Chapter 2

Bioinformatics and Mutations Leading to Exon Skipping

F.O. Desmet and C. Béroud

Abstract

Our knowledge about human genes and the consequences of mutations leading to human genetic diseases has drastically improved over the last few years. It has been recognized that many mutations are indeed pathogenic because they impact the mRNA rather than the protein itself. With our better understanding of the very complex mechanism of splicing, various bioinformatics tools have been developed. They are now frequently used not only to search for sequence motifs corresponding to splicing signals (splice sites, branch points, ESE, and ESS) but also to predict the impact of mutations on these signals. We now need to address the impact of mutations that affect the splicing process, as their consequences could vary from the activation of cryptic signals to the skipping of one or multiple exons. Despite the major developments of the bioinformatics field coupled to experimental data generated on splicing, it is today still not possible to efficiently predict the consequences of mutations impacting splicing signals, especially to predict if they will lead to exon skipping or to cryptic splice site activation.

Key words: Mutations, Exonic splicing enhancer, Bioinformatic tools, Exon skipping, Splicing, Genetic diseases

1. Introduction

Mutations responsible for human genetic diseases are quite variable from one gene to another, e.g., with large deletions accounting for up to 65% of mutations of the *DMD* gene (1, 2), while missense mutations account for 77% of mutations of the *LMNA* gene (1, 3). If we take into account the 102,433 unique mutational events reported in 3,804 human genes collected in the Human Gene Mutation Database (HGMD) in Cardiff (http://www.hgmg.cf.ac.uk, HGMD Professional release 2010.2) (4), missense mutations account for 44.92% of cases, small deletions for 15.7%, nonsense mutations for 11.28%, splice sites for 9.56%, small insertions for

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6.47%, and large deletions for 6.25%. Other mutations correspond to rare events such as mutations in repeated sequences, complex rearrangements, or mutations located in regulatory regions.

For many years, it has been believed that mutations affecting the coding sequence directly impacted the protein at the amino acid level either by disrupting the reading frame, by introducing a premature termination codon or by an amino acid substitution. Only intronic mutations affecting donor or acceptor splice sites were recognized as potentially affecting mRNA processing. This has been called into question with improved understanding of the splicing machinery and associated splicing signals.

Today, many splicing signals have been identified both in introns and exons, which are used by the cellular machinery to properly splice introns through a polyproteic complex known as the spliceosome. This complex, which catalyzes the sequential phosphodiester transfer reactions and contains more than 100 core proteins coupled to five small nuclear RNAs (snRNAs), is believed to be one of the most complex machines in the cell (5). Two types of introns have been well characterized (6, 7), which require different snRNAs. On one hand, U2 snRNP-dependent introns represent more than 99.9% of all introns and are excised by a spliceosome containing the U1, U2, U4, U5, and U6 snRNPs. On the other hand, U12 snRNP-dependent introns are usually shorter and correspond to the minor class of introns. They are excised by a spliceosome containing U11, U12, U4atac, U6atac, and U5 snRNPs (8). The cellular machinery recognizes both intron types thanks to sequence motifs localized at their extremities. These motifs are degenerated in humans and higher eukaryotes, and the study of thousands of known exons and surrounding intronic sequences has led to the definition of consensus motifs both for donor (5′ splice site (ss)) and acceptor (3′ss) splice sites (9, 10). Note that U2 and U12 introns are characterized by different consensus motives: GT/AG and AT/AC, respectively. A third motif the branch point sequence (BPS) also participates to the splicing reaction (11). The three motifs (5′ss, 3′ss, and BPS) constitute the core splicing signals (12) that will be used during the early stage of exon definition. Following the initial splice sites recognition, a series of structural rearrangements will activate the spliceosome. Nevertheless, because the high degeneration of the core splicing signals is exacerbated for the BPS (9, 11), it was unclear how the cell can efficiently distinguish true splice sites from “decoy” splice sites that could define “pseudoexons” (9). This has been elucidated by in vitro analysis showing that the core splicing signals contain only about half of the signals required to accurately define exon–intron boundaries (13). The additional signals (also known as auxiliary splicing sequences (14)) are now classified as exonic splicing enhancers (ESEs) or silencers (ESSs), which respectively promote or inhibit the exon recognition. Similarly, enhancer (ISE) or silencer (ISS)

