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CHAPTER 2

Pre-mRNA splicing in cancer: the relevance in oncogenesis, treatment and drug resistance

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ABSTRACT

Introduction

Aberrant pre-mRNA splicing in cancer is emerging as an important determinant of oncogenesis, response to treatment and anticancer drug resistance. At the same time, the spliceosome has become a target for a novel class of preclinical chemotherapeutics with a potential future application in cancer treatment. Taken together, these findings offer novel opportunities for the enhancement of the efficacy of cancer therapy.

Areas covered

Herein we present a comprehensive overview of the molecular mechanisms involved in splicing and review the current developments regarding splicing aberrations in relation to several aspects of cancer formation and therapy. We delineate the identified mutations in the various components of the spliceosome and their implications for cancer prognosis. Moreover, the contribution of abnormal splicing patterns as well as deregulated splicing factors to chemoresistance is discussed, along with novel splicing-based therapeutic approaches.

Expert opinion

Significant progress has been made in deciphering the role of splicing factors in cancer including carcinogenesis and drug resistance. Splicing-based prognostic tools as well as therapeutic options hold great potential towards improvements in cancer therapy. However, gaining more in-depth molecular insight into the consequences of mutations in various components of the splicing machinery as well as of cellular effects of spliceosome inhibition is a prerequisite to establish the role of splicing for tumor progression and treatment options respectively.

Keywords: cancer therapy, drug resistance, hematological malignancies, oligonucleotides, prognosis, spliceosome, splicing factors

INTRODUCTION

The coding sequence of viral genes was originally found to be interrupted by non-coding regions almost four decades ago by Phillip Allen Sharp and Richard J. Roberts.[1,2] These non-coding segments, later termed introns, must be removed from the nascent precursor messenger RNA (pre-mRNA) transcript, in order to obtain the mature mRNA, which serves as a template for protein translation. The process of intron removal and joining of the coding regions (exons) together is termed splicing. Nowadays, it is recognized that a single human gene can give rise to multiple transcripts, some of which result in different protein isoforms, by the means of alternative splicing.[3,4] Conceivably, this phenomenon is a major contributor to proteome diversity in the cell, and thereby can influence a plethora of biological functions, including regulation of cell cycle, apoptosis and differentiation.[3,4] Many mechanistic and functional aspects of splicing have been identified, hence contributing to better understanding of the role of splicing in malignant and non-malignant diseases. The knowledge of splicing aberrations in respect to disease pathology, most notably cancer, is continuously increasing with significant progress achieved over the past two decades. The importance of splicing alterations in oncogenesis and resistance to chemotherapy is rapidly emerging. However, the unprecedented complexity of splicing makes it apparent that a more in-depth molecular understanding of its regulation and the exact role of various splicing alterations in cancer is warranted. Here, we discuss recent progress in delineating the role of splicing aberrations in drug resistance and cancer treatment, as well as its implications to prognosis, therapeutics and treatment of cancer.

PRE-mRNA SPLICING

Pre-mRNA arising during transcription by RNA polymerase II (RNAPII) contains the exons interrupted by non-coding introns.[5] Most human genes contain multiple exons with an average length of approximately 50 - 250 base pairs (bp), which is much shorter than that of the introns frequently consisting of thousands of bp.[6] Therefore, the long pre-mRNA requires conversion to mature mRNA by intron excision via splicing. Since deviations in this important process could have detrimental consequences on protein structure and function, splicing is carried out by complex ribonucleoprotein machinery – the spliceosome.

The discrepancy between the total number of genes and the total number of proteins found in humans has led to the discovery that many human genes are alternatively spliced.[4] Alternative splicing results in different mature mRNAs derived from the same gene, and it is a common phenomenon for the vast majority (95-100%) of human genes.[3] Moreover, it was shown that more than 80% of alternatively spliced genes result in different protein isoforms, sometimes with opposite functions.[7] As multiple regions of a single gene can undergo alternative splicing, in extreme cases hundreds of isoforms can arise from the same gene. Alternative splicing greatly expands the coding capacity of the genome[8], and consequently the complexity of the proteome and is a subject to a complex regulation.

Regulation of splicing

Alternative splicing is regulated by an interplay between sequence elements embedded within the pre-mRNA (*cis*-acting elements) and the splicing factors (*trans*-acting elements – Figure 1),

which direct the spliceosome to the correct splice sites.[4,9] Recognition and binding of specific *cis*-acting elements by splicing factors drives exon definition followed by spliceosome assembly that influences both constitutive and alternative splicing.[10,11]

Cis-acting elements

The *cis*-acting elements include relatively short sequences appearing both in exons and introns. The exon/intron boundaries are marked by the splice sites. Each exon is flanked by the 5'-splice site in the downstream intron, and the 3'-splice site in the upstream intron, accompanied by a branch point sequence (BPS) and a polypyrimidine tract (PPT).[9] These sequences are recognized by the spliceosome (Figure 1). An additional regulatory class of *cis*-acting elements can enhance or repress

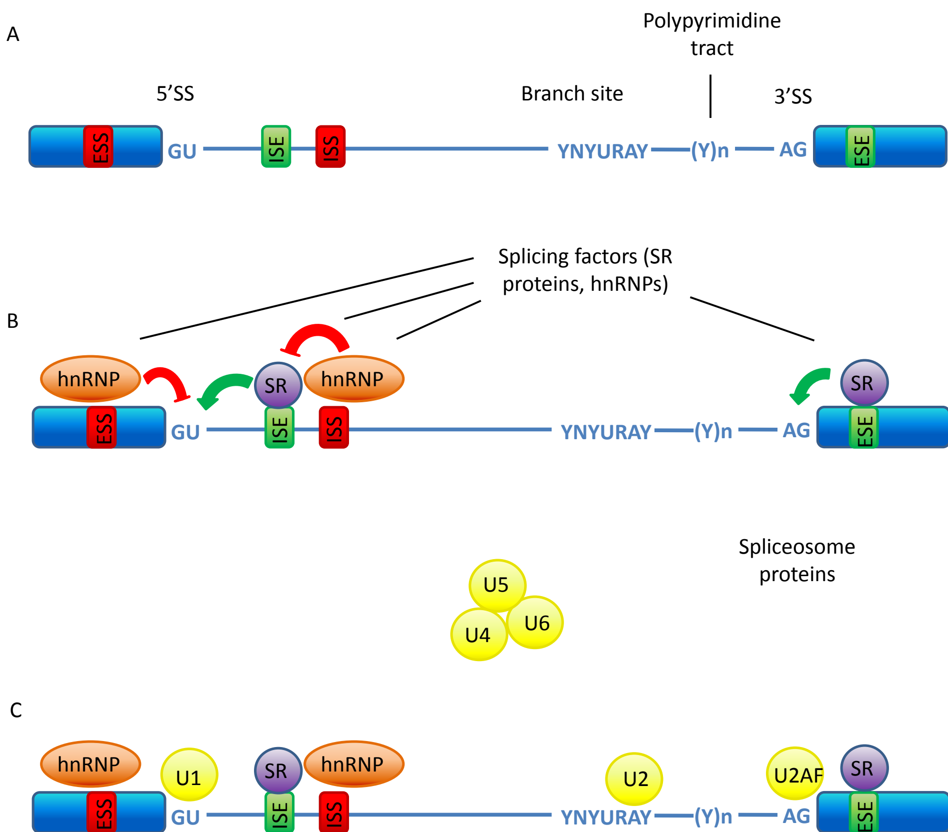

Figure 1. Overview of factors involved in splicing regulation. Splicing decisions are made based on interactions between the sequences present within the target mRNA (*cis*-acting elements) and splicing regulators (*trans*-acting elements). **A** – the *cis*-acting elements including the basic sequences - 5' and 3' splice sites (5'SS and 3'SS), the branch point and the polypyrimidine tract, as well as additional regulatory sequences (ESE - exonic splicing enhancers, ISE - intronic splicing enhancers, ESS -exonic splicing silencers, ISS - intronic splicing silencers); **B** – interaction of the splicing factors with *cis*-acting elements, including SR - serine-arginine proteins and hnRNP - heterogeneous nuclear ribonucleoproteins; **C** – interaction of the spliceosome components (yellow circles) with cis-acting elements. U1, U2, U4-U6 – small nuclear ribonucleoprotein particles (snRNPs) U1, U2, U4-U6; U2AF - U2 auxiliary factor.

