Alternative splicing in acute leukemia-relevance in treatment response

Wojtuszkiewicz, A.M.

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LIST OF ABBREVIATIONS

AAI - anti-apoptosis index
ALL - acute lymphoblastic leukemia
AML - acute myeloid leukemia
AraC - cytarabine
BCL-2 - B-cell CLL/lymphoma 2
BCL-X - BCL2 like 1
BM - bone marrow
CFSE - carboxyfluorescein succinimidyl ester
CI - combination index
CR - complete remission
Dex - dexamethasone
DHFR - dihydrofolate reductase
DXR - doxorubicin
EV - extracellular vesicle
FACS - fluorescence-activated cell sorting
FPGS - folylpolyglutamate synthetase
hnRNP - heterogenous nuclear ribonucleoprotein
IC - inhibitory concentration
Intron 8 PR - intron 8 partial retention
LC - lethal concentration
MAMB - meayamycin B
MCL-1 - myeloid cell leukemia 1
MRD - minimal residual disease
MTT - 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide
MTX - methotrexate
PB - pladienolide B
PBS - phosphate buffered saline
PCR - polymerase chain reaction
PG - polyglutamate
RFC - reduced folate carrier
SF3B1 - splicing factor 3 subunit 1
SRSF - serine/arginine-rich splicing factor
TS - thymidylate synthase
TSIA - thymidylate synthase inhibition assay
VCR - vincristine
WT - wild type

CHAPTER 1

General introduction

INTRODUCTION

This thesis focuses on mRNA splicing in acute leukemia, with an emphasis on its role in drug resistance and its potential as novel treatment modality by targeting the spliceosome. This chapter provides some relevant background information related to this topic.

Acute leukemia

Acute leukemia is a hematological malignancy derived from the bone marrow precursor cells. The processes of differentiation and maturation are blocked in leukemic cells, which together with their unharnessed proliferative capacity eventually lead to overcrowding of the bone marrow and consequently abnormal hematopoiesis. Acute leukemia can originate from two types of white blood cell precursors, of the myeloid or the lymphoid lineage, giving rise to either acute myeloid leukemia (AML) or acute lymphoblastic leukemia (ALL). ALL is the most common type of cancer in children, while AML predominantly affects adults.[1,2] The treatment outcome of pediatric ALL has tremendously improved over the years with a 5-year event-free survival reaching currently as high as 85%.[2,3] This great achievement is largely attributed to better supportive care together with a deepened understanding of the disease biology, which translated to improved risk stratification and therapy regimens.[3] However, the treatment results for AML are less impressive.[4] Moreover, still about 20% of childhood ALL and 40% of pediatric AML patients eventually have to face a relapse, which comes with a dismal prognosis.[4–6] Since, cellular drug resistance is among the most common causes of relapse, it is imperative to further unravel its mechanisms, in order to circumvent it or offer alternative treatments, resulting in therapy better adjusted to the risk of relapse of individual patients.

Acute lymphoblastic leukemia - genetic background and treatment outline

Proper assessment of the risk of relapse for ALL patients is a critical step in selection of optimal therapy, which ideally combines maximised efficacy against leukemia with minimal toxicity to normal tissues. Current risk classification takes into account readily apparent clinical characteristic of patients as well as biological features of leukemic cells.[2] Based on these characteristics most current treatment protocols stratify patients into standard, high (or intermediate/average) and very high risk groups, which receive appropriate risk-adjusted treatment. Among the clinical characteristics the white blood cell count, age at diagnosis as well as lineage (precursor B-cell or T-cell ALL) have proven to be strong indicators of prognosis in ALL.[2] Over the course of time a growing number of genetic abnormalities have been found to dictate prognosis for ALL patients. These include chromosomal aberrations, often resulting in gene fusions, as well as genetic polymorphisms and point mutations. Essential cellular processes, such as hematopoiesis, proliferation and signalling are amongst the pathways frequently perturbed by various genetic lesions in many ALL subtypes.[3] The most common genetic alterations, appearing in about 20-25% of childhood ALL patients, include hyperdiploidy (>50 chromosomes per cell) and *ETV6-RUNX1* (*TEL-AML1*) fusion, which are both associated with a favourable treatment outcome.[2,7] In contrast, hypoploidy (<45 chromosomes in a cell), *MLL*-rearrangements and *BCR-ABL1* fusion confer poor prognosis.[2] Interestingly, in some cases *BCR-ABL1*-negative precursor B-cell ALL samples were characterised by *BCR-ABL1*-like gene expression profile, which was also associated with high risk of relapse.[7] The usefulness of transcriptome profiling in ALL prognostication was further demonstrated in 2 studies showing that

