

Alternative Splicing, a Driving Force in Acute Myeloid Leukaemia and How to Target it

Job Smink, BSc

Abstract

The majority of human genes are alternatively spliced, which is a process that allows cells to produce multiple protein with different functions from a single gene. However, aberrations in alternative splicing are frequently involved in tumour pathogenesis and progression of many cancers, including acute myeloid leukaemia. The influence is so immense that each of the cancer hallmarks is affected. Splicing dysfunctions are often linked to occurrence of driver mutations in splicing related genes such as splicing factors and the spliceosome. But epigenetic influences have recently come up as additional regulators, for example, hypoxia-induced alternative splicing as consequence of the tumour microenvironment. Abnormality in alternative splicing leads to the expression of different protein isoforms with contradicting functions and changes in total expression of the canonical protein. The alteration in the transcriptome landscape makes the cancer cells distinctively different from normal cells, presenting them as a perfect target for immunotherapies. In this review, we provide any overview of the various mechanisms involved in tumour driven alternative splicing and dive into its potential in prognostic use and anticancer treatment. We also discuss remaining open questions, therapy resistance, and pitfalls in alternative splicing targeted therapy.

Layman summary

A cell contains a genetic code called DNA that stores all the biological information. To produce proteins from DNA messenger RNA (mRNA) is needed. Initially, mRNA is transcribed as a primary transcript called pre-mRNA, which has multiple coding regions separated by non-coding regions. In order to translate a protein from pre-mRNA, the non-coding regions have to be spliced out first. Splicing is performed by a complex of different proteins called the spliceosome, which consists of a few core proteins and splicing factors. After additional RNA processing, the mRNA is ready for translation into a protein.

The human genome comprises roughly 20.000 to 25.000 protein-coding genes, however, together they encode over 90.000 different proteins. The diversity is caused by a process called alternative splicing (AS), where the non-coding and coding regions are spliced differently. This entitles that either coding regions are alternatively included/excluded or non-coding regions are retained, resulting in a mRNA isoform. These isoforms are then translated into different versions of the same protein (protein isoforms). Aberration in AS is often involved and critical for different cancers, such as acute myeloid leukaemia. This is a malignant disorder of the myeloid line of blood cells and is characterized by rapid expansion of abnormal blood cells and interferes with normal blood cell production. It is the most prevalent form of acute leukaemia, with around 20.000 cases and 12.000 deaths in the United States alone. Dysfunction of AS in cancer is often associated with mutations in splicing-regulating genes and can drive tumour pathogenesis and progression. Besides these mutations, recent research has shown that epigenetic influences play a role in the splicing aberrations. For example, tumour growth causes hypoxia due to lack of oxygen in the tumour microenvironment, which induces hypoxia dependent AS. The consequence of AS dysregulation is the generation of different isoforms of proteins exclusively expressed in cancer. But the expression of proteins that we see in a healthy cell is also altered and shifted to different isoforms not exclusive to cancer. The specific splicing pattern we see in cancer and the transcriptome landscape can form excellent targets for prognosis and anticancer treatment because of their distinction from healthy cells. In this review, we will explain how AS works, the exact effect it has on acute myeloid leukaemia, and its use in prognosis and therapy. We will also talk about the unanswered questions on AS in cancer, how treatment resistance is built up due to AS, and the current problems of targeting AS for potential anticancer treatment.

Introduction

Acute myeloid leukaemia (AML) is a malignant disorder of haemopoietic stem cells, characterized by clonal expansion of abnormally differentiated myeloid progenitor cells, due to chromosomal rearrangements and genetic abnormalities¹. AML is the most prevalent form of acute leukaemia with an estimated 19,940 new cases and 11,180 deaths in the United States of America alone per 2020². The overall survival rate is the lowest amongst leukaemia, making up for about 50% of deaths in 2020². Although AML was previously incurable, multiple therapies have been developed, resulting in long-term cures of 30% to 40% in young adults and approaching 70% in paediatric AML^{3,4}. However, this number decreases as age increase to numbers as low as 10-15% 5-year survival rates in patients older than 60 years^{4,5}. Rapid and accurate diagnosis of patients is needed to determine the optimal immunotherapy strategy, especially in rapidly fatal cases such as fast proliferation of malignant blasts accompanied by tumour lysis syndrome or disseminated intravascular coagulation⁶. Developing more effective drugs requires a better understanding of the mechanism underlying AML and its driving forces.

Mutations drive AML by transcriptional programmes, which are influenced by different mechanisms in the body. RNA processing has slowly come to light as an important participant in this process, which includes 5' capping, polyadenylation, and splicing. Initially, mRNA is transcribed as pre-mRNA containing both coding and non-coding regions. The non-coding regions are removed by a protein complex called the spliceosome during splicing and results in mature mRNA. Alternative splicing (AS) is a post-transcriptional process that allows expression of multiple RNA splice variants and protein isoforms with different functions from a single gene⁷. Dysregulation of AS contributes to tumorigenesis, drug resistance and can potentially be a driving force in AML⁸⁻¹¹. There are different forms of alteration in splicing. First, true alternative splicing involves dysregulation in the transcript bodies by a change in splice site selection, resulting in differential exon usage and intron retention. Second, alternative promoter usage causes isoform with altered 5' ends. Third, alternate polyadenylation site selection, involved in determination of the last exon. Currently, there is an increasing interest and recognition for AS in tumorigenesis and cancer progression, even to the point where some suggest that it is the 11th hallmark of cancer.

In conclusion, AS contributes additional layers of complexity to the leukaemia-driving transcriptome, which is an under-researched subject. This review will introduce the general mechanisms of splicing, outline the manipulation of AS as a driving force for AML, and highlight its potential role as a diagnostic marker and therapeutic target. Finally, remaining open question, drug resistance, and the pitfalls in therapeutic targeting of AS will be discussed.

RNA Splicing

The majority of eukaryotic genes are transcribed as pre-mRNA, consisting of both coding (exons) and non-coding (introns) regions. Before translation of mRNA, it is first processed by splicing, removing the introns. The end product will consist of exons and form the mature mRNA. Multiple introns, with a mean length of 1kb¹², can be present in the RNA, which is why correct splicing is very difficult and important for correct expression of proteins. An intron comprises three important conserved sites for splicing: the 5' splice site (SS) or donor site, branch point (BP), and 3'SS or acceptor site (Fig. 1)¹³. First, the 5'SS, located at the 5' end of the intron, has a conserved GURAGU sequence important for site recognition. Second, the branch point, typically located 15-30 nucleotides upstream of the 3'SS, contains an internal adenosine residue that is critical for splicing and is followed by a polypyrimidine track. Last, the 3'SS contains a conserved YAG sequence important for site recognition. Splicing is performed by a molecular machine, termed the spliceosome, in two distinct catalytic steps, branching and exon ligation. Although the biochemical two-step phosphoryl transfer mechanism behind splicing

