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Review Article

The missing puzzle piece: splicing mutations

Marzena A Lewandowska[1,2]

[1]*Molecular Oncology and Genetics Unit, Department of Tumor Pathology and Pathomorphology, The Franciszek Lukaszczyk Oncology Center, Dr I. Romanowskiej 2, 85-796, Bydgoszcz, Poland;* [2]*Department of Thoracic Surgery and Tumors, Ludwik Rydygier Collegium Medicum, Bydgoszcz, Nicolaus, Copernicus University, Torun, Poland*

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Abstract: Proper gene splicing is highly dependent on the correct recognition of exons. Among the elements allowing this process are the "cis" (conserved sequences) and "trans" (snRNP, splicing factors) elements. Splicing mutations are related with a number of genetic disorders and usually induce exon skipping, form new exon/intron boundaries or activate new cryptic exons as a result of alterations at donor/acceptor sites. They constitute more than 9% of the currently published mutations, but this value is highly underestimated as many of the potential mutations are located in the "cis" elements and should be confirmed experimentally. The most commonly detected splicing mutations are located at donor (5') and acceptor (3') sites. Mutations at the branch point are rare (only over a dozen are known to date), and are mostly searched and detected when no alteration has been detected in the sequenced exons and UTRs. Polypyrimidine tract mutations are equally rare. High throughput technologies, as well as traditional Sanger sequencing, allow detection of many changes in intronic sequences and intron/exon boundaries. However, the assessment whether a mutation affects exon recognition and results in a genetic disorder has to be conducted using molecular biology methods: *in vitro* transcription of the sequence of interest cloned into a plasmid, with and without alterations, or mutation analysis via a hybrid minigene system. Even though microarrays and new generation sequencing methods pose difficulties in detecting novel branch point mutations, these tools seem appropriate to expand the mutation detection panel especially for diagnostic purposes.

Keywords: Molecular pathology, mutations, branch point, aberrant splicing

Introduction

Proper gene splicing is highly dependent on the correct recognition of exons that are usually composed of 300 bp and are interspersed by introns of an average size of 145 bp [1], in which many sequences resembling those of 5' and 3' termini are present. Among the elements allowing the identification of exons and their correct joining are the "cis" and "trans" elements. The "cis" elements include conserved sequences such as donor sites, acceptor sites, branch point and polypyrimidine tract, as well as auxiliary elements, such as enhancers and silencers. The "trans" elements may be divided into spliceosome snRNP and splicing factors, either repressors or activators of splicing. The signal sequences at the 5' and 3' termini are recognized by spliceosome components, small nuclear RNAs (snRNAs), which form complementary RNA-RNA complexes. At the first step of this process, complementary binding of U1snRNP to the AG-GU sequence at the donor site (or U12snRNP binding to the non-canonical sequence AU-AC) [2] as well as binding of the splicing factor U2AF35 to the 3' terminus and of U2AF65 to the polypyrimidine sequence at the acceptor site, lead to the formation of the early spliceosomal complex E. Subsequently, U2snRNP recognizes adenine at the branch point, which induces the formation of spliceosomal complex A. In the following step of the process (spliceosomal complex B), the 2'-OH group of the adenine nucleotide at the branch point attacks the first nucleotide of the intron (5' terminus), both of which are in close proximity due to the interactions between U2, U6 and pre-mRNA. Afterwards, U5 snRNA forms exon loops and positions exons in a way that facilitates the second nucleophilic attack, in which the 3'-OH group from the released exon attacks the last nucleotide of the intron at the

3' terminus. Finally, the last spliceosomal complex C stabilizes the regrouped RNA: the exons are joined and the intron is released as a lasso [3-5].

