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An Insight into the Role of Spliceosomal Mutations in Myelodysplastic Syndromes

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Abstract

Objectives: The identification of altered splicing signatures in Myelodysplastic Syndromes (MDS) could likely provide key markers for diagnosis, prognostication and development of novel therapeutics.

Methods: This review presents an insight into role of spliceosomal gene mutations in the pathogenesis of MDS, emphasizing on their clinical and prognostic significance. We also discuss emerging studies delineating the functional consequences of these mutations and pointing towards the emergence of a new leukemogenic pathway involving spliceosomal dysfunction.

Results: The current update demonstrates the importance of RNA splicing at different stages of MDS develop¬ment and highlights an intriguing connection between DNA damage response pathway, altered splicing outcomes and differentiation in the myeloid lineage. Together, the data discussed suggests a mechanistic link between mutant spliceosomal proteins, alterations in the splicing of key regulatory genes and impaired hematopoiesis.

Discussion: A careful scrutiny of abnormal splicing mechanisms is required to unravel unidentified splicing scenarios in MDS. Further studies could possibly identify functionally important targets of mis-splicing and also genes which cooperate with spliceosomal gene mutations, leading to a refined molecular classification of MDS.

Conclusion: The most challenging goal for the future would be, integrating the different layers of gene-expression regulatory pathways altered in MDS as a consequence of aberrant splicing and acquire a systematic view of the many molecular mechanisms contributing to the pathophysiology of this complex disease.

Keywords: Myelodysplastic Syndromes; Splicing Mutation; *SF3B1; U2AF1; SRSF2*

List of abbreviations: snRNP: Small Nuclear Ribonucleoprotein; AS: Alternative Splicing; SF1: Splicing Factor 1; U2AF: U2 Auxiliary Factor; SREs: cis-acting Splicing Regulatory Elements; RBPs: RNA-Binding Proteins; ESEs or ESSs: Silencers; ISEs: Intronic Splicing Enhancers; ISSs: Intronic Splicing Silencers

Introduction

Myelodysplastic Syndromes (MDS) are clinically and phenotypically heterogeneous clonal haematopoeitic stem cell disorders characterized by ineffective hematopoiesis, peripheral blood cytopenias and risk of leukemic transformation. Somatic mutations in early progenitor cells contribute to MDS pathogenesis although no single genetic defect has been shown to be exclusively associated with MDS. Genomic instability is the hallmark of leukemic transformation and it manifests as genetic defects including chromosomal aberrations, gene mutations, copy-number alterations and aberrant gene expression which are common events in MDS. Mutations in a broad range of genes involved in regulation of gene expression such as epigenetic modifiers, transcription regulators, signal transducers and pre-mRNA splicing factors have been identified in MDS, implicating complex molecular mechanisms in its pathogenesis and evolution. Spliceosomal genes *SF3B1, U2AF1* and *SRSF2* are the most common targets (~45-80%) of somatic mutations in MDS though they have been identified in other myeloid and lymphoid neoplasms [1-4]. The role of these alterations as disease alleles is compelling as majority of these lesions are recurrent, heterozygous and mutually exclusive; defining clinical features closely related to MDS [5-10]. The mutual exclusivity of these alterations with respect to each other and

the fact that they are involved in the same function in pre-mRNA splicing i.e. the recognition of 3' intronic splice sites, suggests that their co-occurrence could be lethal for the cell and that spliceosomal dysfunction as a result of disruption of 3′ splice-site recognition and related chain of events could be implicated in MDS pathogenesis although validatory studies are essential [11].

Spliceosomal dysfunction due to mutations in core spliceosomal genes could result in defective small nuclear ribonucleoprotein (snRNP) complex assembly on the pre-mRNA, deregulated global and alternative mRNA splicing, faulty nuclear-cytoplasmic export and unspliced mRNA degradation [12-16]. Consequently, a change in the expression of critical downstream genes occurs, as observed in a few recent studies on MDS [6,17-21]. The persuasive nature of these mutations coupled with recent reports suggesting the role of altered splicing in different stages of MDS development, defines the scope of this review [18,22-24]. Herein, we summarize the current understanding of the molecular machinery involved in splicing and its regulation and discuss the recurrent genetic lesions in splice factor genes, their functional impact and the possibility for novel therapeutic intervention in MDS.

The Intricate Process of Pre-mRNA Splicing by the Spliceosome

The removal of introns and exon joining is mediated by the process of pre-mRNA splicing which primarily occurs co-transcriptionally and constitutively. It is carried out in the nucleus by the spliceosome, a complex macromolecular machinery consisting of five small nuclear ribonucleoproteins (U1, U2, U4, U5 and U6 snRNAs) and > 150 proteins [25-27]. A majority (95%) of human genes undergo alternative splicing (AS), an indispensible splicing mechanism responsible for increasing proteomic diversity by generating multiple mRNA isoforms from a single gene through mechanisms enlisted in Figure 1. It is a tightly