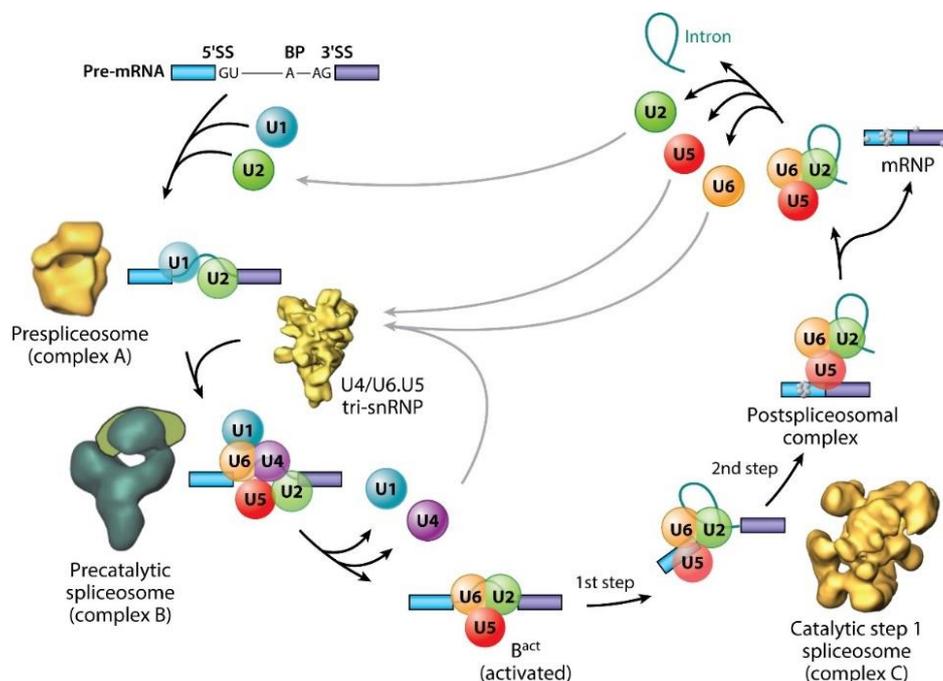


Fig. 1 RNA splicing by two consecutive transesterification reactions. Introns contain three important sites for splicing, the 5' splice site, branch point (in red), and 3' splice site. The mechanism of splicing happens in a two-step transesterification reaction, first between the 5' splice site and the branchpoint, resulting in a cleaved 5' exon and a lariat-intron 3'. Followed by transesterification between the 5' splice site and 3' splice site, leading to removal of the lariat-intron and ligation of two exons. ¹⁴

is simple, the biological process is not (Fig. 1). The first chemical reaction takes place at the 5'SS and BP. The phosphodiester bond at the 5'SS, also known as the splice donor, is attacked by the 2' hydroxyl of the adenosine residue of the BP. This results in cleavage at the 5'SS site and ligation of the 5' end of the intron to the adenosine residue, producing a lariat-intron 3' exon intermediate. During the second reaction, ligation of the 5' exon and 3' exon takes place. The now exposed 3' hydroxyl at the 5' exon attacks the phosphodiester group of the 3'SS, also known as the splice acceptor, ligating the exons together and releasing the lariat intron.

The Splicing Machinery. The spliceosome mainly consists of five small nuclear RNAs: U1; U2; U4/U6; U5; and their associated protein, collectively called small nuclear ribonucleoproteins (snRNPs) ¹⁵. Furthermore, it contains many more polypeptides not directly bound to the snRNPs ¹⁶. The spliceosome is a highly dynamic machine that constantly changes its composition and structure over the course of splicing (Fig. 2) ¹⁶. Splicing starts with binding of the U1 snRNP to the 5' site of an intron by base pairing of a conserved nucleotide sequence surrounding the 5'SS via the U1 small nuclear RNA, stabilized by members of the serine-arginine rich (SR) protein family and U1 snRNP proteins. The early phase of spliceosome formation also includes binding of the U2 snRNP to the 3'SS, after binding of multiple proteins to the BP. At this point, it is called the prespliceosome or complex A, which is followed by binding of the remaining three snRNPs U4/U6 and U5, resulting in the B complex. This complex is still catalytically inactive and requires compositional rearrangements to facilitate the first transesterification step. During activation, U1 and U4 are destabilized or released from the complex, resulting in an activated spliceosome capable of undergoing the first catalytic step in the splicing process. After additional rearrangements, the second and last catalytic step takes place, and the spliceosome is released from the RNA, now called mRNA ¹⁷.

The binding of U1 and U2 happens in a co-transcriptional fashion, in which RNA polymerase II mediates binding through its carboxy-terminal domain (CTD) ¹⁸. Specifically, the binding of U2 is also assisted by the splicing factor (SF)1, U2AF proteins and the SF3b complex (consisting of seven proteins). SF1 initially binds to the branch site, while U2AF binds to the polypyrimidine tract and the AG dinucleotide at the 3' SS, together forming the E complex ^{16,19}. The SF3b complex is recruited to the U2 snRNP and recognises the branch points adenosine and U2AF, facilitating an interaction between U2 and the BP sequence ²⁰. Mutations in these splicing factors are frequently associated with cancer, including AML ²¹.



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Fig. 2 Splicing by the spliceosome. The spliceosome is a highly dynamic machine involved in splicing of pre-mRNA. It consists of five different small nuclear RNAs U1, U2, U3, U4/6 and U5 and numerous additional proteins, together called nuclear ribonucleoproteins (snRNPs). The first step in splicing is binding of the U1 snRNP and U2 snRNP to the 5'SS and 3'SS via their small nuclear RNAs respectively, resulting in the prespliceosome. This complex recruits the tri-snRNP, consisting of the remaining three snRNPs, forming the precatalytic spliceosome. Subsequently to release of U1 and U4 the complex is activated and ready for the first catalytic step. After additional rearrangement complex C is formed and the second catalytic step can be performed. This results in the postsplisomal complex which releases from the RNA. ²²

Alternative Splicing. The human genome contains roughly between 20.000 and 25.000 protein-coding genes, which together encode over 90.000 different proteins ²³. This phenomenon is mainly caused by a process called alternative splicing, which takes place in 92-94% of all human genes ²⁴. It is a deviation of the aforementioned splicing process, in which exons and introns are differently retained, resulting in mRNA splicing isoforms. There are five main types of AS: Exon skipping (ES), intron retention (RI), Mutually exclusive exons (MXE), alternative 5' splice site (A5SS) and alternative 3' splice site (A3SS) (Fig 3A). In addition, there are several less obvious AS events, such as alternative first (also called alternative promoter) and last exons (also called alternative terminator). The selection of exons that are kept during splicing is defined by interactions between *cis*-acting elements and *trans*-acting factors (Fig 3B). *Cis*-acting elements include different regions surrounding the 5'SS, BP and 3'SS: exonic/intron splicing enhancers and exonic/intronic splicing silencers. Enhancers interact with positive *trans*-acting factors, such as SR proteins ²⁵, while silencers interact with negative factors, such as heterogeneous ribonucleoproteins (hnRNPs) ²⁶. *Trans*-acting factors mostly consist of splicing factors, which have two classic types of proteins: SR proteins and hnRNPs, besides this there are tissue specific splicing factors. Interaction of the SR proteins, for example SRSF2, with the enhancers causes inclusion of exons by interacting with the U1 and U2 snRNP, while the hnRNPs that bind the silencers inhibit binding between SR proteins and snRNP proteins ^{27,28}. Enhancers mostly act at canonical splicing sites, while silencers are more involved in alternative splicing sites ²⁹. In summary, the *cis*-regulatory elements and *trans*-acting proteins are important for regulation of splicing and AS.