exon inclusion into the mature mRNA or promote the usage of alternative splice sites. These elements include exonic splicing enhancers (ESE), exonic splicing silencers (ESS), intronic splicing enhancers (ISE) and intronic splicing silencers (ISS), which are recognized and bound by splicing factors.[4]

Trans-acting elements

There are two main classes of splicing factors, acting either as activators or repressors of exon inclusion. The first class consists of serine-arginine (SR) proteins which play an important role in the assembly of the spliceosome (Figure 1). SR proteins can bind to ESE, thereby enhancing exon inclusion by recruiting the spliceosome to the correct splice sites.[4,10] SR proteins are extensively phosphorylated by SR protein kinases (SRPKs) 1 and 2, as well as by several other kinases (i.e. Cdc2-like kinases or AKT).[12] Phosphorylation status of splicing factors is an important determinant of their subcellular localization and activity. SR protein family contains a well-studied serine/arginine splicing factor 1 (SRSF1), SRSF2 and U2 auxiliary factor (U2AF).[10]

The other class of *trans*-acting splicing factors - heterogeneous nuclear ribonucleoproteins (hnRNPs), mostly act as splicing silencers (Figure 1). ISS and ESS are commonly bound by hnRNPs thereby repressing splice site recognition and subsequently exon inclusion into the mature mRNA. Well-studied members of the hnRNP family include hnRNPA1, hnRNPH1 and polypyrimidine-tract binding protein (PTB).[10]

According to the canonical view, SR proteins are splicing enhancers, while hnRNPs act as splicing silencers, however, the current opinion on splicing regulation points to its context dependency.[13] Several SR proteins as well as hnRNPs could either repress or promote exon inclusion depending on the sequence they bind.[14] These observations suggest, that it is rather the interaction of splicing factors with particular binding positions as well as each other that determines their function in a cell type-specific manner.[13,14]

The spliceosome and the mechanism of splicing

Splicing is catalyzed by the spliceosome – a large ribonucleoprotein complex consisting of five small nuclear ribonucleoprotein particles (snRNP U1, U2, U3, U5 and U4/U6) and more than 150 proteins.[10] This major spliceosome complex excises the vast majority of introns from pre-mRNA molecules. In most *Eukaryotae*, including human cells, an additional - minor - spliceosome exists, containing functional analogues of snRNPs present in the major spliceosome (snRNP U11/U12, U4atac/U6atac and U5 snRNP shared by both spliceosomes).[5] The minor spliceosome is involved in removal of an infrequent atypical class of introns (U12 type) from pre-mRNA [5].

The spliceosome is assembled on the pre-mRNA and undergoes a sequence of dynamic stepwise changes, beginning with complex E, which is subsequently transformed into complex A and finally the large spliceosome forms.[4] Additional rearrangements result in the formation of the active spliceosome[15], which then catalyzes removal of the intron by 2 phosphoryl-transfer reactions, resulting in joining the exons together and the release of the intron in a form of a branched lariat structure.[4,9]

Modes of alternative splicing

Alternative splicing generates a variety of transcripts from a single gene, by differential usage of exon and intron sequences. Exons present in the predominantly expressed mature transcript are termed

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constitutive exons, as opposed to alternative exons, which are only included in certain transcripts.[9] Alternative splicing is based on 5 basic mechanisms.[16] The most common is exon skipping, where a selected exon is excluded from the mature mRNA. Inclusion of a complete sequence of an intron in the transcript is termed intron retention. Alternative 3' or 5' splice site selection leads to the inclusion of a part of an intron or exclusion of a partial sequence of an exon, thereby resulting in exons with a different size compared with their constitutively expressed counterparts. The last mechanism is the usage of mutually exclusive exons. Such exons, despite being coded by the same gene, never coexist together in a single transcript but are differentially included only in specific splice variants.[12] An overview of the different alternative splicing mechanisms is depicted in Figure 2A. The decisions on which splice sites are recognized by the spliceosome are guided by the interaction between the *cis*-acting elements and splicing factors. Moreover, a growing body of evidence supports the impact of transcription and chromatin structure on splice site selection and the efficiency of splicing.

Transcription and chromatin structure in regulation of splicing

Pre-mRNA splicing was documented to occur both co- and post-transcriptionally. Posttranscriptional splicing proved to be predominant in mouse macrophages, as indicated by a recent RNA sequencing study, which detected unspliced RNA species associated with chromatin.[17] On the other hand, a number of other studies indicate that the majority of intron-containing genes in humans and yeast are spliced co-transcriptionally.[18,19] The proportion of fully spliced exons was shown to increase towards the 5' end of the mRNA, suggesting that splicing is progressively carried out in parallel to active transcription.[18] In addition, pausing of RNA polymerase in terminal exons was detected in yeast and its duration was estimated to allow effective splicing before termination of transcription.[19] One of the mechanisms underlying the enhancing effect of transcription on splicing involves the carboxyl-terminal domain (CTD) of RNA polymerase II, of which deletion resulted in inefficient splicing.[20] Indeed, phosphorylated CTD domain could directly bind U2AF2, which promoted recruitment of U2AF2 and PRPF19 to the nascent pre-mRNA and consequently enhanced splicing.[21] Splicing was also shown to be influenced by the rates of transcription, which in turn were linked to nucleosome positioning and specific distribution of histone modification within a gene.[22] In addition, histone modifications were involved in the direct recruitment of splicing factors to nascent mRNA.[22]

THE ROLE OF SPLICING IN CANCER PATHOGENESIS AND TREATMENT

Alternative splicing regulates multiple cellular processes, including cell division, differentiation of cells and tissues and development of organisms.[4] Considering the complex nature of splicing, it is not surprising that alterations in this process play an important role in many disorders including cancer. The mechanisms causing altered splicing in cancer affect both *cis*- and *trans*-acting elements. These aberrations were found to disrupt splice site recognition, create new splice sites or interfere with splicing regulatory elements, which can result in altered function of the normal protein. Somatic mutations can cause cancer by affecting splicing of oncogenes and tumor suppressors or other cancer-related genes.[23] Finally, splicing factors themselves can be deregulated in cancer, driving tumor formation.

