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Alternative splicing in acute leukemia-relevance in treatment response

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2016

document version

Publisher's PDF, also known as Version of record

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citation for published version (APA)

Wojtuszkiewicz, A. M. (2016). *Alternative splicing in acute leukemia-relevance in treatment response*. [PhD-Thesis - Research and graduation internal, Vrije Universiteit Amsterdam].

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CHAPTER

**General discussion
and future perspectives**

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Therapy resistance continues to pose a major obstacle to efficacious treatment of both acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) patients and therefore, the mechanisms underlying this phenomenon warrant further exploration. Pre-mRNA splicing has recently come to attention in hematological malignancies as a novel prognostic tool and contributor to pathogenesis as well as drug resistance (**chapter 2**).¹⁻¹⁶ Taking into account the unprecedented complexity of pre-mRNA splicing, it remains a great challenge to understand how disturbances in the mechanisms that govern splicing regulation can lead to therapy resistance. Herein, we set out to explore the relevance of pre-mRNA splicing in leukemia from different angles (Figure 1). Firstly, we investigated whether aberrations in folypolyglutamate synthetase (*FPGS*) splicing can contribute to methotrexate (MTX) resistance in childhood ALL. In this analysis, we employed an *in vitro* cell line model, in which we explored this phenomenon, followed by a clinical validation of our findings in a childhood ALL patient cohort. Secondly, as 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. The latter defect may potentially result in multidrug resistance and can possibly be transferred between cancer cells residing in the same microenvironment. Finally, we evaluated whether the pre-mRNA splicing is a suitable target for therapeutic interventions in pediatric leukemia using novel spliceosome inhibitors - pladienolide B (PB) and meayamycin B (MAMB).

MTX resistance

Over the past seven decades MTX has proven to be of essence in achieving cures in childhood ALL treatment.¹⁷ Several studies documented better anti-leukemic effect of MTX as well as favourable 5-year event-free survival (EFS) in childhood ALL patients whose leukemic cells accumulated higher levels of MTX polyglutamates.¹⁸⁻²⁰ In **chapter 3**, we extended on these reports by corroborating that MTX continues to be a crucial determinant of the long-term outcome of childhood ALL treatment. The most important factor associated with 10-year overall survival (OS) and EFS of ALL patients treated with MTX-containing regimens was the level of MTX polyglutamates accumulated in leukemic cells, particularly the long-chain MTX polyglutamates. Intracellular concentration of these active metabolites of MTX was previously shown to be affected by a number of factors, including impaired reduced folate carrier (RFC) function, enhanced expression of dihydrofolate reductase (DHFR) and thymidylate synthase (TS) together with mutations diminishing their affinity towards antifolates, as well as overexpression of specific drug efflux transporters of the ATP-binding cassette transporter family.²¹⁻²⁹ In line with previous reports³⁰, *FPGS* activity was the most prominent determinant of MTX resistance (as defined by the level of *ex vivo* inhibition of one of its target enzymes, thymidylate synthase - TS) in this study. Moreover, the activity of *FPGS* was also associated with OS, hence underlining its importance in the clinical setting.

Impaired *FPGS* activity is indeed an established mechanism of resistance to MTX and other polyglutamable antifolates in leukemic cells.^{15,30-40} Of notice, in human leukemic cell line models *FPGS*-dependent antifolate resistance was often induced by selection schedules using intermittent pulses of high-dose polyglutamable antifolates (MTX, raltitrexed, and pemetrexed).^{32,33} This clinically relevant mode of selection yielded antifolate-resistant cell lines displaying 90% to 99% loss of *FPGS* activity, but strikingly no coinciding substantial decrease in *FPGS* mRNA level.^{33,34,40} Consistently,