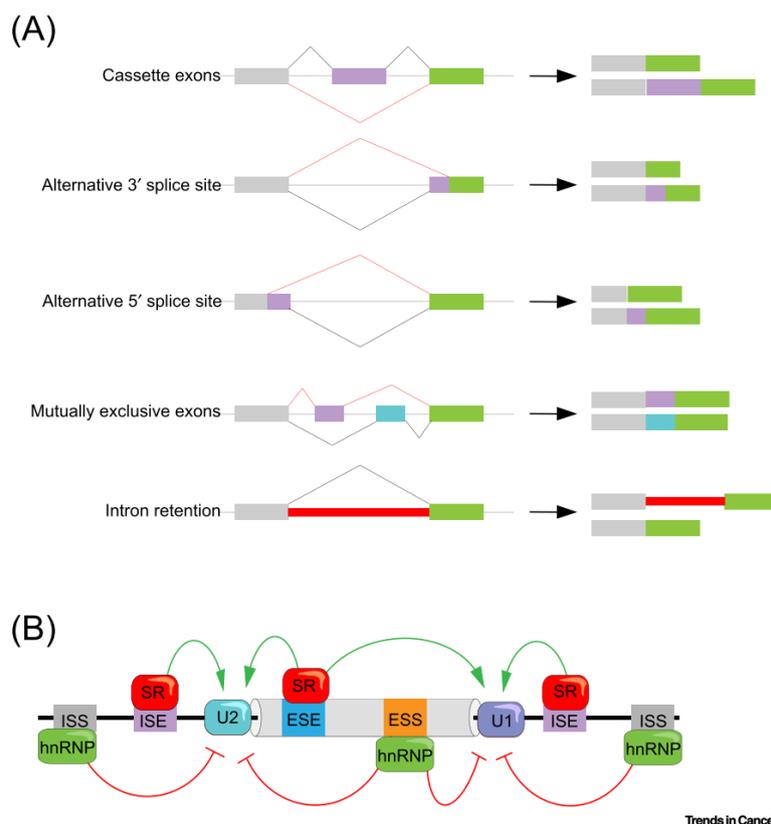


Fig. 3 The mechanism of alternative splicing. (A) There are five main types of alternative splicing. Cassette exons or exon skipping, the most common event in which an exon is skipped. Alternative 3' or 5' splice site uses an alternative splicing acceptor or donor respectively, leading to either partial exclusion of an exon or inclusion of an intron. Mutually exclusive exons is a form of AS where one of two exons is spliced out if the other is retained. Last, intron retention is the inclusion of an intron. The red and black lines represent different splicing events; the grey line represents introns; the red box represents a retained intron; the grey, purple, green, and blue boxes represent different exons. (B) The selection of exons that are included in mature mRNA is decided by interactions between cis-acting elements in the RNA and trans-acting factors. There are four main types of cis-acting RNA sequences, exonic/intron splicing enhancers and exonic/intronic splicing silencers, which are involved in the recruitment of RNA-binding protein splicing factors. Serine/arginine-rich proteins are typically recruited to enhancers and promote exon inclusion via interactions with U1 and U2, while heterogeneous nuclear ribonucleoproteins bind to silencers to inhibit this interaction and repress the splice site.³⁰

As mentioned before, splicing can occur co-transcriptional, as it was shown that snRNPs are recruited to active genes during transcription³¹. One part of the transcription machinery is the RNA polymerase II, which coordinates transcription and RNA processing with its CTD³². It also affects RNA splicing, where it actively promotes splicing when its CTD domain is unmodified. On the contrary, it inhibits splicing when the CTD domain is hyperphosphorylated by influencing early steps in the assembly of the spliceosome and recruitment of splicing factors^{33,34}. The elongation rate of RNA polymerase II, which affects the speed at which splice sites and regulatory sequences become available, is also involved in the alternative splicing process^{35,36}. At a slower elongation speed, there is an increased inclusion rate of cassette exons. At an increased speed, for example caused by elongation-promoting transcription factors, there is an elevated amount of ES³⁵. In addition, epigenetic mechanisms, such as DNA methylation and histone modification, play a role in co-transcriptional RNA processing through control of elongation speed and splicing factor recruitment³⁷. They affect AS by alteration in the chromatin structure, for example inducing an open chromatin state that facilitates RNA polymerase II elongation^{35,38,39}. DNA methylation can increase elongation speeds, by inhibiting binding of proteins involved in RNA polymerase II pausing, causing exon exclusion⁴⁰. However, epigenetic regulation also plays a role in AS in a non-co-transcriptional manner by microRNAs and long noncoding RNAs⁴¹.

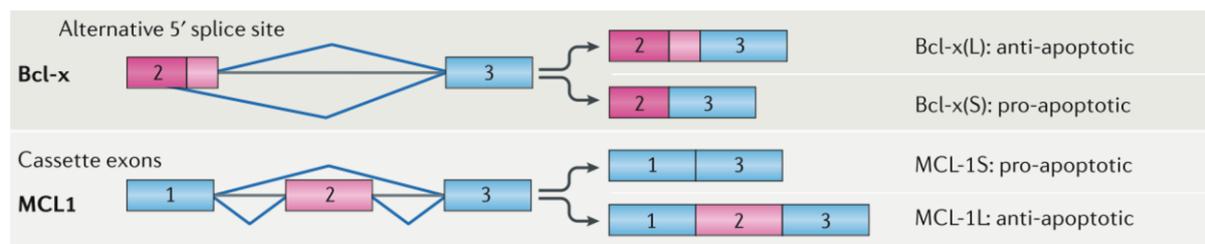


Fig. 4 Alternative splicing of BCL-2 genes. The BCL-2 family genes, *Bcl-X* and *MCL-1* both express two contradicting isoforms. *Bcl-X* expresses the anti-apoptotic isoform *Bcl-X_L* and the pro-apoptotic isoform *Bcl-X_S*, due to A5SS. *MCL-1* expresses the anti-apoptotic isoform *MCL-1L* and the pro-apoptotic isoform *MCL-1S*, due to ES of the second exon. ¹⁴

The outcomes of AS are different isoforms of proteins, which have diverse and even antagonistic functions ⁴². For example, the *BCL-2* family is a group of genes that play a role in apoptosis as both inhibitors and inducers of cell death, mainly by regulating and mediating the intrinsic apoptosis pathway ⁴³. All proteins in the family contain BCL-2 homology (BH) domains, which determines their interaction with each other and indicated whether they are anti-apoptotic or pro-apoptotic ^{44,45}. The *Bcl-2*-like 1 gene (*BCL2L1* or *Bcl-X*) encodes two well-known isoforms, *Bcl-X_L* and *Bcl-X_S*, with contradicting anti-apoptotic and pro-apoptotic functions due to inclusion or exclusion of the BH1 and BH2 domains respectively ⁴⁶. This difference is caused by AS, specifically, a different 5'SS in the first exon. For *Bcl-X_L* the distal end of the 5'SS is used, while *Bcl-X_S* uses the proximal end (Fig.4). Another example is the myeloid leukaemia sequence 1 (*MCL-1*) gene, which also has two major isoforms, *MCL-1L* and *MCL-1S*, with anti-apoptotic and pro-apoptotic functions respectively ⁴⁷. *MCL-1L* mRNA consist of three exons and translated it contains a BH1, BH2, BH3, and transmembrane domain, while *MCL-1S* consists of just the BH3 domain, because of ES of the second exon (Fig. 4) ^{47,48}. In addition, one new pro-apoptotic isoform was discovered, called *MCL-1SE*, which comprises the BH1, BH2, BH3, and transmembrane domain ⁴⁹. On the other hand, mRNA isoforms can also be degraded through nonsense mediated decay, contributing another layer to post-transcriptional gene expression. One third of AS events contain a premature termination codons, leading to degradation of transcripts with nonsense mediated decay *cis*-elements in their 3' untranslated region ⁵⁰.

Alternative Promoter Usage. At least 52% of the human genes contain more than one promoter, giving additional depth to the complexity of gene structure ⁵¹. Use of alternative promoters is associated with different types of cancer, including AML ^{8,52}. Promoters are DNA sequences, located at the transcriptional start site of both protein-coding and non-coding genes. They are involved in the assembly of the transcription machinery and initiation of transcription. The transcriptional start site (TSS) of a gene is imbedded in the promoter and thus dictates where transcription starts ⁵³. Genes that contain alternative promoters are more likely to generate AS transcripts, with a positive relationship between the amount of alternative promoters and the number of AS transcripts ^{54,55}. This is further supported by the fact that studies found that usage of different promoters results in changes in alternatively spliced exon inclusion and the involvement of promoter architecture in AS ⁵⁶⁻⁵⁸. The first and most obvious way alternative promoters affect splicing is by selection of a different first exon ⁵¹. Transcription will start at the alternative promoter, which might exclude a certain amount of exons which are upstream of the alternative start site. Second, the speed of transcription by RNA polymerase II, which plays a role in splicing as described above, is another mechanism. Using alternative promoters can alter the elongation speed by promoter specific transcriptional activators, resulting in different AS transcripts ⁵⁹. Last, promoters can also recruit factors with dual functions in transcription and splicing