signals can also be located within introns. These *cis*-acting splicing regulatory elements (SREs) then recruit *trans*-acting splicing factors that activate or suppress the splicing process (15). Few of these *trans*-acting splicing factors have been cloned and their activity demonstrated by a variety of approaches including minigenes (see Chapter 3). They include members of the serine–arginine (SR)-rich protein family (16) who target ESEs, while proteins of the heterogeneous nuclear ribonucleoparticles (hnRNP) family repress splicing by targeting ESS or ISS motifs and directly antagonize the recognition of splice sites or interfere with the binding of proteins to ESE motifs (17).

Considering the importance of core and auxiliary splicing signals in the pre-mRNA splicing process, it is now recognized that all types of mutations (missense, nonsense as well as small insertions or deletions) can indeed result in splicing defects either by disrupting or creating signals. The demonstration of a potential impact on splicing requires the study of mRNA, which is not always feasible in diagnostics because of the unavailability of the target tissue. Various efficient in vitro methods using minigenes have been developed to circumvent this problem (see Chapter 3). Because many mutations can lead to splicing defects, it is useful to first select the most relevant ones before in vitro validation, which is expensive and time-consuming. To do so, one can use various bioinformatics tools available through the Web. In this chapter, we review the most popular of these bioinformatics resources and present their contribution to real diagnostic situations using a specific dataset we generated for this purpose.

2. Methods

2.1. Bioinformatic Resources to Identify Splicing Signals

As anticipated by Wang and Burge, given the complexity of splicing regulation, the splicing code will not have the simple tabular form of the genetic code, but instead will include a variety of tables and subtables that are applicable in different circumstances (12). Various bioinformatics resources have been developed to predict core and/or auxiliary splicing signals. The most advanced systems are used to predict 5′ss and 3′ss. To do so, six methods have been developed (18) and have been included in various softwares (for review, see ref. 19). Their efficiency has been evaluated using various sets of mutations (19, 20). The prediction of BPS is also available through the Human Splicing Finder (9) or the Sroogle tools (21) and various softwares are now available to predict ESE and/or ESS (21–29). To predict mutations leading to exon skipping, we focused on tools able to predict 5′ss and 3′ss as well as ESE/ESS (Table 1).

Table 1
Selected software to predict core splicing signals (5′ss and 3′ss) and ESE/ESS

Software	Type	URL
Human Splicing Finder	5′ss, 3′ss, ESE, ESS	http://www.umd.be/HSF/
MaxEnt Scan	5′ss, 3′ss	http://genes.mit.edu/burgelab/maxent/
SROOGLE	5′ss, 3′ss, ESE, ESS	http://sroogle.tau.ac.il/
ESE Finder	ESE	http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home
RESCUE ESE	ESE	http://genes.mit.edu/burgelab/rescue-ese/
Fas-ESS	ESS	http://genes.mit.edu/fas-ess/
PESX	ESE, ESS	http://cubweb.biology.columbia.edu/pesx/

2.2. Databases of Mutations

Concomitantly to bioinformatics predictions, the researcher can access a wide variety of databases related to the collection of mutations from human genes. The most extensive ones are the Locus Specific DataBases (LSDBs) that collect mutations from a single gene. They include both published and unpublished mutations and a curation process by experts from the specific fields to ensure their quality (30, 31). They have different structures and contents, but thanks to the availability of generic software, their content should get harmonized in the near future (3, 32, 33). A regularly updated list of such LSDBs can be found at the Human Genome Variation Society Web site (HGVS): http://www.hgvs.org/dblist/glsdb.html.

Data about mutations are also available at central databases such as the HGMD (4), the National Center for Biotechnology Information (NCBI) http://www.ncbi.nlm.nih.gov/, or the European Bioinformatics Institute (EBI) http://www.ebi.ac.uk/. Other resources such as the Online Mendelian Inheritance in Man (OMIM) (34) and the Universal Protein Resource (UniProt) (35) allow a rapid access to some mutations associated with human genes. In contrast to LSDBs, only few annotations are available for each entry and no indication about potential exon skipping impact is available.