differential gene expression profiles were associated with resistance to core chemotherapeutics used in ALL (including prednisone, vincristine and L-asparaginase) and were strong predictors of the treatment outcome.[8,9] Current treatment protocols for ALL are based on combination chemotherapy administered in three phases - remission induction, consolidation/intensification and maintanance.[1,3] The essential therapeutic backbone in contemporary ALL treatment protocols includes next to dexamethasone (Dex), vincristine and L-asparaginase, the folate antagonist - methotrexate (MTX). MTX is a continuously important pillar in ALL treatment administered within the central nervous system (CNS) directed prophylaxis of relapse as well as during consolidation and maintenance therapy.[2,3] It is one of the oldest chemotherapeutics used in ALL treatment and substantial knowledge on its mechanism of action is available.

Antifolate metabolism

MTX acts as an antagonist of folates (vitamin B9) - essential vitamins, which are used as one-carbon donors in a plethora of crucial biosynthetic processes, including biosynthesis of purines and thymidylate, amino acid conversion and mitochondrial protein synthesis.[10,11] MTX exerts its function by blocking folate-dependent enzymes (with its primary target being dihydrofolate reductase - DHFR), leading to inhibition of the nucleotide biosynthesis.[12,13] This in turn results in blocked DNA replication and consequently cell death.[14] Under physiological pH, MTX is predominantly taken up by leukemic cells via the reduced folate carrier (RFC/SLC19A1), after which it undergoes a unique reaction – polyglutamylation (Figure 1).[10,11] Due to this metabolic conversion, catalysed by the folylpolyglutamate synthetase (FPGS), intracellular retention of MTX is largely enhanced with a concomitant increase in MTX-mediated inhibition of a number of folate-dependent enzymes (i.e. thymidylate synthase - TS/TYMS, and aminoimidazole carboxamide ribonucleotide transformylase - AICARTFase).[10,11] Consequently, polyglutamylation substantially augments the pharmacological activity of MTX and decreased accumulation of this imperative metabolite was shown to be related to poor response to MTX treatment in ALL patients.[15,16] As MTX has proven to be a cornerstone in ALL therapy, the mechanisms of resistance to this drug need to be further delineated to improve treatment outcome of ALL patients.[3,15] Next to impaired FPGS activity, several other alterations in MTX metabolism have been shown to result in MTX resistance and increased risk of relapse. This includes inactivating mutations in or down-regulation of *RFC* gene, elevated levels of DHFR and TS enzymes as well as polymorphisms in *RFC*, *TS* and *DHFR* genes.[10,17–24] As illustrated by these examples, drug resistance of leukemic cells can result from molecular aberrations of various types and much is known about changes in DNA as well as transcription regulation leading to decreased therapy responsiveness. However, the contribution of aberrant splicing to drug resistance remains currently poorly characterised.

mRNA splicing in drug resistance of leukemia

Drug resistance in leukemia can originate from molecular lesions affecting genes involved in drug metabolism or regulation of apoptosis – the process of programmed cell death.[10,25,26] Disturbances in these phenomena can lead to decreased responsiveness of leukemic cells to treatment or an outgrowth of drug resistant clones and a consequent disease refractoriness or relapse, respectively.[27] One of the important novel mechanisms affecting drug metabolism and apoptosis regulation resulting in the occurrence of drug resistance is aberrant splicing.