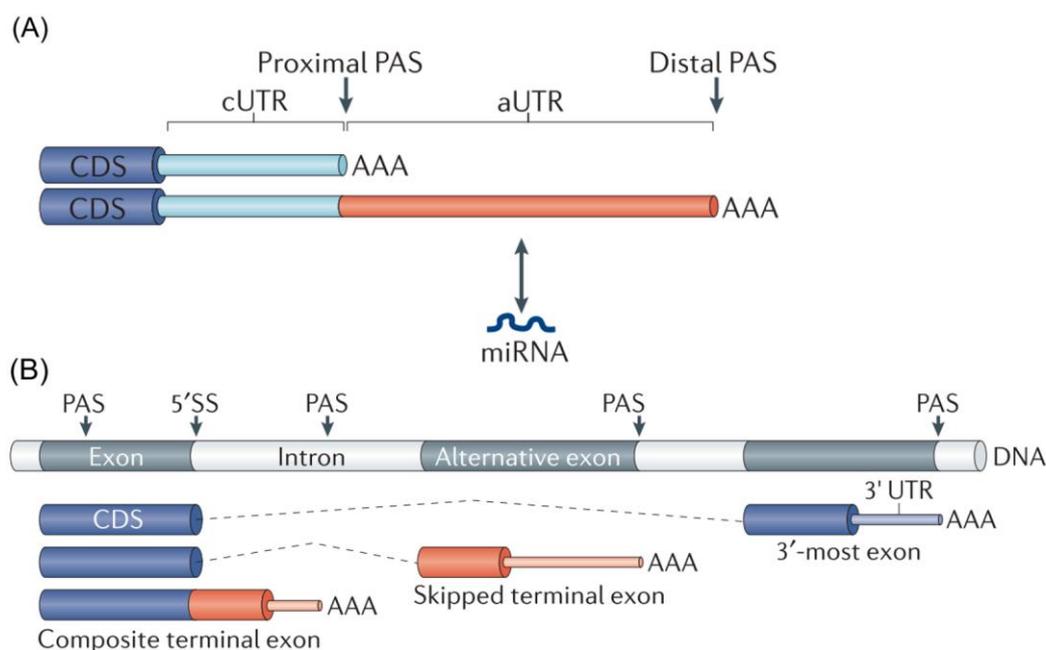


Fig. 5 Alternative polyadenylation. Alternative polyadenylation is caused by polyA signals (PAS) located at alternate locations in the mRNA. There are two different locations for alternative PASs: in the 3' untranslated region (UTR) or upstream of the terminal exon. (A) PASs in the 3' UTR generate two different mRNA isoforms with a different length UTR. This results in the same protein but can alter, for example, protein expression via microRNA (miRNA), normally involved in gene expression via mRNA silencing. (B) PASs located upstream of the terminal exon results in mRNA isoforms with different terminal exons. The two main types are skipped terminal exon, in which a PAS is used that is located in a non-canonical exon, and composite terminal exon in which a part of an intron is included. ⁷¹

⁶⁰. For example, the thermogenic coactivator PGC-1 interacts with RNA polymerase II and the splicing factor SRp40, but this protein is only recruited to the promoter if it has a DR-1 domain ⁶¹. If an alternative promoter missing a DR-1 domain is selected, the lack of SRp40 recruitment could result in an alteration of the mRNA.

Alternative Polyadenylation. Polyadenylation is required for maturation of mRNA and is involved in determination of the terminal exon ^{62,63}. It consists of two steps: cleavage of the pre-mRNA at the 3'-end and polymerization of a poly(A) tail. As the first and last exon are flanked by only one splicing site, they are recognized different from internal exons. Identification of the terminal exon is mediated by a complex of proteins that consist of different recognition, cleavage, and polyadenylation factors that act upon a polyA signal (PAS). This is usually a hexanucleotide consensus element, AAUAAA, followed by a U or G/U rich sequence ⁶⁴. However, additional downstream elements and a terminal splice acceptor site are needed to select the correct AAUAAA element ⁶⁵. Mutations in either the PAS sequence of terminal splice acceptor disrupt splicing and 3' end formation ^{65,66}. In addition, splicing related proteins, such as SF3B1, U2AF, U2, and U1A, contribute in assisting recognition of the terminal exon ⁶⁷⁻⁷⁰. Furthermore, terminal exon definition occurs co-transcriptionally ⁶⁶.

Alternative polyadenylation (APA) is a process in which an altered PAS is used (Fig. 5). Just like AS and alternative promoter usage, APA allows organism to enhance their transcriptome diversity, complexity, and regulation of gene expression ⁷¹. Alternative PASs can be present in two different locations, either upstream of the last exon, resulting in a different protein isoforms, or in the 3' untranslated region, resulting in shorter or longer mRNA isoform, producing the same protein for example at a different expression level ^{72,73}. 3' untranslated region APA is involved in different types of cancer, including AML ^{74,75}, as miRNAs in this region are involved in regulation of gene expression by mRNA silencing (Fig. 5A) ⁷⁶. The upstream exon APA leads to alternative terminal exons, which depend on the splicing relative to the location of the PAS. There are two main types, skipping of a

terminal exon, due to splicing or composite terminal exons, generated by extension of an internal exon with an adjacent intron, due to inhibition of the 5'SS (Fig. 5B) ⁷¹. Another, less frequent type is PASs located in an internal exon, resulting in the lack of an in-frame stop codon and degradation via the non-stop decay pathway ⁷⁷.

Coupling between splicing and APA is limited to defining the last exon ⁷⁸. On the contrary, in case of intronic PASs, they are more likely to compete. Tian *et al.* found that weak 5'SS and large introns are determining factors for undergoing polyadenylation at internal PASs ⁷⁹. Although, studies showed that splicing and APA are connected in removal of the last intron, data did not suggest involvement of a coupled mechanism with AS ⁷⁸.

The Effect of Alternative Splicing in AML

Even though AS benefits humans by expanding the transcriptome diversity from a limited number of genes, it can have a negative effect and even play a role in cancer. The role of aberrant splicing and its contribution to tumorigenesis, cancer progression and resistance to therapy has increased over the past years. The importance of AS is ever increasing as it is essential for understanding every hallmark of cancer and is suggested to be a new cancer hallmark ^{14,80,81}. Currently, there are 10 different hallmarks of cancer, each affected by aberrant splicing in their own way. For AML, roughly 86% patients have two or more driver mutations, with 18% of patients having a driver mutations in genes encoding chromatin or RNA splicing regulators ⁸².

One important hallmark that is affected by AS is cell death resistance. There are a number of different apoptosis-regulating genes that have isoforms with contradicting functions, like *Bcl-X* and *MCL-1* described above. Multiple types of cancer utilize this to escape the intrinsic apoptosis pathway and gain resistance against certain types of cancer treatment ⁸³. *Bcl-X* for example produces the isoforms *Bcl-X_L* and *Bcl-X_S*, associated with anti-apoptotic and pro-apoptotic functions, respectively. In many cancers, the transcription of the *Bcl-X_L* is upregulated and is even associated with chemotherapy resistance and cancer cell stemness ⁸⁴. *MCL-1* has one anti-apoptotic and two pro-apoptotic isoforms, *MCL-1L*, *MCL-1S*, and *MCL-1SE*. The anti-apoptotic function of the *MCL-1* has been suggested in tumour survival ⁸⁵⁻⁸⁷. For AML specifically, it is even suggested *MCL-1* is critical for survival, pathogenesis and expansion of human and mice AML cells ^{88,89}. Sustaining proliferation signalling is another fundamental trait of cancer cells to uphold chronic proliferation. AS plays a role in this process, as shown by increased production of CD44 variant 6, an isoform that promotes cell proliferation. Overexpression of CD44 variant 6 is linked to tumorigenesis and cancer progression ⁹⁰.

Until recently, a lot of research has focused on the expression and mutations of genes in cancer as a mode of malignant dysregulation of the transcriptome. Studies about AS focused on the different mutations in splicing factors or splicing related genes. However, more researchers started to include genome wide transcriptomic analysis by, for example, total RNA sequencing (RNA-seq) data, which are affected by post-transcriptional RNA processing like AS and degradation. Detection of AS events is often performed using RNA-seq in combination with bioinformatics analysis of the data ⁹¹. Advancements in the depth of sequencing have increased the amount of sequencing data available in public databases. These provide a resource for RNA-seq analysis using different methods. In most cases, more than one analysis is used on a data set and, if possible, multiple databases are used. Unfortunately, there are no universal and perfectly reliable bioinformatic tool for identification of AS events using RNA-seq data, thus some studies use several algorithms ⁸. Additionally, methods that only use quantitative data from either mRNA or proteins neglects the production of inactive protein isoforms, excluding genes which could be involved in the disease progression. Overall, in the different types of cancer, AS events happen in many different genes. One study found 27,833 AS events in 8337 different genes, with ES being the predominant type of splicing in AML ¹⁰.