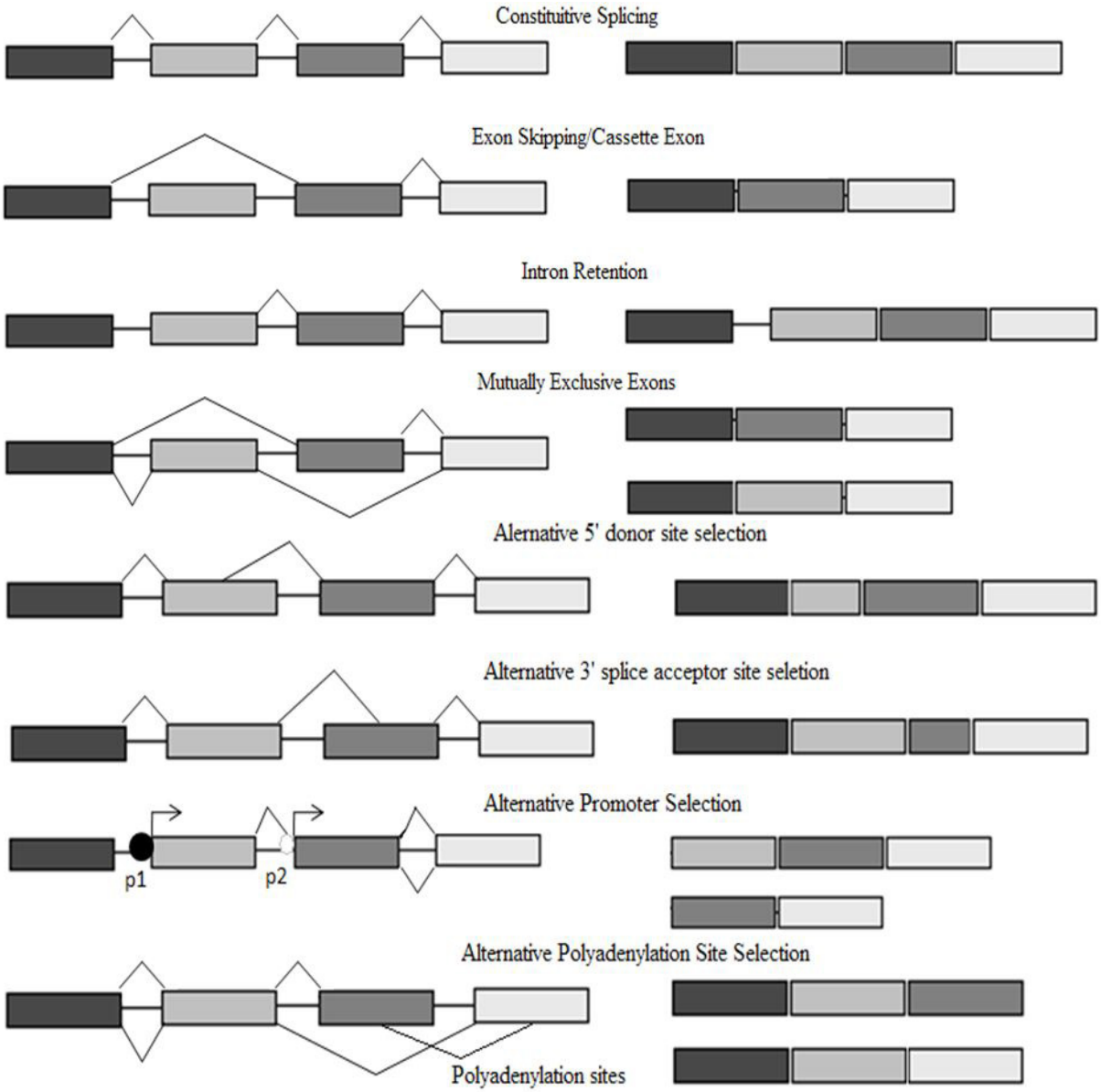


Figure 1: The different modes of alternative splicing. In this figure the boxes denote the coding exons while the lines denote the intron and P1/P2 stand for promoters

regulated process resulting in cell-type, developmental stage and gender specific mRNA isoform expression in response to external stimuli or intracellular signals.

The spliceosome performs the two primary functions of splicing: recognition of the intron/exon boundaries (5'/3' splice sites) and catalysis of the transesterification reaction to excise out non-coding intron as a lariat followed by ligation of exons to form mature mRNA. Splice site recognition is governed by the cis-regulatory elements typically located in the exons or their surrounding intronic sequences including the 5' and 3' splice sites and the branch point. These sequence elements are recognized by trans-acting splicing factors which act in tandem to recruit the spliceosome to the correct splice sites and block nearby pseudo-splice sites [28]. The sequential binding and release of snRNPs and the numerous auxiliary protein factors as well as precise RNA–RNA, protein–RNA and protein–protein interactions result in tissue-specific expression of mature mRNA [29].

In most eukaryotes, including humans, there are two classes of introns: the common U2 type, representing more than 99% of human introns and the rare U12 type, differing in their consensus splice-site sequences (Table 1). The "major" or U2-type spliceosome consisting of U1, U2, U4, U5, and U6 snRNPs, catalyzes the vast majority of transcript splicing events whereas the "minor" or U12-type spliceosome utilizes the U12 snRNP to mediate splicing of approximately 800 specific transcripts [29-31]. U12 introns are spliced by the same mechanism as U2 introns although, utilizing a different but homologous set of snRNPs (U11, U12, U4atac, and U6atac).

U1 snRNP (U1snRNA)	U2 snRNP (U2snRNA)	U4 snRNP (U4snRNA)	U5 snRNP (U5snRNA)	U6 snRNP(U6snRNA)
Sm Proteins (SmB/SmB', SmD1, SmD2, SmD3, SmE, SmF and SmG) **U1-specific proteins** (U1-70K, U1-A and U1-C)	**Sm Proteins** **U2-specific proteins** U2A', U2B" **Additional Proteins** SF3a120, SF3a66, SF3a60 SF3b155, SF3b145, SF3b 130, SF3b 49,SF3b 14a/p14, SF3b14b, SF3b10	**Sm Proteins** and Prp3 Prp4 Prp31 CypH Snu13	**Sm Proteins** and Prp8 Snu114 Brr2 Prp6 Prp28 40K 52K Dib1	**LSm Proteins** (LSm2-8) Prp 24

