulation of proliferation, quiescence, and differentiation of HSCs [17]. It has been reported that TGF-β upregulates the cyclin-dependent kinase inhibitor, p57KIP2, leading to cell cycle arrest in human hematopoietic cells [18]. CXCL12 is a homeostatic chemokine which plays a vital role in different processes such as angiogenesis, inflammation, and induces migration of hematopoietic precursors. In BM, CXCL12 is expressed by osteoblasts and binds to C-X-C chemokine receptor type 4 (CXCR4) receptor resulting in retention of hematopoietic progenitor cells in the BM. CXCR4 is expressed by Hematopoietic progenitor cell (HPC) and HSC [19]. Mice lacking either

CXCL12 or CXCR4 die due to BM failure [20]. Other cytokines including Interleukin-3 (IL-3) and Interleukin-7 (IL-7) are essential in regulating myeloid and erythroid cells development [21, 22].

Signaling pathways involved in hematopoiesis

Maintenance of HSC self-renewal and differentiation depends on complex interactions with the BM microenvironment. In addition to growth factors and cytokines, several signaling pathways play a critical role during the hematopoiesis development. For example, RAS/extracellular signal-regulated protein kinases (ERK), phosphoinositide-3-kinase (PI3K)/protein kinase B (AKT), Janus kinase-signal transducer and activator of transcription (JAK/STAT), and Notch signaling pathways. Receptor tyrosine kinases (RTKs), such as KIT and FMS like tyrosine kinase 3 (FlT3) are playing essential role in the development of hematopoietic precursors. The KIT has been reported to be expressed in all stages of hematopoiesis [23]. Binding KIT with its ligand SCF results in activation of its intrinsic kinase activity and autophosphorylation of several tyrosine residues and thereby activating multiple signaling pathways such as RAS/ERK, PI3K/AKT and JAK/STAT pathways [24]. KIT has been reported to be expressed in early hematopoiesis [25].

FLT3 expression is essential for differentiation of the multipotent progenitors toward myeloid and lymphoid cells. Moreover, JAK/STAT signaling pathway has been shown to play a role in transducing the activity of cytokines and growth factors in embryonic development, hematopoiesis, and stem cell maintenance [26, 27]. Constitutive activation of JAK-STAT pathway is linked to the development of different malignancies in humans such as sarcomas and lymphomas [28] and mutation and activation of JAK2 is commonly occurring in polycythemia vera [29]. Downmodulating STAT activation is important to maintain cellular homeostasis, and constitutive hyperactivation of STATs particularly STAT3 and STAT5 has been implicated in the development of different types of leukemias as well as solid tumors [30].

Within the BM niche, self-renewal of HSCs is regulated by Notch signaling. Notch is known as a major mediator of cell fate determination during development by regulating different cellular functions such as differentiation, proliferation, and survival. Notch signaling is required during HSCs development as well as in T cell development [31]. Mutation of β-catenin in BM environment results in overexpression of Notch ligand, Jagged 1, and induces AML development with chromosomal alterations [32].

These signaling pathways play a crucial role in the regulation of normal hematopoiesis as well as HSCs quiescence and self-renewal. Dysregulation of these signaling pathways leads to HSC functional defect and can give rise to hematopoietic malignancies or BM failure. Therefore, better understanding on the role of BM niche in regulating the HSC fate and malignancy through intrinsic and extrinsic signaling pathways is key to develop effective treatment of hematological malignancies.

Chapter 2

Leukemia

Introduction

Leukemia is a life-threatening malignant disorder of the blood and BM. The word leukemia, originally *leukämie* in German, is derived from the Greek words "leukos" meaning white and "haima" meaning blood, known as "white blood cells" [33]. It is characterized by uncontrolled proliferation of developing leukocytes cells which replace the normal functional cells leading to anemia, thrombocytopenia, and granulocytopenia. These blood findings are usually associated with clinical symptoms such as weakness, shortness of breath, bleeding tendency, and compromised immune system leading to frequent susceptibility to infections. According to GLOBOCAN, leukemia is the 11th most commonly diagnosed cancer and the 9th leading cause of cancer death worldwide in 2020. The etiology of leukemia remains unknown, but it can occur as a result of a combination of genetic and epigenetic alterations/translocations which can trigger genes responsible for the differentiation and proliferation of hematopoietic cells in the BM. Other risk factors have been documented to be associated with developing leukemia such as exposure to radiation (therapeutic or occupational), chemotherapy, family history, age, and some viral infections [34, 35].

Classification of leukemia

Leukemia can be classified into myeloid or lymphoid based on the cell origin, and acute or chronic based on the progression of the disease. Thus, four main subtypes of leukemia are categorized as follows: Acute myeloid leukemia (AML), Chronic myeloid leukemia (CML), Acute lymphocytic leukemia (ALL), and Chronic lymphocytic leukemia (CLL) **(Figure 2)**. Acute

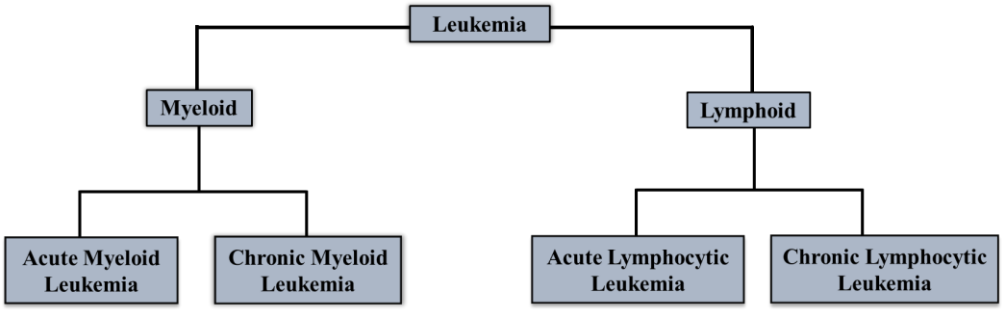

Figure 2. Classification of leukemia according to cell origin and disease progression.

leukemias are characterized by acute onset of symptoms and the presence of dysfunctional immature cells called (blasts) and can progress rapidly and fatally if untreated. Conversely, chronic leukemias are defined by the existence of more functional mature or relatively mature cells which expand slowly and may take several months to years to develop clinical symptoms [35, 36].

Diagnosis and treatment of leukemia

The diagnosis of leukemia is usually based on some clinical characteristic features, patient's history, and panel of invasive and non-invasive diagnostic tests ranging from initial clinical examination and routine laboratory tests to more specific and advanced investigations to confirm and to identify the stage the of disease. These investigations include BM morphology assessment from aspirate and/or biopsy, immunophenotyping by multi-parametric flow cytometry and/or immunohistochemistry, molecular evaluation of genetic aberrations, cytogenetic analysis, and/or next-generation sequencing [37]. The precise evaluation and classification of leukemia are very crucial steps in patient clinical management. Treatment of leukemia depends on many factors including type of leukemia, age, cytogenetic and molecular findings. The treatment options may include chemotherapy, radiation, monoclonal antibodies, hematopoietic stem cell transplantation, and tyrosine kinase inhibitors (TKIs) [38]. These therapeutic options can be conducted as a mono or combination therapy based on many factors such as the stage of the disease, location, and age.

Chapter 3

Acute myeloid Leukemia

Introduction

Acute myeloid leukemia (AML), also called acute myelogenous leukemia, is a clonal hematopoietic disorder of myeloid progenitors in the BM. It is the second most common type of leukemia in adults in which the incidence increases with age [39]. AML represents approximately 80% of adult leukemias with median incidence age from 66 to 71 years, and 15-20% of childhood leukemias [40-42]. In Europe, 3.7 new cases of AML per 100,000 inhabitants are diagnosed yearly [43]. It is characterized by excessive proliferation of abnormal immature blood cells, mostly blasts, which constitute more than 20% of the BM cells and display a high degree of heterogeneity [44, 45]. Once the disease is progressed, the blast cells accumulate in the BM, blood and organs and interfere with normal blood cell production leading to fatal consequences due to infection, bleeding, and organ infiltration if left untreated within one year after the diagnosis [46, 47]. The diagnosis of AML requires identification of 20% or more blasts in the BM or peripheral blood [48]. AML is further classified based on morphology such as the presence or absence of Auer rods, or by immunophenotyping using specific panel of cell surface antigen markers. Assessment of BM aspirate and biopsy morphology, immune-phenotype, and genetics/cytogenetics examinations remain an essential clinical routine practice for the diagnosis and classification of AML [49].

Etiology

For many patients, the direct causes of AML remain unknown. However, there are many risk factors implicated in the development of AML including chemotherapy, radiation therapy, family history, smoking, and other

environmental exposures. Patients who are exposed to chemotherapeutics agents are at risk for developing therapy-related AML [50]. One study showed that patients received chemotherapy for a primary cancer have displayed 4.7 folds high risk of developing AML compared to the general population [51]. Moreover, patients with existing clonal hematologic disorders such as myelodysplastic syndrome (MDS) and myeloproliferative neoplasms (MPNs) are at higher risk to be transformed into secondary AML [52]. In addition, some inherited disorders like Down syndrome and Fanconi anemia can increase the risk of AML development whereas previously healthy individuals who develop AML may be related to *de novo* AML causes [42, 53].

Classification

Determination of the AML subtype can be crucial as it impacts both the treatment option and the clinical outcome. Two major classification systems have been developed for AML: The French American British, also called FAB classification and the World Health Organization (WHO) classification system [54, 55] **(Table 1)**. FAB classifies AML according to the cytochemistry and morphology of leukemic cells into eight subtypes, from M0 – M7. Although FAB classification still commonly used to divide AML, WHO classification becomes the system of choice because it takes into consideration the diversity of genetic alterations presents in AML which carries more prognostic information than the FAB system. The WHO system includes multiple recurrent genetic aberrations found in AML that can be used for following up such as Nucleophosmin 1 (NPM1) as well as other factors related to AML development such as history of other hematological malignancies or therapy-induced AML.

Pathophysiology

AML is a highly heterogeneous blood disease which can result from different genetic mutations and chromosomal rearrangements leading to uncontrolled proliferation, prolonged survival, and impaired hematopoietic cell differentiation [56]. Genetic mutations are counted for 97% of AML cases [57].

