Alternative splicing in acute leukemia-relevance in treatment response

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CHAPTER 9

English/Dutch/Polish summary

SUMMARY

Introduction

The term cancer comprises a group of over 100 diseases characterized by abnormal cell growth involving various tissues. While the growth of normal cells in our body is a subject to stringent surveillance, in cancer some cells manage to escape this mechanism and start dividing in an uncontrolled manner. This unharnessed proliferative potential leads to overcrowding of normal tissues with cancerous (or malignant) cells, which often have the ability to spread across the body and invade other tissues.

Acute lymphoblastic leukemia (ALL) is a malignancy which arises in the bone marrow affecting immature lymphocytes - a subtype of white blood cells - which comprise the immune system. Immature leukemic blasts divide uncontrollably and accumulate in the bone marrow, thereby impairing its normal hematopoietic function. ALL constitutes approximately 25% of all cancer diagnoses in children under 15 years of age with the peak incidence observed at the age of 2-3 years. The treatment outcome of pediatric ALL has improved over the past decades with a current 5-year event-free survival (EFS) reaching as high as 85%.[1,2] Still about 20% of childhood ALL patients eventually face a relapse, which comes with a dismal prognosis. Therapy resistance continues to pose a major obstacle to efficacious treatment, hence the mechanisms underlying this phenomenon warrant further exploration. Pre-mRNA splicing - one of the essential steps in gene expression - has recently been described as novel potential prognostic tool and contributor to pathogenesis of haematological malignancies as well as drug resistance.[3,4]such as chemorefractoriness. While investigating the coding genome of fludarabine-refractory CLL, we observed that mutations of SF3B1, encoding a splicing factor and representing a critical component of the cell spliceosome, were recurrent in 10 of 59 (17% Therefore, in this thesis we set out to explore the relevance of alternative splicing in acute leukemia, including its role in therapy resistance as well as novel treatment options based on inhibition of the spliceosome - the complex machinery containing RNA and proteins, which catalyzes splicing.

Pre-mRNA splicing in cancer

Compared to other steps of gene expression, pre-mRNA splicing has been relatively understudied with respect to both its mechanism and the role it plays in cancer. Recent years have brought a growing number of publications addressing both of these issues. In **chapter 2** we comprehensively discussed the molecular mechanisms behind splicing and current developments regarding splicing aberrations in cancer pathogenesis and prognosis as well as spliceosome-targeted therapy approaches. We reviewed the novel mutations recently identified in cancer affecting various components of the splicing machinery, including SF3B1 and U2AF1, together with their potential use in prognosis of cancer therapy, in particular in hematological malignancies. Moreover, the contribution of splicing aberrations to chemoresistance was discussed with respect to genes involved in both drug metabolism and apoptosis regulation. Although the role of splicing in regulation of apoptosis is well documented, it remains unclear how it precisely translates to chemotherapy resistance in leukemia. Moreover, several genes implicated in drug metabolism in ALL and in a different acute leukemia subtype - acute myeloid leukemia (AML) - have been described to

be aberrantly spliced, resulting in decreased activity of chemotherapeutics.[5–7]autoimmune disease, and cancer. The traditional view that glucocorticoids act through a single glucocorticoid receptor (GR In this respect, aberrant splicing of folylpolyglutamate synthetase (*FPGS*) has been associated with methotrexate (MTX) resistance in adult ALL.[8]methotrexate; MTX MTX – a folate antagonist - continues to be an important pillar of ALL treatment used in prophylaxis of central nervous system relapse and during maintenance therapy.[1,2] Its efficacy is highly dependent on the activity of FPGS - the enzyme, which catalyzes polyglutamylation of MTX (sequential addition of multiple glutamate residues), thereby enhancing its intracellular retention and target enzyme inhibitory potency. As MTX has proven to be of great essence in ALL therapy, further delineation of the mechanisms of resistance to this drug might help to improve the treatment outcome of ALL patients.

MTX resistance and aberrant FPGS splicing

While many molecular mechanisms of MTX resistance in ALL have been well-defined and characterized in an *in vitro* setting, their clinical relevance is often unclear. In **chapter 3** we investigated the underlying basis of MTX resistance in a cohort of 235 childhood ALL patients, for whom clinical data with a long term follow-up were available. The analyzed samples, obtained at the time of diagnosis, were also characterized with respect to potential MTX resistance parameters, including MTX-polyglutamate levels in leukemic cells, MTX sensitivity as determined by the inhibition level of one of its target enzymes - thymidylate synthase (TS) and FPGS catalytic activity assays. Beyond this, mRNA expression was measured for several enzymes involved in (anti)folate metabolism (FPGS; TS; gamma-glutamyl hydrolase - FPGH; dihydrofolate reductase - DHFR) and transport (reduced folate carrier - RFC). Extending on previous reports, our analysis showed that leukemic blast cell accumulation of high levels of long-chain MTX polyglutamates were strongly associated with longer survival of ALL patients. Similar relations were observed for the total accumulation of polyglutamylated MTX and FPGS catalytic activity. Moreover, FPGS activity was the most prominent determinant of MTX resistance (as defined by TS inhibition assay - TSIA) in this study emphasizing its importance in the clinical setting.