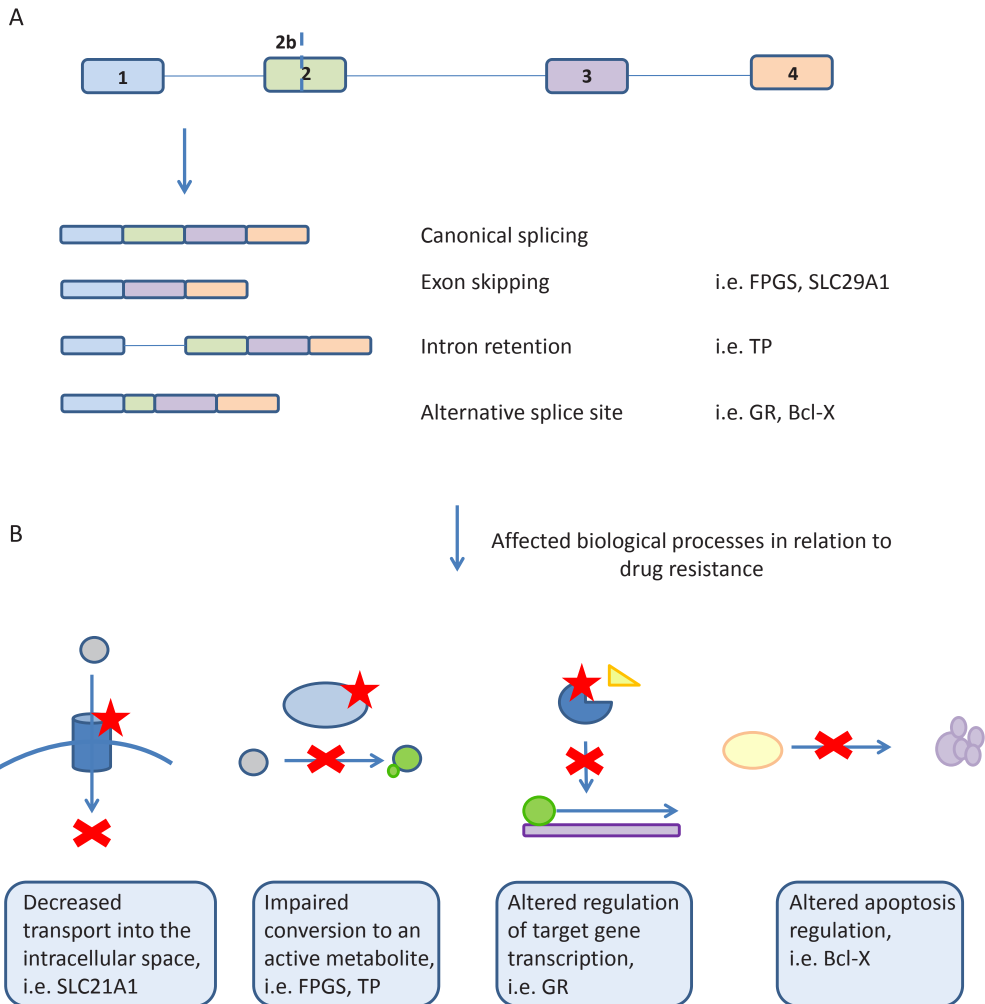

Figure 2. Modes of alternative splicing and their possible biological consequences resulting in drug resistance. **A** - A schematic overview of different modes of alternative splicing (including exon skipping, intron retention and alternative splice cite usage), together with examples of genes, which if affected by a specific splicing alteration can contribute to drug resistance. **B** - processes involved in cellular response to chemotherapy, which were reported to be affected by aberrant splicing, resulting in drug resistance (at the level of drug transport, conversion to active metabolite, altered regulation of gene expression and apoptosis modulation). FPGS - folylpoly-γ-glutamate synthetase, SLC29A1 - solute carrier family 29 member 1, GR -glucocorticoid receptor, TP - thymidine phosphorylase.

Aberrant splicing in oncogenesis

Many oncogenes and tumor suppressors are known to be aberrantly spliced in cancer (Table 1).[24,25] c-Kit , a well-studied oncogene - is a member of the type III receptor tyrosine kinase family, and constitutive activation of Kit was associated with gastrointestinal stromal tumors.[26] A study conducted by Chen et al., in gastrointestinal cancer patients has identified a deletion in Kit localized to the 3' splice site of exon 10, which at the same time created a novel 3' splice site in exon 11. As a result, a constitutively active mutated Kit protein was expressed.[27]

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Table 1. Overview of oncogenes and tumor suppressors related to splicing.

Gene	Oncogene/tumor suppressor	Tumor type	Mechanism	Ref.
SRSF1	oncogene	various	splicing regulation of multiple oncogenes/tumor suppressors (including RON, BIN1)	24,29–31
hnRNPH1	oncogene	glioma, cervical cancer	splicing modulation of IG20/MADD and RON	24
hnRNPA2/B1	oncogene	glioblastoma, lung cancer, breast cancer	splicing modulation in numerous genes	24
PTB1	oncogene	glioblastoma multiforme	exon skipping of FGFR-1	32
RBM4	tumor suppressor	various	splicing modulation of Bcl-X, CD44, antagonism against SRSF1	33

p53, the most commonly inactivated gene in cancer is a transcription factor and a key player in the maintenance of genetic stability. The complex regulation of p53 isoforms expression is described in detail by Khoury et al.[28]: nine different isoforms of this gene exists resulting partially from alternative splicing. This includes differential splicing of intron 9 leading to 3 variants of the carboxy-terminal domain (long p53α, shorter isoforms β and γ), as well as alternative splicing of intron 2, resulting in either inclusion or deletion of the first 40 amino acids of the p53 protein.[28] Several alternatively spliced variants of the p53 gene have been reported to be associated with loss of p53 function, aberrant regulation of apoptosis and cancer. The abnormal cancer-related patterns of p53 isoform expression showed tissue specificity. For example, the loss of short p53 isoforms was reported in AML, while the overexpression of p53β as well as other short p53 isoforms was observed in melanoma cell lines.

Evidence supporting the role of aberrantly expressed splicing factors in oncogenesis is constantly growing. Interestingly, many splicing factors were found to be upregulated in cancer but only a few are known to be downregulated.

Archetypic splicing factor SRSF1 and its role in regulation of mRNA splicing is very well documented.[29] SRSF1 can act as a potent proto-oncogene, and it has been shown to be upregulated in 20-50% of the cancers studied.[23,29] Consistently, overexpression of SRSF1 resulted in tumor formation in nude mice.[12] Interestingly, even a mild overexpression of SRSF1 was able to drive oncogenic transformation of immortalized rodent fibroblasts and human mammary epithelial cells, which were able to form tumors in mice.[29–31] One of the mechanisms underlying oncogenic properties of SRSF1 involves aberrant splicing of several cancer-associated genes, including the tyrosine kinase receptor and also an oncogene - Ron.[46] SRSF1 binds to a regulatory sequence in exon 12 of Ron, thereby stimulating exon 11 skipping, resulting in production of a constitutively active protein isoform.[46]

The splicing factor PTB1 was also found to play a role in cancer. PTB1 controls the splicing of fibroblast growth factor receptor 1 (FGFR1), towards a higher affinity receptor.[47] PTB1 was shown to directly interact with, and thereby promote the skipping of, an α exon in malignant glioblastomas. Expression of the FGFR1 lacking the α exon was strongly increased in tumor samples compared with normal controls, suggesting its possible contribution to glial cell malignancy.[32]