2.3. Dataset of Mutations Leading to Exon Skipping

In order to evaluate the various bioinformatics tools related to the prediction of splicing signals, we searched for published mutations using the following keywords: “mutation,” “exon skipping,” and restricted the dataset to publications from year 2009. The selected publications were then filtered to select point mutations with experimental validation of the full exon skipping. A total of 71 mutations were thus selected (Table 2). All mutations have been renamed according to the international nomenclature system using the cDNA (c.) nomenclature (36). In addition, genes have been described with the official gene symbol approved by the HUGO Genes Nomenclature Committee (HGNC) (37).

Table 2
Selected mutations resulting in an exon skipping

Gene	Mutation	Skipping of exon	References
SLC46A1	c.1082-1 G>A	3	(52)
LDLR	c.313+6 T->C	3	(53)
LDLR	c.2389 G>T	16	(53)
BEST1	c.256 G>A	4	(54)
BEST1	c.707A>G	6	(54)
BEST1	c.715 G>A	7	(54)
ABCB11	c.500 C>T	7 (mild, 80% wild-type splicing)	(55)
ABCB11	c.557A>G	7 (moderate, 60% wild-type splicing)	(55)
ABCB11	c.957A>G	10 (mild, 95% wild-type splicing)	(55)
ABCB11	c.1388 C>T	13 (mild, 90% wild-type splicing)	(55)
ABCB11	c.2776 G>C	22 (mild, 90% wild-type splicing)	(55)
ABCB11	c.3084A>G	24 (severe, 5% wild-type splicing)	(55)
ABCB11	c.3329 C>A	25 (mild, 90% wild-type splicing)	(55)
ABCB11	c.3346 G>C	25 (mild, 80% wild-type splicing)	(55)
ABCB11	c.3382 C>T	25 (mild, 85% wild-type splicing)	(55)
ABCB11	c.3383 G>A	25 (mild, 90% wild-type splicing)	(55)
ABCB11	c.3556 G>A	26 (mild, 90% wild-type splicing)	(55)
ABCB11	c.3691 C>T	27 (severe, 5% wild-type splicing)	(55)
POMT1	c.280+1 G>T	4 (complete)	(56)
PAH	c.707-2delA	7 (complete)	(57)
APOB	c.3697-1 G>C	24 (complete)	(58)
SETX	c.6106 G>A	15 (complete)	(59)
SETX	c.6208+1_6208+2insT	16 (complete)	(59)
SETX	c.6546+5 G>A	19 (complete)	(59)
SETX	c.7287+5 G>A	25 (complete)	(59)
COL6A2	c.1770delG	23 (complete)	(60)
SLC35D1	c.392+3A>G	4 (complete)	(61)
APC	c.1918 C>G	14 (moderate)	(62)
PYCR1	c.797 G>A	6 (complete)	(63)
DMD	c.3432+1 G>A	25 (complete)	(64)
CDC73	c.237+1 G>C	2 (moderate, 50% wild-type splicing)	(65)

(continued)

Table 2 (continued)

Gene	Mutation	Skipping of exon	References
CDC73	c.132-1 G>A	3 (moderate)	(65)
CDC73	c.729+1 G>C	7 (moderate)	(65)
FBN1	c.2168-1 G>T	18 (complete)	(66)
COG1	c.1070+5 G>A	6 (complete)	(67)
HPS1	c.398+5 G>A	5 (complete)	(68)
HPS1	c.988-1 G>T	12 (complete)	(68)
PMM2	c.256-1 G>C	3 or 3–4 (moderate, 50% wild-type splicing)	(69)
NF1	c.4577+1 G>C	27a (complete)	(70)
ECGF1	c.1300+1 G>C	9 (complete)	(71)
ECGF1	c.215-1 G>C	2–3	(71)
COL7A1	c.6900 G>A	87 (complete)	(72)
COL7A1	c.6847_6873del	87 (complete)	(72)
COL7A1	c.6862_6877del	87 (complete)	(72)
COL7A1	c.6899A>G	87 (complete)	(72)
COL7A1	c.6900+2_6900+5del	87 (complete)	(72)
COL7A1	c.6900+4A>G	87 (complete)	(72)
ABCA1	c.720+6 T>C	7 (complete)	(73)
ABCA1	c.4560-1 G>C	31 (complete) or cryptic ss	(73)
LHCGR	c.537-3 C>A	7 (complete)	(74)
GNPTG	c.610-2A>G	9 (complete) or cryptic ss	(75)
SPTB	c.3776+1 G>A	16–17	(76)
SPTB	c.3777-2A>G	16–17	(76)
TFR2	c.614+4A>G	4 (complete)	(77)
FECH	c.68-2A>G	2 (complete)	(78)
SLC12A1	c.724+4A>G	5 (complete)	(79)
PKHD1	c.53-3 C>A	3 (complete)	(79)
TG	c.274+2 T>G	3 (complete)	(80)
COL6A2	c.801+3A>C	5 (complete)	(81)
MR	c.2511-2A>C	7 (complete)	(82)
CDH3	c.1431-1 G>T	11	(83)