Table.1 Classification of AML

FAB classification

M0 Undifferentiated acute myeloblastic leukemia
M1 Acute myeloblastic leukemia with minimal maturation
M2 Acute myeloblastic leukemia with maturation
M3 Acute promyelocytic leukemia
M4 Acute myelomonocytic leukemia
M5 Acute monocytic leukemia
M6 Acute erythroid leukemia
M7 Acute megakaryoblastic leukemia

WHO classification

AML with recurrent genetic abnormalities

- AML with t(8;21)(q22;q22) RUNX1/RUNX1T1
- AML with inv(16)(p13.1q22) or t(16;16)(p13.1;p22) CBFB/MYH11
- Acute promyelocytic leukemia with t(15;17)(q22;q12) PML/RARA
- AML with t(9;11)(p22;q23) MLLT3/MLL
- AML with t(6:9)(p23;q34) DEK/NUP214
- AML with inv(3)(q21q26.2) or t(3.3)(q21;q26.2) RPN1/EVl1
- AML (megakaryoblastic) with t(1:22)(p13;q13) RBM15/MKL1
- AML with mutated NPM1
- AML with mutated CEBPA

AML with myelodysplasia-related change

Therapy-related myeloid neoplasms

Acute myeloid leukemia, not otherwise specified

- AML with minimal differentiation AML without maturation
- AML with maturation
- Acute myelomonocytic leukemia
- Acute monoblastic/monocytic leukemia Acute erythroid leukemia
- Pure erythroid leukemia Erythroleukemia, erythroid/myeloid
- Acute megakaryoblastic leukemia Acute basophilic leukemia
- Acute panmyelosis with myelofibrosis Myeloid sarcoma
- Myeloid proliferations related to Down syndrome

In 2002, Kelly and Gilliland have proposed a 'two-hit' model for leukemogenesis. According to this model, AML is the consequence of at least two different interplaying classes of mutations. Class I mutations which activate signal transduction pathways and therefore induce proliferative and survival advantages. Class II mutations are those which affect transcription factors of cell cycle machinery components and cause impaired cell differentiation and resistance to apoptosis **(Figure 3)** [58, 59].

The two-hit model hypothesis is supported by the observation that a single mutation alone is insufficient for the development leukemic transformation. Mouse studies with high transgene expression of the fusion protein AML1/ETO, t(8;21), also known RUNX1/RUNX1T1, did not develop leukemia [60]. In contrast, add-on mutational events such as FLT3 length mutations promoted the development of leukemia in an AML1/ETO mouse model [61].

Other studies demonstrated that combined mutations between FLT3 and CEBPA accelerated the development of AML in mouse model [62]. The fact that many AML patients have more than one mutation in their leukemic cells supports the two-hit model hypothesis [63]. However, recent studies have identified other group of mutations that cannot be classified under the two-hit hypothesis.

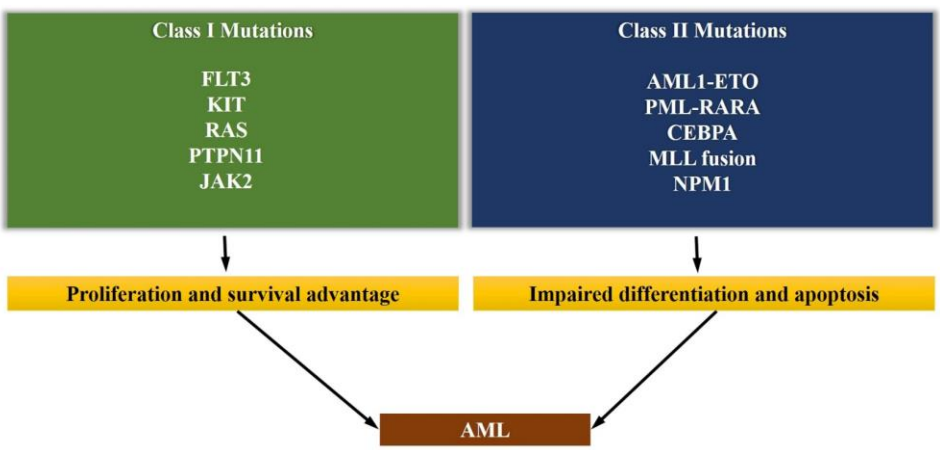

Figure 3. The Two-Hit hypothesis of AML. This model outlines different mutations associated with the appearance of AML.

Theses mutations mostly alters genes that are involved in epigenetic regulation which include the DNA methylation, histone modification, and chromatin remodeling in AML [64].

In a whole genome study of 200 AML patients conducted by the Cancer Genome Atlas Research Network has shown that AML is characterized by multiple somatically acquired mutations as shown in (**Figure 4**) [65]. In this thesis, I will focus on mutation class I, mainly mutations in FLT3, as nearly 30% of AML patients harbor oncogenic FLT3 mutations making it one of the most common mutated genes in AML.

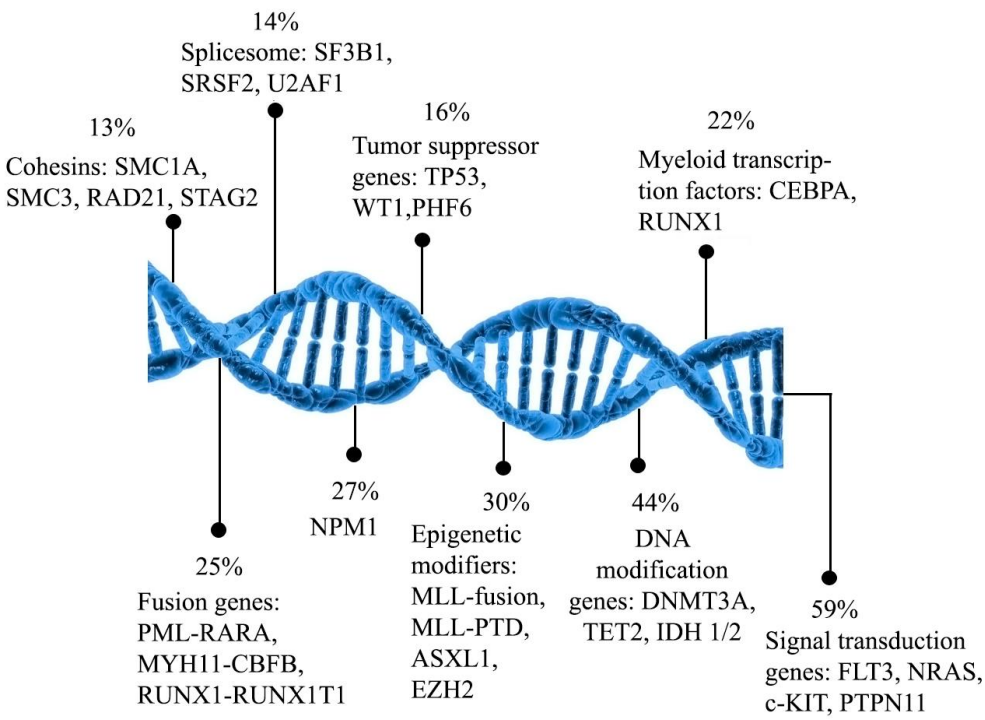

Figure 4. Recurrent mutations associated with *de novo* AML. Mutated genes and their frequencies of appearance are listed according to their functional groups or pathways involved in AML. (Data obtained from The Cancer Genome Atlas Research Network, 2013).

Treatment

The treatment of AML has been well improved over the past few years due to an improved understanding on the molecular heterogeneity of the disease which aid in better therapy stratification and prognostication for patients with AML. Several treatment options can be used for patients with AML. Chemotherapy is the main treatment, also known as induction therapy. Combination of cytarabine and anthracyclines are used in induction therapy. This regimen is usually administered in a dose of 100-200 mg/m^2 of cytarabine continuous infusion for 7 days with idarubicin at 12 mg/m^2 for 3 days or daunorubicin at doses of 45-60 mg/m^2 for 3 days. This therapeutic regimen is commonly referred to as 7 + 3 [66, 67].

The goal of the induction therapy in AML is to clear blood and BM from malignant blast cells and bring complete remission (CR) over 7 days of treatment. Drugs at this phase of treatment are targeting the DNA replication machinery of the cancer cells. It is worth mentioning here that drug tolerance varies between age groups and mutational status. For example, 60-80% of the patients below 60 years of age achieve CR while elderly patients undergo cytarabine chemotherapy with low doses and display around 40-55% who achieve CR [68]. On the other hand, patients with cytogenetic or intermediate prognosis markers require more aggressive doses of cytarabine.

Patients who do not respond to initial therapy can be offered hematopoietic stem cell transplantation treatment. This type of treatment has improved the outcomes in patients with AML who fail primary induction therapy. Targeted therapy is another treatment that uses monoclonal antibodies directed against specific cell antigens or small molecules inhibitors that target tyrosine kinase mutations in cancer cell such as Gemtuzumab (anti-CD33) and FLT3 inhibitors, respectively [69, 70]. This type of targeted therapy may be added to the induction chemotherapy regimen for patients with AML who have certain genetic mutations like those found in FLT3. Other targeted therapy includes epigenetic modulators and mitochondrial inhibitors [71].

Chapter 4

Receptor tyrosine kinase: FMS-like tyrosine kinase 3 (FLT3)

Introduction

Receptor tyrosine kinases (RTKs) are signaling enzymes which catalyze the transfer of the adenosine triphosphate (ATP) γ-phosphate to the tyrosine residues of substrates. RTKs play a crucial role in regulating different cellular processes such as growth, differentiation, and metabolism [72]. Around 90 tyrosine kinase genes are identified in the human genome in which 56 genes encode transmembrane tyrosine kinase receptors [73]. Based on protein homology and structure, the RTKs family can be divided into 20 subfamilies including Vascular Endothelial Growth Factor Receptor (VEGFR), Epidermal Growth Factor Receptor (EGFR), Platelet-Derived Growth Factor Receptor (PDGFR), and Fibroblast Growth Receptor (FGR) [74, 75]. These RTKs are cell surface membrane proteins and share a similar protein structure which composed of a ligand-binding extracellular domain, a transmembrane domain, a juxtamembrane region, a tyrosine kinase domain, and a carboxy (C-) terminal tail. The ligand-binding domains of the extracellular region differ in their overall structure based on the receptor subfamily [76]. In addition to their central role in normal cellular processes, RTKs have been demonstrated to be implicated in a variety of human diseases, most notably cancers [77]. Understanding RTKs and their downstream signaling effect on different cellular functions allowed the development of novel targeted drug therapies such as TKIs with significant improvement in clinical outcomes.

FLT3

Structure and expression

The FMS-like tyrosine kinase 3 (FLT3) gene, also known as the murine fetal liver kinase 2 (FLK2) and human stem cell kinase-1 (STK-1), is located on chromosome 13q12 in human, and encodes a membrane-bound RTK that has an important role in the growth, survival and differentiation of hematopoietic stem cells [78, 79]. FLT3 gene consists of 993 amino acids in length and exists as two forms; one more glycosylated, mature plasma membrane expressed form of 155-160 kDa, and a comparatively less glycosylated immature form of about 130-140 kDa [80]. FLT3 belongs to the type III RTK family together with platelet-derived growth factor α and β receptors (PDGFRA and PDGFRB), KIT, and colony-stimulating factor 1 receptor (CSF1R). They are composed of an extracellular part with five immunoglobulin-like domains of which some bind the ligand, a transmembrane region, a juxtamembrane domain (JMD), and a bipartite

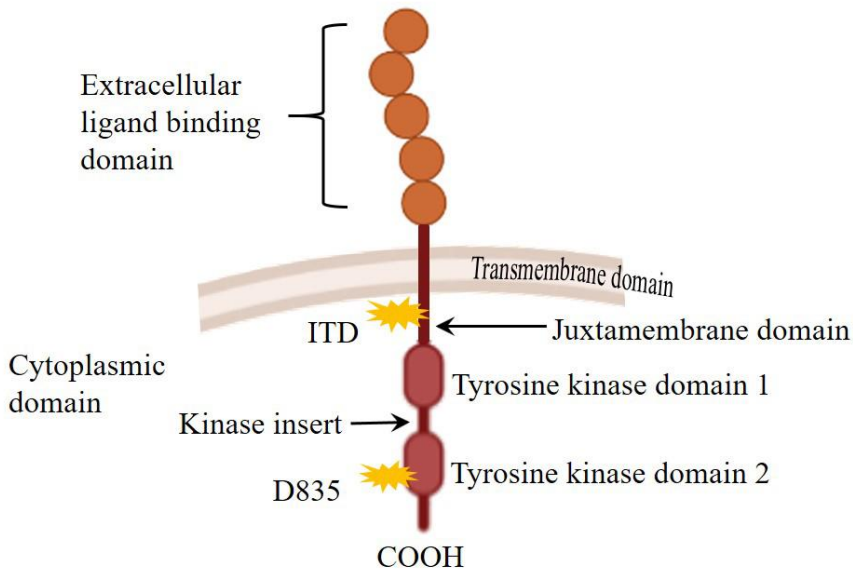

Figure 5. Schematic representation of FLT3 structure. ITD: Internal tandem duplication in the juxtamembrane domain which is the most common mutation in FLT3 and D835: point mutation of aspartic acid 835 in tyrosine kinase domain.

tyrosine kinase domain (TKD1 and TKD2) separated by a kinase insert region (KI) and a C-terminal tail [81] **(Figure 5)**.