Interestingly, RBM4 - another splicing factor, was recently found to have a tumor suppressor function.[33] Ectopic overexpression of RBM4 in several types of cancers including lung, breast and prostate, resulted in lower proliferation rates using both colony formation and soft-agar assays. These results obtained *in vitro* were paralleled by decreased tumor growth in nude mice.[33] This effect was partially mediated by regulation of both splicing and translation of multiple targets. Most prominent alterations were noted in splicing of Bcl-X and CD44, as well as splicing patterns of several genes implicated in tumorigenesis (i.e. RON in breast and colon tumors, BIN1 in melanoma).[33]

A recent study evaluated alternative splicing events in two independent AML patient cohorts in comparison to healthy donors.[48] This study brought to light an additional level of complexity in AML in the form of widespread splicing alterations, including oncogenes and tumor suppressors as well as an array of genes involved in apoptosis, proliferation, differentiation and splicing itself.[48]

Impact of aberrant splicing on cancer treatment prognosis

A wave of recent whole exome/genome studies demonstrate new somatic spliceosomal mutations which occur in both myeloid and lymphoid malignancies (Table 2), most prominently in myelodysplastic syndromes (MDS) and chronic lymphocytic leukemia (CLL).[49] Mutations in splicing regulators are now estimated to be one of the most frequent molecular aberrations in MDS and affect most notably splicing factor 3 subunit b1 (SF3B1), U2 small nuclear RNA auxiliary factor 1 (U2AF1), SRSF2 and ZRSR1.[45]

Interestingly, mutations in the *SF3B1* gene - one of the components of U2 snRNP, conferred a favorable prognosis upon MDS patients, in contrast to CLL patients, who displayed a poor prognosis.[34–36,50] Similarly, mutations in SRSF2, U2AF1 and ZRSR2 in MDS were predictive of more aggressive disease

Table 2. Splicing factors with potential prognostic significance in cancer.

Gene	Alteration	Prognosis	Tumor type	Ref.
SF3b1	mutations	in MDS longer overall survival and leukemia free survival; in CLL faster disease progression and shorter overall survival	MDS, CLL, breast cancer, ovarian cancer, pancreatic cancer, melanoma	34–36
PRPF8	mutations or hemizygous deletions	more aggressive disease, progression to AML	MDS, AML	37
U2AF1	mutations	worse overall survival in CMML, increased progression to AML in MDS	MDS, CMML	38–40
SRSF2	mutations	shorter overall survival and more frequent progression to AML in MDS, possible favorable overall survival in TET2 mutated CMML patients	MDS, CMML	40–44
ZRSR2	mutation (accompanied by WT TET2)	shorter overall survival and more frequent progression to AML	MDS	40,45

Abbreviations: MDS - myelodysplastic syndrome; CLL - chronic lymphocytic leukemia; AML - acute myeloid leukemia; CMML - chronic myelomonocytic leukemia

with increased rates of progression to AML, as well as with shorter overall survival.[38,41,45] Remarkably, mutated SRSF2 was found to be an independent adverse prognostic marker in multivariate analysis in the context of several other molecular markers.[41] In addition, multivariate analysis revealed that patients carrying a mutation in ZRSR1 accompanied by the wild-type TET2 had an inferior overall survival and a higher rate of transformation to AML.[45] PRPF8 was recently identified to be mutated or deleted in MDS and related diseases.[37] Defects in the PRPF8 gene were predominantly detected in primary or secondary AML, suggesting an association with leukemogenesis.[37] This observation is supported by the fact that PRPF8 knockdown lead to aberrant splicing of genes involved in hematopoietic differentiation together with increased proliferation and clonogenicity.[37]

Factors involved in the splicing pathway were also found mutated in chronic myelomonocytic leukemia (CMML), with U2AF1 and SRSF2 having a potential clinical significance.[40] U2AF1 was associated with worse overall survival[38,39], while the prognostic significance of SRSF2 is still unclear. Some studies showed that SRSF2 predicted inferior overall survival in CMML, however it did not remain an independent prognostic factor in multivariate analysis.[42] In contrast, another group found that mutated SRSF2, U1AF1 and SF3B1 have no impact on overall nor leukemia free survival in CMML patients.[43] Interestingly, despite the lack of prognostic significance in the whole population of CMML patients, in the TET2 mutated subgroup of patients SRSF2 mutation seemed to have a favorable effect on overall survival.[44]

ALTERED SPLICING AND ANTICANCER DRUG RESISTANCE

The increasing recognition of the role that pre-mRNA splicing plays in cancer pathobiology, is paralleled by the progress in understanding its contribution to anticancer drug resistance (Figure 3).[51] Aberrant pre-mRNA splicing can promote cell survival in response to chemotherapy by deregulation of apoptosis or alterations in drug metabolism. Interestingly, several recent publications indicate that cell-to-cell transfer of splicing factors might be a part of intercellular communication, possibly resulting among other alterations in increased drug resistance.[52]

Altered splicing in genes involved in apoptosis regulation

Apoptotic cell death is a crucial mechanism determining cell survival. Its complex regulation is based on the balance between the expression of pro- and anti-apoptotic factors and if impaired can result in diminished drug sensitivity.[53] Many genes involved in apoptosis are regulated by splicing, which often results in 2 isoforms with opposite functions (e.g. Bcl-XL and Bcl-XS; Mcl-1L and Mcl-1S, reviewed in [53]).

The *Bcl-X* gene can be alternatively spliced into two isoforms: anti-apoptotic Bcl-XL and pro-apoptotic Bcl-XS. The two variants arise from alternative splicing at two competing 5' splice sites in exon 2. Cancer cells often display elevated expression levels of Bcl-XL, resulting in apoptosis inhibition. Similarly, caspase-2 is alternatively spliced to produce two isoforms: pro-apoptotic caspase-2L and caspase-2S. Caspase-2 is often aberrantly spliced in cancer, resulting in higher expression levels of caspase-2S and enhanced survival of cancer cells.[62] Aberrant splicing was also shown to affect one of the death receptors involved in apoptosis - the pro-apoptotic transmembrane protein FAS. An isoform of FAS lacking the transmembrane domain, due to exon 6 skipping, was found to act as an anti-apoptotic soluble decoy receptor, antagonizing the full length FAS and thereby allowing cancer cells to escape apoptosis.[63] Survivin 3B (S3B), an alternative splice variant of an apoptosis inhibitor