Table 1: The protein composition of the major human spliceosomal snRNPs. Each snRNP consists of a small uridine rich small nuclear RNA (snRNA), a common set of seven Sm proteins (B/B', D3, D2, D1, E, F, and G) and other particle specific proteins. LSm (Like Sm proteins)

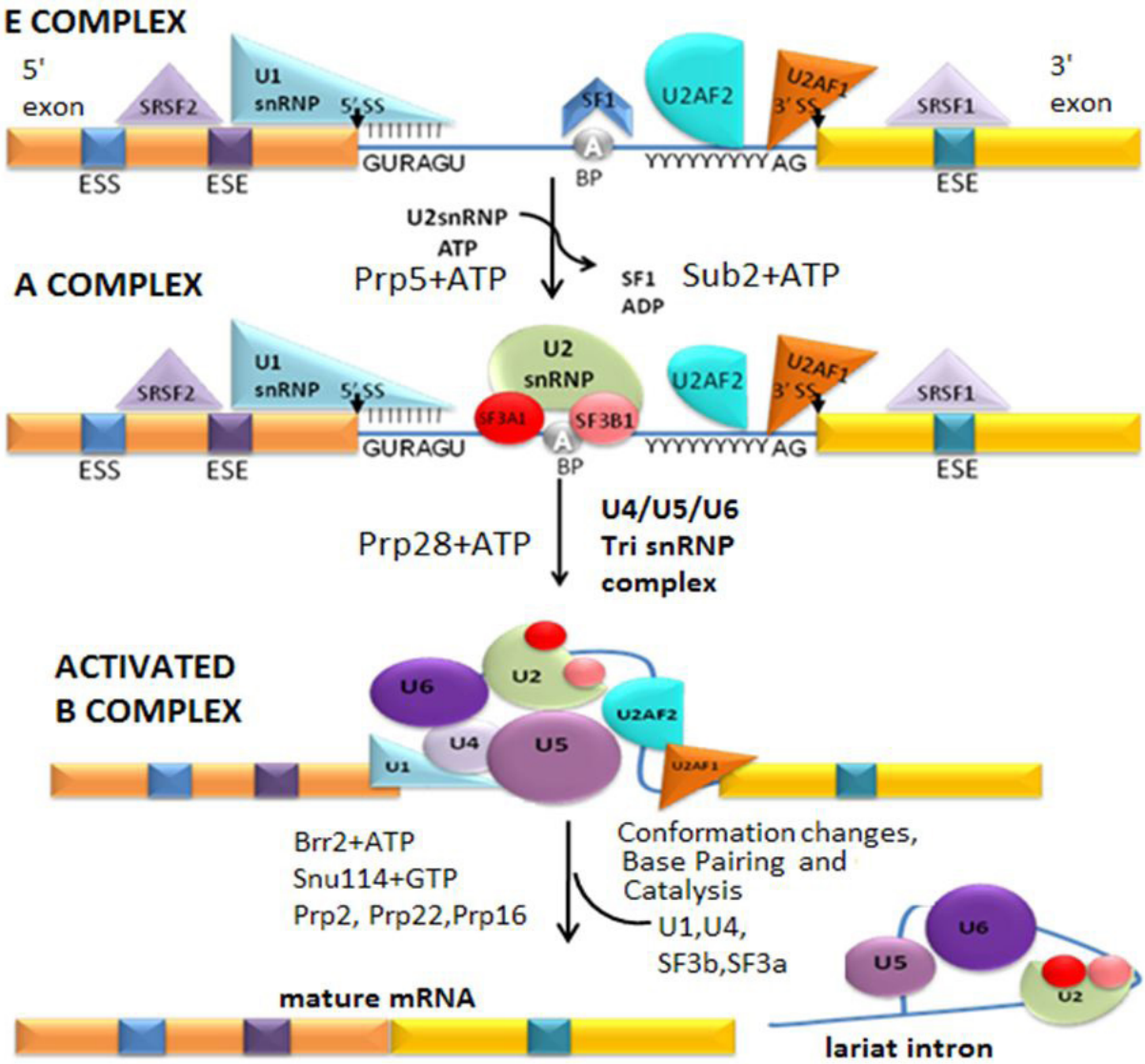
The process of splicing by the major spliceosome is initiated in the spliceosome assembly phase which includes the sequential formation of three complexes: an early ATP independent "E" complex, an ATP dependent "A" complex and a "B" complex, reviewed in detail by Wahl *et al.* [26] (Figure 2). DExD/H-box RNA dependent helicases/ATPases are required to mediate structural changes at various steps in the spliceosomal assembly pathway and also for fidelity control of the splicing reaction [26]. "E" complex formation begins with the sequential binding of a U1 snRNP to the 5' splice site, splicing factor 1 (SF1) to the branch point and of the U2 auxiliary factor (U2AF) heterodimer, consisting of U2AF1 (also called U2AF35) and U2AF2 (or U2AF65) subunits, to the AG splice acceptor dinucleotide at the 3' splice site of the target intron and the polypyrimidine tract, respectively [32]. SRSF1 or SRSF2 (Ser-Arg regulatory proteins or SR proteins) splice factors, are also recruited to the polypyrimidine tract located between the branchpoint and the 3' splice site, for prevention of exon skipping and regulating AS [28,32]. ZRSR2, RNA Binding Motif and Serine/Arginine Rich 2 splice factor also participates in the recognition of 3′-splice site during the early stages of spliceosome assembly [32].