In normal human cells, FLT3 is expressed predominantly by early myeloid and lymphoid progenitor cells. Other organs such as placenta, brain, gonads, and liver also express FLT3 [79, 82]. It has been reported that 90-98% of patients with AML and pre-B ALL express FLT3 [83].

Role of FLT3 in normal hematopoiesis

FLT3 is an essential growth factor receptor required for proliferation, differentiation, and survival of hematopoietic stem cells [84]. It has been documented that FLT3 is required for efficient production of some immune cells, such as dendritic cells. Knock out mouse studies showed lethal development due to the lack of adequate hematopoietic cell lineage production including dendritic cells [85]. Studies have also demonstrated enhanced cell proliferation of HPCs cells after FLT3 coordination with other growth factors such as SCF and IL3 [86]. FLT3 receptor exists as an inactive monomeric form in the plasma membrane. Structural biology studies showed that FLT3 has a "closed" activation loop which blocks the access to phosphoryl active sites and ATP-binding site in the monomeric form. The JMD functions as an autoinhibitory loop preventing dimerization as well as the exposing key substrate binding sites [87]. Binding of FLT3-WT receptor to its ligand, FL, leads to dimerization of the receptor. Dimerization of the receptor activates its intrinsic tyrosine kinase activity and phosphorylates tyrosine residues within the receptor intracellular domain. Tyrosine phosphorylation creates docking sites for signaling proteins and induces downstream signal transduction followed by a rapid homodimerization, internalization, and degradation of the receptor [88]. FL is expressed by wide variety of tissues including hematopoietic organs, prostate, ovary, lung, kidney, heart, colon, and placenta. Expression of FL by most tissues in contrast to limited expression of FLT3, that is mainly found in early hematopoietic progenitor cells, indicate that FLT3 expression is a rate limiting step in determining the tissue specificity of FLT3 signaling pathway [89]. Previous studies have reported that exogenous FL increases blast proliferation in patients with FLT3-WT and in patients with oncogenic FLT3.

Therefore, FL-mediated triggering of FLT3 appears to be important for both WT and the mutant FLT3 signaling [90].

Downstream signaling pathways of normal FLT3

FLT3-WT is capable of activating multiple signaling pathways when stimulated by FL resulting in receptor autophosphorylation at tyrosine residues and activation of multiple cytoplasmic molecules. The FLT3 cytoplasmic domain interacts and phosphorylates the p85 subunit of PI3K, growth factor receptor-bound protein 2 (GRB2), and SRC family tyrosine kinase. Activation of PI3K/protein kinase B (AKT) and mitogen-activated protein kinase (MAPK) leads to different cellular functions such as proliferation and cell survival **(Figure 6**) [79, 91]. MAPK or ERK pathway is one of the best kinase cascades studied so far. These signaling are involved in different cellular responses including differentiation, migration, and survival. ERK phosphorylation can occur through interaction of GRB2/SOS or GRB2/GAB2 to tyrosine residues 768, 955, and 969 in FLT3 upon FLT3 stimulation. GRB2/GAB2 association recruits SHP2 and results in ERK phosphorylation [92]. It should also be noted that SHP2 can interact directly with FLT3 through Y599 and Y842 [93, 94]. Moreover, FLT3 is unable to bind to the p85α subunit of PI3K in human but it can activate the PI3K pathway through association or phosphorylation of GAB1 and GAB2 [95]. PI3K-mediated activation of AKT is implicated in different oncogenic signaling pathways including FLT3. This transduction pathway can lead to cell cycle arrest and apoptosis through inactivation of FOXO3a by FLT3-FL dependent activation. Moreover, activation of PI3K/AKT-mTOR (mammalian target of rapamycin) signaling pathway has been reported in drug resistance as part of parallel activation pathways in AML [96, 97].

FLT3 mutations in AML

FLT3 gain-of-function mutations have been reported in 30% of AML patients and in a small subset of patients with ALL. Internal tandem duplication (ITD) is the most common mutation in FLT3 and found in 25-35% of adult AML patients and 10-15% of pediatric AML [82, 98].

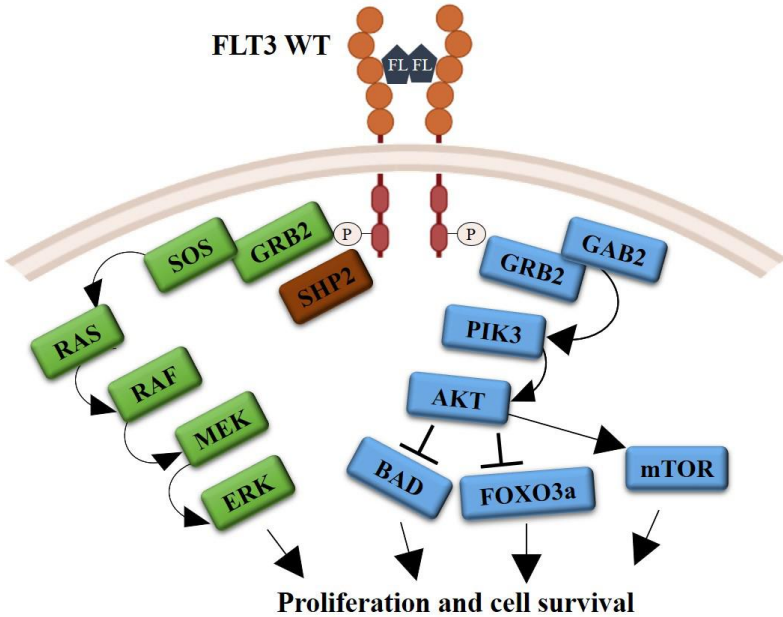

Figure 6. Downstream signaling pathways of wild-type FLT3 (FLT3-WT). Binding of FL to FLT3 activates the FLT3 dimerization and leads to activation of the PI3K and the MAPK pathways triggering cell proliferation/survival and inhibits apoptosis.

Point mutation within the tyrosine kinase domain (TKD) is the second common type of mutated FLT3 and present in approximately 7-10% [99]. FLT3-ITD and FLT3-TKD mutations are ligand-independent and lead to constitutive activation of FLT3 signaling resulting in inhibition of apoptosis, differentiation, and inducing cellular proliferation [100]. Mutations in the kinase domain is considered less severe than the ITD mutations [101]. These mutations are usually associated with poor prognosis in AML [99, 102] **(Figure 5**).

The ITD mutation has been firstly identified by Nakao et al in 1996 [103]. It is described by the duplication of a segment of the JMD of FLT3, which results in ligand-independent constitutive activation of FLT3. The ITD mutations always occur with reading frame maintained, and range in size from 3 to >400 bp [104]. The majority of ITD mutations are found in residues

589-599 [105]. The size of the ITD is negatively correlated with 5-year overall survival of AML patients [106].

In FLT3-WT, the JMD exerts an autoinhibitory function by preventing the activation loop conformational change. Crystallization studies of FLT3/ITD have demonstrated that this autoinhibitory function is lost due to JMD and kinase domain interaction disruption caused by the ITD mutation, and therefore the receptor activity is maintained [87].

The mechanism by which FLT3-ITD mutations are formed still poorly understood. However, Kiyoi et al. suggested that the reason behind FLT3-ITDs formation is a DNA replication error caused by a DNA palindromic intermediate sequences between amino acid 593 and 602, and thereby inducing the tandem duplication [107]. Around 95% of FLT3-ITD mutations in patients have duplication of at least one amino acid in the tyrosine rich region Y591 to Y599 [108].

FLT3-ITD mutated AML patients have higher rates of relapse and short overall survival. The prognostic value is influenced by both mutant allele frequency and presence of co-existing mutations [109]. For example, high FLT3-ITD ratio is associated with higher risk of relapse while low FLT3-ITD ratio is linked to favorable outcomes in patients with a co-existing NPM1 mutations. FLT3-ITD allele ratio is generally defined as the ratio between FLT3-ITD to FLT3-WT of ≥ 0.5 [110]. The observations displayed by poor prognosis in patients with FLT3-ITD mutations have flagged the demands to develop new treatment strategies to improve patient's outcomes.

Other less frequently occurring mutations include point mutations of aspartic acid 835 in the activation loop of TKD2. FLT3-TKD is found in approximately 7-10 % of AML patients and occurs by a substitution of aspartic acid 835 for a tyrosine or other amino acids such as histidine, glutamate, or valine [111]. Other insertion mutations have been also reported including the insertion mutation in exon 20 in a small subset of AML patients where glycine and serine residues are inserted between 840 and 842 amino acids [112, 113]. Unlike TDK2, TKD1 exhibits mutations to lesser extent, for example, mutations in residues N676 and F691 [114]. Interestingly, *in vivo* studies have shown mutational tendency towards specific hematopoietic

lineage. For example, mice with ITDs are associated with myeloproliferative disorders while mice with TKD mutants are linked to oligoclonal lymphoid disorders [115]. Although the presence of FLT3-TKD mutation does not alter the AML risk assessment, the prognostic relevance of FLT3-TKD mutations is speculated to be dependent on the frequency of the mutations and cytogenetic changes [99].

Several point mutations associated with FLT3 such as smaller insertions or/and deletions have also been reported within the TKD and JMD constituting 2% of patients with AML. For example, Stirewalt et al. have identified the novel point mutations V579A, V592A, F590G, and Y591D in the FLT3 JMD of AML patients [116]. Moreover, Reindl et al. has identified additional mutations such as F594L, and Y591C and observed that these mutations lead to reduced stability of the autoinhibitory JMD, activate STAT5, and upregulate Bcl-xL leading to increased resistance to apoptosis [117].