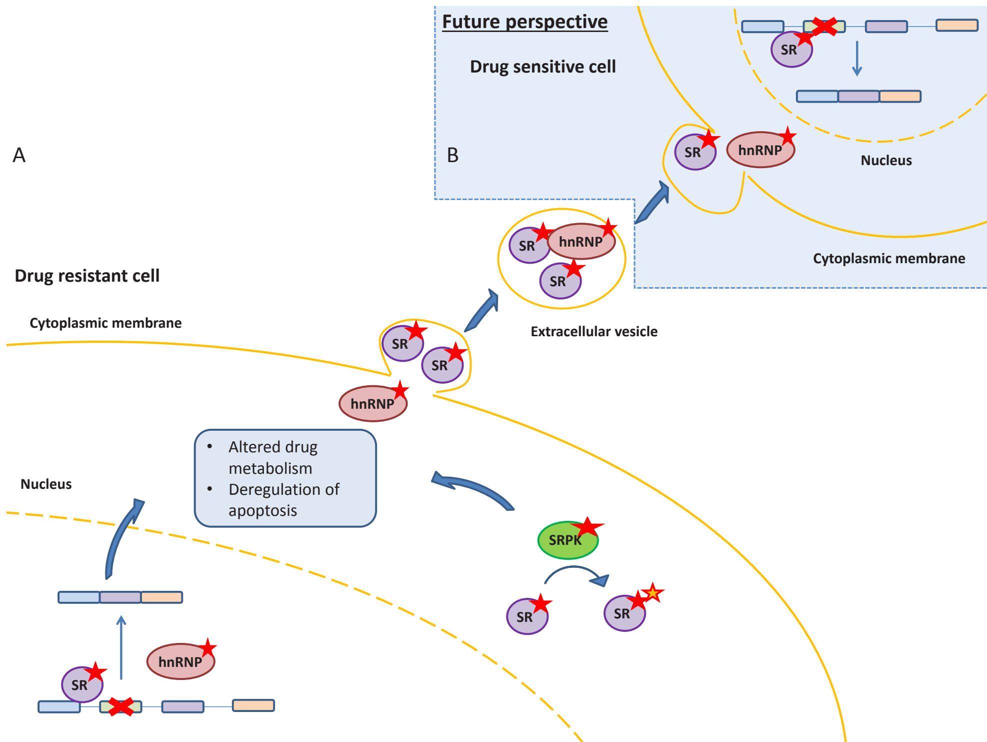

Figure 3. Overview of the mechanisms of aberrant splicing contributing to anticancer drug resistance. A – Currently documented mechanisms of altered splicing. Aberrant elements are labelled with asterisks (red asterisks – altered activity due to i.e. abnormal expression levels or mutations, orange star with red lining - aberrant phosphorylation status) and include alterations in the *cis*-acting elements, splicing factors (SR proteins and hnRNPs) and SR protein kinases. The alterations described can result in altered drug metabolism or deregulated apoptosis. Factors involved in splicing were found to be secreted in association with aggressive disease phenotype and apoptosis-resistance. **B** – A future perspective on a vesicle-mediated transfer of splicing factors as a mechanism of intercellular communication contributing to drug resistance. An actual uptake of functional splicing factors by drug sensitive cells, leading to acquisition of drug resistance via altered splicing patterns is an intriguing concept; however this putative mechanism has not been proven yet.

survivin was reported to be associated with cancer. S3B was able to mediate staurosporin resistance through interactions with procaspase-6. Specifically, S-3B binding to procaspase-6 inhibited its activation despite mitochondrial depolarization and caspase-3 activation.[64] Moreover, a recent report by the Childhood Oncology Group showed that expression of two additional isoforms of survivin – S2B and SΔ2, lacking exon 2 was associated with treatment outcome in AML patients;[65] a high ratio of S2B/SΔ2 expression was predictive of refractory disease and inferior survival, however its clinical relevance warrants further validation.

Altered splicing of genes involved in drug metabolism

Resistance to specific chemotherapeutics can be caused by alterations in splicing of genes involved in their respective metabolic pathways. Such genes involved in conversion of prodrugs to active metabolites, translocation of drugs into the intracellular space as well as efflux have been reported (Figure 2 and Table 3).

Table 3. Splicing alterations in target genes associated with drug resistance

Gene	Function	Splicing alteration	Drug resistance	Tumor type	Ref.
FPGS	conversion to active metabolite	exon 12 skipping	methotrexate	ALL	[54]
TP	conversion to active metabolite	unsplicing	5-fluorouracil (capecitabine)	metastatic colorectal and breast cancer	[55]
dCK	conversion to active metabolite	skipping of exon 5, exon 3-4, exon 3-6, exon 2-6, altered splicing of exon 2 and 3	cytarabine	AML, ALL	[56–58]
SLC29A1	drug transport	exon 13 skipping	cytarabine	ALL cell lines	[58]
GCR (isoforms β, γ, A, P)	transcription factor	alternative splice site in exon 9 (β), exon 3 (γ), skipping of exon 5- exon 7 (A), skipping of exon 8 and 9 (P)	glucocorticoid	small cell lung carcinoma (γ), ALL, CLL (β, γ), multiple myeloma (A, P), corticotroph adenomas (γ)	[59,60]
MRP 1	efflux transporter	various partial and full length exon skipping and partial intron retention	doxorubicin	ovarian cancer	[61]

ALL - acute lymphoblastic leukemia; AML - acute myeloid leukemia; CLL - chronic lymphocytic leukemia

Loss of folylpoly-γ-glutamate synthetase (FPGS) activity via aberrant mRNA splicing was shown to be associated with resistance to the antifolate methotrexate (MTX) in acute lymphoblastic leukemia (ALL). Stark et al. found that MTX–resistant cell lines display a range of aberrantly spliced *FPGS* mRNA species (including intron retention and exon skipping), resulting in loss of FPGS activity which is a well-documented contributor to MTX resistance. The existence of these FPGS splice variants could also be confirmed in adult ALL patient samples.[54] Thymidine phosphorylase (TP) is an enzyme essential for the pharmacologic activity of the prodrug capecitabine, which is converted to 5-fluorouracil and is used in the treatment of metastatic colorectal cancer and breast cancer. Resistance to this drug was associated with loss of TP protein, which was due to lack of splicing of *TP* mRNA.[55] The nuclear localization of hnRNP H1/H2 and hnRNP F was the mechanism underlying this type of drug resistance (being absent in parental sensitive cell lines). In this study the binding of *TP* mRNA by hnRNP H1/H2 was demonstrated upon immunoprecipitation assays.

Deoxycytidine kinase (dCK) converts cytarabine to its active metabolite and alterations of *dCK* mRNA splicing including skipping of several exon combinations, was identified in AML patients displaying cytarabine resistance.[57] These alternatively spliced forms were neither detectable in sensitive AML cells nor in normal cells obtained from healthy donors. These variants were later shown *in vitro* to be devoid of dCK activity but did not inflict a dominant negative effect on the wild type dCK protein.[56] However, upon expression in cells deprived of the wild type dCK, these alternatively spliced dCK variants induced cytarabine resistance. In another study, exon 2 and exon 3 skipping of dCK were detected in cytarabine-resistant CCRF-CEM (T-ALL) cell line, obtained by a stepwise selection to gradually increasing concentrations of cytarabine.[58] This cell line displayed

decreased dCK activity, as compared with the parental cells. Interestingly, the same group identified exon 13 skipping of another gene involved in cytarabine metabolism - solute carrier family 29 member 1 (SLC29A1). The detected splicing alteration in SLC29A1 caused by an intronic mutation resulted in decreased uptake of cytarabine.[58]

The glucocorticoid receptor (GR) is a ligand-dependent transcription factor important for the efficacy of glucocorticoids, such as dexamethasone. GR expression is regulated by splicing thereby resulting in several isoforms.[60] The function of the full-length transcript encoding GRα, was shown to be antagonized by alternatively spliced GR variants in some cases with a dominant negative function (including GRβ, GRγ, GRA and GRP).[60] These alternative GR isoforms were found elevated in several cancers including ALL, CLL and lung cancer.[60] Additional splice variants were detected in various cancerous and non-malignant tissues, including GRδ, GR-S1, GR-NS-1 and GR-DL-1, as well as alternative forms of exon 1,[73] which might potentially display an altered function. However, their role in cancer and glucocorticoid resistance requires further investigation.