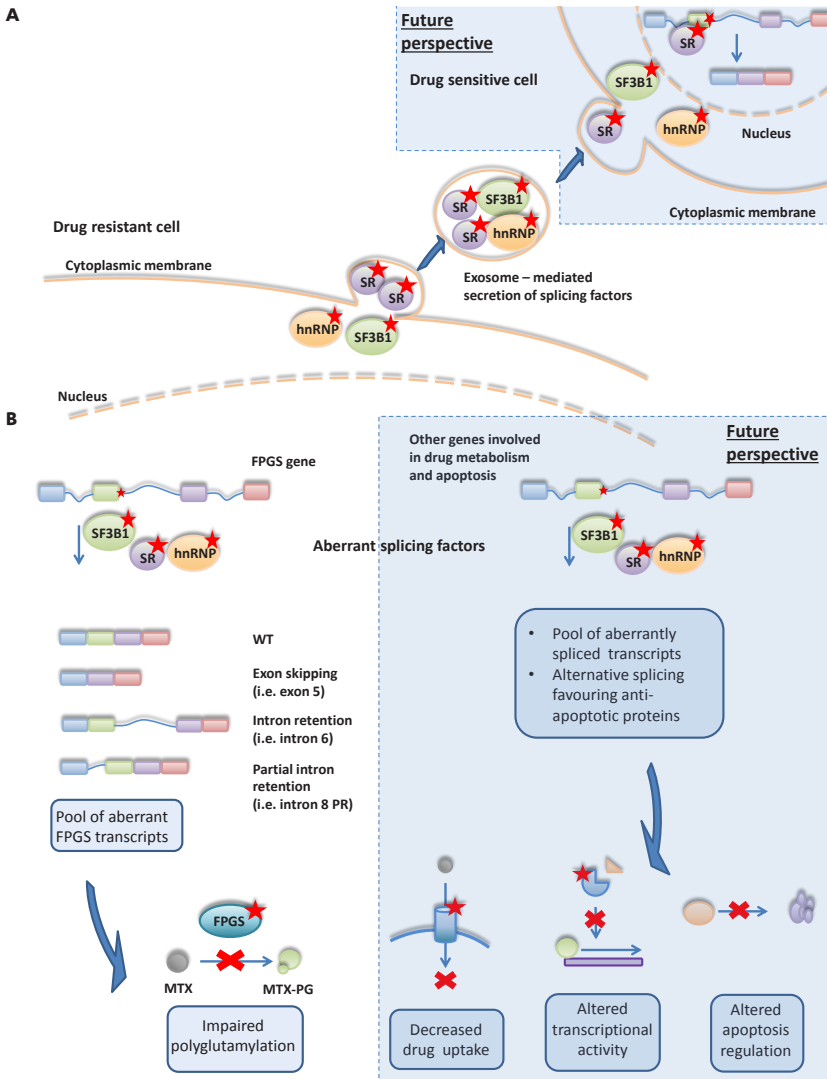


Figure 1. Schematic overview of aberrant splicing contributing to drug resistance. The figure depicts an overview of drug resistance related aberrant splicing events based on literature data and results described in this thesis along with the potential future developments of the current findings. **A** - Splicing factors were found in this study to be secreted by AML cells in association with apoptosis-resistance. The blue box represents a future perspective on the vesicle-mediated transfer of splicing factors as a mechanism of intercellular communication contributing to drug resistance. Actual uptake of functional splicing factors by drug-sensitive cells leading to acquisition of drug resistance via altered splicing patterns is a proposed concept requiring experimental confirmation and validation. **B** - Aberrant splicing of folypolyglutamate synthetase (FPGS) resulting in impaired polyglutamylation of antifolates (including MTX), uncovered in MTX-resistant cell lines and ALL patient samples. The splicing factors responsible for this phenomenon are currently under investigation but potentially regulate splicing of multiple genes. The blue box depicts an outlook on the influence of aberrant splicing factors influencing genes engaged in drug metabolism and apoptosis, resulting in chemotherapy resistance (at the level of drug transport, conversion to active metabolite, altered regulation of gene expression and apoptosis modulation). Red asterisks represent aberrant activity due to e.g. altered expression or mutations; SR - serine/arginine rich splicing factor; hnRNP - heterogeneous nuclear ribonucleoprotein; SF3B1 - splicing factor 3 subunit B1; WT - wild type; FPGS - folypolyglutamate synthetase; MTX - methotrexate; PG - polyglutamate chain.