(continued)

Table 2 (continued)

Gene	Mutation	Skipping of exon	References
LDLR	c.1705+1 G>T	11 or 11–12	(84)
LDLR	c.1705+1 G>A	11 or 11–12	(84)
LDLR	c.1584+5 G>A	10 (moderate, 50% wild-type splicing)	(84)
LDLR	c.191-2A>G	3	(84)
LDLR	c.314-1 G>A	4	(84)
LDLR	c.2389+1 G>T	16	(84)
LDLR	c.1988-1 G>A	14	(84)
EXT1	c.962+1_962+4del	2	(85)
EXT2	c.537-18_559del	3	(85)
EXT2	c.939+2 T>G	5	(85)

2.4. Bioinformatics Prediction of Splicing Signal Alteration

For each mutation affecting a 5′ss or a 3′ss (intronic or exonic), the impact on the consensus value (CV) was evaluated by the Human Splicing Finder and the MaxEnt Scan tools (Table 1). A positive prediction was annotated when it corresponded to a strong alteration of these signals. When using the HSF algorithm, we used a threshold for 5′ss and 3′ss of 65 with a pathogenic ΔCV of 10% except for position +4 where it was set to 7% as recommended (9). For MaxEnt (38), the minimal ΔCV was fixed to 10%. Only a single result was given, as both tools agreed for all mutations.

For exonic mutations distant from the exon boundaries and therefore not predicted to affect the 5′ss and the 3′ss, we searched for the alteration of an existing ESE using the ESE-Finder (22), the RESCUE ESE (23), the PESE (26), and the HSF-ESE (9G8 and Tra2-β) (9) matrices/tools and for the creation of an ESS using the Sironi's motifs (24), the Wang's decamers (25), the Fas-ESS hexamers (12), the PESS (26), and HSF-ESS (hnRNPA1) (9). For all analyses, the default parameters from the various software were used.

3. Results

From the 71 selected mutations published in 2009 and experimentally proven to result in an exon skipping, 19 were localized in exons at a distance from exon–intron boundaries and therefore were affecting critical auxiliary splicing sequences. The remaining 52 were localized at the exon–intron boundaries and thus directly

affected the core splicing signals (5′ss or 3′ss). Among these mutations, 20 affected the 3′ss and 32 the 5′ss. Only five exonic mutations were affecting the 5′ss. They all involved the last base of the exon. No exonic mutation resulted in the inactivation of the 3′ss.

The 52 mutations were all predicted to strongly affect the wild type splice site by both prediction tools, except for mutation c.720+6 T>C (*ABCA1* gene) that was only predicted by the MaxEnt tool and the c.724+4A>G (*SLC12A1* gene) that was only predicted by the HSF tool (Table 3). If the disruption of a 5′ss or a 3′ss could result in the skipping of the corresponding exon, it could also lead to the activation of a cryptic site. It has been proposed that this site should be located within 50 bp from the wild-type splice site in order to be activated (39). We thus searched for 5′ cryptic splice sites using the HSF algorithm with a CV threshold of 70. For 21 mutations, no cryptic site was detected while for 11 mutations the presence of a candidate cryptic splice site was detected. For 3′ss, another core splicing signal should be taken into account: the BPS. We used the HSF algorithm to detect such signals. For 16 out of the 19 mutations disrupting the 3′ss, no cryptic site was predicted within the 50 bp range and compatible with the BPS. For three mutations, a candidate cryptic 3′ss was predicted. At least for one of them, the activation of this cryptic site was reported concomitantly to the exon skipping.