The impact of aberrant splicing on multidrug efflux can be exemplified by the ATP-driven multidrug efflux transporter of the ABC superfamily - multidrug resistance protein 1 (MRP1/ABCC1). This multidrug extrusion pump was long known to be implicated in drug resistance to various cancer chemotherapeutics. A recent study found more than 20 splice variants of MRP1 in ovarian cancer, as compared with the matched healthy tissues.[61] Forced overexpression of the detected splice variants of MRP1 showed that some of them were able to confer resistance to doxorubicin, a well-established substrate of ABCC1.[61]

Impairments in splicing regulators resulting in drug resistance

A growing number of factors involved in regulation of splicing have been implicated in induction of drug resistance (Table 4). Mutations in the SF3B1 gene in CLL patients were shown to be associated with resistance to fludarabine treatment. Seventeen percent of the fludarabine-refractory cases

Table 4. Splicing regulators involved in drug resistance

Splicing regulator	Function	Target gene mediating drug resistance	Drug resistance	Ref.
SRSF1	splicing factor	unknown	carboplatin, paclitaxel	[66]
hnRNPH1/H2	splicing factor	TP	capecitabine (5-fluorouracil)	[55]
RBM17	part of the spliceosome complex; catalysis of the second step of splicing	possible targets: FAS, estrogen receptor β	multidrug resistance (doxorubicin, etoposide, carboplatin, vincristine, mitoxantrone)	[63,67,68] reviewed in [69]
SRPK1	SR protein kinase (SRSF1, SRSF9, SRSF3, SRSF5, SRSF4)	BAX, RLP17	cisplatin, gemcitabine	[70]
SF3B1	component of the spliceosome	unknown	fludarabine refractoriness	[50]
SRSF9	splicing factor	GR	glucocorticoids	[59,71]
SRSF3, SFPQ	splicing factors	unknown	gemcitabine, cytarabine	[72]

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harbored mutations in the SF3B1 gene, with a frequency significantly greater than observed in CLL patients responding to this antitumor agent (5%).[50]

Overexpression of RBM17 in cervical and ovarian cancer cells was shown to induce resistance to multiple chemotherapeutics with various mechanisms of action including doxorubicin and vincristine.[67,68] The underlying basis of the resulting drug resistance is still poorly understood but involves, at least in part, the induction of exon 6 skipping of the FAS receptor (see paragraph 4.1).[69] The splicing factor SRSF9 was shown to switch splicing of the GR promoting drug resistance associated with the β isoform in neutrophils.[59] The SRSF1 oncogene was shown to mediate resistance to carboplatin and paclitaxel in lung adenocarcinoma.[66] H358 lung tumor cells with stable overexpression of SRSF1 were selectively resistant to carboplatin and paclitaxel but not to etoposide, which was accompanied by an acquisition of an epithelial-to-mesenchymal transition (EMT) phenotype.[66]

Next to alterations in splicing factors themselves, alterations in serine-arginine protein kinases (SRPK) can also contribute to drug resistance. SRPK1 regulates the localization and function of SR proteins by phosphorylation and was shown to be overexpressed in many tumors including pancreatic cancer.[69] Interestingly, downregulation of SRPK1 by siRNA in pancreatic cancer cells resulted in increased apoptosis and sensitivity to cisplatin and gemcitabine.[70] This cytotoxic effect was accompanied by alterations in the phosphorylation status of several SR proteins, as well as increased expression of the pro-apoptotic protein BAX and RPL17 – a ribosomal protein associated with multidrug resistance.

Intercellular communication level

Little is known about intercellular communication involving functional cell-cell transfer of the splicing machinery components. The presence of nuclear proteins including splicing-related factors, was previously detected in secretomes of cancer cells.[74,75] The novel functional implications of these secretome findings are currently emerging.[76] In this respect, enrichment of nuclear proteins including splicing factors was recently found in secretome obtained from colorectal cancer tissues when compared with healthy individuals.[74] In addition, several splicing regulators (including SF3B1 and SRSF1) were detected in exosomes secreted by polarized Madin-Darby canine kidney cell line MDCK transformed with oncogenic H-Ras.[75] The vesicle-mediated transfer of the detected splicing factors was suggested to play a functional role in induction of EMT, resulting in a more aggressive phenotype.[75] Proteins involved in splicing were the major functional cluster found in the secretome of apoptosis-resistant primary AML cells[52] and could also be detected in exosomes derived from apoptosis-resistant cells.

SPLICING AS A NOVEL TARGET FOR CHEMOTHERAPY

Recent insights in the field of pre-mRNA splicing may lead to new therapies for cancer patients. At the moment, different therapies are being developed to target splicing aberrations, including inhibitors of the spliceosome (Table 5), as well as oligonucleotides directed at specific genes displaying altered splicing (Figure 4).

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Table 5. Spliceosome inhibitors as modulators of drug resistance.

Inhibitor	Target	Effect	Tumor type	Ref.
meayamycin B	SF3B	sensitization to Bcl-XL inhibitors	lung cancer, head and neck squamous cell carcinoma, multidrug resistant derivative of Chinese hamster lung cancer cells (DC3F), breast cancer, colon cancer, prostate, cervical cancer	77,78
spliceostatin A	SF3B3 and/or SF3B1	inhibition of splicing of multiple genes and nuclear retention of pre-mRNA	cervical cancer, breast cancer, lung cancer	79,80
pladienolide B	SF3B3 and/or SF3B1	inhibition of splicing	colon cancer, colorectal cancer, cervical cancer	80,81
thailanstatins A-C	SF3B	inhibition of splicing	prostate cancer, non-small-cell lung cancer, triple-negative breast cancer, ovarian cancer	82
sudemycins C1, D6, E, F	SF3B	modulation of splicing of apoptotic genes; changes in chromatin structure (E)	rhabdomyosarcoma, cervical cancer, primary skin fibroblasts, metastatic melanoma, prostate cancer, neuroblastoma	83,84
amiloride	dephosphorylation of SRSF1, changes in expression of several splicing factors (hnRNPs, SRSF3)	modulation of splicing of cancer related genes (Bcl-X, survivin, BCR/ABL)	leukemia (including imatinib sensitive and resistant cell lines), hepatocellular carcinoma, colon cancer, glioblastoma	85,86

Spliceosome inhibitors

The two archetypic spliceosome inhibitors include pladienolide and an analogue of FR901464 – spliceostatin A. Both compounds are natural products of bacteria - *Streptomyces platensis* (pladienolide) and *Pseudomonas sp.* (FR901464). An approach combining chemical probes with subsequent immunoprecipitation and mass spectrometry revealed that spliceostatin A and pladienolide both target the SF3B subunit of U2 snRNP.[79–81] Recent experimental evidence point to either SF3B3 or SF3B1 as well as to an interface of both as the binding site of pladienolide and spliceostatin A.[79–81] Spliceostatin A mediated inhibition of splicing *in vitro* leads to pre-mRNA accumulation and results in cell cycle arrest in G1 and G2/M phases.[79] The mechanism underlying this inhibition is still poorly understood with some studies reporting splicing inhibition and nuclear retention of transcripts, while others postulate differential usage of splice sites and alterations in the expression of particular genes.[79,80] Another derivative of FR901464 - meayamycin B, was able to switch Mcl-1 splicing towards the pro-apoptotic isoform Mcl-1S and thereby sensitized several solid tumor cells to another agent, ABT-737, which inhibits Bcl-2 and Bcl-XL. Meayamycin B was effective in a range of solid tumors, including multidrug resistant cells.[77,78,87]