Table 1. Frequent splicing factor mutations in acute myeloid leukaemia.

Cancer type	Splicing factor	Prevalence (%)
AML with myelodysplastic features ⁹⁴	U2AF35	9,7
	SRSF2	6,5
	SF3B1	4,8
	U2AF1	3
	ZRSR2	1,6
	SF3A1	1,6
	PRPF40B	1,6
De novo AML ^{21,115}	SRSF2	10,2
	SF3B1	2,8-3
	U2AF1	2,1-4
	U2AF35	1,3
	ZRSR2	1
	HNRNPK	1
	SF3A1	0,7
	PRPF40B	0,7
	SF3A1	0,1
	U2AF2	0,1
	SF1	0,1

Alteration in the Core Spliceosome Proteins and Splicing Factors. Mutations in components of the spliceosome occur in nearly 50% of secondary AML cases evolved from myelodysplastic syndrome (MDS)⁹²⁻⁹⁴ and in around 7% of de novo AML (Table 1)^{92,94}. In AML, there are three frequently occurring mutated genes, *SRSF2* (6-10%), *U2AF1* (3-4%), and *SF3B1* (3-5%); which are all involved in the recognition of the 3'SS, they are mutually exclusive mutations (tested in MDS) and mainly heterozygous^{17,21,25,95,96}. Although they share this involvement, they have distinct differential isoform expression profiles⁹⁷.

SF3B1 is the most commonly mutated splicing factor in cancer and was first detected in DMS patients^{95,98-100}. In AML, it is mostly accompanied by other recurrent mutations, like *PTK2*, a protein involved in AML^{97,101}. Mutations in *SF3B1* alter the function of snRNP U2 by promoting alternative BP usage, and cryptic 3'SS selection, most often resulting in framing errors^{98,99}. The most common mutation is located at K700, and to a lesser extent, K666, H662 and E622, which cause missense mutations in the HEAT domain⁹⁴. This domain functions as the central scaffold within the SF3b complex and interacts extensively with the other components and clamps the U2/BP complex together²⁰. One example of defect splicing is the protein ABCB7. Due to alternative 3'SS usage, a premature termination codon is introduced, decreasing the protein levels and causes an iron deposition, a feature associated with malignancy in MDS^{102,103}. In addition, a multitude of different proteins are affected by these alterations. The consequence mostly consists of downregulation of protein expression because of nonsense mediated decay of the aberrantly spliced mRNAs (50%)⁹⁹. AS of two other genes mentioned before, *Bcl-X* and *MCL-1*, are influenced by unaltered *SF3B1*. Within the second exon of *Bcl-X* a *cis*-element is located that binds *SF3B1*¹⁰⁴. Downregulation of *SF3B1* increased the expression of *Bcl-X_S* and decreased the expression of *Bcl-X_L*¹⁰⁵. Furthermore, downregulation of *SF3B1* upregulated the expression of pro-apoptotic version, *MCL-1S*¹⁰⁶. However, it was shown that *MCL-1*, but not *Bcl-X*, was sensitive to targeting of *SF3B1* with small-molecule splicing modulators in tumour cells¹⁰⁷. A potential explanation could be that *Bcl-X* isoforms are

determined by alteration in the 5'SS, while mutations in *SF3B1* mostly affect cryptic 3'SS selection, additionally other splicing factors also modulate the AS of *Bcl-X*^{46,106}.

Even though *SF3B1* mutations are present in cancer, some studies found that they are associated with better overall survival of MDS patients and lower chance of disease progression to AML^{108–110}. However, contradicting studies showed no significant effect of the mutations on clinical outcomes^{111,112}. One study even discovered that the specific mutation K666N is associated with high-risk MDS and AML¹¹³. Interestingly, K666N has a different splicing pattern from other *SF3B1* mutations, including mutations on the same hotspot, which adds to the story of studies that did not identify any difference or even a better overall survival rate of MDS patients. Most studies have either grouped all the mutation variants together or taken a select number of hotspots, which would exclude the results from less common mutations such as K666N when compared to, for example, K700. An explanation for this difference could be that the K666 hotspot is involved in the tertiary structure of *SF3B1*, while K700 is not¹¹⁴. Another explanation could be that favourable prognosis seen in MDS patients with *SF3B1* mutations is due to association with the favourable prognosis in MDS patients with ring sideroblasts in general¹¹⁰.

Environmentally Driven Alternative Splicing. During tumour progression, tumour cells experience hypoxia due to the inadequate vascularity as a consequence of overgrowing. Tumour-hypoxia plays a role in regulation of the different hallmarks by adaptation to low-oxygen conditions, mainly caused by hypoxia-inducible factors (HIFs)¹¹⁶. Different mechanisms can cause tumour-hypoxia: perfusion-hypoxia from decreased blood flow, diffusion-hypoxia caused by an increased O₂ diffusion distance, and anaemic hypoxia because of a decreased oxygen transport capacity¹¹⁷. A decrease in available oxygen causes accumulation of HIF transcription factors resulting in HIF-target gene expression, but more importantly AS in HIF-targeted or non-targeted genes^{118,119}. Hypoxia directly and indirectly influences AS in several ways. It has a direct effect on the splicing machinery by de-regulation of spliceosome assembly; alteration of splicing factor activity, expression, intracellular localization, and interaction; activation and expression of SR protein kinases; altering the expression of miRNAs and induces the expression of master long noncoding RNA regulators involved in splicing⁸⁰. The indirect effect is caused by promotion of cytosolic stress granules, causing an accumulation of splicing factors, splice regulating proteins and miRNAs¹²⁰. Thus, environmental changes, for instance, hypoxia, influences AS through alterations in splicing associated processes and epigenetics. Hypoxia-induced AS is suggested as the 11th hallmark of cancer and has shown to affect all 10 hallmarks of cancer, demonstrating its immense effect and driving force in tumour pathogenesis and progression⁸⁰. The main contributor is the sheer number of protein isoforms involved in tumour progression, produced as a result of hypoxia. However, while information on the effect of hypoxia on AML is available, the role of hypoxia-induced AS is lacking^{121–123}. The exact mechanism used for AS in AML remains elusive, but some suggestions have been made, as mentioned before. One could argue the potential role of hypoxia-induced AS. For example, tropomyosin receptor kinase A (*TrkA*), a high-affinity receptor of neural growth factor, has a hypoxia-induced oncogenic alternatively spliced isoform called *TrkAIII* that lacks exons 6,7, and 9, resulting in a constitutively active protein¹²⁴. AML cells containing the *RUNX1/RUNX1T1* fusion gene express this specific isoform, indicating a connection^{125,126}. Furthermore, one study found a lack of correlation between splicing patterns and mutations/expression of the same gene, but a strong co-correlation of several splicing variations across AML populations¹²⁷. These results suggest that there might be common upstream *trans*-acting factors, which could, for example, include hypoxia-induced AS. More research on the potential role in AML is needed and would give an additional aspect of the mechanism of AS.