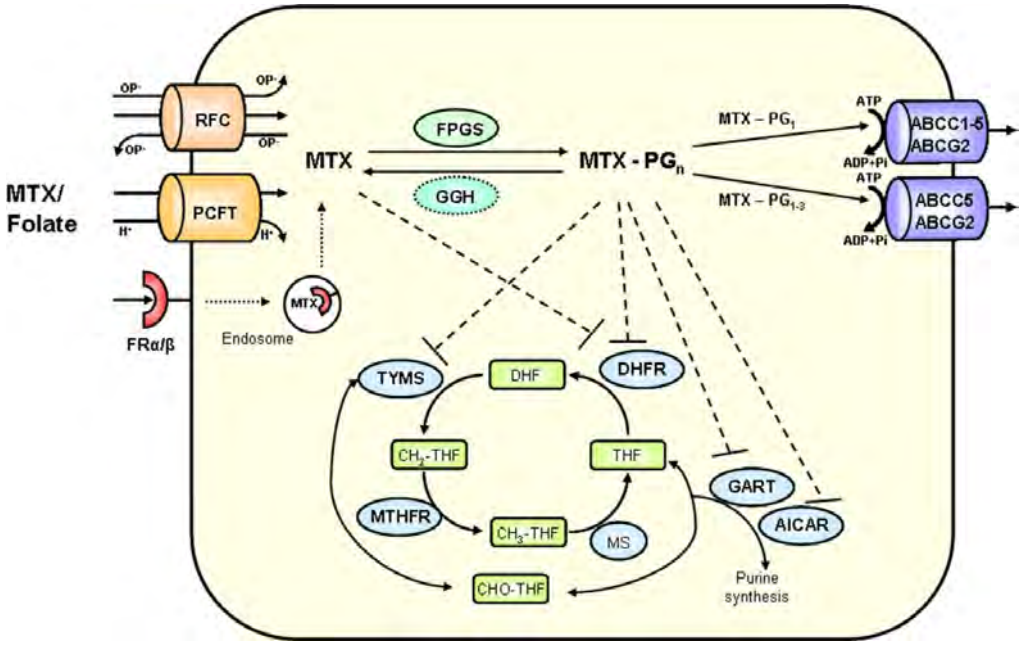

Figure 1. Major components involved in folate/MTX metabolism. The figure depicts proteins engaged in uptake, extrusion and metabolism of folate/MTX with MTX-inhibited enzymes highlighted. The influx transporters are indicated on the left and include Reduced Folate Carrier (RFC), proton coupled folate transporter (PCFT), as well as folate receptor (FR) α and β isoforms. The enzymes metabolizing folates as well as MTX comprise folylpolyglutamate synthetase (FPGS) and gamma-glutamyl hydrolase (GGH, compartmentalized in lysosomes). The folate dependent enzymes involved in folate cycling include dihydrofolate reductase (DHFR), thymidylate synthase (TYMS), aminoimidazole-carboxamide ribonucleotide transformylase (AICARTF/ATIC), glycinamide ribonucleotide transformylase (GART), methylenetetrahydrofolate reductase (MTHFR) and methionine synthase (MS). The MTX efflux transporters, members of the ABC (ATP binding cassette) transporter family include ABCC1-5 and ABCG2. Inhibition of folate dependent enzymes by methotrexate is depicted with the long dashed line. MTX – Methotrexate; PG - polyglutamate; OP - Organic Phosphate; H+ - Hydrogen; ATP - Adenosine-5′-triphosphate; ADP - Adenosine diphosphate; Pi - phosphate; DHF - Dihydrofolate; THF - Tetrahydrofolate; CH3-THF - 5-methyl-tetrahydrofolate; CH2-THF - 5,10-methylene-tetrahydrofolate; CHO-THF - 10-formyl-tetrahydrofolate (adapted from Blits *et al*, 2013[10]).

The vast majority of human genes consists of the protein coding regions (exons) interrupted by the non-coding sequences (introns).[28] Hence, to allow proper translation into proteins, the introns are excised from pre-mRNA via a process termed spicing, resulting in mature mRNA consisting exclusively of exons (Figure 2). Splicing is catalysed by a multicomponent ribonucleoprotein complex - the spliceosome.[29] Abnormalities in the process of mRNA splicing have recently started to gain attention in the context of hematological malignancies.[30] Mutations in several factors involved in splicing, including splicing factor 3b, subunit 1 (SF3B1), U2 small nuclear RNA auxiliary factor 1 (U2AF1) and serine/arginine-rich splicing factor 2 (SRSF2) were found in several cancers of the hematopoietic system and hold promise as prognostic tools as well as contributors to pathogenesis.[30,31] Nonetheless, our understanding of the role of splicing in drug resistance is still very limited, especially with respect to genes involved in drug metabolism. The role of splicing in