Downstream signaling pathways of oncogenic FLT3

The activation of FLT3-WT requires its respective ligand FL. However, FLT3-ITD is ligand-independent and can constitutively and selectively activate STAT5 besides PI3K/AKT and MAPK/ERK pathways **(Figure 7)**. [89]. In contrast to FLT3-ITD, FLT3-WT and FLT3-TKD cannot activate the STAT5 signaling pathway [118]. Activation of STAT5 results in stimulation of several specific downstream targets that are key mediators of cell cycle progression and antiapoptotic signaling such as cyclin D1, BAD, c-Myc and the protooncogene Pim-1 [119, 120]. FLT3-ITD mutations-induced phosphorylation of STAT5 contributed to Pim-1-mediated overexpression of CXCR4 which in turn contributes to chemotherapy resistance and disease relapse [121]. Rocnik et al. has identified that tyrosine residues Y589 and Y591 play a crucial role in STAT5 activation by FLT3-ITD. Substitution of these two sites to phenylalanine has abolished the phosphorylation of STAT5 and reduced the myeloproliferative disease potential in FLT3-ITD mice [105]. Moreover, FLT3-ITD duplication of Y591in AML blasts has been associated with high BCL-2 levels, a transcriptional target of STAT5 [122].

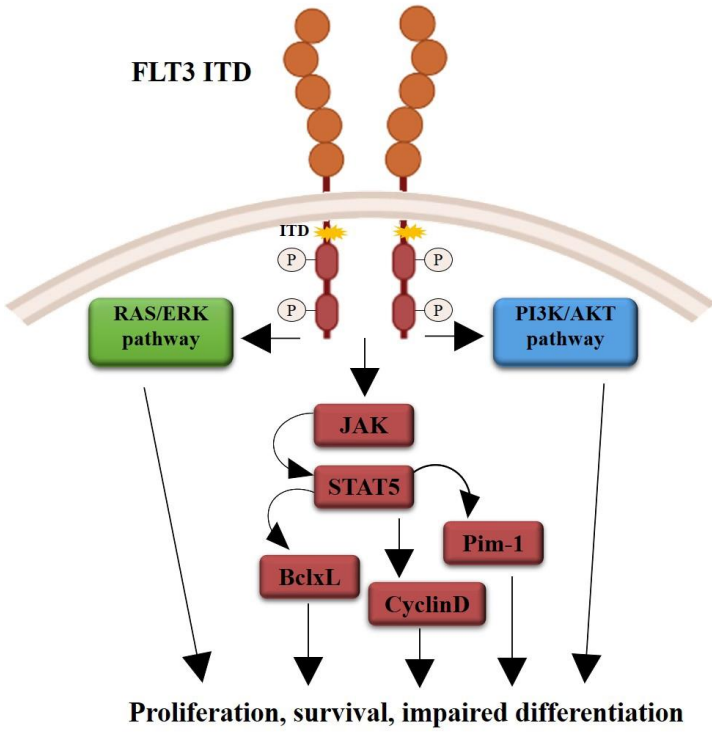

Figure 7. **Schematic view of FLT3-ITD signaling.** FLT3-ITD signals STAT5 pathway as well as RAS/ERK and PI3K/AKT pathways leading to increase cell proliferation and survival.

This signaling renders FLT3-ITD expressing cells resistant to apoptosis which may explain at least in part the poor outcomes for those patients having these types of mutations.

FLT3 inhibitors

FLT3 mutations emerged as one of the most common mutations in AML which are associated with high risk of relapse and poor prognosis. Therefore, FLT3 is a promising therapeutic target for treatment of AML with FLT3 mutations. The breakthrough of TKI imatinib in the treatment of BCR-ABL1 in CML has led to the development of more than 20 inhibitors directed against mutated FLT3 [123]. Although multiple FLT3 inhibitors have been developed for the treatment of FLT3-mutated AML allowing fast entrance of these compounds to the clinical trials, only two

inhibitors (midostaurin and gilteritinib) are currently approved for distinct clinical indications.

Based on their mechanism of action, FLT3 inhibitors can be divided into two main types. type I inhibitors which act on both active and inactive forms of the mutated kinase and thereby preventing autophosphorylation and subsequent activation of downstream signaling, and type II inhibitors which bind only inactive kinase molecules.

Midostaurin (Rydapt), also known as PKC-412, is a powerful type I multi-kinase inhibitor that exerts a potential inhibitory effect on multiple signaling pathways involved in both ITD and TKD mutations such as VEGFR, protein kinase C, KIT, and PDGFR-β [124]. This inhibitor was approved by FDA and EMA in 2017 for the clinical therapy of newly diagnosed FLT3-mutated AML adults in combination with standard cytarabine and daunorubicin, and for maintenance therapy of AML patients who are not eligible for allogeneic hematopoietic stem cell transplantation (ASCT) [125, 126]. Midostaurin has been characterized from Streptomyces staurosporeus bacterium and was initially developed as an inhibitor of protein kinase C [127]. It has been shown that treatment with midostaurin reduced FLT3 autophosphorylation and diminished downstream signaling through p38, MAPK, and STAT5 [128]. A clinical study showed that midostaurin monotherapy in relapsed/refractory (R/R) AML reduced blasts in 71% of patients with FLT3-mutant AML and 42% of patients with FLT3-WT [129].

Gilteritinib (Xospata, ASP2215) is another approved FLT3 type I inhibitor used as a single-agent therapy for adults with R/R FLT3-mutated AML [130]. Gilteritinib has a dual effect on both FLT3 and AXL [131]. AXL expression has been linked to some FLT3 inhibitors resistance such as midostaurin and quizartinib [132]. Gilteritinib has displayed an impressive result in preclinical studies in FLT3 and AXL-mutant tumor models by decreasing tumor size, blocking the activation of cellular survival pathways, and restoring the apoptotic pathway [133]. Moreover, clinical trials in patients with R/R AML, including FLT3-WT and FLT3-mutated, displayed better outcomes in FLT3-mutated compared to FLT3-WT patients [134].

Crenolanib inhibitor has been shown to overcome the secondary resistance through targeting both FLT3-ITD and FLT3-TKD mutated receptors. [135]. A phase III clinical trial of crenolanib *vs* midostaurin combined with chemotherapy in newly diagnosed FLT3-mutated AML is currently recruiting patients (NCT03258931).

Lestaurtinib is another TKI that has been investigated in several clinical trials to target both FLT3-WT, FLT3 mutants as well as JAK2. However, lestaurtinib has been discontinued from clinical development due to limited response. In a phase III clinical trial, lestaurtinib showed no difference in OS when combined with frontline induction and consolidation chemotherapy in patients with FLT3-mutated AML [136, 137].

Sorafenib is a type II multi-kinase inhibitor (RAF, PDGFR, VEGFR, KIT) that targets FLT3-ITD but not other oncogenic FLT3 mutants. A clinical study showed that combination of sorafenib with induction chemotherapy displayed 93% CR rate [138]. A randomized phase II clinical trial using sorafenib as a maintenance therapy led to reduce risk of relapse and death after ASCT in patients with FLT3-ITD mutant AML [139]. It is worth mentioning here that sorafenib has been approved by the FDA for the treatment of hepatocellular and renal cell carcinomas.

Quizartinib is an FLT3-selective type II TKI (AC220) that can selectivity inhabit FLT3 ITD but not TKD mutations. Quizartinib has demonstrated activity against other RTKs such as KIT and PDGFR [140]. Combinatorial clinical studies of quizartinib with hypomethylating drug (5-azacitidine) or low-dose cytarabine resulted in an overall response rate of 75% in patients with R/R AML harboring the FLT3-ITD mutation [141]. In 2019, Japan has approved the use of quizartinib for R/R AML patients with FLT3-ITD.

Mechanisms of resistance of FLT3 inhibitors

The promising preclinical effect of FLT3 inhibitors have allowed their fast entry to the clinical trials. Although several FLT3 inhibitors have shown promising results, the vast majority of these inhibitors have displayed limited clinical benefits. The most common reason attributed to low therapy response

is the development of therapy resistance. Resistance to TKIs can be classified into two main categories namely primary and secondary, also known acquired, resistance based on their mechanisms.

TKIs primary resistance to AML therapy is originated from different mechanisms. FLT3 mutations, persistent activation of compensating survival pathways, and BM-stromal cells derived resistance have been reported to be implicated in the TKIs resistance. For example, point or compound mutations in FLT-3TKD as well as co-occurrence with FLT3-ITD mutations in the same blast clones may develop primary resistance to several FLT3 inhibitors [142, 143]. In addition, FLT3-ITD627E and FLT3-ITD-TKD dual mutations have been shown to induce Mcl-1 and Bcl2-mediated resistance to apoptosis respectively [144, 145]. Moreover, the role of BM niche has been postulated as another mechanism of primary resistance. Expression of FL by BM stroma after chemotherapy stimulates AML cells with FLT3 mutations and activate ERK signaling pathway as well as expression of CXCR4 contributing to resistance development of FLT3 inhibitors [146-148]. Co-existence of some other mutations. which are not related to FLT3, such as those in cyclin D3 have been reported with FLT3-ITD-positive AML patients who developed resistance to the FLT3 inhibitor PLX3397 [143].

On the other hand, secondary resistance might arise due to some specific mutations that alter the conformational change of the active site of the receptor and thereby preventing TKI binding. Single amino acid substitution at (N676K) within the FLT3 kinase domain displayed resistance to midostaurin [149]. Point mutations have been also reported to mediate secondary resistance of different TKIs. For instance, mutation at gatekeeper residue (F691) or at codon 835 of the activation loop of the FLT3 receptor have been documented in FLT3-ITD AML patients [150, 151]. Acquired mutations in JAK1, JAK2, or JAK3 in patients with FLT3-ITD mutations have been linked to sorafenib, midostaurin, or quizartinib resistance [152].

Activation of parallel signaling pathways has been suggested to mediate secondary TKIs resistance. A study conducted by Zhang et al. has demonstrated that phosphorylated FLT3 was not able to induce a significant inhibition of ERK, AKT, S6K, and STAT downstream effectors in sorafenib-

resistant cell lines with acquired point mutations in the TKDs of the FLT3 gene. This might be explained at least in part by counter activation of MEK/ERK and/or AKT/S6K pathways [153]. In light with these findings, Yang et al. has also shown that FLT3 mutant cells co-cultured with BM stroma or exogenous FL exhibited ERK phosphorylation that could not be inhibited with doses of quizartinib or sorafenib mediated full inhibition of AKT and FLT3 phosphorylation. Another report has pointed out the aberrant expression of PI3K/mTOR pathway in developing secondary resistance to sorafenib [97]. FLT3-ITD cells treated with FLT3 inhibitors displayed increased phosphorylation of the RTK AXL thereby activating STAT5 signaling pathway leading to FLT3 inhibitor resistance [132].

Although TKIs have provided a new class of novel therapeutic approach, resistance is still the main dilemma that should be addressed to further boosting this type of treatment strategy. Understanding the mechanism of developing resistance against FLT3 inhibitors would allow the development of better inhibitors or combination therapies that can overcome drug resistance.

Chapter 5

Paper I

Src-like adaptor protein 2 (SLAP2) binds to and inhibits FLT3 signaling

Aim

This paper aims to investigate the role of SLAP2 in regulating FLT3 stability and activation as well as effects on the downstream signaling in acute myeloid leukemia.