The Influence of RUNX1/RUNX1T1 on Alternative Splicing. One of the most common cytogenetic abnormalities in patients with AML is the chromosomal rearrangement t(8;21)(q22;q22), occurring in roughly 4-7% of adults (typically young adults)^{82,128} and an even higher rate in children at 17%¹²⁹. The chromosomal translocation causes the generation of the RUNX1/RUNX1T1 fusion protein. *RUNX1* is a transcription factor essential for normal haematopoiesis¹³⁰, while *RUNX1T1* is a transcriptional co-repressor^{131,132}. The fusion protein consists of the conserved runt homology domain from *RUNX1* and almost the whole *RUNX1T1* gene, resulting in repression of *RUNX1* target genes¹³³. Besides this, a recent study showed its effect on the production of RNA isoforms⁸. They proposed two different mechanisms: direct control of the TSS in target genes and direct/indirect control of expression of genes involved in splicing. However, this study identified multiple different splicing related processes involved. First, RUNX1/RUNX1T1 affects the local chromatin status at its binding sites, altering the distribution of RNA pol II and transcription rates^{8,134,135}. As discussed before, transcription kinetics affect AS. Second, even though most splicing events are co-transcriptional, in some cases intron removal can be delayed until release of the pre-mRNA^{136,137}. They found that delayed splicing regulation is affected by RUNX1/RUNX1T1, a process involved in regulated AS^{8,136}. Third, RUNX1/RUNX1T1 causes RNA isoform generation by controlling alternative promoter usage. Bindings sites of the fusion proteins are more often located in promoter regions and intronic regions, implicating a direct effect on the transcription^{134,138,139}. As mentioned earlier, the local chromatin status is influenced, subsequently the accessibility of promoters is altered, including alternative ones. This leads to transcriptional repression or, less frequently, activation of alternative TSSs⁸. Last, AS is controlled by differential expression of splicing factors and mRNA surveillance genes by RUNX1/RUNX1T1 directly binding to the genes or indirect by transcription factor expression⁸. One example is the regulation of AS in PTK2B via RBF2.

When we compare the methods of AS manipulation of *RUNX1/RUNX1T1* with, for example, hypoxia-induced AS, we can see resemblances in the mechanisms. Both affect AS via a form of epigenetic regulation, alterations in the expression level of splicing related genes, and the produced isoforms. However, an apparent difference is the direct influence of RUNX1/RUNX1T1 on the chromatin status and the use of alternative TSSs as a result. Although, histone modification and promoter methylation are affected by hypoxia^{140,141}. Research on the role of alternative TSSs in AS of AML is lacking, more information could potentially help with the development of therapeutics.

Cancer Related Protein and mRNA Isoforms. As a consequence of aberrant splicing, different mRNA and protein isoforms are produced with distinct functions or even expression levels. As mentioned before, mutations in, for example, splicing factors, but also the fusion protein RUNX1/RUNX1T1, can alter the production of certain isoforms. Most of the effects we see of AS on cancer are a subsequence of the expression of the different isoforms related to carcinogenesis in one way or another. Tyrosine kinase 3 (*FLT3*) is one of the most common genetically altered genes in AML and is over expressed in the majority of AML blasts^{82,142,143}. The FLT3 pathway is constitutively activated in 30% of AML cases, due to internal tandem duplicates and point mutations in the tyrosine kinase domain²¹. *FLT3* is mis-spliced in AML, as result from skipping of ≥ 1 exon or cryptic splicing sites¹⁴⁴. Although it is not known how this contributes to pathogenesis of AML, it is expected to play a role. In addition, there is no research on the correlation between specific mutations in, for example, splicing factors and the mis-splicing. As we have seen that cryptic splicing is a known effect of mutations in *SF3B1*, it would be interesting to uncover a potential link^{98,99}. Important to note is that, as has been shown before, research on specific protein mutations has to be performed with separation between mutations within the same gene.

Non-oncogenic Dependencies of Splicing Factors. The majority of cancers depend on genetic alterations in key oncogenes and repression of key tumour suppressors¹⁴⁵. The state of dependency on these oncogenes is called oncogene addiction and tumour suppressor gene hypersensitivity^{146,147}. However, cancer also has an increased dependency on regular cellular functions that are not oncogenic themselves, this is called non-oncogene addiction¹⁴⁸. Although non-oncogenes are required for maintenance, unlike oncogenes, they rarely undergo significant genomic alterations¹⁴⁹. One emerging driver of non-oncogenic addiction is RNA-binding protein (RBP) splicing factors. These are a type of protein that interacts with RNA via their RNA-binding domain and affect splicing. Non-oncogene RBPs are differentially expressed in cancer cells when compared to healthy cell types and tissues, leading to dysregulation of splicing¹⁵⁰. The role of different RBPs as non-oncogene has been shown in AML, including the *RBM39* gene^{151–153}. *RBM39* is involved in recognition of the 3'SS by interactions with U2AF1. Recent research has found that this protein is required for maintenance of AML through mis-splicing of *HOXA9* target genes, an important transcriptional network in AML¹⁵¹. Loss of *RBM39* results in intron retention of multiple core splicing factors in cell with spliceosome mutations. In addition, the role of non-oncogenic RBPs has also been implicated in different types of cancer¹⁵⁴. Altogether, this indicates an important role of hijacking the expression of RBP splicing factors for tumour maintenance.

The Clinical Relevance of Tumour Driven Alternative Splicing

The overall survival rate of paediatric AML patients is as high as 68%, but this rate decreases as age increases to 30-40% in young adults and as low as 10% in people above 60^{2,4}. This calls for an urgent improvement of both risk stratification of AML, to prolong survival, and therapies used to combat it. The misregulation of AS is involved in tumorigenesis of multiple malignancies and creates specific splicing patterns, implicating its potential role in prognosis and targeting in therapy. In addition, at this moment a broad range of different anticancer treatments are available, however malignant cells hijack the splicing machinery to gain drug resistance by AS of genes involved in drug metabolism, cell death and cellular proliferation¹⁵⁵. Interestingly, the involvement of AS in drug resistance makes it a potent candidate for combination therapies.

Alternative Splicing as a Prognostic Marker. Prognostic markers are an objectively measurable biological characteristic that can be used to evaluate treatment stratification and predict a patient's risk of a future outcome. This includes, for example, specific gene variants and expression levels of particular proteins. Recent studies showed that AS can be a potential prognostic marker for risk stratification and classification^{9,10,156–158}. AS events happen in several genes in AML, with two studies reporting approximal 30.000 mRNA splicing events in 8.500 genes^{10,156}, while another reported up to 100.000 events¹⁵⁷. The first two studies found around 3.000 survival related AS events in 2.000 genes. Approximately 900 events with a hazard ratio greater than one, and 1.800 with a hazard ratio below one. The latter study identified 7.033 significant prognostic AS events in 3.861 host genes. Interestingly, up to six different AS events can occur on the same gene¹⁰. For example, in the *PILRB* gene four splicing events (A3SS, A5SS, MXE and SE) are associated with overall survival of AML patients¹⁵⁷.

Most studies used a similar method to establish the prognostic value of AS in AML. First, the top prognostic AS events, with the highest risk score, were identified. Second, a multivariate Cox regression analysis is used to comprehensively evaluate the prognostic use of the selected events. Chen *et al.* established an alternative donor site model that identifies the favourable and poor outcome of patients with AML and serve as a moderate and independent prognostic indicator¹⁵⁶. Another study, by Zhang *et al.*, used independent prognostic AS events of different types for their

prognostic predictors models and showed that A3SS had the best predictive performance¹⁰. While Jin *et al.* found that A3SS, A5SS, MXE, RI and ES can distinguish high-risk AML groups, with a low survival time, from low-risk groups, with a relatively prolonged survival time¹⁵⁷. However, they state that a robust prognostic predictor should avoid the bias of using specific AS event types and include as many types as possible. This resulted in the AS-15 signature prognostic model, consisting of a mix of MXE, ES and A5SS events. The model is shown to be a reliable independent prognosticator in adult and paediatric AML, and outperforms gene expression-based scoring. They explain that the performance difference could be due to not only the relation between AS and the expression of genes, but also its reflection on upstream regulation. In addition, the AS-15 signature is able to improve the European LeukemiaNet risk classification of AML, the current gold standard¹⁵⁹. In conclusion, the studies above demonstrate the potential AS events have as a prognostic marker for AML, with different possible splicing signatures. More importantly, it might be an essential addition for prognosis in diseases with high heterogeneity, like AML with mutations in chromatin and RNA-splicing regulators, as prediction based on cytogenetic and mutational analyses are limited¹⁶⁰.

Alternative Splicing Based Therapy. As more research is done on the role of AS in cancer, its potential and interest as a therapeutic target has increased. The different mechanics involved in AS provide a variety of different targets. For example, the core spliceosome proteins and splicing factors are often mutated in cancer, making them an ideal target. In addition, the different protein isoforms expressed due to cancer specific AS are frequently involved in tumorigenesis and thus a potential target. There are several therapies which take advantage of AS, which include small-molecule splicing modulators and splicing-modifying antisense oligonucleotides.