For the 19 exonic mutations affecting auxiliary splicing sequences, we only considered the inactivation of an existing ESE or the creation of an ESS as these two events could result in an exon skipping event (Table 4). For 18 out of the 19 mutations, at least one ESE-matrix/tool predicted the disruption of an ESE (average of 1.6), while for 15 mutations at least one ESS-matrix/tool predicted the creation of an ESS (average of 1.7). Because of the highly degenerated motifs of ESE and ESS, these sequences overlap frequently, thus resulting in a competition for binding of ligand-proteins (40, 41). This is illustrated here as all mutations predicted to create an ESS are also predicted to disrupt an ESE. This could also reinforce the effect of these mutations as they result in a loss of an enhancer signal coupled with the creation of a silencer signal. For mutations predicted to result in the disruption of an enhancer signal only, it could be postulated that this signal plays a critical role in the recognition of this exon by the cellular machinery, as adjacent core splicing signals are weak. The evaluation of 5′ss and 3′ss CVs does not support this hypothesis (data not shown). In addition, the exon recognition process by the spliceosome is not a yes or no system and some mRNA molecules could escape the effect of the mutation (exon skipping), resulting in the production of a certain amount of wild-type transcript from the mutant allele. For most exonic mutations described here, the exon skipping efficiency has been evaluated by in vitro assays and it has been shown that the resulting exon skipping could range from

Table 3
Bioinformatics predictions of mutations affecting 3′ and 5′ splice sites

Gene	Mutation	3′ss	5′ss
ABCA1	c.4560-1 G>C	✓	–
ABCA1	c.720+6 T>C	–	✓[m]
APOB	c.3697-1 G>C	✓	–
CDC73	c.132-1 G>A	✓	–
CDC73	c.237+1 G>C	–	✓
CDC73	c.729+1 G>C	–	✓
CDH3	c.1431-1 G>T	✓	–
COG1	c.1070+5 G>A	–	✓
COL6A2	c.1770delG	–	✓
COL6A2	c.801+3A>C	–	✓
COL7A1	c.6900+2_6900+5del	–	✓
COL7A1	c.6900+4A>G	–	✓
COL7A1	c.6900 G>A	–	✓
DMD	c.3432+1 G>A	–	✓
ECGF1	c.1300+1 G>C	–	✓
ECGF1	c.215-1 G>C	✓	
EXT1	c.962+1_962+4del	–	✓
EXT2	c.537-18_559del	✓	–
EXT2	c.939+2 T>G	–	✓
FBN1	c.2168-1 G>T	✓	–
FECH	c.68-2A>G	✓	–
GNPTG	c.610-2A>G	✓	–
HPS1	c.398+5 G>A	–	✓
HPS1	c.988-1 G>T	✓	–
LDLR	c.1584+5 G>A		✓
LDLR	c.1705+1 G>A	–	✓
LDLR	c.1705+1 G>T	–	✓
LDLR	c.191-2A>G	✓	–
LDLR	c.1988-1 G>A	✓	–
LDLR	c.2389+1 G>T	–	✓

(continued)

Table 3 (continued)

Gene	Mutation	3′ss	5′ss
LDLR	c.2389 G>T	–	✓
LDLR	c.313+6 T>C	–	✓
LDLR	c.314-1 G>A	✓	–
LHCGR	c.537-3 C>A	✓	–
NF1	c.4577+1 G>C		✓
NR3C2	c.2511-2A>C	✓	–
PAH	c.707-2delA	✓	–
PKHD1	c.53-3 C>A	✓	–
PMM2	c.256-1 G>C	✓	–
POMT1	c.280+1 G>T	–	✓
PYCR1	c.797 G>A	–	✓
SETX	c.6106 G>A	–	✓
SETX	c.6208+1_6208+2insT	–	✓
SETX	c.6546+5 G>A	–	✓
SETX	c.7287+5 G>A	–	✓
SLC12A1	c.724+4A>G	–	✓[h]
SLC35D1	c.392+3A>G	–	✓
SLC46A1	c.1082-1 G>A	✓	–
SPTB	c.3776+1 G>A	–	✓
SPTB	c.3777-2A>G	✓	–
TFR2	c.614+4A>G	–	✓
TG	c.274+2 T>G	–	✓