Introduction

FLT3 inhibitors have shown promising results in treating AML patients in clinical trials. However, many patients relapse and develop resistance after short-term of treatment. Resistance linked to FLT3 is well documented, and therefore a better understanding of FLT3 downstream signal transduction pathways is key to identify an alternative target for the treatment of AML patients carrying oncogenic FLT3. Signal transducing adaptor proteins are essential intracellular transmembrane molecules that provide an important scaffold to initiate cascade of key signaling pathways. Autophosphorylation of several tyrosine residues as a result of FLT3-ligand binding provides docking sites for several adaptor proteins containing SH2 domains [82, 154]. For example, GRB2-FLT3 interaction provides a docking site for GAB2 and results in downstream signaling [92]. On the other hand, FLT3 binding to the suppressor of cytokine signaling 6 (SOCS6) initiates ubiquitination followed by degradation of FLT3 and therefore inhibits the downstream signaling.

SRC-like adaptor protein 2 (SLAP2) is an adaptor protein belongs to the SLAP family proteins. SLAP2 consists of 261 amino acids and shares 36% structural similarity with its homolog SLAP [155]. SLAP and SLAP2 have similar structures with SRC family kinases (SFKs) [156]. They consist of SRC homology 2 domain (SH2), SRC homology 3 domain (SH3), N-terminal region, and a unique C-terminal tail that mediates association with the CBL ubiquitin E3 ligase but lacks tyrosine kinase domain [157]. The SH2 and SH3 are essential for interaction with multiple proteins. For instance, SLAP associates with the type III RTKs FLT3, KIT, PDGFRB, and CSF1R after stimulation with their respective ligands [158]. This association takes place through binding of phosphorylated tyrosine residues in the receptor to the SH2 domain of SLAP. SLAP plays a fundamental role in the regulation of T- and B-cell development [159, 160] while SLAP2 has been shown to be involved in the regulation of different signaling pathways; for example, associating with CSF1R through its SH2 domain which leads to downregulation of the receptor [161]. A study conducted by Pandey et al. has demonstrated that SLAP2 can negatively regulate T cell receptor signaling transduction pathway [156]. SLAP2 is expressed in different types of hematopoietic cells and tissues including leukocytes, monocytes, platelets, T- and B-cells as well as in lung, spleen, and the thymus [162, 163]. However, the role of SLAP2 in regulating FLT3 signaling in AML has not been revealed yet. Therefore, we hypothesized that SLAP2 might take part in regulating the signaling of the RTK FLT3.

Results and discussion

Previous reports indicated that activation of FLT3 results in phosphorylation of FLT3 on several tyrosine residues which recruit SH2 domain-containing signaling proteins. To identify novel FLT3 interacting proteins, we used a panel of SH2 domain-containing proteins including VAV2, SLAP2, CRK, ITK, TEC, NCK2, and CRKL. Then we have transfected COS-1 cells either with plasmids for FLAG-tagged of these panel of adaptor proteins or FLT3-WT and empty vector. Immunoblotting results exhibited strong SLAP2 association with FLT3 following ligand-stimulation. Several studies have shown that SLAP, a close homolog of SLAP2, associates with FLT3 in a

phosphorylation-dependent manner as well as interacts with proximal components of the TCR and BCR signaling complexes. This association is mediated through the SH2 domain of SLAP and tyrosine-phosphorylated residues of the receptors [164, 165]. In order to examine the interaction between SLAP2 and FLT3, we transiently expressed SLAP2 and FLT3 in COS-1 cells. We observed that FLT3-SLAP2 interaction was a ligand-dependent in FLT3-WT cells while oncogenic FLT3-ITD association with SLAP2 was ligand-independent. These results suggest that FLT3-SLAP2 interaction is dependent on FLT3 activation.

Several tyrosine residues in the intracellular domain of FLT3 get phosphorylated when its ligand binds to the receptor, leading to creating docking sites for predominantly SH2 domain-containing signaling proteins [166]. In order to identify the SLAP2 binding sites in FLT3, synthetic phosphopeptides corresponding to known FLT3 tyrosine phosphorylation sites were used in peptide fishing assay. We found that SLAP2 association with FLT3 occurs through different phosphotyrosine residues namely: pY589, pY591, pY599, and pY919 with stronger association being detected with pY589 and pY591. To verify our finding, we checked the SLAP2-FLT3 association using a double phosphorylated peptide, pY589/pY591, which displayed higher affinity compared to either pY589 or pY591 alone. Moreover, mutation in pY589/pY591 residues significantly decreased this association. Since Y589, Y591, and Y599 were previously reported as SRC binding sites in FLT3 [93, 118], this suggest that SLAP2 might compete with SRC for binding to these sites, and loss of SLAP2 expression activates FLT3 signaling through SRC. These results indicate that SLAP2-FLT3 association mostly occurs through two phosphotyrosine residues: pY589 and pY591. To examine whether the SLAP2 SH2 domain has a role in the association with the phosphotyrosine residues, we generated an SH2 domain mutant of SLAP2 that does not bind phosphotyrosine (SLAP2-R121E). Immunoprecipitation experiments showed that FLT3 and SLAP2 interaction in the SLAP2 SH2 domain mutant was eliminated compared to the Wild SLAP2 which was able to interact with ligand-stimulated FLT3-WT indicating that SLAP2 SH2 domain is essential for the interaction with FLT3.

FLT3 plays a vital role in controlling different cellular processes such as proliferation and differentiation [167]. To assess the biological role of SLAP2-FLT3 interaction, we generated Ba/F3 and 32D cells expressing FLT3-ITD along with an empty control vector or a vector expressing SLAP2. Initially, we checked whether SLAP2 plays a role in FLT3-ITD-mediated cell proliferation. We observed that cells expressing SLAP2 significantly decreased FLT3-ITD-dependent cell proliferation in both Ba/F3 and 32D cell compared to the empty vector-transfected cells. However, SLAP2 expression showed no effect on the level of apoptosis upon FL depletion. These results suggest that SLAP2 expression reduces FLT3-ITD-mediated cell proliferation without inducing apoptosis. Next, we sought to understand the influence of SLAP2 in FLT3-ITD mediated cellular transformation *in vitro* and *in vivo* models. Our results showed that SLAP2 expression significantly reduced FLT3-ITD-dependent colony formation in semi-solid medium, tumor volume, and tumor weight in the xenograft mouse model. Thus, we concluded that SLAP2 acts as a negative regulator of FLT3.

To determine whether SLAP2 is implicated in FLT3-ITD-induced aberrant global gene expression, we analyzed microarray data for mRNA expression of FLT3-ITD/empty vector and FLT3-ITD/SLAP2 cells. SLAP2-expressing cells have demonstrated specific gene signature, which is associated with the loss of STK33, ALK or PDGFR indicating that SLAP2 is involved in controlling oncogenic signals from FLT3-ITD. Furthermore, using AML patient data, we found that SLAP2 expression increased in AML patients with FLT3-ITD mutation and patients who have low SLAP2 expression displayed intermediate or poor prognosis indicating that SLAP2 plays a crucial role in FLT3-ITD driven AML.

It has been shown that association of adaptor proteins to the activated FLT3 receptor results in activation or inhibition of downstream signaling. For instance, association of GRB10 and SRC family kinases to FLT3 positively regulate FLT3 downstream signaling while SOCS2 and LNK inhibit FLT3 signaling [168-171]. To study the effect of SLAP2 on FLT3 signaling, we stably transfected Ba/F3 and 32D cells expressing FLT3-WT with an empty control vector or a vector expressing SLAP2. Thereafter, we examined

RAS/ERK, PI3K/AKT and p38 signaling pathways using western blot. Interestingly, SLAP2 expression significantly reduced ERK and AKT phosphorylation as well as p38 phosphorylation. Moreover, because several studies have shown that FLT3-ITD mediates phosphorylation and activation of STAT5 [172, 173], we sought to examine the impact of SLAP2 expression on STAT5-mediated oncogenic FLT3 signaling. We used cells expressing FLT3-ITD/empty vector and cells expressing FLT3-ITD/SLAP2. Our results showed a substantial decrease in STAT5 phosphorylation in SLAP2 expressing cells compared to empty vector-transfected cells. These findings suggest that SLAP2 negatively regulates FLT3 signaling by inhibiting the phosphorylation of signal transduction molecules of the receptor.

Our group has previously found that SLAP modulates FLT3 and KIT stability [158, 165]. Therefore, we asked whether SLAP2 has a role in the regulation of FLT3 stability. Ubiquitination and degradation assays were performed and demonstrated that SLAP2 expression increased FLT3 degradation through enhancing ubiquitination. This is in line with other findings where SLAP2 downregulates CSF1R signaling by recruiting the ubiquitin E3 ligase CBL to the receptor leading to accelerating ubiquitination and degradation [161]. These data demonstrate that SLAP2 expression decreased FLT3 stability which might explain the effect on cellular signaling.

In our current study, we propose a mechanism of FLT3 regulation by SLAP2 ubiquitin ligase. SLAP2 SH2 domain associates with FLT3 through phosphotyrosine residues Y589 and Y591 in FLT3 and results in increase FLT3 ubiquitination and degradation as well as inhibits ERK, AKT, and STAT5 phosphorylation. Taken altogether, we show that SLAP2 acts as a negative regulator of FLT3-mediated oncogenic signaling and this can be explained by competition with SRC and destabilization of FLT3. Thus, modulation of SLAP2 expression levels could potentially synergize FLT3 inhibitors to treat FLT3-ITD positive AML patients. Moreover, identification of novel interacting proteins will contribute to our better understanding of FLT3 downstream signaling and will provide an alternative approach to develop novel therapy for FLT3-ITD positive AML.

Chapter 6

Paper II

ABL2 suppresses FLT3-ITD-induced cell proliferation through negative regulation of AKT signaling

Aim

The aim of this paper is to examine the role of ABL2 in oncogenic FLT3 signaling.

Introduction

Although several FLT3 inhibitors have been developed and displayed promising results in clinical trials against acute leukemia, many patients develop drug resistance and have a poor prognosis. The development of drug resistance such as the acquisition of point mutations in the kinase domain and upregulation of alternative signaling pathways remains the major obstacles to the successful management of targeting FLT3 [174, 175]. It has been known that FLT3 signaling is tightly regulated by associating proteins including protein kinases, protein phosphatases, and adaptor proteins [162, 176]. For example, protein kinases such as FYN [177] enhance the oncogenic FLT3-ITD signaling while the protein kinase CSK partially inhibits the mitogenic signaling [178]. Furthermore, binding FLT3 to SOCS2 adaptor protein leads to inhibit FLT3 downstream signaling whereas the interaction with GRB10 positively regulates downstream signaling [169, 179]. This line of evidence demonstrates the important role of the associating proteins in

regulating FLT3. Thus, gaining more knowledge about different adaptor proteins and their roles controlling FLT3 signaling might be an alternative approach to target mutated FLT3 in AML.