Recently, impaired splicing of *FPGS* has been shown to potentially contribute to MTX resistance in adult ALL patients.[8]methotrexate; MTX In **chapter 4**, we evaluated the profiles of aberrant splicing in the *FPGS* gene in both adult and childhood ALL, and assessed its impact on FPGS function and MTX resistance, using *in vitro* cell line models. The occurrence of *FPGS* splicing alterations was first investigated in an initial set of ALL patients using a PCR-based assay spanning the entire sequence of *FPGS* transcripts. This comprehensive splice variant screening showed a plethora of splicing alterations occurring in the *FPGS* gene in childhood ALL patient samples, reaching beyond the initial expectations. The impact of aberrant *FPGS* splicing on FPGS function was explored using 3D protein modelling and *in vitro* FPGS activity assay. Moreover, modulation of *FPGS* splicing in response to treatment with MTX and other chemotherapeutics was determined. These analyses demonstrated that *FPGS* transcripts harboring splicing alterations result in disrupted FPGS function. The most prominent *FPGS* splice variant noted in all the examined samples was intron 8 partial retention (intron 8 PR). This *FPGS* splice variant was particularly modulated in the human FPGS-deficient MTX-resistant T-ALL cell line – CCRF-CEM/R30dm in response to MTX exposure, indicating that dynamic changes in *FPGS* splicing might facilitate cell survival under antifolate-induced stress.

To validate our findings in an *ex vivo* setting we then determined *FPGS* splicing aberrations in a large pediatric ALL patient cohort (91 patients), wherein we investigated its association with MTX resistance and clinical outcome. The levels of *FPGS* splicing alterations were semi-quantified using PCR combined with fragment analysis (**chapter 5**). Additionally, the sensitivity to other chemotherapeutics was assessed in the MTT assay for these patient specimens. Since many genes in the cell share the same splicing regulators, we also addressed the question whether aberrant *FPGS* splicing might be an indication of a broader underlying defect in the splicing machinery, which potentially results in multidrug resistance. Supporting our observations *in vitro*, intron 8 PR appeared again the most relevant *FPGS* splice variant in the *FPGS* splicing screen performed in our cohort of childhood ALL patients. In a subset of patients with suboptimal concentration of MTX polyglutamates high levels of *FPGS* intron 8 PR were predictive of inferior survival. The relation between *FPGS* intron 8 PR and survival remained significant in multivariate analysis, which included known prognostic factors such as white blood cell count, lineage and age. Interestingly, in this subgroup of patients, high levels of *FPGS* intron 8 PR correlated with higher MTX resistance as determined by the TSIA assay and lower accumulation of long chain MTX polyglutamates. Moreover, high levels of intron 8 PR were also associated with resistance to glucocorticoids. Remarkably, we found that an MTX-resistant FPGS-deficient subline of CEM/R30dm harbours high level of intron 8 PR and, concomitantly, a markedly high dexamethasone (Dex) cross-resistance (350-fold). These observations suggest that aberrant *FPGS* splicing might constitute a part of a broader phenomenon resulting from defects in the splicing machinery and affecting multiple genes. This hypothesis warrants further exploration and implies that patients displaying aberrant splicing-based drug resistance could potentially benefit from splicing modulating therapeutic strategies.

Potential of splicing-directed therapies

Several splicing-directed therapies currently exist, including target specific oligonucleotide-based strategies as well as general spliceosome inhibition using small molecules. The latter agents primarily target SF3B1, which is one of the subunits of the spliceosome, and showed promising results in solid tumors as well as in chronic lymphocytic leukemia (CLL).[9–12] Based on these observations, we first evaluated whether pre-mRNA splicing is a suitable target for therapy also in pediatric acute leukemia using novel spliceosome inhibitors - pladienolide B (PB) and meayamycin B (MAMB). The response of leukemic cell lines to both agents was assessed using the MTT assay. Remarkably, both ALL and AML cell lines were responsive to very low (low nanomolar) concentrations of PB and MAMB. The observed growth inhibition was associated with cell cycle arrest in G_1 and G_2/M phases as well as apoptosis induction with concomitant splicing changes in several apoptosis-related genes, including *MCL-1* and *BCL-X*. Interestingly, even leukemic sublines resistant to various conventional chemotherapeutics, including MTX, Dex, bortezomib and imatinib remained sensitive to SF3B1 inhibitors. In line with our *in vitro* leukemia cell line models, both primary ALL and AML samples showed a remarkable sensitivity to PB and MAMB in the MTT assay, while the non-malignant BM cells obtained from healthy children were significantly less sensitive (though still in the nanomolar range) These findings suggest that SF3B1 inhibitors could be further evaluated as a novel therapeutic option for patients displaying drug resistant disease. Our results do suggest that spliceosome inhibition might be able to sensitize MCL-1-dependent Dex resistance in ALL patients, as suggested