Proper identification of the conserved dinucleotides at the 5' terminus (the donor site AG/GURAGU, where R is a purine and Y is a pyrimidine) and the 3' terminus (the acceptor site YAG/RNNN), as well as the branch point (composed of the sequence YNYURAC) with the polypyrimidine tract are essential for the correct joining of exons [6]. Exonic and intronic splicing enhancers (abbrev. ESE and ISE, respectively), and silencers (ESS and ISS) help the process of joining exons. The enhancers usually contain a conserved sequence specifically dedicated to the binding of serine- and arginine-rich SR proteins. The proteins are composed of one or two copies of RNA recognition motif and the characteristic C-terminal domain with a high content of arginine and serine (RS domain). The SR proteins bind to ESE and ISE via the RNA recognition motif and the RS domain interacts with splicing enhancers [7]. The SR proteins promote the step of early spliceosomal complex E formation including U1 snRNP, but are also involved in the recruitment of U4/U6.U5 tri-snRNP and promote the second step of transesterification [8, 9]. Recent reports of the Human Epigenome Project indicate that methylation also plays a role in gene splicing. A bioinformatics data analysis revealed that the hypermethylation of CpG islands (>80%) often occurs at the sites of alternative mRNA splicing (particularly the noncanonical ones), but does not occur in alternative promoters. An increase in the methylation of sequences containing many putative ESE was also noticed [10].

Splicing mutations

The most common splicing mutations are those that induce exon skipping, form new exon/intron boundaries or activate new cryptic exons as a result of alterations at donor or acceptor sites. It is estimated that the very high number of currently unclassified alterations may be due to the abnormalities of gene splicing. For example, in the HGMD database, more than 9% of over 141,000 published mutations are considered splicing mutations, but the percentage varies between specific genes (e.g., 33% for the *MAPT* gene, 23% for *BRCA2*, only 9% for *BRCA1*, 18% for *ATM*, and only 4% for *SMN1/2*) (http://www.hgmd.org/). However, the results are still highly underestimated as many of the potential mutations are located in the "cis" elements and should be confirmed experimentally. Moreover, a growing number of laboratory kits used for routine molecular diagnostics cover splicing mutations, e.g., kits for analysing mutations within the *CFTR* gene usually detect the mutation 3849+10 kb C>T. Yet the identification and characterization of novel splicing mutations, unless they are located at the canonical 5' or 3' termini, is quite difficult, therefore bioinformatics software, such as SpliceView, Analyzer Splice Tool, Human Splicing Finder, SpliceReport or Cryp-Skip, and the DBASS3/DBASS5 repositories help to determine, whether the potential alteration occurs at a donor site, acceptor site or branch point [11]. However, in the case of mutations located deep within an intron, further bioinformatics analyses, such as those conducted using ESEfinder, should necessarily consider splicing regulatory elements (SREs) [12] and those conducted using mFold or pFold should consider the alterations in the secondary structure of RNA. Nonetheless, these predictions should be confirmed with molecular biology methods, either in an *in vitro* transcription procedure using a previously prepared plasmid containing the sequence of interest with and without alterations, or by employing mutation analysis via a hybrid minigene system, in which a fragment of the gene of interest derived from a patient and a healthy subject is amplified and cloned into the previously prepared plasmid. However, such an analysis is time consuming, using either *in vitro* transcription or in vivo plasmid with a minigene. Another analysis may be conducted based directly on the RNA isolated from patient cells or from a cell line derived from the patient, but this procedure is performed rarely. For example, the cell line CFP15a derived from a nasal polyp from a carrier of the C->T mutation at 3849+10 kb generates a new donor site and leads to the inclusion of 84 nucleotides from intron 19 along with the stop codon [13].

Splicing mutations at donor (5') and acceptor (3') sites

The 1525-1G>A mutation in the *CFTR* gene identified 20 years ago is an interesting splicing mutation that removes the normal AG acceptor

site in intron 9 and thus allows the use of alternative acceptor sites. On the identification of the mutation, it was suggested that 1525-1G>A removes a reading frame by skipping exon 10 and different alternative acceptor sites in intron 9 were proposed [14]. An analysis of *CFTR* transcripts conducted 10 years later revealed that apart from the transcript lacking exon 10 (as expected), two additional transcripts using acceptor sites in exon 10 at positions 1610-1611 and 1678-1679, were detected [15]. A bioinformatics analysis of potential ESE sites and the estimation of the "strength" of potential acceptor sites allowed Ramahlo et al. to note that the sequence fragment 1610-1679 of the second additional transcript is so "weak" that it is not recognized as a potential acceptor site. On the other hand, based on the analysis by Fairbrother et al. [16], they predicted the presence of ESE in both exon 10 and 11; the former exon (ex10, 1589-1594) in particular is located within 15 bp from the first alternative acceptor site and 85 bp from the second one, which explains the formation of the two transcripts with an acceptor site in exon 10 instead of intron 9 [15].