The mammalian Abelson (ABL) kinases, ABL (ABL1), and Arg (ABL2) are non-receptor tyrosine kinases which have been demonstrated to play important roles in various biological processes including cell proliferation, survival, morphology, and apoptosis [180]. ABL kinases have been shown to be involved in regulating cell proliferation downstream signaling of the T- and B-cell receptors [181]. The BCR–ABL1 fusion gene has been identified in different types of leukemias, including CML, ALL, and rarely in AML [182, 183]. Furthermore, the oncogenic form of ETV6-ABL2 has been identified in T-ALL and to less extent in AML [180, 184]. Accumulating studies found that expression of ABL family kinases is upregulated in solid tumors; for instance, pancreatic cancer, anaplastic thyroid cancer, and colorectal cancer [185-187]. Several studies have shown that ABL family kinases are implicated in cancer cell invasion, proliferation, and survival by regulating EGFR, IGFR, and HER2 [188, 189]. However, the impact of ABL family kinases on FLT3 signaling has not been investigated yet. Therefore, we hypothesized that ABL2 might play a role in FLT3 signaling.

Results and discussion

Activation of RTKs results in phosphorylation of different residue sites which serve as binding sites for SH2 domain-containing signaling proteins [190]. Using SH2 domain array screening, we have identified ABL2 as an FLT3 binding protein. Moreover, ABL2 showed affinity to different binding sites in FLT3 including pY726, pY793, and pY842. To confirm the association between ABL2 and FLT3, we co-expressed FLAG-tagged ABL2 with FLT3-WT or FLT3-ITD in COS-1 cells. We observed that FLT3 ligand stimulation exhibited a strong ABL2-FLT3 association while the association with FLT3-ITD was ligand-independent. Consequently, our results suggest that FLT3 kinase activity is essential for the interaction with ABL2, and this association was through the ABL2 SH2 domain.

Previous studies published by our group identified the pY793 as FLT3 autophosphorylation site and found that GRB10-FLT3 association through pY793 residue phosphorylates GRB10 which binds p85 which in turn leads to activation of PI3-kinase and activation of AKT pathway [166, 179]. Thus, this suggests that binding ABL2 to FLT3 through the binding site Y793 might influence activity of the PI3K pathway.

It is well known that ABL kinases activate downstream signaling of growth factor receptors which mediates many cell responses, such as proliferation, migration, and cell transformation [187]. To examine the effect of ABL2 in oncogenic FLT3-ITD-mediated biological functions, we generated Ba/F3 cells stably expressing FLT3-ITD and ABL2 or empty vector and performed cell viability, apoptosis, and colony formation assays. Compared to the empty vector, cells expressing ABL2 displayed significantly reduced cell viability as well as colony formation. On the other hand, ABL2 expression neither increased nor decreased the fraction of apoptotic cells. These data suggest that ABL2 expression negatively regulates FLT3-ITD-mediated cell viability and colony formation. Our findings are consistent with other reports showed that loss of ABL2 enhanced cell proliferation leading to accelerating tumor growth in vivo in breast cancer while depletion of ABL2 reduced cell growth in non-small cell lung carcinoma cell lines [191, 192]. These data indicate that the effect of ABL2 kinase in regulating cell survival and cell proliferation might be cell or context dependent.

It has been reported that activation of the oncogenic BCR–ABL1 results in activating many signaling pathways including RAS, STAT3, and the PI3K/AKT signaling pathways [180]. To assess the role of the ABL2 in regulating the FLT3-induced signaling pathways, we generated Ba/F3 cell lines expressing FLT3-WT and ABL2 or empty vector. Immunoblotting data showed that expression of ABL2 selectively reduced AKT phosphorylation without affecting the ERK or p38 pathway. These data indicate that ABL2 expression negatively regulates AKT phosphorylation. Since most of the associating proteins are implicated in the activation and stability of interacting receptors via recruiting the ubiquitination machinery [74, 165], we sought to examine the role of ABL2 in regulating FLT3 protein stability.

Our results showed no significant difference in FLT3 degradation in cells expressing ABL2 compared with the control cells. In addition, we did not observe any effect of ABL2 expression in the phosphorylation or ubiquitination of FLT3. Taken together, our findings suggest that ABL2 expression does not influence FLT3 stability but partially suppresses FLT3 downstream signaling through PI3K/AKT pathway. In this context, it is worth mentioning here that we did not study the mechanism behind PI3K/AKT regulation, but this can be explained by the ability of ABL2 to compete for binding FLT3 with proteins essential for activation of the AKT pathway or it could be that ABL2 can directly target FLT3 downstream signaling proteins. Therefore, further study is needed to investigate the mechanism behind PI3K/AKT regulation.

In summary, this paper show that overexpression of ABL2 significantly decreased FLT3-ITD induced cell proliferation and colony formation. In contrast to ABL1 close homology, ABL2 found to be a negative regulator of FLT3 signaling through the regulation of AKT pathway.

Chapter 7

Paper III

Tyrosine 842 in the activation loop is required for full transformation by the oncogenic mutant FLT3- ITD

Aim

The aim of this study is to investigate the role of the Y842 residue in FLT3 signaling.

Introduction

The activation loop is a short and well conserved amino acid located in the kinase domain [193]. The activation loops usually contains one to three tyrosine residues that can function as phosphorylation sites [194]. It is well known that phosphorylation of several tyrosine residues is critical for activating RTK signaling in many cases. For instance, phosphorylation of activation loop tyrosine residues increases substrate phosphorylation of the fibroblast growth factor receptor and activates insulin receptor [195]. However, although it has been shown that activation loop has no role in the activation and phosphorylation of some type III RTKs such as KIT, it reduces the transformation capacity of the oncogenic D816V mutant [196]. Point mutations in the TKD of FLT3 is found in 7–10% of AML patients, most commonly at D835, and the resulting mutants are resistant to some FLT3 TKI [197]. Moreover, point mutations in TKD within FLT3/ITD allele is considered as one of the most important sources of resistance to sorafenib or

AC220 therapy [153, 198]. This might be due to the mutation-induced conformational changes of the catalytic domain which decreases its affinity for the FLT3 inhibitor [122]. One *in vivo* study conducted by Williams et al showed that FLT3/ITD Y842C mutation causes resistance to TKIs such as sorafenib and sunitinib [199]. Another study has demonstrated that FLT3-Y842H mutation in Ba/F3 FLT3-ITD cells induces resistance to the TKI SU5614 [175]. In primary AML blast cells, the detection of Y842C mutation within the kinase domain of FLT3 constitutively activates FLT3 [113]. Although Y842F mutation seems to be rare in AML patients, it is still useful to detect and study different mutations in TKD at different residues which might help in developing FLT3 inhibitors that target TKD.

Results and discussion

Oncogenic mutations in FLT3 result in abnormal activation of survival and proliferation signaling [200]. To study the role of the activation loop Y842 in FLT3, we generated a Y-to-F mutant (tyrosine to phenylalanine Y842F) and ectopically expressed in 32D myeloid cells. We observed that cells expressing mutant FLT3-ITD/Y842F displayed a significant reduction in cell proliferation compared to FLT3-ITD cells indicating that phosphorylation of the FLT3 activation loop is important for maintenance cell viability. Unlike FLT3-ITD cells, cells expressing FLT3-ITD/Y842F showed increased apoptosis. This is in line with previous findings showed that Y823F mutation of KIT, the mutation corresponding to Y842F in FLT3, decreased cell proliferation [201].

Several tyrosine residues have been implicated in oncogenic cellular transformation [202]. To this end, we have investigated the role of Y842F in FLT3- ITD-mediated cellular transformation. We used colony formation assay and found that expression of Y842F significantly reduced the number of colonies as well as the colony size compared to cells have only the FLT3-ITD mutation. To verify our findings *in vivo,* we developed a mouse xenograft model and found that tumor formation was delayed concomitantly with reduced tumor weight in mice injected with mutant Y842F compared to the control mice. These results suggest that the phosphorylation of the activation loop tyrosine is important in FLT3-ITD-mediated transformation.

Given that Y842F mutation reduced cell viability, colony formation and tumor formation in FLT3-ITD cells, we hypothesized that activation loop mutation Y842F might impact FLT3-ITD-related genes expression. Interestingly, gene expression studies demonstrated that the expression of Y842F in cells led to suppression of anti-apoptotic genes. Furthermore, in a gene set enrichment analysis (GSEA), we found downregulation of several oncogenic pathways including KRAS, SRC and loss of p53 in FLT3-ITD/Y842F cells compared to cells have only FLT3-ITD mutation. These data indicate that FLT3-ITD/Y842F has an impaired oncogenic capacity.

Activation of FLT3 upon ligand stimulation activates intracellular signaling pathways including the Ras/ERK and PI3K/AKT leading to cell proliferation and activation. Because the oncogenic FLT3 is constitutively active, it induces the same signaling pathways as FLT3-WT plus STAT5 signaling [82]. To investigate how the Y842F affects signal transduction, the phosphorylation of AKT and ERK were examined using western blot. We found that cells expressing Y842F suppressed the RAS/ERK pathway. However, we have not seen any difference of AKT phosphorylation between cells expressing Y842F mutant and cells expressing only FLT3-WT/FLT3-ITD. Moreover, there was no reduction of STAT5 phosphorylation in cells expressing Y842F mutant. Since the activation loop Y842 in FLT3 corresponds to activation loop Y823 in KIT, we checked the effect of Y842F on FLT3 activation. As expected, no change was detected in the kinase activity of FLT3 in cells expressing Y842F similarly to previous findings of Y823F in KIT [196]. However, Y823F decreased ERK, AKT, and P38 signaling while Y842F only suppressed ERK signaling.

It is known that phosphorylation of ERK by FLT3 can be induced mainly through GAB2 and SHP2 [203]. Therefore, we checked the GAB2 and SHP2 phosphorylation in presence of Y842F mutant and found that cells expressing Y842F mutant substantially decreased SHP2 phosphorylation but not GAB2 phosphorylation. Since SHP2 can associate with phosphotyrosine Y599 in FLT3 [93], we examined the FLT3-Y599 phosphorylation in cells expressing FLT3-WT or Y842F mutant. No decrease in FLT3-Y599 phosphorylation has been detected in cells expressing Y842F indicating that the activation loop tyrosine does not regulate FLT3-Y599 phosphorylation.

However, it could be speculated that the activation loop Y842 in FLT3 functions as a secondary binding site. Collectively, these data suggest that Y842 in the activation loop is important for SHP2 activity and thereby regulates the RAS/ERK pathway.

In summary, our findings show that activation loop tyrosine residue Y842 in FLT3 plays a crucial role in SHP2 in FLT3-ITD-mediated transformation suggesting that drugs targeting SHP2-Y842 binding or SHP2 activity might improve the outcomes of patients with acute leukemia. Although Y842F mutation seems to be rare, we have added Y842F mutation to the list of mutations in FLT3 that warrant additional investigations to develop future targeted therapy in AML patients.