Further, the "E" complex is converted into the ATP-dependent, pre-spliceosomal "A" complex with the replacement of SF1 by U2 snRNP at the branch point, bringing together the proximal and distal exons. ATP hydrolysis is required for this 5' and 3' splice site pairing, which locks the splice sites into a specific splicing pattern for facilitating subsequent steps of spliceosomal assembly. U2 snRNP consists of a 12S RNA subunit and the SF3a and SF3b multiprotein complexes, the SF3B1 splice factor, mediates the interaction of U2 snRNP with the intronic branchpoint sequence.

Subsequently, the U4/U6-U5 pre-assembled tri-snRNPs are recruited, generating the catalytically inactive "B" complex, with all the snRNPs bound to the pre-mRNA strand at this stage. Major compositional and conformational rearrangements take place resulting in the release of U1 and U4 snRNPs and the binding of U5 snRNP to the exon sequences near the splice sites, juxtaposing the neighboring exons. This is followed by the replacement of U1 snRNP by U6 snRNP which base pairs with the 5'ss through the conserved ACAGA box of the snRNA component. Additionally, extensive base pairing and structural rearrangements happen between U6 and U2 snRNPs resulting in catalytic activation and generation of the intermediate activated spliceosome or the activated B complex (B*).

The subsequent splicing events involve the dissociation of SF3a and SF3b complex proteins, exposing the branch-point 2'OH group for a nucleophilic attack on the 5' splice site which results in a free 5' exon and a lariat intron intermediate (C1 complex). Lastly, the cross linking of the U5 and U6 snRNP occurs followed by the attack of 3' OH of the 5' exon on the 3'ss, forming the C2 complex which results in the ligation of exons. The remaining snRNPs are disassembled and a mature protein encoding mRNA is formed. Most of the interactions involved in splicing are usually weak and strengthened by multiple interactions, providing the splicing machinery with accuracy and remarkable flexibility [26,33]. The nuclear mRNA surveillance mechanism accounts for fidelity of

splicing by ensuring only fully functional mRNA are committed to cytoplasmic transport for subsequent translation [32].



Intron–exon boundaries are marked by the highly conserved dinucleotide sequences GU and AG, found immediately within the intron at the 5' and 3'ss (splice site). Other sequences that are crucial for identification of splice sites and mRNA processing include the intron branch site (BP) and polypyrimidine tract directly upstream of the 3'ss. Splice site enhancer (ESE) and suppressor sequences (ESS) have been shown in the figure

Figure 2: The process of Spliceosomal Assembly

Regulation of Pre-mRNA Splicing

The regulation of AS involves the activity of several regulatory molecules in tandem to allow for tissue specific expression of a particular mRNA isoform. The recognition of appropriate splice sites by the spliceosome is the key point for regulation of pre-mRNA splicing/AS. Traditionally, it has been thought to be regulated by the short, degenerate RNA sequences located either in exons or introns called cis-acting splicing regulatory elements (SREs) as well as trans-acting RNA-binding proteins (RBPs) which bind to these elements [33-35]. The exons that end up in the mature mRNA during the process of AS, is entirely defined by the interaction between the cis-acting elements and the trans-acting factors. The splice site sequences alone cannot provide the degree of control needed for correct exon selection. The splice site strength or how well it matches the consensus sequence determines the efficacy with which spliceosome components will bind to it and whether or not these sites will be used in splicing [36].

The cis-regulatory elements are crucial for accurate splice site recognition [34,36-38]. These elements are classified as exonic splicing enhancers (ESEs) or silencers (ESSs) which promote or inhibit the inclusion of the exon they reside in, and as intronic splicing enhancers (ISEs) or silencers (ISSs) which enhance or suppress the usage of adjacent splice sites or exons from an intronic location. These SREs function by recruiting trans-acting splice regulatory factors or RBPs which activate or suppress splice site recognition and or spliceosome assembly through various mechanisms [38,39]. Ultimately, the organization of regulatory sequences within pre-mRNAs (ESEs, ESSs, ISEs and ISSs) and the relative ratios of different regulatory proteins, determine which splice sites are used in the splicing reaction [38].

The classical RBPs influencing the choice of splice site include proteins of two families: serine/arginine-rich (SR) proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs). These proteins positively or negatively regulate inclusion of a particular exon, though few instances of antagonistic functions are reported [38-40]. Many of these regulatory proteins show the presence of two functional domains: an amino terminal RNA-binding domain and a carboxy terminal protein–protein interaction domain. The best characterized RNA-binding domains are the RNA recognition motif (RRM) and K-homology (KH) domains. Most ESEs are recognized by SR proteins, which contain one or more RRM domains and an arginine/serine-rich (RS) protein–protein interaction domain. SR proteins enable the recruitment of the U1 snRNP and U2AF to the neighboring 5' and 3' splice sites, by binding to an ESE and directly interacting with protein targets, promoting the inclusion of that exon into the mature transcript. Recently, these proteins have been hypothesized to mediate cross-intron interactions between splicing factors bound to the 5' and 3' splice sites and are also required for cross-exon interactions in both constitutively and alternatively spliced pre-mRNAs [41-45].

The best characterized ESSs and ISSs are recognized by members of the hnRNP family. These proteins are highly abundant RBP's dispersed in the nucleus lacking an RS domain. Several family members contain an arginine/glycine-rich domain that may be involved in both RNA binding and interactions with other proteins. These complexes bind to ISSs and ESSs, blocking the recruitment of the U1 snRNP and U2AF by a mechanism that has not been fully defined. Inhibition of splice site recognition is achieved by sterically blocking the access of snRNPs or of positive regulatory factors through the proximity of splicing silencers to splice sites or to splicing enhancers [25,42].