Much like cystic fibrosis, the rare Pompe disease (glycogenosis type II) is acquired via autosomal recessive inheritance. Genetic analyses of the acid alpha-glucosidase (*GAA*) gene revealed the missense mutation p.R600C and the new splicing mutation c.546G>T [17]. The latter was initially analysed as a neutral mutation of an exon, which does change the amino acid sequence. However, further studies demonstrated that this mutation alters the donor site of exon 2 and while the correct transcript is produced, it is of limited quantity (10%). Other examples of splicing mutations of the same *GAA* gene are the following: c.1194+2T>A, c.2646_2646+1delTG, c.692+1G>C and 1326+1G>A. These mutations occur at the donor site and an analysis of the first two examples conducted using the minigene system revealed that the mutations lead to the skipping of exons 7 or 18 of the *GAA* gene [18], whereas the mutated transcript in patients carrying the mutation c.692+1G>C [19] or 1326+1G>A [20] was undetectable in vivo.

Other examples are the melanoma-predisposing mutations at the donor site of the gene *CDKN2A* in exon 1 (c.149A->C) or exon 2 (IVS2+1G->T; c.457G->T) [21]. An RT-PCR analysis of the former mutation AGgt ->CGgt revealed two different transcripts of the gene *CDKN2A*. The first transcript contained the correctly recognized exon 1 and 2, yet with an amino acid change from glutamine to proline. The second, abnormal transcript was 69 bp shorter due to the activation of a cryptic donor site in exon 1. The resulting protein was shorter by 23 amino acids forming the constitutive fragment of ankyrin repeats necessary for the interactions with the proteins $p16^{INK4A}$ and CDK4 [21].

The previously described mutations at donor or acceptor sites regarded classic dinucleotide sequences GT-AG at the 5' and 3' termini recognized by the U2 complex of small nuclear ribonucleoproteins. Nonetheless, some introns have different, noncanonical sequences of their donor and acceptor sites with the following 5' and 3' termini: AT-AC or GT-AG. The excision of these introns depends on U12-snRNPs and while the complete removal of the U12-dependent introns is critical for the correct gene expression, little is known about the mechanism of their recognition and removal. Therefore it was crucial not only to discover the mutation IVS2+1A->G in intron 2 of the gene *LKB1*, encoding a serine-threonine kinase, which alters the sequence necessary for the recognition of the donor site [22], but more importantly, to perform a more profound functional analysis and find correlations between this molecular alteration in intron AT-AC and Peutz-Jeghers syndrome. Two out of eight transcripts generated as a result of the mutation IVS2+1A>G correspond in both the minigene study and the analysis of RNA isolated from the cells derived from Peutz-Jeghers syndrome patients. One of the transcripts was produced due to the use of an acceptor site located one nucleotide downstream of the normal 3' terminus. The second product of *LKB1* exon joining in the presence of the mutation IVS2+1A>G is based on the mix of RNAs, in which both the 1 nt and 4 nt downstream positions are used as new cryptic acceptor sites. These results suggest that the abnormal process of exon joining is a result of the U12-dependent exon joining, when the cryptic acceptor site is located very close to the original acceptor site. Further analyses of the *LKB1* exon joining products for the first time revealed that in the absence of U12snRNA, the U2-dependent spliceosome may use the U12snRNP donor site [23].