A consensus pharmacophore shared by both spliceostatin A and pladienolide was used in a novel class of potent spliceosome modulators – sudemycins, which displayed increased activity in tumor cells

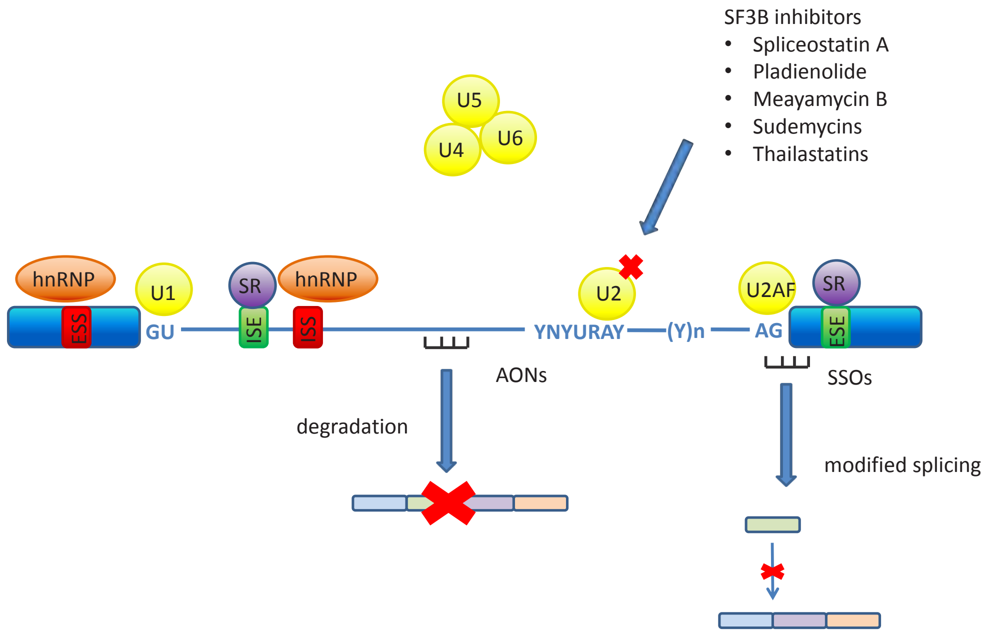

Figure 4. Splicing-based therapeutic options. Currently available splicing-based therapies involve targeting of specific abnormal splice variants or inhibition of the SF3B subunit of the spliceosome. Cancer-associated splice variants can be targeted using antisense oligonucleotides (AONs), which result in transcript degradation or splice switching oligonucleotides (SSOs) that modify splicing decisions. A growing number of compounds inhibit the SF3B subunit of the U2 snRNP, hence causing alterations in the splicing of multiple genes.

compared with normal cells.[83] Sudemycins had a selective effect on splicing patterns, which included caspase 2 and caspase 9 but not Bcl-X.[83] Interestingly, sudemycins also appeared to induce alterations in chromatin structure – namely changes in methylation of H3K36 – resulting in, next to reversible splicing alterations, changes in gene expression, followed by cell cycle arrest and apoptosis.[84]

A representative of a different class of compounds – amiloride – has been found to alter splicing patterns in leukemic cells and might improve the treatment of CML with Imatinib.[85] Owing to its pH altering properties, amiloride was used for a long time in treatment of hypokalemia, hypertension and edema.[88] However, a recent study showed that amiloride also induced an altered phosphorylation status of SRSF1 and changes in expression of several splicing regulators (hnRNPs and SR proteins).[85,86] These alterations were paralleled by altered splicing of multiple genes, including Bcl-X, survivin and the fusion gene BCR/ABL. Consequently, a decreased viability of both solid tumors and leukemic cells was observed.

Despite promising results in pre-clinical studies, clinical experience with this class of chemotherapeutics is still very limited. A novel drug, which targets the spliceosome, E7107 is a derivative of pladienolide B and the only spliceosome inhibitor tested in clinical setting thus far. Upon E7107 treatment of patients with solid tumors an increase in the levels of unspliced pre-mRNA corresponding to selected genes (i.e. DNAJB1 and EIF4A1) was observed. Although overall E7107 was well tolerated with predominantly manageable side effect, an unpredictable ophthalmologic

toxicity was observed in two patients, of which the mechanism is poorly understood.[89,90] This study constitutes an important step in development of clinically relevant spliceosome inhibitors, pointing to the necessity to make further advances in understanding the mechanism of action of these compounds in a tissue specific manner.

Oligonucleotides

Many genes involved in both tumorigenesis and chemoresistance are potential targets for oligonucleotide treatment.[91] Novel strategies utilizing oligonucleotides are being developed to target specific splice sites in cancer related genes.[92] One of the current approaches to target usage of a specific splice site involves antisense oligonucleotides (AONs).[93] AONs inhibit gene expression by targeting a specific sequence resulting in degradation of mRNA through RNase H and the RISC complex. They can be designed to block splice sites as well as other regulatory sequences, thereby preventing snRNPs and splicing factors from binding to the pre-mRNA.[93] Several promising studies document the successful application of AONs.[93,94] Signal transducer and activator of transcription 3 (STAT3) is an established activator of numerous oncogenic pathways and an inhibitor of apoptosis.[94] Its truncated isoform STAT3β lacks the C-terminal transactivation domain and promotes apoptosis. A shift in splicing from STAT3α to STAT3β was induced by the administration of modified AONs targeted to a splicing enhancer, which controls exon 23 alternative splicing. This change was associated with cell cycle arrest and increased apoptosis in a panel of cell lines (including breast cancer, cervical cancer and leukemia) and tumor regression in a xenograft mouse model of metastatic melanoma.[94]

A different approach utilized the splicing switching oligonucleotides (SSOs) which modify pre-mRNA splicing.[91] SSOs sterically block sequences in pre-mRNA without inducing degradation, thereby modulating pre-mRNA splicing. RNA degradation is blocked by alterations introduced in the oligonucleotide sugar-phosphate backbone. This approach can be used to switch splicing of Bcl-X, by targeting the 5' splice site of exon 2. A decrease in the expression of Bcl-XL accompanied by an increased expression of Bcl-XS was induced by SSOs in metastatic melanoma cells as well as in xenograft models, which resulted in reduction of the tumor load.[95] The tyrosine kinase receptor HER4 plays a complex role in cancer pathogenesis and progression. The cytoplasmic region of this gene can be alternatively spliced resulting either in the CYT1 isoform that binds to and activates phosphoinositide 3-kinase (PI3K) or in the CYT2 isoform, which lacks the domain responsible for PI3K interaction. A splice switching oligonucleotide mediating a shift from CYT1 to CYT2 expression resulted in decreased cell growth of breast cancer cells both *in vitro* and in *in vivo* xenograft mouse models.[96] Further optimization of approaches for effective delivery and minimization of adverse effects of these compounds are also currently under development.[97,98]

Several clinical trials testing oligonucleotides in cancer treatment are currently in progress.[99] A recent clinical trial showed that the usage of splicing modulating morpholinos is a very promising approach in Duchenne mascular dystrophy, where the modified oligonucleotides effectively corrected the underlying genetic defect in this disease leading to clinical benefits in the treated patients.[100] However, the usage of oligonucleotides in cancer treatment still faces many challenges, including uniform delivery to various regions of tumors as well as genomic instability of the malignant cells resulting in phenotypic plasticity.[99]

CONCLUSION

In the recent years much progress has been made in deciphering the role of pre-mRNA splicing in several aspects of cancer treatment. Mutations in spliceosome genes were recently reported in both myeloid and lymphoid malignancies and the prognostic relevance of these lesions is emerging. Mutant components of the spliceosome, i.e. SRSF2, ZRSR2 and U2AF1, are mostly associated with poor outcome in cancer patients. Moreover, aberrant splicing patterns are linked to drug resistance. Altered function of splicing regulators, as well as mutations in splice motifs in target genes can result in abnormal variants, affecting cellular response to chemotherapy. This can result in the impaired balance in apoptosis regulation, leading to decreased induction of cell death (i.e. Bcl-X, FAS, caspases) or alternatively in changes in several aspects of drug metabolism, including drug influx transporters (e.g. SLC29A1), conversion into active metabolites (i.e. FPGS, TP, dCK), as well as drug extrusion (e.g. MRP1).