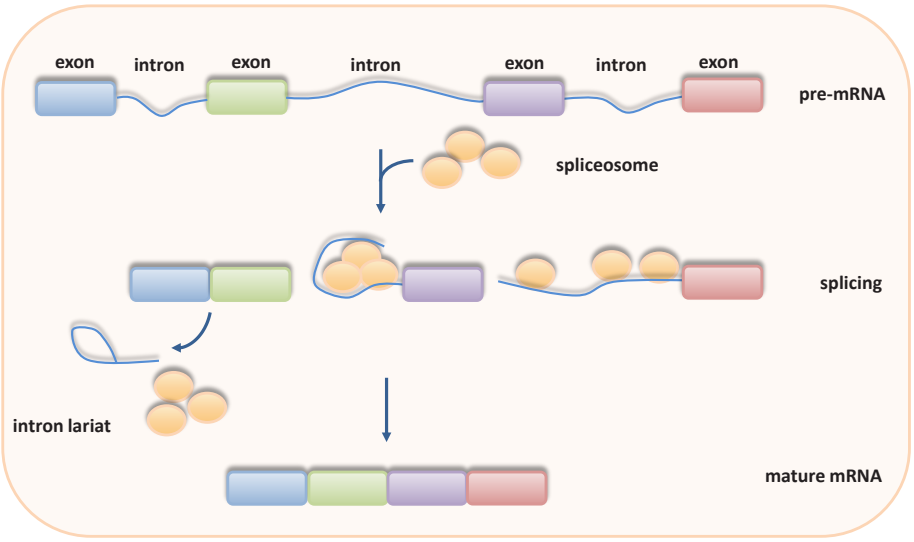

Figure 2. Schematic overview of the pre-mRNA splicing process. In the process of pre-mRNA splicing the introns (non-coding regions) are recognized by the multicomponent complex - the spliceosome. The spliceosome then catalyzes excision of introns, which are released from mRNA in a form of lariat structures, coupled with joining the exons together. The resulting mature mRNA molecule consists of exons alone.

regulation of apoptosis is overall quite well documented.[31–33] Several genes, including BCL2-like 1 (BCL-X), Myeloid Cell Leukemia 1 (MCL-1) and Caspase 2, were shown to be differentially spliced into 2 isoforms with antagonistic functions in apoptosis.[31–33] Elevated levels of the anti-apoptotic isoforms of these genes were associated with enhanced cell survival and decreased sensitivity to treatment. Still, how the changes in splicing patterns of genes engaged in apoptosis translate precisely to chemotherapy resistance in leukemia remains to be determined. Up to date, several genes implicated in drug metabolism in ALL and AML have been described to be aberrantly spliced, resulting in decreased activity of chemotherapeutics. This includes deoxycytidine kinase (dCK)[34–36], human equilibrative nucleoside transporter 1 (hENT1)[36] and glucocorticoid receptor (GR).[37,38] Recently, impaired splicing of folylpolyglutamate synthetase (FPGS) has been evidenced as a potential contributor to MTX resistance in adult ALL.[39] The occurrence of aberrant FPGS splicing in childhood ALL, its exact contribution to MTX resistance, as well as the clinical relevance of this phenomenon requires further experimental exploration.

Targeting the spliceosome in ALL

The gradually unravelling role of splicing in the pathogenesis and drug resistance of leukemia comes with novel therapeutic opportunities for patients harboring this type of lesions. Since, the knowledge of splicing regulation and its impact on disease biology is still rather limited, targeting this process as well as overcoming splicing-based drug resistance is challenging. In this respect, several therapeutic strategies are currently under study (Figure 3), including oligonucleotides specifically targeting abnormally spliced gene products[40–42], as well as molecules inhibiting SF3B -