✓ = The wild type ss is inactivated by the mutation
✓[m] = The inactivation of this site was only predicted by the MaxEnt tool
✓[h] = The inactivation of this site was only predicted by the HSF tool

a mild status (>70% of wild-type transcript is present) to a severe or complete status (<10% of wild-type transcript is present) (Table 2). No difference was observed between the prediction of ESE or ESS motifs within the three groups (mild, moderate, and severe). Note that for mutation c.256 G>A of the *BEST1* gene, no prediction of the alteration of auxiliary sequences motifs has been found.

The 71 mutations from this study are located in 31 genes (Table 1). Among these genes, 25 are included in the HGMD

Table 4
Bioinformatics predictions of splice site signals

Gene	Mutation	ESE_1	ESE_2	ESE_3	ESE_4	ESS_1	ESS_2	ESS_3	ESS_4	ESS_5
ABCB11	c.500 C>T	✓	✓	–	–	–	–	–	–	✓
ABCB11	c.557A>G	–	✓	–	–	✓	–	✓	–	–
ABCB11	c.957A>G	–	✓	–	✓	✓	–	–	–	–
ABCB11	c.1388 C>T	✓	–	✓	–	–	–	✓	✓	✓
ABCB11	c.2776 G>C	✓	–	–	–	✓	–	–	–	–
ABCB11	c.3084A>G	–	✓	–	–	✓	–	–	–	–
ABCB11	c.3329 C>A	✓	–	–	–	✓	–	✓	–	✓
ABCB11	c.3346 G>C	✓	–	–	–	–	–	–	–	–
ABCB11	c.3382 C>T	✓	–	–	–	–	–	✓	✓	–
ABCB11	c.3383 G>A	✓	–	–	–	–	–	–	✓	–
ABCB11	c.3556 G>A	–	✓	–	–	–	–	–	–	–
ABCB11	c.3691 C>T	✓	–	–	–	✓	–	✓	–	–
APC	c.1918 C>G	✓	–	–	–	✓	–	✓	–	–
BEST1	c.256 G>A	–	–	–	–	–	–	–	–	–
BEST1	c.707A>G	–	–	✓	✓	✓	–	✓	–	–
BEST1	c.715 G>A	✓	–	–	–	✓	–	–	✓	✓
COL7A1	c.6847_6873del	✓	✓	✓	✓	–	–	–	–	–
COL7A1	c.6862_6875del	–	✓	✓	✓	✓	–	–	–	–
COL7A1	c.6899A>G	✓	✓	–	–	–	–	–	–	✓

ESE_1: ESE-Finder; ESE_2: RESCUE ESE; ESE_3: PESE; ESE_4: HSF-ESE; ESS_1: Sironi motifs; ESS_2: Wang's decamers; ESS_3: Fas-ESS hexamers; ESS_4: PESS; ESS_5: HSF-ESS
✓ = Creation of an ESS or disruption of an ESE

database, but only 10 mutations out of the 65 expected (15.4%) have previously been reported according to this database. As expected, no indication about the impact on splicing was given. Going through the HGVS listing of available LSDBs through the Internet, we have been able to identify LSDBs for 12 genes. These LSDBs included 14 mutations out of the 23 from this review (60.9%). In addition, for almost all mutations an indication about the impact of these mutations on splice sites was given either using manual annotation (33) or embedded prediction algorithms coupled to manual annotation (3). Nevertheless, no information about in vitro validation of exon skipping was available.