Therapies aiming at cancer specific abnormal splice variants include the AONs or SSOs. This approach exhibited encouraging results in both *in vitro* and *in vivo* studies, causing effective shifts in the splicing profile of target genes paralleled by growth inhibition of the tumor cells. An alternative approach involves compounds directly targeting the spliceosome. This includes SF3b inhibitors - spliceostatin A, pladienolide and a group of derivative compounds. The effectiveness of these molecules in splicing modulation has been demonstrated *in vitro* and *in vivo* in several types of solid tumors, resulting in cell cycle arrest and apoptosis as well as sensitization to other drugs.

EXPERT OPINION

The rather impressive progress in understanding the contribution of splicing aberrations to cancer pathobiology, therapeutic efficacy and drug resistance has illuminated several exciting avenues.

Discovery of frequent and previously unknown mutations in the splicing pathway has opened new opportunities in prognosis and treatment of hematological malignancies including MDS, CLL and CMML. Most of those alterations are poor prognostic factors and are likely to have a substantial impact on administration of cancer therapy and the treatment outcome. Moreover, an increasing number of reports are recently pointing to aberrant splicing as an important and common mechanism of anticancer drug resistance. Consequently, cancers displaying extensive aberrant splicing, as well as abnormal splicing patterns of single genes may serve as attractive druggable targets for novel therapeutic approaches. The ultimate goal of this field is to design therapeutic interventions specifically targeting and manipulating cancer associated aberrant splicing to enhance the curative effect of contemporary regimens. However, new developments in achieving this aim come with the realization that there is still a great deal of knowledge to gain and many challenges to face.

The main obstacle in the way to controlled use of splicing-based therapeutics is the unprecedented complexity of the splicing phenomenon and the variety of its interactions with the intracellular environment. Our current understanding of how exactly mutations in the spliceosome or impaired activity of splicing factors affect gene expression profiles, which in turn translate to the specific phenotype of cells, is still very limited. Splicing factors are often implicated in other aspects of RNA metabolism and therefore predicting the consequences of their mutations and/or inhibition can pose problems. In addition, it is becoming clear that the interface between aberrant

splicing and other genetic lesions resulting in cancer with often aggressive phenotypes must be taken into consideration. For example, mutations in SF3B1 were predictive of either favorable or unfavorable prognosis, depending on the type of disease in which they occurred (MDS and CLL, respectively).[36] This illustrates that the interaction of mutated spliceosome components with the genetic background of the disease can modify their phenotypic effect. These insights can pave the way for the development of new diagnostic procedures as well as novel therapies for patients presenting with spliceosome mutations.[101]

Several potential therapeutic options utilizing splicing are available including oligonucleotides against specific aberrant splice variants as well as spliceosome inhibitors. Much progress is being made in the design of therapeutic oligonucleotides, including improved stability ensuring elevated *in vivo* effectiveness, as well as novel delivery techniques. Yet, several clinical trials using oligonucleotides targeting cancer specific genes revealed substantial limitations of this approach. This includes off target effects, unspecific modes of action as well as ineffective delivery methods. Hence, these issues will need to be addressed in future studies to improve the therapeutic effect of oligonucleotides.[99] Some of the studies involving SF3B inhibitors point to an enhanced cytotoxicity in tumor cells compared with normal cells, suggesting that an optimal therapeutic window exists for these compounds. Interestingly, cells harboring mutations and deletions in the PRPF8 gene displayed increased sensitivity to meayamycin B, when compared with normal bone marrow cells.[37] In addition, recently published results of phase I clinical trials have confirmed the splicing inhibitory activity of pladienolide derivative - E7107 and point to good tolerability of this drug.[89,90] However, despite overall easily manageable side effects two patients displayed severe unpredictable toxicities underlining the necessity for more extensive pre-clinical research in the development of this class of drugs. The exact role of SF3B subunit in expression of particular genes in a tissue specific manner needs to be determined, in order to understand all the potential effects of its inhibition. In addition, as the current spliceosome inhibitors seem to be rather unspecific, more tumor targeted compounds might be required to avoid severe toxicities to normal tissues. A recent study suggests that depending on the dose used, spliceosome inhibitors can either modulate splicing patters in cells or exert a cytotoxic activity.[102] This implies that the potential therapeutic use of spliceosome inhibitors is not limited to single agent cytotoxic activity but extends to modulation of splicing patterns of specific genes, resulting in sensitization of cells to chemotherapy. The resulting findings may open new avenues for a novel class of (spliceosome targeted) chemotherapeutics in cancer treatment and point to patient characteristics which may favor their efficacious application.

Interestingly, a large-scale screen has shown that traditional chemotherapeutics can shift splicing of Bcl-X and other genes associated with apoptosis in MCF-7, HeLa and PC-3 cells.[103] The effect of various compounds on splicing patterns of apoptosis-related genes varied between tumor cell lines, pointing to cell type specificity. Moreover, different classes of compounds tended to induce alterations in splicing of different apoptotic genes, suggesting that certain combinations of chemotherapeutics may enhance their curative effect.[103] Therefore, the knowledge of splicing based on the response to traditional chemotherapy may also help to fine-tune their combinatorial application in order to achieve an optimal therapeutic effect.

Finally, several studies point to the secretion of splicing regulators via extracellular vesicles, as an intercellular communication pathway to mediate more aggressive disease phenotypes, as

well as therapy resistance (Figure 3B). The role of the microenvironment in sustaining a malignant phenotype is yielding increasing amount of evidence. Although, the vesicle-mediated transfer of functional splicing regulators between the neighboring cells is an intriguing concept, it still requires support by a substantial body of evidence. If further corroborated, this concept implies that analysis of selected factors in patient serum next to the investigation of the malignant cells themselves, may give a more complete disease characteristic, which can translate to a better tailored treatment. On the other hand, detection of such secreted factors is dependent on tumor load and therefore the sensitivity (next to specificity) of detection could be an issue.

In general, several exciting possibilities arise from the gain of insight into splicing aberrations. The understanding of how classical chemotherapeutic agents modulate splicing patterns could indicate more optimal drug combinations. Moreover, development of novel therapeutic options based on general spliceosome modulation as well as targeting specific splice variants holds promise for patients harboring tumors that are resistant to conventional chemotherapy. Taken together, deciphering abnormal splicing patterns may guide future clinical decisions, resulting in more effective personalized therapies.

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