Small-molecule splicing modulators have been tested in the past and several are being tested in clinical trials at the moment. Currently, the most common strategy is small molecules that directly bind SF3B1. The first SF3B1 modulators were isolated from *Pseudomonas sp.* (FR901464) and *Streptomyces* (pladienolide B and GEX1). From these bacterial fermentation products, different synthetic derivatives were made. These compounds bind to SF3B1 on its HEAT repeat domain in the branch point adenosine-binding pocket, stalling the protein in an open conformation and thus preventing the recognition of the branch site^{161,162}. Although the mechanism of the inhibitors is known, how they selectively lead to antitumour effects instead of general splicing inhibition is unknown. Older compounds have been tested in clinical phase trials (e.g., E7107), but due to adverse effects, like loss of vision, were terminated early^{163,164}. However, novel derivatives of pladienolide B, H3B-8800 and 17S-FD-895 showed potent antitumor effect with low cytotoxicity in preclinical studies. H3B-8800 is undergoing clinical trials in various leukaemia, including AML (NCT02841540)^{165,166}. A recent study reported that splicing modulation of the *BCL2* family is an essential mechanism in cytotoxicity induced by E7107¹⁰⁷. Although proteins such as MCL-1, BCL2L2 and BCL2A1 were sensitive to E7107 splicing modulations, Bcl-X was not. They demonstrated that a combination of E7107 with Bcl-X_L inhibitors greatly enhances cytotoxicity¹⁰⁷. This highlights the potential of combination therapies using SF3B1 splicing modulators. But more importantly, it provides a mechanism-based therapeutic strategy for targeting of small-molecule splicing modulators.

Besides SF3B1 targeting molecules, there are proteolysis targeting chimeras (e.g. indisulam and E7820) that promote recruitment of RBM39 to the E3 ligase substrate receptor DCAF15, causing ubiquitylation and thus degradation of the protein^{167,168}. Recently, it was shown that both indisulam and E7820 recruit RBM39 (and closely related protein RBM23) via its RRM2 domain to DCAF15^{169,170}. Wang *et al.* found that RBM39 is crucial for AML maintenance by mis splicing *HOXA9* target genes¹⁵¹. Genetic or pharmacologic targeting of the protein caused repressed cassette exon inclusion and promoted RI in genes preferentially required in AML and showed antileukemic effects. Interestingly, indisulam has been tested in clinical phase II in a variety of tumours. The combination of indisulam

with chemotherapy only yielded a low response rate in non-small cell lung cancer patients¹⁷¹. However, relapsed and/or refractory AML or high-risk MDS patients had a 1-year overall survival of 51% compared to 8% in non-responders, highlighting the potential benefit¹⁷². The general concept of proteolysis targeting chimeras is very interesting and not limited to one specific RBM, more research on other derivatives of current compounds could prove useful.

Another relatively new therapeutic approach to splicing is antisense oligonucleotides (ASO), specifically splice-modulating oligonucleotides (SSO). ASOs are used in RNA-based therapies and directly target specific RNA molecules. They are synthesized single-stranded nucleic acids, typically 12-30 nucleotides in length, that bind to RNA via base-pairing, modulating the RNA target. The mechanism of modulation depends on the design of the oligonucleotide but can broadly be divided into mechanisms promoting RNA cleavage and degradation or steric blocking. SSOs are part of the latter, it disrupts splicing by binding to complementary *cis*-acting sequences on the pre-mRNA. As a result, the spliceosome and regulatory factors are unable to recognize these sequences and splice sites are switched¹⁷³. Due to the length of the oligonucleotide specificity is very high. The main drawback of this therapeutic is delivery to specific target tissue because of accumulation in the liver and kidney¹⁷³. However, ASOs have been used successfully in Duchenne muscular dystrophy and spinal muscular atrophy¹⁷⁴⁻¹⁷⁸. This success led to research on its application in cancer. In uveal melanoma with *SF3B1* mutations, it was demonstrated that using ASOs targeting *BRD9*, a potent tumour suppressor, caused exclusion of a poison exon in *BRD9*, resulting in a strong suppression of tumour growth¹⁷⁹. In addition, in some cancers, the *Bcl-X* gene is alternatively spliced to express upregulated anti-apoptotic *Bcl-X_L*, associated with chemoresistance, and down regulated pro-apoptotic *Bcl-X_S*¹⁸⁰. Using SSO-induced *Bcl-x* splice-switching (delivered using lipid nanoparticles) in metastatic melanoma shifted expression in favour of *Bcl-X_S* and resulted in reduced tumour load¹⁸¹. Higher expression of *Bcl-X_S* also increases chemosensitivity¹⁸². Last, RNA oligonucleotides can be used as decoy to target splicing factors and inhibit splicing and biological activities¹⁸³. Research of ASOs in AML is limited, but as seen in uveal melanoma, *SF3B1* mutated cells are sensitive to ASOs binding *BRD9*. This splicing factor mutation is one of three mutations frequently occurring in AML and shows the potential of this specific ASO in AML. However, more research on ASOs in general is needed and the applicability in AML could lead to finding new potential therapeutics.

As the use of splicing modulators to combat cancer is emerging, different methods are explored to expand the therapeutic window, favourably shifting the therapeutic index. For example, by application of low doses combined with other antitumor agents. For chronic lymphocytic leukaemia, it was found that a combination of the splicing modulator sudemycin and the Bruton's tyrosine kinase (Btk) inhibitor ibrutinib had a favourable effect, especially in cells with mutations in the spliceosome-RNA processing machinery¹⁸⁴. The effect induced by sudemycin involves the splicing modulation of multiple target genes important for tumour survival and is related to an AS switch of MCL-1 towards its proapoptotic isoform. The actual enhanced effect in the combination therapy is suggested to be related to the induction of ES in the inhibitor of Btk (*IBKT*), which subsequently increases the phosphor-BTK levels. High levels of phosphor-BTK increase effectivity of ibrutinib in AML patients¹⁸⁵. Recently it was shown that mutations in the splicing factors *SF3B1*, *SRSF2* and *U2AF1* (S34F) increase R-loop formation in leukaemia cell lines, resulting in DNA damage, replication stress and activation of the ATR-Chk1 pathway¹⁸⁶⁻¹⁸⁸. Different combination therapy suggestions have come forth from these results. First, ATR inhibitors cause further DNA damage, promoting cell death in *U2AF1* (S34F)-expressing cells. The splicing modulation compound E7107 enhanced this effect by further inducing R loop accumulation and inducing an ATR response, rendering the cells more sensitive to ATR inhibitors¹⁸⁷. Second, *SF3B1* (K700E) mutations were sensitive to ATR inhibitors (VE-821) and Chk1 inhibition (UCN-01), a critical substrate for ATR¹⁸⁸. In addition, the combination of UCN-01 or VE-821 with

splicing modulator sudemycin D6 had an enhanced effect. Thus, the effect of combination therapies has been shown in MDS cells, but could have an effect in other myeloid malignancies, like AML.

Neoantigens as Biomarkers and Application in Immunotherapy. The effect of alternative splicing can manifest in different ways, including the expression of a protein isoform. Most of the isoforms produced by cells are known, but novel isoforms have been identified due to different mutations in AML^{8,156}. Cancer-specific protein and RNA isoforms can generate neoantigens offering opportunities for design of immunotherapies¹⁸⁹. In most cases, tumour-specific neoantigens are generated by mutations in the genes of tumour cells, but can also be produced as a result of AS^{190,191}. RI splicing events typically encode neoantigens¹⁹², because of nonsense mediated decay¹⁹³. This process generates small peptide substrates, which can be presented on MHC type I¹⁹⁴. Although, in AML RI only accounts for a small percentage (7-0.2%) of the detected AS events^{10,157}. Shen *et al.* found that RI events in the context of frameshift neoantigens are less frequent than mis-splicing of exons¹⁹⁵. A recent comprehensive analysis of AS in 8.705 cancer patients identified multiple predicted putative neoantigens as a result of AS¹⁹¹. In addition, splice site disruption due to splice-site-creating mutations leads to unique predicted neoantigen peptides¹⁹⁶. For AML, Grinev *et al.* discovered an unknown isoform of the *PARL* gene⁸. Whether this could form a potential neoantigen is unknown and requires additional research, as determining the most immunogenic epitopes is a critical hurdle in immunotherapy. Altogether, these studies highlight the potential of AS in the generation of neoantigen.