The precise interactions between cis-acting elements embedded in pre-mRNA and trans-acting factors, the splice site strength, concentration, composition and activity of splice regulatory proteins as well as the cross-exon communication between the trans-acting factors, allows the exon to be recognized as a unit (exon definition) prior to intron removal [38,40,41]. In addition to ubiquitous RBP's, tissue-specific regulatory proteins also have a role to play in AS [38]. The other factors such as the rate of transcription, chromatin conformation and histone modifications, pre-mRNA secondary structure, small nucleolar RNAs (snoRNAs) and non-coding RNAs are also involved in regulation of splicing [46-58].

Mutations in components of core spliceosomal machinery and their Functional Impact

Aberrant splicing due to alterations in the cellular concentration, composition, localization and activity of regulatory splicing factors has been associated with several diseases including cancer.

The maintenance of high-fidelity mRNA splicing is important, as the translation of mis-spliced mRNAs into proteins with aberrant function would have disastrous consequences on the cell. Not only the presence of different transcripts, but also the levels and/or ratios of the different mRNA isoforms could result in a disease condition. AS is primarily regulated by cis elements within the RNA and the trans RNA binding factors. Therefore, mutations in splicing enhancer/silencer elements and RNA binding trans factors could result in the expression of undesirable isoforms, which in turn can induce a disease condition. There are a large number of examples of cis-acting splicing mutations that are associated with disease conditions. However, the dearth of mutations in core spliceosomal components suggests, that mutations in these genes could be lethal during embryonic development, if not at the level of individual cells [35-38,59].

Recently, there has been an increase in reports of disease-causing mutations within genes encoding components of core-spliceosomal machinery particularly in hematological malignancies (Table 2). Yoshida *et al.* and Makishima *et al.* suggest that these mutations could contribute to a new leukemogenic pathway involving spliceosomal dysfunction [5,8]. These mutations likely disrupt several processes downstream, because an altered spliceosome may cause abnormal transcription as well as altered splicing outcomes such as exon skipping, intron retention, and cryptic splice site activation with truncated (or elongated) exon [6,8,13,18-24]. Mutations affecting core spliceosomal proteins also impact alternative splicing. Auxiliary splicing factors, such as *SRSF2* are known to interact with the basal spliceosome machinery and modulate alternative splicing of specific genes. As a consequence, mutations affecting these factors could cause alteration in alternative splicing of at least a subset of genes, which has been observed upon knockdown of these factors *in-vitro* [60]. The prognostic value of the most common mutations and their phenotypic association in the clinical setting is currently under investigation (Table 2). There is no clarity on the whether spliceosomal gene mutations lead to gain or loss of function and the specific mechanism (s) by which splicing factor mutations lead to diseased state remains to be determined. Thus, spliceosomal mutations may, depending on the pattern of affected proteins, lead to similar functional effects on tumour suppressor genes as chromosomal deletions, epigenetic silencing or inactivating/hypomorphic mutations. However, the observed pattern of occurrence of highly specific missense mutations in these factors, coupled with the lack of nonsense mutations and deletions, implies a gain-of-function or better gain-of-dysfunction mechanism as suggested by Makishima *et al.* [8].

The targets for the majority of spliceosome-associated mutations in MDS are components of the "E" and "A" splicing complexes [59]. Multiple independent somatic mutations have been identified in the genes *SF3B1, U2AF1, SRSF2* and *ZRSR2* involved in recognition and binding of the 3′ splice site [61]. *U2AF1* is exclusively required for splicing of U2 introns, whereas *ZRSR2* is required for splicing of both U2 and U12 introns [13,62]. *SF3B1* and *SRSF2* are expected to participate in the splicing of both U2 and U12 introns [13]. Mutations in these genes most likely reflect defects in 3′ss recognition during RNA splicing. The defects in 3′ss recognition (but with normal 5′ss recognition) can result in two 5′ss competing for one 3′ss, an AS pattern that resembles

Spliceosomal Gene	Role in Splicing	Frequently mutated amino Acid	Frequency in MDS without RS(ref)	Frequency in MDS with RS (ref)	Prognosis
SF3B1 (splicing factor 3b, subunit 1, 155kDa;2q33.1)	The SF3b1 complex is part of the functional form U2 snRNP that binds to the branch site near the 3' end of introns and helps to specify the site of splicing.	K700 R625 K666	7% [76] 1.8% [77] 6.5% [5] 3.3% [17]	NA [76] 4/5 [77] 75.3% [5] 75.6% [17]	Favorable And low Risk of Leukemic Transformation
U2AF1 (U2 small nuclear RNA auxiliary factor1; 21q22.3)	U2AF1, the small subunit of U2 auxiliary factor (non-snRNP protein) binds to the 3′ AG splice acceptor dinucleotide of the pre-mRNA target introns helping in the early steps of 3' splice site recognition. U2AF1 forms a heterodimer with U2AF2 (U2AF65) resulting in constitutive and regulated RNA splicing by directly mediating interactions between the U2AF2 protein and other splicing regulators such as SRSF1 and SRSF2.	S34 Q157	7.8% [76] 19.6% [77] 11.6% [5] 5.6% [17] 7.8% [9]	NA [76] 1/5 [77] 0% [5] 4.9% [17] 10.34% [9]	Inferior Overall survival (76,9) Increased risk of progression to AML
SRSF2 (serine/arginine-rich splicing factor 2; 17q25.1)	It belongs to the serine/arginine (SR)-rich family of pre-mRNA splicing factors containing an RNA recognition motif (RRM) for binding RNA and an RS domain for binding other proteins. It binds to splicing regulatory sequence elements in pre-mRNA transcripts and to components of the spliceosome helping in splice-site selection, spliceosome assembly, and both constitutive and alternative splicing. It is also involved in mRNA export from the nucleus and in translation.	P95	10% [76] 14.3% [77] 11.6% [5] 13.3% [17] 15% [9]	5.5%[5] 2.4% [17] 10.34% [9]	Inferior overall survival and a more rapid and more frequent progression to AML. [9]
ZRSR2 (zinc finger CCCH type, RNA-binding motif and serine/arginine rich 2;Xp22.1)	ZRSR2 associates with the U2AF heterodimer, required for the recognition of a functional 3' splice site in pre-mRNA splicing, and may play a role in network interactions during spliceosome assembly.	NA	7.7% [5] 13.8% [17] 4.34% [9]	1.4% [5]	No impact on OS