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in the investigated childhood ALL cohort *FPGS* mRNA expression did not show a correlation with *FPGS* activity, pointing to the involvement of post-transcriptional processes in the regulation of *FPGS* function. Diminished rates of *FPGS* protein synthesis were previously reported to diminish its activity in murine leukemic cell lines selected with edatrexate.⁴¹ Studies by Stark et al have previously shown, that aberrant *FPGS* splicing is a possible cause of MTX resistance in leukemic cell lines and adult ALL patients¹⁵, which set the stage for further investigations described in this thesis.

Aberrant *FPGS* splicing in MTX resistance

We have systematically documented an assortment of splicing alterations occurring in the *FPGS* gene in childhood ALL patient samples (**chapter 4** and Figure 1B). These observations further extend our previous findings in adult ALL and leukemic cell line models,¹⁵ supporting altered *FPGS* splicing as a factor contributing to MTX resistance. Several different splice variants of *FPGS* resulting from differential translation initiation⁴² as well as alternative splicing of exon 1 have been previously described in leukemic cells.^{43,44} In **chapter 4** we show that the plethora of *FPGS* splicing alterations is beyond the initial anticipations and possibly includes various combinations of different splice alterations in a single transcript. This surprising complexity poses difficulties in understanding the contribution of particular alterations to the extent of decrease in *FPGS* activity. *FPGS* transcripts harboring splicing alterations will definitely result in disrupted *FPGS* function, as indicated by our *in vitro* *FPGS* catalytic activity assays. In a pilot analysis we showed that aberrant splice variants did not have a dominant negative effect over the wild type *FPGS*, however only a limited number of variants was tested. Moreover, it is possible that the multiplicity of detected splicing alterations together results in an overall decrease of *FPGS* activity. Similarly, skipping of a number of exons in deoxycytidine kinase (*dCK*), an enzyme crucial to cytarabine efficacy, was found in cells of AML patients displaying cytarabine resistance, as opposed to cytarabine-sensitive cells.⁴⁵ Transcripts carrying these alterations were devoid of activity as determined *in vitro*, however did not inflict a dominant negative effect over the wild type *dCK* protein.⁴⁶ Exon 2 and exon 3 skipping of *dCK* was also found in cytarabine-resistant CCRF-CEM cell line generated by a stepwise selection to gradually increasing concentrations of cytarabine.⁴⁷ This cell line displayed decreased *dCK* activity as compared to the parental cells. Therefore, aberrant splicing of *FPGS* is likely to cause diminished activity via an overall decrease in protein expression rather than a dominant negative effect.

Unexpectedly, we found that *FPGS* splicing alterations are common not only in ALL but can be also found in non-malignant bone marrow cells. Non-malignant human hematopoietic cells have been previously reported to express 4 splice variants of *FPGS* exon 1,^{43,44} pointing to alternative splicing of this gene being a part of intracellular regulation of *FPGS* expression. Conceivably, individuals displaying abundant alternative splicing of the *FPGS* gene might be intrinsically more prone to develop MTX resistance upon treatment. Interestingly, lower levels of alternative *FPGS* splice variations appeared in peripheral blood samples of healthy individuals. At the same time equivalent *FPGS* splicing profiles were shared by different cell subpopulations examined in a non-malignant bone marrow sample, suggesting that the bone marrow microenvironment plays a role in the regulation of *FPGS* splicing. It has to be emphasized, that the number of non-malignant samples examined in this study was limited and therefore our findings need to be further verified in more substantial sample numbers.