Mutations at the branch point

Mutations at the branch point are very rare, and are mostly searched and detected when the entire coding fragment along with the 5' and 3' termini of each exon, as well as the 5' and 3'UTRs, have already been sequenced, but no alteration has been detected. To date, only over a dozen branch site mutations have been published and their consequences include abnormal exon skipping, retention of the entire intron or its fragment due to the activation of cryptic 3' splice sites. First mutations at the branch point were identified almost 20 years ago, as during the analysis of mutations in the fibrillin 1 and 2 (*FBN* 1 and 2) genes, an abnormal exon 31 excision was noticed in both genes. In the fibrillin 1 gene, deletions in exon 31 [24] and abnormal splicing of exon 32 [25] are related with Marfan syndrome. The mutation T->G in intron 30 of the fibrillin 2 gene was identified in a patient with Beals syndrome and is located several base pairs from a sequence corresponding to the branch point (located between positions -21 and -15 and a very short polypyrimidine sequence at the 3' terminus [26]. Molecular analysis using the nonisotopic RNase Cleavage Assay demonstrated that exon 31 is partially skipped. It was also estimated that while only 25% of the transcript derived from the mutated allele is produced, this quantity is sufficient for the phenotype of congenital contractural arachnodactyly (CCA) to be recognized. More profound molecular diagnostics of the mutations in FBN2, including detection of the mutation IVS30: T-26G, was conducted in 30 members of the patient's family (5 generations, 18 patients with the CCA phenotype) and cosegregation of the branch point mutation with the CCA phenotype was demonstrated in the analysed lineage.

Another example of branch site mutation is IVS32: T-25G of the *COL5A1* gene. This substitution is located 2 bp upstream of a highly conserved adenosine residue at the branch point As a consequence, 45 base pairs of exon 33 are "skipped" in 60% transcripts of the mutated *COL5A1* [27]. A similar branch site mutation, IVS4: T-22C, was identified in the lecithin–cholesterol acetyltransferase (*LCAT*) gene [28, 29]. The analysis of the *LCAT* mutation IVS4: T-22C using an in vitro strategy with a minigene system, as well as the analysis of transcripts isolated from the patient's leukocytes, revealed the presence of a zero (muted) allele, i.e. a copy of the gene that lost its function due to intron retention [28, 29]. Subsequently, Li and Pritchard prepared the expression plasmid pcDNA3.1 containing the strong CMV promoter and inserted exons 1-4 into the multiple cloning site along with the 83-nucleotide intron and, further downstream, exons 5 and 6 of the *LCAT* gene. The investigated branch point was located between positions -19 and -25 bp from the 3' terminus of exon 5. The above studies employing branch point-directed mutagenesis confirmed that the mutations of adenosine to other nucleotides completely disable the normal splicing process of joining exons 4 and 5, and that the reintroduction of the correct sequence in a minigene system restores the correct exon joining; further studies of the mutation IVS4: T-22C demonstrated that enhancing the branch point via point-directed mutagenesis inducing a G->A mutation partially restores the normal splicing process [30].

Niemann-Pick disease type 1 is a lysosomal storage disease characterized by the intracellular accumulation of nonesterified cholesterol and other lipids in various tissues in patients with mutations in the gene *NPC1*. The analysis of transcripts combined with the sequencing of the *NPC1* gene (isolated from fibroblasts) revealed the alteration c.882-28A>G. To assess the effect of the alteration, further functional analysis employing the minigene strategy was conducted, in which the sequences spanning from the 3' terminus of intron 5 to intron 8 (including WT or mutant intron 6) were inserted into a pTarget plasmid and transfected into the Cos1 cell line. The WT minigene generated mRNA of the expected size and sequence, while the *NPC1* minigene carrying the mutation c.882-28A>G generated a shorter transcript lacking exon 7, that was also observed in an analysis of RNA derived from patient's fibroblasts [31].

Another -28A>G substitution was identified in intron 9 of the *KCNH2* gene as a pathogenic mutation causing LQT syndrome. Using the splicing minigene assay, it was demonstrated that the substitution of adenine for cytosine or thymine at the branch point also contributes to the incorrect identification of the acceptor site of intron 9 and affects the splicing of the *KCNH2* gene. Nevertheless, the presence of thymine or cytosine at the branch point, in con-

trast to the newly discovered mutation IVS9-28A>G, results not only in the production of the aberrant transcript, but also in a much higher production of the correct transcript. Furthermore, the polypyrimidine tract was modified to a stronger one (three purines were mutagenized to polypyrimidine), which reinforced the acceptor site of *KCNH2* intron 9. This contributed to the proper recognition of the 3' splice site even in the presence of the -28A>G mutation [32].

Another example of a mutation at a branch site is the newly identified mutation c.661-31T->G in the uroporphyrinogen III synthetase gene, found in patients with autosomal recessive congenital erythropoietic porphyria (CEP). The *UROS* mutation c.661-31T->G considerably decreases the production of the WT transcript (as much as 90%) and decreases the activity of the enzyme. It is the first branch point mutation found in the last intron and this localization may potentially affect the observed 100% intron retention without exon skipping [33].