Chapter 8

Paper IV

The ALK inhibitor AZD3463 effectively inhibits growth of sorafenib-resistant acute myeloid leukemia

Aim

The aim of this paper is to investigate the efficacy of the ALK inhibitor ADZ3463 in FLT3 in acute myeloid leukemia

Introduction

Patients with AML FLT3-ITD mutation have a high relapse rate and poor overall survival after chemotherapy treatment and stem cell transplantation. Several FLT3 inhibitors have been identified and tested in clinical trials. However, FLT3 inhibitors did not achieve robust clinical outcomes due to acquired resistance after treatment [99]. Acquired secondary mutations in FLT3-TKD at D835 have been identified in FLT3-ITD patients relapsed after sorafenib therapy [151]. ALK inhibitor AZD3463 is an anaplastic lymphoma receptor tyrosine kinase inhibitor. One study showed that AZD3463 inhibits neuroblastoma growth by overcoming crizotinib resistance [204]. Recently, another study showed that AZD3463 sensitizes breast cancer cells to rapamycin and leads to cancer cell apoptosis [205]. However, the function of this inhibitor has not been examined in relation to FLT3 in AML. Here, we identify ALK AZD3463 as a novel inhibitor targeting FLT3 ITD as well as overcoming sorafenib resistance in AML.

Results and discussion

The aim of this study is to identify a novel therapy for FLT3-ITD-dependent AML and to overcome the secondary resistance exerted by sorafenib in AML patients carrying FLT3-ITD mutations. It has been reported that aberrant activation of the PI3K/mTOR pathway induces drug resistance in leukemia [206]. Our group has previously reported that sorafenib-resistant cells acquired a secondary mutation in the kinase domain of FLT3 (D835Y) and displayed upregulation of the PI3K/mTOR pathway [97]. To this end, we have generated resistant cells derived from the AML cell line, MOLM-13, by subjecting the cells to long-term treatment with sorafenib. In order to characterize the sorafenib-sensitive and -resistant MOLM-13 cells, Peptide Kinase Profiling assay was performed. We observed upregulation of kinase activity in sorafenib-resistance cells for peptide substrates that are selective to PDGFRB, CSK, and FES compared to sorafenib-sensitive cells. In addition, treating cells with sorafenib for 16 hours inhibits tyrosine phosphorylation of those peptide substrates in sorafenib-sensitive cells but not in sorafenib-resistant cells. These findings suggest that tyrosine kinases phosphorylate several substrates selective for PDGFRB, CSK, and FES that are involved in sorafenib resistance.

In order to determine the kinase-dependency of MOLM-13-sorafenib-sensitive and-resistance cells, cells were treated with a panel of 378 protein kinase inhibitors using different concentrations of kinase inhibitors and the viability of cells was measured using PrestoBlue fluorescence assay. MOLM-13-sorafenib-sensitive and-resistant cells exhibited significant reduction in viability at concentrations (100 and 1000 nM). Besides AML cell lines, we also used a lymphoid cell line, Jurkat cell line, to exclude non-specific inhibition on AML. We found that many inhibitors targeting protein tyrosine kinases including ALK inhibitor AZD3463 selectively inhibited the growth of both sorafenib-sensitive and-resistant cells at 100 nM and 1000 nM concentrations. To verify our finding, we checked the EC_{50} of AZD3463 for both sorafenib-sensitive and-resistant cells. AZD3463 displayed an EC_{50} value around 31 nM and 26 nM respectively. Given that both cells exhibited similar effective inhibition by AZD3463, it was therefore selected for further

studies based on its lowest EC50 values among other inhibitors on MOLM-13-Sorafenib resistant cells. Previous findings have shown that AZD3463 is a promising therapeutic agent against activating ALK mutations in neuroblastoma [204]. Therefore, we checked the ALK expression in human MOLM-13, MV4-11, THP-1 and murine Ba/F3 or 32D AML cell lines. Using western blot, we found that ALK is expressed in the human cell lines but not in the murine cell lines. Therefore, we used the Ba/F3 cell line lacking ALK expression as a control for further experiments.

AZD3463 has been previously shown to decrease proliferation and induce apoptosis in neuroblastoma cells carrying ALK mutation [204]. Moreover, a recent publication found that combination of AZD3463 with rapamycin-induced apoptosis in breast cancer cells [205]. These findings are in line with ours where AZD3463 inhibited the growth of both sorafenib-sensitive and-resistant MOLM-13 cells as well as induced apoptosis in dose-dependent manner suggesting that AZD3463 plays a crucial role in cancer cell survival. Furthermore, treated FLT3-ITD primary AML cells with AZD3463 induced apoptosis. This suggests that AZD3463 is an active drug against FLT3-ITD-dependent AML.

Because Tyrosine kinases share a high degree of structural homology in the kinase domain, inhibitors targeting the ATP-binding site may not be selective for a single kinase. Therefore, a specific inhibitor that targets FLT3 ATP-binding site is increasingly needed. Provided that MOLM-13 cells are dependent on oncogenic FLT3-ITD signaling, and AZD3463 induced apoptosis as well as growth inhibition, we hypothesized that AZD3463 might inhibit FLT3. Using molecular docking, we were able to demonstrate that AZD3463 occupies the ATP-binding site of FLT3 as much as it does with the selective FLT3 inhibitor AC220. To verify the specificity of AZD3463 for FLT3, we hypothesized that AZD3463 might inhibit FLT3 in MOLM-13 cells carrying oncogenic FLT3-ITD.

To this end, we have stably transfected FLT3-ITD in Ba/F3 cell line. Expression of FLT3-ITD was verified by western blotting. We used Ba/F3 cells expressing mutated ALK (ALK-F1174L) as a positive control then we

treated the cells with different concentrations of AZD3463 for 48h and found that Ba/F3-FLT3-ITD cells were more sensitive to the drug compared to Ba/F3-ALK-F1174L cells. These data suggest that AZD3463 is a potent inhibitor of FLT3-ITD.

Next, we wanted to evaluate the specificity of AZD3463 on FLT3-ITD. A panel of AML cell lines expressing FLT3-ITD or FLT3 -WT namely: MOLM-13, MV4-11, PL-21, GDM-1, MOLM-16, NOMO-1, THP-1, KG-1, HL-60, and SKM-1 were examined for cell growth after AZD3463 treatment. We detected selective inhibition for AZD3463 in MOLM-13 and MV4-11 cells which both express FLT3-ITD indicating that AZD3463 is a selective inhibitor of FLT3-ITD. Moreover, treatment of MOLM-13, Ba/F3-FLT3-ITD and THP-1 with different concentrations of AZD3463 reduced cell proliferation in cells expressing FLT3-ITD but not THP1 cells expressing FLT3-WT.

It is well known that FLT3-WT is an important key player for normal hematopoiesis [207]. To check if AZD3463 has an inhibitory effect on FLT3-WT, we first stably transfected FLT3-WT in Ba/F3 cell line. We then examined the role of AZD3463 in signaling downstream of FLT3-WT as well as of FLT3-ITD (including AKT, ERK, and p38) in MOLM-13, MV4-11, THP-1 as well as Ba/F3 cells transfected with FLT3-WT treated with the AZD3463 by western blotting. We found that AZD3463 blocked the phosphorylation of FLT3 and inhibited the activation of AKT, ERK1/2, and p38 signaling pathways in both MOLM-13 and MV4-11 cell lines in a dose-dependent manner. However, AZD3463 was unable to inhibit ligand-induced FLT3 activation, as well as downstream signaling, in MOLM-13, THP, or FLT3-WT expressing Ba/F3 cells. This effect can be explained by the fact that MOLM-13 cells contain one copy of FLT3-WT in addition to the FLT3-ITD mutation while MV4-11 cells carry only the FLT3-ITD mutation. This indicate that AZD3463 selectively inhibits oncogenic FLT3-ITD but not FLT3-WT.

Single agent treatment by FLT3 inhibitors showed limited anti-leukemic activity in clinical studies and displayed secondary resistance and relapse

[208, 209]. Several studies highlighted that combination therapy is a way to enhance the treatment efficiency and overcome the resistance. For example, using midostaurin in combination with intensive chemotherapy has been shown to be associated with improved remission rates [210]. Similar data were also found in a clinical trial that a combination of crenolanib combined with chemotherapy increased the CR rate in FLT3-mutated AML patients [211]. In our study, we have investigated the efficacy of AZD3463 in combination with conventional chemotherapy agents. We combined different concentrations of the chemotherapeutic agents; cytarabine, daunorubicin, vincristine, cyclophosphamide, methotrexate, 6-mercaptopurine and doxorubicin HCL or dexamethasone and AZD3463 in MOLM-13, MV4-11 cells, and using PL-21 cells as a control. We observed parallel effect when AZD3463 combined with cytarabine, daunorubicin, or vincristine in reducing the cell growth, while the rest of chemotherapeutic agents did not show any noticeable effect.

To test the effect of AZD3463 on cell proliferation *in vivo*, we used animal models with xenografts, where we injected MOLM-13 cells subcutaneously. After one week, mice were treated by injection of 15 mg/kg AZD3463 or vehicle for 6 days. Interestingly, mice treated with AZD3463 showed significant delay of tumor growth and reduced tumor weight and volume compared to the vehicle group.

Taking all together, we showed in this study that AZD3463 selectively inhibits the activation of FLT3-ITD and does not affect FLT3-WT downstream signaling. Furthermore, we showed that AZD3463 effectively inhibited FLT3-ITD in AML cells that were resistant to sorafenib. Collectively, this study suggests that AZD3463 is a promising inhibitor to target FLT3-ITD positive AML. However, more studies should be performed *in vitro* including gene expression for the cells treated with AZD3463 as well as the survival advantage and toxicity *in vivo* and investigate the possibility of developing acquired resistance to AZD3463 would give a better understanding about the inhibitor.

Concluding remarks

Our understanding of AML biology has comprehensively increased over the last decade. While the development of FLT3 inhibitors has substantially improved the outcomes of FLT3-mutated AML patients, the emergence of resistance addresses a significant challenge. Mutations as well as persistent activation of downstream signaling pathways of FLT3 contribute to resistance to FLT3 inhibitors. Understanding the oncogenic signaling at multiple levels of AML is key to develop novel FLT3-targeted therapies.

In this thesis, we highlighted the importance of associating proteins in regulating the FLT3 signaling pathways. We have successfully identified SLAP2 and ABL2 as a potent FLT3 interacting proteins and found that they act as negative regulators of FLT3-mediated oncogenic signaling. Our findings suggest that targeting FLT3 receptor indirectly by modulation of receptor stability, activation, and downstream signaling using adaptors proteins can provide an alternative approach to develop novel therapy for FLT3-ITD positive AML.

We have revealed the role of activation loop Y842 in FLT3 signaling and found that Y842 in the activation loop is important for binding and regulation of SHP2 activity and thereby regulating the RAS/ERK pathway. The Y842 mutation is less frequent in AML patients but some studies reported that additional Y842 mutation to FLT3-ITD leads to development of resistance to FLT3 drugs. Our findings suggest an important role of the activation loop tyrosine residue Y842 in SHP2-FLT3-ITD-mediated malignant transformation addressing the possibility of targeting SHP2-Y842 binding or SHP2 to improve the outcomes of patients with acute leukemia.

Finally, we have identified ALK inhibitor AZD3463 as a novel target therapy for FLT3-ITD-dependent AML. Moreover, AZD3463 was found to inhibit the signaling in FLT3-ITD in AML naïve cells and sorafenib-resistant cells. Thus, AZD3463 displayed a promising effect that underscore its potential use in FLT3-ITD AML and warrants more investigations for further clinical evaluation.