The most prominent *FPGS* splice variant noted in all examined leukemic specimens was intron 8 partial retention (intron 8 PR). This *FPGS* splice variant was also modulated in *FPGS*-deficient MTX-resistant cell line - R30dm in response to MTX exposure, indicating that dynamic *FPGS* splicing changes facilitates cell survival under antifolate-induced stress. It was recently shown that, in a range of solid tumors, hypoxia induced a reduction of intracellular purine and pyrimidine levels leading to cell-cycle arrest, which acts as a resistance mechanism to cell cycle dependent drugs (including antifolates).⁴⁸ Therefore, it is conceivable that MTX-induced changes in *FPGS* splicing might lead to diminished *FPGS* activity resulting in intracellular decrease in purine and pyrimidine pools, which in turn cause a reversible cell cycle arrest. This hypothesis, however, warrants further experimental exploration.

Validation of our *in vitro* observations was performed in a cohort of 91 childhood ALL patients, revealing intron 8 PR as the most relevant *FPGS* splice variant in the *FPGS* splicing screen (**chapter 5**). In a subset of patients with suboptimal concentrations of MTX polyglutamates, high levels of *FPGS* intron 8 PR were predictive of inferior OS and EFS. Similar associations were found in multivariate analysis including WBC, lineage and age, pointing to the potential clinical relevance of this phenomenon. These findings strongly suggest that aberrant *FPGS* splicing contributes to MTX resistance in ALL, although direct evidence documenting a causative relationship between *FPGS* splicing alterations, to the extent to which they are observed in patient cells, and MTX resistance is still lacking. The clinical relevance of this alteration warrants further validation in a larger study cohort, as the numbers of patients in this analysis, especially in certain patient subgroups, were limited. Moreover, the underlying mechanisms of *FPGS* splicing alterations remain to be characterized. In this respect, current studies are concentrating on the role of splicing factors/spliceosome. It is conceivable that a deranged splicing machinery will affect more than one gene implicated in drug resistance, thereby provoking resistance to multiple chemotherapeutics (Figure 1B - future perspectives box).

Alternative splicing as a contributor to drug resistance

In our *in vitro* model, the level of intron 8 PR in R30dm cells was unexpectedly elevated upon exposure to doxorubicin and cytarabine, as opposed to dexamethasone (Dex) and vincristine. Remarkably, R30dm cells showed moderate cross-resistance to doxorubicin and cytarabine in cytotoxicity assays⁴⁹ but not to vincristine.⁴⁹ Since doxorubicin and cytarabine are both DNA replication dependent, we hypothesised that these drugs (similar to MTX) can lead to a reversible cell cycle arrest assuring resistance to chemotherapy in leukemic cells under stress conditions. Alternatively, these findings might indicate that an enhanced level of intron 8 PR is a result of disturbed splicing regulation, which also affects other genes causing resistance to a number of chemotherapeutics. In fact, R30dm cells showed a remarkable level of resistance to Dex (over 350-fold), which did not induce changes in *FPGS* splicing. Strikingly, in our childhood ALL patient cohort intron 8 PR was indicative of individuals displaying high resistance to both Dex and prednisone. Although we had a limited number of samples, this finding suggests that intron 8 PR is not only a reflection of a broader splicing defect but also holds relevance in the clinical setting.

Several examples of splicing aberrations contributing to drug resistance in leukemia (and other tumor types) are already known and can result from mutation in regulatory sequences embedded