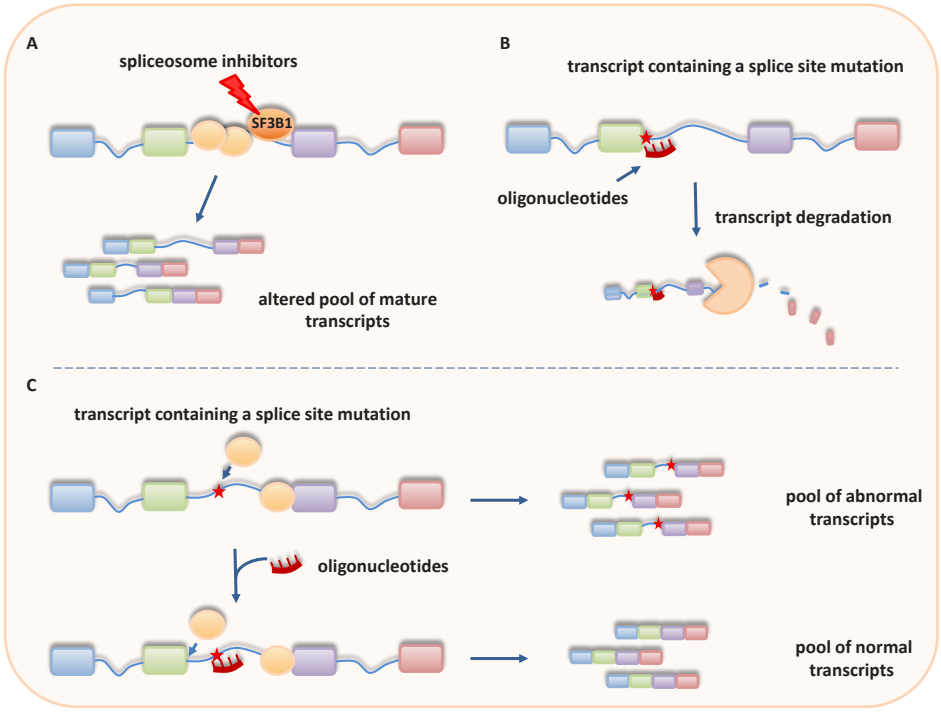

Figure 3. Currently available therapies targeted at splicing. The figure depicts current strategies targeting either the spliceosome itself (**A**), or specific abnormal transcripts resulting from aberrant splicing (**B** and **C**). **A** – compounds targeting the spliceosome mainly include inhibitors of the SF3B subunit of the spliceosome; their clinical relevance is currently under investigation. **B** – Targeting aberrant transcripts includes the usage of oligonucleotides, which bind to abnormal transcripts initiating their degradation (**B**) or block the mutated splice sites, preventing their recognition in the splicing process (**C**).

one of the spliceosome subunits.[43–45] SF3B inhibitors were shown to modify splicing profiles of target cells, leading to cell cycle arrest and apoptosis.[43–45] Interestingly, these compounds display enhanced selectivity for tumor cells compared to normal tissues, rendering them attractive candidates as novel class of cytotoxic agents in cancer treatment.[46] Spliceosome inhibitors were so far evaluated for their therapeutic potential in a range of tumors, including lymphoma and chronic lymphoblastic leukemia. Several *in vitro* and *in vivo* studies showed promising results with spliceosome inhibitors being effective as single agents as well as in combination with other drugs.[43–51] Together, as disruption of splicing in acute leukemia is becoming evident, experimental exploration of potential splicing-modulating therapies is warranted in these malignancies.

AIM OF THE THESIS

The aim of this thesis was to explore the role of splicing in drug resistance as well as novel treatment options for acute leukemia, based on spliceosome-inhibition. We first evaluated the extent of aberrant splicing in the FPGS gene in ALL, followed by the assessment of its impact on FPGS function and MTX resistance, using *in vitro* leukemic cell line models. To validate our findings in an *ex vivo* clinical setting, we then determined the impact of FPGS splicing aberrations in a large pediatric ALL patient cohort with respect to associations with MTX resistance and the clinical outcome. Moreover, we explored the efficacy of novel class of compounds - spliceosome inhibitors, including pladienolide B and meayamycin B – as a single agent as well as a tool to sensitize leukemic cells displaying splicing-based drug resistance. Finally, we investigated whether the intracellular impact of splicing on drug resistance is paralleled by its potential role in intercellular communication between leukemic cells. In this respect, we determined whether splicing regulating factors were among the proteome composition of the secretome as well as extruded extracellular vesicles of apoptosis-resistant primary AML cells.

INTRODUCTION TO THE CHAPTERS

Chapter 2 covers a review on several aspects of pre-mRNA splicing in cancer treatment. This chapter discusses the current status of the molecular mechanisms and relevance of splicing for drug resistance and prognosis in hematological malignancies. Moreover, it summarizes current options for splicing-targeted therapies, including splice variant-specific approaches utilizing oligonucleotides as well as more general splicing modulation with a novel class of chemotherapeutics – spliceosome inhibitors.

Chapters 3 – 5 focus on disturbed FPGS splicing and its role in MTX resistance in pediatric ALL. **Chapter 3** presents the clinical significance of MTX resistance in pediatric ALL patients treated with combination chemotherapy. In **chapter 4** we assessed the occurrence of splicing alterations in FPGS in adult and pediatric ALL patients. The impact of aberrant FPGS splicing on FPGS function was explored together with its modulation in response to treatment with MTX and other chemotherapeutics. Finally, the occurrence of FPGS splicing aberrations was determined in a large pediatric ALL patient cohort. The relation of detected FPGS splicing alterations with MTX resistance and the clinical outcome of ALL patients is described in **chapter 5**.

Chapter 6 reports on a novel splicing-utilizing therapeutic approach in leukemia. In this study, we explored the response of a panel of leukemic cell lines (including drug resistant cells) and patient samples to spliceosome inhibitors, including pladienolide B and meayamycin B. Moreover, the sensitivity of primary leukemic cells was compared to normal bone marrow samples. Lastly, we assessed the ability of spliceosome inhibitors to re-sensitize MTX as well as Dex resistant leukemic cells.

Finally, in **chapter 7** we evaluated splicing as a possible mediator of apoptosis resistance in AML. In this study, we characterised the proteome profile of the secretome as well as extracellular vesicles extruded by apoptosis-resistant primary AML cells. The major functional protein clusters potentially involved in intercellular communication-dependent chemotherapy resistance are described.

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