Popular science summary

Blood is formed predominantly by bone marrow during the entire life of an individual adult. The formation of blood is called hematopoiesis. Hematopoiesis can give rise to different types of an early-stage blood cells to which we call it immature blood cells and reside mainly in the bone marrow after formation, and more mature cells including the so-called white blood cells which play a very important role in the body's immune system and circulate in blood vessels, lymph nodes, and tissues. These cells are controlled by the DNA which regulates all cellular functions such as cell division, movement, programed cell death, differentiation, etc. Any defect in the DNA might result in uncontrolled cell division which is basically known as cancer.

Leukemia is a type of blood cancer characterized by multiple genetic alterations results from certain damage of the DNA at specific point of the hematopoiesis process. Leukemia can be classified into two main types according to the cell origin or the disease progression. Acute and chronic myeloid leukemia, AML and CML respectively as well as acute and chronic lymphoid leukemia ALL and CLL, respectively.

Among all leukemias, our work is concerned about the AML type. AML is an aggressive blood cancer of immature blood cells that has complex mix of different genetic defects called mutations. This type of disease is mainly occurred in elderlies but can also be found in other ages in a low incidence. The reason behind AML is not fully understood but it is believed that any source with potential risk to damage the DNA such as radiation, certain chemotherapeutic drugs used in cancer therapy can increase the risk to develop AML.

In my thesis, I shed the light on a protein called FLT3 which represents one of the most common genetic mutations in AML. FLT3 is a cell membrane tyrosine kinase receptor that functions to transduce signals to immature blood cells to become mature. This signal is transported through different signaling pathways that are comprised of various cellular proteins to eventually

translate the signal into cell functions, for instance, cell differentiation and division. In normal state, FLT3 works by binding to its respective ligand (FL) and initiates a signal transduction cascade. Mutations in the FLT3 such as internal tandem duplication (ITD) result in a ligand-independent constitutive activation of FLT3 and thereby abnormal non-stopped signals are created and transduced leading to uncontrolled cell differentiation and survival.

Despite the major advancement in leukemia therapy over the past few years, disease recurrence, also known as relapse, remains the main obstacle. Although the development of targeted therapy such as tyrosine kinase receptor inhibitors including those used against FLT3 mutations has revolutionized AML therapy, patients usually develop drug-resistance shortly after treatment. Therefore, another approach to develop novel therapy against FLT3-resistant AML is increasingly needed. It is believed that the heterogeneity of AML reflected by its complexity with different mutations in FLT3 are the main drivers of targeted therapy related failure.

Since activation of FLT3 is regulated by associating proteins which help transducing the FLT3 signals to the cells, it is of high significance to understand how associating proteins mediate FLT3 signaling. In our research work, we have identified SLAP2 as an interacting protein that displayed higher affinity to bind FLT3. Further studies using different biochemical and molecular biological techniques used to define the role of SLAP2 in controlling FLT3-mediated signaling. We found that SLAP2 controlled tumor cell growth signals and reduced tumor cell transformation via regulation of FLT3 receptor activity. Moreover, we have also identified another FLT3 associating protein called ABL2 and studied its role in regulating FLT3 signaling. Our results showed that the presence ABL2 with FLT3-ITD in cells decreased tumor growth and blocked a particular FLT3 signaling called PI3K/AKT. This is of particular interest because targeting interacting proteins could be a potential alternative to target AML.

In addition, we have also demonstrated through mutagenesis studies the role of amino acid called tyrosine (Y) located at the 842 position of the FLT3 receptor (Y842) in regulating FLT3 signaling. We found that mutant Y842 has an ability to decrease survival of AML cells and reduced FLT3 signaling

particularly ERK signaling as well as decrease the binding of FLT3 to SHP2. SHP2 is a potent binding partner of FLT3 required for mediating FLT3-ERK signaling. Our work elucidates the important role of Y842 for FLT3-mediated RAS/ERK signaling and cellular transformation.

Interestingly, using a screening panel of inhibitors for AML cells, we found that ALK inhibitor AZD3463, an inhibitor used preclinically for neuroblastoma with ALK mutation, blocked the FLT3 signaling pathways and selectively killed FLT3-ITD positive AML cells besides those cells who developed secondary resistance against sorafenib, an FLT3 inhibitor.

In summary, we have demonstrated several targeting strategies and identified a novel inhibitor by which FLT3-ITD positive AML can be treated.

Populär sammanfattning

Blod bildas huvudsakligen i benmärgen under hela livet i en vuxen individ. Bildandet av blodceller kallas hematopoes. Hematopoesen kan ge upphov till olika blodceller som i det tidiga stadiet av utveckling, är så kallade omogna blodceller. De förekommer huvudsakligen i benmärgen efter att de bildats och mer mogna celler, inklusive de så kallade vita blodkropparna som spelar en viktig roll i kroppens immunsystem, cirkulerar i blodkärl, finns i lymfnoder och i vävnader. Dessa celler kontrolleras av sitt DNA som reglerar alla cellulära funktioner, såsom celldelning, rörelse, programmerad celldöd, differentiering etc. En defekt i DNA:t kan resultera i okontrollerad celldelning (vad som i princip är känt som cancer).

Leukemi är en typ av blodcancer som kännetecknas av flera genetiska förändringar som beror på skador på DNA:t vid specifika tidpunkter under hematopoesen. Leukemi kan delas in i fyra huvudtyper beroende på cellernas ursprung eller på sjukdomsprogressionen. Akut och kronisk myeloisk leukemi, AML respektive CML, samt akut och kronisk leukemi (ALL respektive CLL).

Bland leukemierna har vårt arbete kretsat kring den typ som kallas AML. Det är en aggressiv blodcancer i omogna blodceller som har en komplex blandning av olika genetiska defekter som kallas mutationer. Detta är huvudsakligen en sjukdom bland den åldrande befolkningen men den förekommer sällsynt även bland yngre. Anledningen till varför vi får AML är inte helt känd men man tror att saker som riskerar att skada DNA:t, såsom strålning och vissa cellgifter som används som cancerterapi, ökar risken av att utveckla AML.

I min avhandling, behandlar jag ett protein som kallas FLT3, vars gen är utgör en av de vanligast muterade generna i AML. FLT3 är ett cell-membran-bundet receptor-tyrosin-kinas som har som uppgift att förmedla signaler till omogna blodceller så att de kan mogna ut. Denna signalering sker genom olika signaleringsvägar vars komponenter utgörs av olika cellulära protein som i slutänden omvandlar signalerna till cellulära funktioner, till exempel

celldifferentiering och celldelning. I normalt fall aktiveras FLT3 genom att binda sin ligand, FL, och en signalkaskad initieras. Mutationer i FLT3 (såsom den så kallade interna tandemduplikationen (ITD) resulterar i ligand-oberoende, konstitutiv aktivering av FLT3 och därigenom förmedlas en abnorm oreglerad signal som leder till okontrollerad celldifferentiering och överlevnad.

Trots stora framsteg inom leukemi-terapi under de senaste åren återfaller patienter i sjukdom vilket är ett hinder för framsteg inom behandlingen. Även om utvecklingen av målriktad terapi, såsom tyrosin-kinas-hämmare (inklusive de som används mot muterad FLT3) har revolutionerat terapin, så utvecklar patienter typiskt resistens mot läkemedelet kort efter att behandlingen har startats. Därför behövs nya sätt att angripa tyrosin-kinas-hämmar-resistent AML. Man tror att heterogenitet i AML (som återspeglas i dess komplexitet med olika FLT3-mutationer) är den huvudsakliga orsaken till misslyckad riktad terapi.

Eftersom aktiveringen av FLT3 signaler är reglerade med proteiner som associerar med receptorn och som hjälper till att fortleda signalen i cellen, så är det mycket viktigt att förstå hur de associerande proteinerna bidrar till FLT3-signalering. I vårt forskningsarbete har vi identifierat SLAP2 som ett protein som binder med hög affinitet till FLT3. Ytterligare studier med hjälp av olika biokemiska och molekylärbiologiska tekniker har hjälpt oss att definieras SLAP2:s roll i att kontrollera FLT3-medierad signalering. Vi fann att SLAP2 kontrollerar tumörens celltillväxtsignaler och minskar tumörcellernas transformation genom att reglera FLT3:s aktivitet. Dessutom har vi identifierat ett annat protein som binder till FLT3, ABL2, och utrett dess roll i FLT3 signalering. Våra resultat visade att närvaro av ABL2 i FLT3-ITD-uttryckande celler minskar tumörtillväxten och blockerar en specifik signaleringsväg, som kallas PI3K/AKT. Detta är av speciellt intresse eftersom man kan tänka sig att attackera associerade signaleringsproteiner som ett sätt att angripa AML.

Dessutom har vi också visat genom mutagenes-studier att aminosyran tyrosin i position 842 i FLT3 (Y842) reglerar FLT3-signalering. Vi fann att om man muterar Y842 till fenylalanin så är receptorn fortfarande aktiv men dess

förmåga att signalera överlevnad i AML har minskat och FLT3-signalering genom speciellt ERK (såväl som bindningen av fosfataset SHP2) har minskat. SHP2 är nödvändigt för att FLT3 ska kunna aktivera ERK. Vårt arbete utredde den viktiga rollen hos Y842 i att mediera aktivering av RAS/ERK signaleringsvägen och cellulär transformation.

Vi screenade en panel av kända hämmare mot våra AML-celler och fann att ALK-hämmaren AZD3463, en hämmare som har använts prekliniskt i neuroblastomceller med ALK-mutation, även hämmade FLT3-signalering och selektivt dödade FLT3-ITD positiva AML-celler inklusive de celler som var resistenta mot tyrosinkinashämmaren sorafenib. Däremot hämmades inte normal, vildtyps FLT3, vilket är bra om man inte samtidigt vill hämma normal hematopoies.

För att summera, så har vi visat på flera strategier för att attackera AML-celler och identifierat en ny hämmare mot FLT3-ITD-positiv AML.

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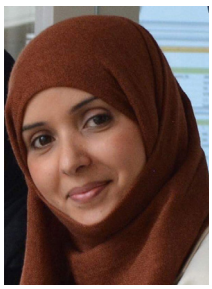
Despite the improvements in leukemia treatment over the past decade, the incidence of leukemia is increasing indicating that leukemia might become a global health concern. While tyrosine kinase inhibitors have dramatically changed the paradigm of leukemia treatment, resistance developed during the course of therapy remains challenging which eventually results in poor clinical outcomes for patients with leukemia particularly acute myeloid leukemia. I believe that the biggest challenge posed by leukemia is the nature of its heterogeneity. Dismantling oncogenic signaling mechanisms is key to understanding therapy resistance with respect to disease heterogeneity in order to develop novel therapies.

About the author



Sausan Moharram is a biomedical scientist who received her university degree from the faculty of medicine at Sanaa University in Yemen. She had several years of experience in working in clinical laboratories especially in transfusion medicine. She moved to Sweden 2010 and received her master's degree in molecular biology with special focus in tumor immunology. She pursued her PhD in translational cancer research focusing on acute leukemias at the faculty of medicine, Lund University.

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