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1 within the target pre-mRNA or disturbances in splicing regulators (**chapter 2**). These alterations
 2 affect splicing of genes involved in drug metabolism or apoptosis regulation (as illustrated in Figure
 3 1B) thereby leading to drug resistance. As mentioned above, alterations in splicing of *dCK* were
 4 shown to be associated with cytarabine resistance.⁴⁵ *dCK* variants lacking either exon 5 or one of 3
 5 exon cassettes (exons 3-4, exons 3-6 or exons 2-6) were found in 7 out of 12 patients with resistant
 6 AML, while only a wild type *dCK* was present in sensitive AML and normal bone marrow controls.
 7 These misspliced variants showed no functional activity *in vitro*, which suggests that they might
 8 contribute to cytarabine resistance in AML patients.⁴⁶ In chronic lymphocytic leukemia (CLL)
 9 patients refractory to fludarabine, mutations in *SF3B1* occurred in higher frequency (10/59, 17%) than
 & in the general CLL population at diagnosis (17/301, 5%, $p=0.002$), suggesting that abnormal activity
 of *SF3B1* contributes to fludarabine resistance.² In THP1 (AML) and U937 (lymphoma) cells acquired
 resistance to capecitabine (precursor of 5-fluorouracil) was reported to be associated with decrease
 in thymidine phosphorylase (TP) activity, leading to inhibited conversion of 5'-deoxyfluorouridine
 to 5-FU. Capecitabine resistance in these cell lines correlated with unsplicing of the *TP* pre-mRNA,
 resulting from nuclear localization of hnRNP1/H2, which was absent from the wild type cells.¹⁶ Where
 most studies so far investigated splicing profiles of unstimulated leukemic cells, we showed that
 exposure of ALL cells to chemotherapy induced substantial changes in splicing of *FPGS* and possibly
 other genes involved in drug metabolism. The dynamic and reversible nature of the splicing shifts
 implies that more attention should be paid to the impact of splicing profiles alterations induced by
 therapy rather than the basal splicing isoforms, thereby avoiding false negative conclusions. Similar
 to genes involved in drug metabolism several studies link altered splicing with multidrug resistance
 induced via disturbed splicing of genes engaged in apoptosis regulation, i.e. the *FAS* receptor⁵⁰
 or *Bcl-X*.^{51,52} Multiple splicing factors, including SRSF1, SRSF9 and *SF3B1*, have been implicated in
 regulation of apoptosis and therefore potentially contribute to therapy resistance.⁵³ Interestingly, a
 recent study reports a landscape of splicing aberrations associated with AML, which illustrates how
 broad this phenomenon is in leukemia compared to non-malignant cells.⁵⁴ Moreover, an association
 was reported between mutations in splicing factors and inferior outcome in *de novo* AML patents.⁵⁵
 This AML-related abundance of alternative splicing makes it an attractive target for novel anti-
 cancer therapies.

Splicing targeted drugs - lessons for novel and conventional cancer therapy

Currently novel splicing-targeted therapeutic strategies are under development, either directed
 at particular aberrant splice variants or at the spliceosome itself. Abnormal splice variants can be
 specifically targeted using antisense or splice switching oligonucleotides, which either result in direct
 degradation of aberrant transcripts or splicing modulation.^{56,57} Promising results at the pre-clinical
 stage have been reported^{56,58-60} and several oligonucleotides against tumor cells are currently in
 clinical trials.⁶¹ However, these studies have disclosed several limitations of this approach, including
 off target effects and inefficient delivery, which need to be addressed to achieve high specificity
 and effectiveness. Thus, further optimization of approaches for effective delivery and minimization
 of adverse effects of these compounds is currently in progress.^{62,63} The successful application of
 splicing-modulating morpholinos in Duchenne Muscular Dystrophy is encouraging for the future of
 this approach in cancer. A recent clinical trial demonstrated that these modified oligonucleotides

were able to correct the underlying genetic defect in Duchenne Muscular Dystrophy resulting in clinical benefits for the patients.⁶⁴ However, a successful application of splicing modulating oligonucleotides in cancer treatment is still facing several challenges, including uniform distribution over various regions within the tumors as well as dealing with phenotypic plasticity of the malignant cells resulting from their genetic instability.⁶¹