In recent years, immunotherapy as treatment for cancer has gained more traction. Immunotherapies have shown effective in multiple different cancers, which previously seemed untreatable¹⁸⁹. Especially T cells play an important role in modern cancer immunotherapies and have demonstrated therapeutic effects¹⁹⁷. However, a major problem is the lack of tumour-specific antigens in different types of cancer¹⁹⁸. The function and importance of neoantigens as tumour-specific antigen has been revealed over the past years. Although most research is focused on mutation-derived neoantigens and neglects the potential of AS. One application of neoantigens has been in therapeutic cancer vaccines. This immunotherapy stimulates the adaptive immune system against tumour antigens, activating and expanding antigen specific CD8⁺ and CD4⁺ T cells¹⁹⁹. The benefit of this is avoiding off-target effects, although clinical trials are largely unsuccessful²⁰⁰, potentially due to suboptimal antigens²⁰¹. Recent advancements generated personalized neoantigen-based therapeutic cancer vaccines, using individualized antigens instead of shared antigens. These vaccines show promising results in different types of cancer²⁰²⁻²⁰⁵.

Other immunotherapies include chimeric antigen receptor (CAR) T cells, which is genetic engineering of T cells. This technique uses autologous T cell that express synthetic recombinant receptors binding a tumour antigen²⁰⁶. CAR T cells have many advantages, such as MHC-independent antigen recognition, programmability and preventing tumour escape²⁰⁷. Anti-CD19 CAR-T cells have been used in B-cell precursor acute lymphocytic leukaemia and showed robust clinical responses and long-lasting anti-tumour activity²⁰⁸. But CD19 is exclusively expressed on the B cell lineage, while most targetable antigens in myeloid malignancies are shared with healthy cells. Although there are ongoing studies using different targets, like CLL-1 (NCT04219163, NCT04884984), CD33 (NCT03971799) and CD123 (NCT04014881), none of these are solely expressed on AML blasts. In addition, due to heterogeneity of AML bearing cells, it is difficult to use single antigen targeting CAR T cells, which led to the design of a CD33-CLL-1 CAR T cell²⁰⁹. This showed effective in treating patients with relapsed/refractory AML and is currently further studied (NCT05016063). Neoantigens would be a huge benefit in the use of CAR T cells in AML because of exclusive expression on the tumour cells and is patient specific. However, due to the heterogeneity of AML and the current lack of AS understanding at a sub clonal level it is unpredictable if it would work, as Patel *et al.* found

considerable cell-to-cell variability in splicing patterns in primary glioblastoma²¹⁰. Another problem with CAR T cell (CD19 and CD22) treatment is therapeutic resistance because of antigen-negative escape^{211–213}. In CD19 CAR T cell escape is caused by AS of exon 2 in *CD19*, which contains the recognized epitope, mediated by SRSF3²¹¹. A solution to this problem could be the earlier mentioned combination CAR T cells, decreasing the chance of development of antigen-negative escape cells or using splicing modulators as a combination technique. Moreover, targeting neoantigens that result from tumour driving AS events could decrease the chance of escape as this would negatively impact cancer cell survival.

Altogether, the identification of tumour-specific neoantigens is important for the development of both cancer vaccines and immunotherapy, however, the therapeutic significance of AS associated neoantigens is unknown. More research is needed on both AS in cancer but most importantly, the generation and the immunogenicity of the neoantigens as a result of.

Conclusion and Future Perspectives

In this review, we highlighted the tremendous effect AS has on tumorigenesis and tumour survival. In recent years, there has been an exponential growth in research involving AS in cancer, which characterized many pathogenic splicing events. It was found that all hallmarks of cancers are affected by AS, by means of many different mechanisms, for example, direct mutations in splicing factors and the core spliceosome proteins. However, splicing abnormalities cannot unanimously be explained by these mutations as they occur irrespectively. Recently, the involvement of epigenetic regulation in cancer driven AS has been researched extensively. The effect of hypoxia-induced AS caused by the tumour microenvironment has even been called the 11th hallmark of cancer. Furthermore, the fusion gene *RUNX1/RUNX1T1* influences aberrant splicing by affecting chromatin status and alters the expression of splicing factors. It has even been shown that mutated splicing factors and mutated epigenetic regulating genes facilitate leukaemogenesis by coordinating effects on the epigenome and RNA splicing²¹⁴. This underlines the importance of understanding the different modes of regulation, including upstream mechanisms involved in gene expression and the epigenome. Expanding knowledge on the different genetic mutations that alter AS either independently or in combination with splicing factor and spliceosome mutations could help with development of new therapies, specifically combination therapies.

We also discussed the potential of AS in prognosis and anticancer therapies. Recently, numerous studies have demonstrated that AS has prognostic values either in addition to other biomarkers or as an independent prognosticator in adult and paediatric AML. The need for new prognostic markers that function in highly heterogenous diseases like AML is urgent and AS has shown its potential. However, as we lack perfectly reliable bioinformatic tools for detection of alternative splicing events, a more universal robust method has to be developed first. In addition, the use of protein isoforms instead of just splicing events for prognostic purpose could potentially be explored.

Using AS in therapy can be performed in different ways, including targeting of splicing directly, targeting splicing indirectly, or using neoantigens produced as a result of AS. At this moment, multiple clinical trials are being performed on different small-molecule splicing modulators and their clinical efficacy and safety toxicity in patients is to be determined. But these therapies had limited efficacy and high toxicity in the past. For most small-molecule splicing modulators, stability, specificity, and delivery are a large issue. Optimizing these characteristics via chemical modification poses a major challenge. In general, it is crucial to determine whether targeting of AS via splicing factors and the spliceosome would reverse pathogenic state or if a wider approach is needed. It is unknown whether mutations in splicing factors act by way of unified mechanisms and targets. Although implications have

been made ¹⁸⁶, research is highly needed. This information could potentially provide general therapeutic approaches that work in a broader manner.

Because only a subset of neoantigens is able to cause a T cells response, screening methods for precise prediction and ranking are crucial. Current algorithms have improved to predict approximately 50% of immunogenic tumour epitopes and filters out 98% of non-immunogenic peptides with a 70% precision ²¹⁵. However, these algorithms excluded neoepitopes from splice-isoforms and aberrant expressed introns on mutant genes as they are difficult to identify using common genomic approaches. Neoantigens derived from AS are important and expanding algorithms to include isoforms is essential to produce a highly specific and more inclusive screening method.

There are multiple problems with personalized neoantigen-based therapeutic cancer vaccines and CAR T cells. First, the development cycle is too long, which increases the cost, but more importantly, it could exceed patient survival. Second, even though neoantigens seem like a significant advantage, being able to target specifically cancer cells, AML has a high heterogeneity. This means not every cancer cell will contain the neoantigen and, even worse, if the original protein is not part of a process required for tumour survival or progression, another sub clonal population takes over. Last, the biggest issue with personalized therapy, like vaccines and CAR T cells, remains the immense cost of the treatment. In order to be a viable option in cancer treatment, processes such as screening, validation, and production have to be optimized.

Drug resistance plays a large role in cancer and is severely affected by AS. In this review, we have discussed multiple ways this occurs, such as shifting of Bcl-X expression to Bcl-X_L resulting in chemoresistance. But also, resistance to CD19 CAR T cell treatment by aberrant splicing of exon 2 in *CD19*. Interestingly, as we have seen with isoform switching of Bcl-X_L to Bcl-X_S by SSOs, chemoresistance is reversed. This indicates that targeting the effect of AS on resistance could complement a lot of existing therapies. For future development, it is important to further explore the role of AS in drug resistance and how to target it. Furthermore, uncovering the mechanisms involved in the resistance against current AS targeted therapies is crucial for development of future treatment.

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