Table 2: The frequency and prognostic impact of common splicing factor genes mutated in MDS.

alternative 5′ ss. A frequent outcome of alternative 5′ ss is the selection of the 5′ ss proximal to the downstream 3′ ss. In support of this speculation, Yoshida *et al.* showed that the expression of mutant *U2AF1* results in large scale (~5%) intron retention in HeLa cell [8]. Because introns are rich in stop codons, retained introns frequently introduce premature termination codons (PTCs) into the mRNA which in turn activates nonsense-mediated mRNA decay (NMD), resulting in changes in mRNA isoform expression as observed in the HeLa cells expressing mutant *U2AF1* [5,62]. Given the importance of these findings we discuss the functional impact of these mutations in MDS enlisting the targets of deregulated/altered splicing (Table 3).

Genes	Genes Differential Expressed/Spliced	Ref
SF3B1	(ASXL1, TP53 and CBL), GATA1/2, iron homeostasis and mitochondrial metabolism (ALAS2, ABCB7GLRX5 and SLC25A37) and RNA splicing/ processing (PRPF8 and HNRNPD).	19,64-67
U2AF1	cell cycle progression (CEP164, EHMT1 and WAC), RNA processing (PTBP1, STRAP, PPWD1, PABPC4, and UPF3B) DNA methylation (DNMT3B), X chromosome inactivation (H2AFY), the DNA damage response (ATR, FANCA), and apoptosis (CASP8), BCOR.	18,69
SRSF2	EZH2, IKAROS, CASPASE 8, BCOR GFI1, CEBPE, HOXB2, GATA1, GATA2, CDKN1A.	23

Table 3: The differentially expressed or spliced genes in MDS functional studies.

SF3B1

The most commonly mutated spliceosomal factor gene is located on chromosome 2q33.1. This gene codes for subunit 1 of the splicing factor 3b protein complex (*SF3B1*), which is involved in the early stages of spliceosomal assembly. The SF3B1 complex is part of the functional form of the U2 snRNP that binds to the branch site near the 3′ end of introns and helps to specify the site of splicing. It cross-links to a 25-nucleotide region in the pre-mRNA located immediately upstream of the intronic branch site [19,63]. *SF3B1* mutations strongly correlate with refractory anemia with ring sideroblasts (RARS) present in over 70% of patients [7,10]. Most mutations in this gene are associated with one of the 22 HEAT (Huntingtin, Elongation factor 3, protein phosphatase 2A and targets of rapamycin 1) repeats present in this polypeptide which mediate its interaction with other proteins [59]. These mutations cluster around residues 625, 666, and 700 present in exons 14, 15 with K700E substitution occurring most frequently (Table 2) [7-10]. In MDS patients harboring *SF3B1* mutations, higher platelet count, increased ring sideroblasts, fewer cytopenias, lower blast count and longer event-free survival have been associated [7].

Several studies have examined the role of *SF3B1* in MDS. RNA-sequencing analysis of *SF3B1* mutants showed differentially used genes relevant in MDS pathogenesis, such as *ASXL1, CBL, EZH,* and *RUNX* families [63]. Dolatashad *et al.* suggest that the aberrant expression and splicing of erythroid-related genes (*GATA1/2*) observed in the CD34+ cells of MDS patients with *SF3B1* mutation may have a role in the ineffective erythropoiesis found in these patients [19]. *SF3B1* knockdown in four myeloid cell lines resulted in inhibition of cell growth and disruption of the cell cycle. Several pathways including cell cycle, RNA processing, mitochondrion and apoptosis/p53 pathway were consistently deregulated in the cell lines with *SF3B1* knockdown [19,64]. Differentially expressed genes at the transcript and/or exon level in *SF3B1* mutant compared to wild-type cases include genes involved in MDS pathogenesis (*ASXL1 and CBL*), iron homeostasis and mitochondrial metabolism (*ALAS2, ABCB7* and *SLC25A37*) and RNA splicing/processing (*PRPF8* and *HNRNPD*). Several genes regulated by a DNA damage-induced BRCA1-BCLAF1-SF3B1 protein complex showed differential expression/splicing in *SF3B1* mutant cases [19,64].

A recent study on transcriptome profiling during early erythroid differentiation showed a marked up-regulation of genes involved in haemoglobin synthesis and oxidative phosphorylation while down-regulation of mitochondrial ABC transporters compared to normal bone marrow was observed. Moreover, mis-splicing of genes involved in transcription regulation, particularly haemoglobin synthesis, confirmed, a compromised haemoglobinization in RARS erythropoiesis [64]. In particular all studies suggest, the mitochondrial gene *ABCB7* is consistently downregulated in RARS patients, suggesting that it may be a key mediator of ineffective erythropoiesis of RARS. It remains to be determined how *SF3B1* mutations result in downregulation of *ABCB7*, although it is possible that intron retention followed by NMD contributes [65-67].