The second approach, aimed directly at the splicing machinery, relies on the use of inhibitors of SF3B1 - one of the major subunits of the spliceosome - including the well-studied spliceostatin A (SSA) and PB.⁶⁵⁻⁶⁷ Several of these compounds, among others MAMB and PB, have shown promising results in the preclinical stage, including both *in vitro* and *in vivo* experimentation.^{65,68-71} Up to our knowledge, our study (**chapter 6**) is the first to assess the effect of SF3B1 inhibitors in acute leukemia. Remarkably, all ALL and AML cell lines evaluated were responsive to low nanomolar or subnanomolar concentrations of PB and MAMB. Even leukemic sublines resistant to various conventional chemotherapeutics remained fully sensitive to spliceosome inhibition. Consistently, MAMB was previously shown to be active against a range of solid tumors, along with multidrug resistant cells.⁶⁸⁻⁷⁰ Similarly, SSA and sudemycins induced potent growth inhibition in chronic lymphoblastic leukemia (CLL) cells both as single agents as well as in combination with other chemotherapeutics, including inhibitors of BCL-2 family members and ibrutinib.^{72,73} Here, we showed that PB/MAMB in combination with Dex exerts a synergistic effect in some Dex-resistant cell lines (**chapter 6**). Interestingly, this combination, while highly synergistic in CEM/R30dm, showed only moderate synergism in CEM-R5C3 and antagonism in CEM-R5 cells. Dex resistance in the latter two cell lines is based on alterations in glucocorticoid receptor (*GR*) with CEM-R5C3 showing ineffective induction of *GR* expression upon Dex treatment and CEM-R5 carrying a hemi or heterozygous mutation in this gene.^{74,75} The underlying cause of Dex cross-resistance in CEM/R30dm remains unknown, although we did note a modestly altered *MCL-1* splicing favouring the long (*MCL-1L*) variant as compared to parental CCRF-CEM WT cells (**chapter 4**). Since, elevated levels of *MCL-1L* protein were previously shown to be associated with prednisolone resistance in ALL patients⁷⁶, spliceosome inhibitors might potentially provide a tool to re-sensitize Dex resistant cells, which rely on *MCL-1L* upregulation. Therefore, SF3B1 inhibitors should be further evaluated as a novel therapeutic option for patients displaying drug resistant disease. However, the current clinical experience with this class of compounds is very limited. E7107, a derivative of pladienolide B, was recently evaluated in phase I clinical trials in patients with solid tumors.^{77,78} Despite, overall good tolerability, E7107 caused unpredictable ophthalmologic side effects indicating that the consequence of SF3B1 inhibition for particular genes in a tissue specific manner needs to be further delineated. In our study, a potential therapeutic window was observed as ALL/AML cells were significantly more sensitive to MAMB than and non-malignant bone marrows. However, given the fact that both types of samples responded to nanomolar concentrations of MAMB, toxicity to normal bone marrow is a possible side effect of this drug. To address this issue, future studies should evaluate the effect of MAMB on leukemia in mouse models with careful consideration of its impact on pre-mRNA splicing patterns in malignant as well as normal tissues. Notably, the exact mechanistic consequences of SF3B inhibition remain unclear as they may include splicing inhibition and nuclear accumulation of unspliced transcripts, as well as differential usage of splice sites and modulation of gene expression.^{65,67} Some reports suggest that spliceosome inhibitors used in high doses can have a different effect on splicing regulation

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and consequently cell fate compared to low doses.⁷⁹ Indeed, we have shown that increasing concentrations of MAMB differentially mediate modulation of *MCL-1* and *BCL-X* splicing in leukemic cells. In particular low dose spliceosome inhibitors might constitute a preferred novel therapeutic option to treat tumors displaying drug resistance based on aberrant splicing, thereby re-sensitizing cells to conventional chemotherapy, and at the same time avoid toxicity to normal cells.

Importantly, insight in splicing profiles of tumor cells raises opportunities not only to develop novel splicing-targeted therapies but also to improve the existent ones. Conventional chemotherapy has been used in cancer treatment for decades with an expanding understanding of the mechanisms of action and resistance to particular drugs. Notwithstanding this fact, the influence of conventional chemotherapy on splicing profiles of their target cells is largely unknown. A recent study elegantly showed that conventional chemotherapy impacts splicing profiles of cancer cells.⁸⁰ The effect exerted by different classes of chemotherapeutics on splicing of apoptosis-related genes varied in a cell-type-specific as well as compound-specific manner. Different types of compounds tended to modulate splicing of different apoptotic genes, indicating that rational combinations of chemotherapeutics may improve their curative effect.⁸⁰ Hence, characterization of splicing based response to conventional chemotherapy may serve to fine-tune their combined application and maximize their curative effect. Interestingly, drug resistance-related splicing aberrations in leukemic cells are not only intrinsic but might also be modulated by the tumor microenvironment, thereby affecting the treatment outcome.