As currently the new generation sequencing methods are more financially available than a few years ago, the sequencing of exons along with their flanking sequences is increasingly performed, but even this approach may not detect the mutations located deep within an intron in a regulator fragment or even at a branch point. Such situation was encountered by Dutch scientists investigating the mutation in the *FGD1* gene (faciogenital dysplasia 1) responsible for Aarskog-Scott syndrome. Conventional Sanger sequencing did not reveal any alteration in the *FGD1* gene. As the following step, a microarray analysis was conducted which confirmed the absence of major deletions or duplications in the genome, again including the *FGD1* gene, but did not indicate the location of the mutation, whose phenotype manifested as Aarskog-Scott syndrome. Exon sequencing via the new generation methods did not provide results until the filtering parameters were modified by accepting the analysis of larger intronic sequences that flanked exons. The presence of a newly identified mutation of the *FGD1* gene, a single nucleotide deletion (c.2016-35del), affects the recognition of the branch point and of the acceptor site of exon 13, which leads to a premature termination of the exon joining process. The novel mutation was not present either in the SNP131 database or among the sequences obtained as part of the project of sequencing 1000 human genomes (Genome 1000) (as of 2011) [34]. The final step was the confirmation of this variant in siblings and the analysis of segregation with ASS.

Polypyrimidine tract mutations

Polypyrimidine tract mutations are as rare as branch point mutations and require many functional analyses as well. Some good examples are the transversions in the polypyrimidine tract at the 3' terminus of the fifth exon of the *FIX* gene, where a pyrimidine (T) was substituted by a purine (G) at position c.392-8T>G (Haemophilia B Mutation Database) or c.392-9T>G [35]. In the presence of both transversions, exon 5 is skipped in the splicing of the *FIX* gene. On the other hand, in vitro analyses using modified U1snRNP, fully complementary with the donor site of exon 5, are very promising for their potential in therapeutic applications. The co-transfection with a minigene containing intron 5 carrying a mutation in the polypyrimidine tract and the modified U1snRNP have a beneficial effect on the recognition of exon 5 and, consequently, its correct inclusion during splicing [36].

Another example of a mutation in the polypyrimidine tract is the lamin A/C (*LMNA*) gene mutation c.937-11 C>G, which results in the inclusion of 40 nucleotides derived from intron 5 during the splicing of the *LMNA* gene [37]. Another interesting discovery was provided by the analyses of the polypyrimidine tract in intron 8 of the *CFTR* gene along with the assessment of the polymorphism of TG repeats that determine the strength of the 3'ss (3' splice site). In CF patients carrying a short polypyrimidine track (up to T5), the risk of developing nonclassic cystic fibrosis and congenital bilateral absence of the vas deferens depends on the number of the TG dinucleotides [38].

Take home message

Proper gene splicing depends on exon recognition allowed by the "cis" (conserved sequences) and "trans" (snRNP, splicing factors) elements. Splicing mutations constitute approximately 10% of human pathogenic mutations. Most known splicing mutations are located at donor (5') and acceptor (3') sites. Mutations at the

branch point are rare (<20 known to date) as well as polypyrimidine tract mutations and searched when no alteration has been detected in the sequenced exons/UTRs. High throughput technologies, as well as traditional Sanger sequencing, allow detection of many changes in intronic sequences and intron-exon boundaries, however, functional analyses or analyses of transcripts from patient cells are necessary to further evaluate the effect of a splicing mutation on exon recognition. Even though microarrays and new generation sequencing methods pose difficulties in detecting novel branch point mutations, these tools seem to be appropriate to expand the mutation detection panel especially for diagnostic purposes.

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The authors have no conflicts of interest that are directly relevant to the content of this article.

Address correspondence to: Marzena Anna Lewandowska, Molecular Oncology and Genetics Unit, Department of Tumor Pathology and Pathomorphology, The Franciszek Lukaszczyk Oncology Center, dr I. Romanowskiej 2, 85-796, Bydgoszcz, Poland. E-mail: lewandowskam@co.bydgoszcz.pl

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