U2AF1

U2AF1 (U2AF35) gene located on chromosome 21q22, belongs to the SR family genes encoding the small subunit of U2 auxiliary factor complex required for recruitment of U2 snRNP to the pre-mRNA branch site of the intron [5,6,18,24]. U2AF1 forms a heterodimer with U2AF2 (U2AF65) resulting in constitutive and regulated RNA splicing by directly mediating interactions between the U2AF2 protein and other splicing regulators such as SRSF1 and SRSF2 [5,6,18,24]. U2AF1 binds to the 3′ AG splice acceptor dinucleotide of the pre-mRNA target intron and U2AF2 binds the adjacent polypyrimidine tract [63]. *U2AF1* mutations are mutually exclusive heterozygous changes, consistently affecting S34 and Q157 residues within the first and second CCCH zinc fingers of the protein [5,6,18,24]. These mutations have been reported at frequencies of upto 8.7% in proliferative phenotypes, including MDS/MPN and high-risk MDS, associated with leukemic evolution and poor prognosis (Table 2). It is also identified in patients with isolated −20/20q- at a higher frequency [6,17]. *U2AF1* mutations have been suggested to cause both alteration/gain of function and loss of function [5,68]. *U2AF1* mutations have been found to be associated with *ASXL1* and *DNMT3A* genes relevant for epigenetic regulation which is especially intriguing as chromatin and histone modifications have been shown to play a significant role in pre-mRNA splicing [9,17]. Early studies using gene reporter assays identified that overexpression of mutant U2AF1 in HeLa cells resulted in dysfunctional splicing marked by frequent inclusion of premature termination codons and intron retention while another early study reported increased exon skipping in a minigene assay following mutant *U2AF1* expression in 293T cells, as well as increased cryptic splice site usage in the *FMR1* gene in MDS samples [5,6].

Several groups have reported splicing changes in cells expressing mutant U2AF1, including cell lines (HeLa, K562), primary human CD34+ cells, and MDS cells [5,6,18,69]. Ilagan *et al.* showed *U2AF1* mutations affecting the first and second zinc fingers alter the preferred 3′ splice site motif in patients in cell culture, and *in vitro* causing highly specific alterations in 3′ splice site recognition and giving rise to different alterations in splice site preference, and largely contributing to distinct downstream splicing events [18]. The differential splicing of tumor-associated genes affecting biological pathways such as cell cycle progression (*CEP164, EHMT1 and WAC*), RNA processing (*PTBP1, STRAP, PPWD1, PABPC4, and UPF3B*), DNA methylation (*DNMT3B*), X chromosome inactivation (*H2AFY*), the DNA damage response (*ATR, FANCA*), and apoptosis (*CASP8*) was identified in few studies suggesting their role in myeloid leukemogenesis [18,69]. However, the downstream targets of mutant U2AF1 identified in these studies are variable, perhaps due to differences in cell types, co-occurring mutations, and experimental methods used.

Shirai *et al.,* showed in vivo hematopoietic consequences of the most common *U2AF1* mutation using a doxycycline-inducible transgenic mouse model [24]. Mice expressing mutant U2AF1 (S34F) display altered hematopoiesis and changes in pre-mRNA splicing in hematopoietic progenitor cells by whole transcriptome analysis (RNA-seq). Integration with human RNA-seq datasets determined that common mutant U2AF1-induced splicing alterations are enriched in RNA processing genes, ribosomal genes, and recurrently mutated MDS and acute myeloid leukemia-associated gene (*BCOR*). An important finding of this study was that perturbations in spliceosome and RNA processing genes occur in three independent mutants *U2AF1* RNA-seq datasets. It raises the possibility that mutation of a spliceosome gene may result in auto regulatory changes in splicing machinery by altering isoform expression. Also, the identification of genes involved in ribosome function and translational processes are an interesting consequence of mutant U2AF1 expression, and these perturbations in the ribosome have been linked to MDS [70,71]. These findings support the hypothesis that mutant U2AF1 alters downstream gene isoform expression, thereby contributing to abnormal hematopoiesis in patients with MDS.

SRSF2

SRSF2 located on chromosome 17q25.2 is important for splice-site selection, spliceosome assembly, and both constitutive and alternative splicing. This protein belongs to the SR splicing regulatory factor family containing an RNA recognition motif (RRM) for binding RNA and an RS domain for binding other proteins. The RS domain is rich in serine and arginine residues and facilitates interaction between different SR splicing factors. These proteins bind to splicing regulatory sequence elements in pre-mRNA transcripts and to components of the spliceosome, and can either activate or repress splicing depending on the location of the pre-mRNA binding site [5,9,20,24]. The proteins' ability to activate splicing is regulated by phosphorylation and interactions with other splicing factor associated proteins. *SRSF2* mutations are stable during disease progression. Pro95 is a commonly identified mutation in *SRSF2*, affecting its binding with the target mRNA's (Table 2). *SRSF2* plays a role in preventing exon skipping, ensuring the accuracy of splicing and regulating alternative splicing. Recently, P95H missense mutation and P95 to R102 in-frame 8 amino-acid deletions have been shown to cause significant changes in alternative splicing of genes involved in cancer development and apoptosis. Also, gene knockout studies have shown that *SRSF2* is essential for the functional integrity of the hematopoietic system likely contributing to pathogenesis of MDS [20]. Although *SRSF2* mutations are particularly associated with CMML, they have been identified in different subtypes of MDS. These mutations not associated with a specific IPSS risk profile or cytogenetic aberration. Previously, they have been found to be associated with mutations in *RUNX1*, a gene coding for a transcription factor, as well as *IDH1*, a gene coding for an enzyme of the citric acid cycle [9].