Alternative splicing in intercellular mediation of drug resistance

In **chapter 7** we showed that among proteins differentially secreted by apoptosis-resistant versus apoptosis-sensitive primary AML cells, those involved in regulation of pre-mRNA splicing were among the top functional protein cluster (Figure 1A). Thus far, the presence of nuclear factors among secreted proteins was often assumed a contaminant and thereby frequently disregarded, however the functional relevance of these findings are currently emerging.⁸¹ In this respect, a recent study reported an enrichment of nuclear proteins, including splicing factors in colorectal cancer-derived secretomes as compared to healthy tissue.⁸² In our study pre-mRNA splicing was also found among the top 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 among other functions in pre-mRNA splicing, which might be responsible for transfer of the resistant profile to neighboring apoptosis-sensitive cells. Vesicle-mediated regulation of gene expression in recipient cells is a phenomenon documented by a growing body of evidence.⁸³ Several splicing regulators (including SF3B1 and SRSF1) were recently identified in exosomes derived from the polarized Madin-Darby canine kidney cell line MDCK transformed with oncogenic *H-Ras* and were proposed to play a functional role in the malignant disease.⁸⁴ The identified splicing factors were linked to induction of epithelial-to-mesenchymal transition (EMT), resulting in enhanced aggressiveness of cancer cells. Vesicles derived from AML blasts were previously shown to mediate functional RNA transfer between cells, thereby affecting several pathogenic features, including proliferative capacity.⁸⁵ Moreover, a recent study documented stroma-derived exosomes loaded with non-coding RNA to facilitate the outgrowth of therapy resistant tumor initiating breast cancer

cells.⁸⁶ Our data suggest that apoptosis-resistant primary AML cells secrete complex nuclear and cytoplasmic regulatory protein networks, with a prominent involvement of splicing factors, which have large potential to impact apoptosis-related protein expression in the neighbouring cells (Figure 1A - the future perspectives box).

It is an intriguing concept that vesicle-mediated transfer of protein networks involved in global modulation of gene expression can confer the induction of drug-resistant phenotype in AML cells. Extruding paramount regulators of gene expression, which have the ability to change levels of multiple targets, instead of transporting the targets themselves is a cunning strategy to effectively induce drug resistance in the recipient cells. However, this concept is still speculative and clearly requires experimental proof and validation. If proven, it could provide novel biomarkers of apoptosis/drug resistance, which might be detected in the blood of AML patients in a proteomic analysis approach. Conceptually, exosome-mediated transfer of drug resistance could be a novel potential therapeutic target. However, since intercellular communication via exosomes is a common phenomenon not only in cancer but also in healthy tissues⁸⁷⁻⁹¹, specific inhibition of this process might be difficult and requires identification of cancer specific vesicle-associated markers.

CONCLUSIONS AND FUTURE PERSPECTIVES

Taken together, our results indicate that aberrant splicing is a highly relevant phenomenon for several aspects of leukemia treatment. Alternative splice variants of the *FPGS* gene seem to have impact on the response of leukemic cells to MTX-containing therapy in the clinic. Therefore their usefulness in guiding clinical decisions or re-sensitization of MTX-resistant cells should be further evaluated. More importantly, the most prominent alteration in *FPGS* splicing - intron 8 PR is likely a reflection of globally disturbed splicing regulation affecting multiple genes. Remarkably, this mode of multidrug resistance seems to be important in the clinical setting, as suggested by the relation between *FPGS* intron 8 PR and cross-resistance to glucocorticoids found not only in a leukemia cell line model but also in cells obtained from childhood ALL patients. These findings warrant further exploration in terms of aberrant splicing patterns underlying pathogenesis and drug resistance in leukemia, together with delineating the intra- as well as inter-cellular mechanisms behind them. Further insights into these processes will contribute to the design of novel therapies targeting defects in splicing or modulating them in order to re-sensitize therapy-resistant disease.

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