by the synergistic effect of both drugs combined in Dex-resistant cell lines. These *in vitro* findings should be further confirmed in mouse models with a careful consideration of potential toxicity to normal tissues. Moreover, *in vivo* SF3B1 inhibitors-mediated modulation of apoptosis-related splicing profiles should be thoroughly assessed, since expression as well as splicing of apoptosis-related factors was previously shown to be influenced by the tumor microenvironment.

Alternative splicing in intercellular communication

Apoptosis-resistance of AML cells at diagnosis is associated with disease-free survival of AML patients and was previously shown to be influenced by microenvironmental factors, including cytokines. In this respect, in **chapter 7** we showed that the expression of apoptosis-related proteins, including anti-apoptotic proteins BCL-2, MCL-1L and BCL-XL as well as pro-apoptotic BAX, is correlated between AML blasts and normal lymphocytes derived from AML patients. Moreover, contact-cultures with apoptosis-resistant cells were able to increase the expression of anti-apoptotic protein BCL-2 in apoptosis-sensitive cells. To gain insight into potential mediators of apoptosis-resistance, we performed comprehensive proteome profiling of both the whole secretome and purified extracellular vesicles (EVs) generated by apoptosis-resistant and apoptosis-sensitive AML cells. Surprisingly, our results showed that among proteins differentially secreted by apoptosis-resistant primary AML cells, regulation of pre-mRNA splicing is the top functional protein cluster, as compared to apoptosis-sensitive AML cells. Interestingly, we found that pre-mRNA splicing was also among the upregulated functional protein clusters uncovered in exosomes secreted by apoptosis-resistant primary AML blasts, in comparison to exosomes of apoptosis-sensitive AML blasts. These observations suggest that apoptosis-resistant cells in AML shed exosomes carrying proteins engaged in pre-mRNA splicing, which might be responsible for transfer of the resistant profile to neighboring apoptosis-sensitive cells. This notion is supported by confocal imaging analyses demonstrating uptake of apoptosis-resistant AML cells-derived EVs in apoptosis-sensitive cells. The impact of this process on the apoptosis-resistance of the recipient cells remains to be determined. Vesicle-mediated transfer of protein networks involved in global modulation of gene expression, conferring the induction of drug resistant profile in AML, is an intriguing concept. Ultimately, it could provide novel biomarkers of apoptosis/drug resistance, which might be detected in the blood of AML patients.

Conclusions

Altogether, our results indicate that aberrant splicing is a highly relevant phenomenon for several aspects of leukemia treatment. Alternative splicing, including *FPGS* splice variants, have an impact on drug resistance as well as the response of leukemic cells to therapy in the clinic. The broad spectrum of splicing profiles indicative of drug resistance warrants further characterization in ALL and AML cells aiming to apply this information in guiding clinical monitoring of drug responses. Deeper insights into these processes could contribute to the design of novel therapies targeting defects in splicing or modulating them in order to re-sensitize therapy resistant disease.

Key Points

- Aberrations in pre-mRNA splicing contribute to both pathogenesis and drug resistance of hematological malignancies, including acute leukemia.

- Elevated intracellular concentrations of methotrexate (MTX) long-chain polyglutamates and higher activity of folylpolyglutamate synthetase (FPGS) are key factors for better long-term treatment outcome of childhood acute lymphoblastic leukemia (ALL).
- Aberrations in FPGS splicing are common in ALL and result in unproductive transcripts or proteins lacking functional activity.
- Intron 8 partial retention of FPGS is related to inferior treatment outcome in childhood ALL patients with decreased accumulation of polyglutamylated MTX as well to dexamethasone resistance, suggesting a broader splicing defect.
- Spliceosome inhibition holds potential as a novel therapeutic option for (drug-resistant) ALL and acute myeloid leukemia (AML) patients, but possible toxicity to normal bone marrow cells warrants further investigation.
- Apoptosis-resistant primary AML cells secrete exosomes containing regulatory protein networks, including splicing factors, which can be taken up and potentially induce apoptosis resistance in primary apoptosis-sensitive cells.

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