Kim *et al.,* 2015 showed that the commonly occurring mutations in the spliceosomal gene *SRSF2* impair hematopoietic differentiation and promote myelodysplasia by altering *SRSF2's* preference for specific exonic splicing enhancer motifs [23]. *SRSF2* mutations change *SRSF2's* normal RNA-binding affinity and specificity *in vitro*, thereby altering the recognition of specific exonic splicing enhancer motifs to drive recurrent missplicing of key hematopoietic regulators. This includes *SRSF2* mutation-dependent splicing of *EZH2*, which triggers nonsense-mediated decay, which, in turn, results in impaired hematopoietic differentiation. In addition to the effects of mutant *SRSF2* on *EZH2* splicing and protein expression, a number of other genes of known importance in hematopoiesis and malignancy were also consistently differentially spliced in isogenic human cells, primary patient samples, and murine cells bearing mutant *SRSF2*. These include additional genes mutated in MDS (such as *BCOR*), genes with an importance in hematopoietic stem cell self-renewal (such as *IKAROS*), and genes critical for cell survival (such as *CASPASE 8*). The expression of several hematopoietic regulators was also altered in *SRSF2* P95H mutant cells, including up regulation of *GFI1, CEBPE,* and *HOXB2*; downregulation of *GATA1, GATA2* and *CDKN1A* [22].

Colla *et al.* used an inducible telomerase reverse transcriptase-estrogen receptor (TERTER) murine model to demonstrate that telomerase deficiency induces myelodysplasia by dysregulating expression of wild-type SRSF2 as well as other 3' spliceosome components. They found that telomerase dysfunction caused decreased expression of genes involved in 3'- mRNA splice site recognition resulting in abnormal splicing outcomes. 40% of the aberrant splicing events in TERTER/ER cells resulted in exon skipping, and 59.5% of aberrant splicing events resulted in exon retention. Aberrant splicing of *DNMT3A* as a result of telomerase dysfunction was identified in the study. These data provide a mechanistic basis for the enrichment of spliceosomal mutations in myelodysplasia and identify altered RNA recognition as an important driver of leukemogenesis.

ZRSR2

This gene is located on chromosome Xp22.2. associates with the U2AF heterodimer, which is required for the recognition of a functional 3′ splice site in pre-mRNA splicing, and therefore, could have a role in network interactions during spliceosome assembly. *ZRSR2* interacts with the 3′-splice site of U2- and U12-dependent pre-mRNAs and promotes different steps in U2- and U12-dependent intron splicing [8,59]. There are no mutational hotspots in *ZRSR2*, with alterations occurring throughout the protein, most of which are amino acid substitutions that affect the RNA binding domains or distal zinc finger motifs, although many nonsense or frameshift mutations that result in protein truncation have been identified [59]. The prognostic impact of mutations in this gene is not clear. Recently, shRNA-mediated knockdown of *ZRSR2* has shown impaired splicing of the U12-type introns and RNA-sequencing of MDS bone marrow reveals that loss of *ZRSR2* activity causes increased mis-splicing. These splicing defects involve retention of the U12-type introns, while splicing of the U2-type introns remain mostly unaffected. *ZRSR2*-deficient cells also exhibit reduced proliferation potential and distinct alterations in myeloid and erythroid differentiation *in vitro* [21].

Therapeutic Implications

The challenge for therapeutics in MDS lies in that MDS manifests clonal heterogeneity and complexity of the clonal architecture and also, it is often unknown as to which mutations are early initiating events and which are later events as a consequence only for a subclone. The spliceosome has become a target for a novel class of pre-clinical chemotherapeutics with a potential for application in cancer treatment through strategies focusing on correction of aberrant splicing resulting from large fraction of disease-causing mutations recently identified [72-74]. Several questions regarding the biological consequences of these novel recurrent mutations and their suitability as targets for novel therapy remain incompletely answered. Further studies elucidating the phenotype associated with these recurrent point mutations and the gene expression pathways affected may enhance the understanding of the repercussions of spliceosomal alterations on neoplastic transformation as well as pave way for therapeutic intervention through novel mechanisms. However, until we decipher the rationale behind the complex clonal heterogeneity in MDS, only through innovations in stem cell therapy can a breakthrough in MDS therapy be expected. The use of modified antisense oligos (ASOs) targeted to specific RNA sequences to redirect splicing and small molecules that act as inhibitor of spliceosomal function, are two of the most recent approaches though in-depth studies are essential to explore their potential in therapy and their discussion in detail is beyond the scope of this review [72-75].

Concluding Remarks and Future Perspective

The findings in recent papers advance toward understanding how alterations in the spliceosome alter the transcriptome, hematopoietic differentiation, and hematopoietic stem cell function in MDS. Recent data demonstrates the importance of RNA splicing at different stages of MDS develop¬ment and highlight an intriguing connection between DNA damage responses, RNA splicing and differentiation in the myeloid lineage. These data also provide a mechanistic link between a mutant spliceosomal protein, alterations in the splicing of key regulators, and impaired hematopoiesis. Further studies using these and other models of MDS will help to identify functionally important targets of mis-splicing as well as the genes that cooperate with spliceosome mutations in MDS. Extensive clinical studies will also contribute to decipher the respective contribution of mutations in splice genes, epigenetic regulators, signaling molecules, and their combinations; leading to a refined molecular classification of myeloid malignancies. A careful scrutiny of abnormal splicing mechanisms is required to thoroughly unravel unidentified splicing scenarios in MDS. Irrespective of whether changes in splicing have a direct causative role in MDS, or act as modifiers or susceptibility factors in the oncogenic process, the identification of splicing signatures is likely to provide important markers for diagnosis, prognosis and/or sensitivity to treatment. However, the most challenging goal for the future will be to integrate the different layers of gene-expression regulation altered in MDS and to acquire a systems-biology view of the many molecular mechanisms that contribute to the pathophysiology of this disease. Together, these studies suggest a novel pathway of importance to myeloid malignancies which may lead to novel therapeutic approaches for MDS patients.

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