4. Discussion

With the development of molecular diagnosis of human genetic diseases, hundreds of genes are routinely scanned to identify pathogenic mutations. These mutations can impact the protein itself through a premature termination codon (nonsense or frameshift mutations) or missense mutations. The importance of the mutations at the transcript level is now well recognized and mutations can impact the transcript stability or the splicing process through the alteration of core or auxiliary splicing signals. These mutations can lead to the skipping of one or more exons but the exact mechanism for multiple exon skipping remains unknown. They can alternatively lead to the activation of cryptic splice sites. In both situations, the altered transcript can produce a shorter protein if the reading frame is conserved or, more often, lead to a premature termination codon (PTC) that will activate the Nonsense Mediated Decay process (NMD) (42). Various experiments have shown that the spliceosome deposits multiple proteins 20–24 nucleotides upstream of exon–exon junction (43); this protein complex has been named the exon junction complex (EJC). During the "pioneer round of translation," if a ribosome encounters a PTC, it will recruit the SURF complex, composed of two translation release factors (eRF2 and 3), Upf1 and the SMG-1 kinase. This complex is activated via a SURF–EJC interaction. If the PTC is located less than 50 nucleotides upstream of an exon–exon junction, this mechanism is inactivated probably because of a steric hindrance. The mutant transcript could also escape this control process if the PTC is localized within the last exon.

The prediction of the consequence of a mutation is now considered both at the transcript and the protein level. The theoretical consequence of the mutation at the protein level is easily and routinely done by various algorithms implemented in major LSDBs generic softwares (3, 33) but can also be addressed for any mutation through the Mutalyzer tool (44). The impact of mutations at

the transcript level is much more complex as mutations can disrupt core splicing signals or auxiliary splicing sequences. Even if such signals are inactivated by the mutation, the prediction of the exact consequence on the transcript and therefore the protein remains a challenge. The first step is to predict if the corresponding exon will be removed from the transcript (exon skipping) or if a cryptic splice site will be inactivated. The second step is the prediction of the consequence at the protein level. In order to evaluate the various mutations leading to an exon skipping, we have extracted 71 mutations published in 2009 for which the impact on mRNA has been experimentally proven by various approaches such as minigenes (see Chapter 3). Among these mutations, 47 were localized in introns and 24 in exons, and they inactivated core-splicing signals (5′ss or 3′ss) in 52 cases or auxiliary splicing sequences (ESE/ESS) in 19 cases. To predict the impact on 5′ss and 3′ss, we used two of the most popular prediction tools: Human Splicing Finder (9) and MaxEnt Scan (38). They both predicted all mutations as pathogenic with only two discrepancies (Fig. 1). In order to evaluate the potential skipping of the corresponding exons, we searched for cryptic splice sites in the surrounding 50 bp from the wild-type splice site as suggested by Krawczak et al. (39). The exon skipping was thus predicted for 66% of 5′ss and 84% of 3′ss. These values are nevertheless too low to be useful for diagnosis. This difference could be due to the creation of multiple mRNA species in the cell carrying a mutation: one corresponding to the transcript carrying the exon skipping, while others correspond to the activation of one or more cryptic splice sites as reported for many mutations. It can also be due to a more complex mechanism that allows the cell to properly select splice sites which will not captured by the present rules. New algorithms taking into account multiple parameters thus remain to be developed in order to efficiently predict the impact of mutations affecting 5′ss and 3′ss.

To evaluate the impact of exonic mutations on auxiliary splicing signals (ESE/ESS), we used nine matrices/tools. For 18 mutations, an average of 1.6 matrices out of 4 predicted the disruption of ESEs, while for 15 mutations, an average of 1.7 matrices out of 5 predicted the creation of ESSs. For only one mutation, no alteration was detected (Fig. 1). Many works have been performed to define ESE and ESS matrices based on SELEX or experimental approaches (15). However, due to technical and conceptual bias, the various sequence sets only share partial homology. A recent study evaluated the efficiency of these matrices to discriminate true signals from false-positive signals and demonstrated that the ESE-Finder ($P=0.0067$), HSF-ESE ($P=0.0017$), and PESE ($P=0.000236$) matrices/tools were the most efficient for ESE predictions (9). Not enough data are available for ESS. Therefore, even if 18 out of 19 exonic mutations were predicted to disrupt enhancers, to create silencers, or to do both simultaneously (15 cases), it is today impossible to predict the outcome of such mutation at the mRNA level.

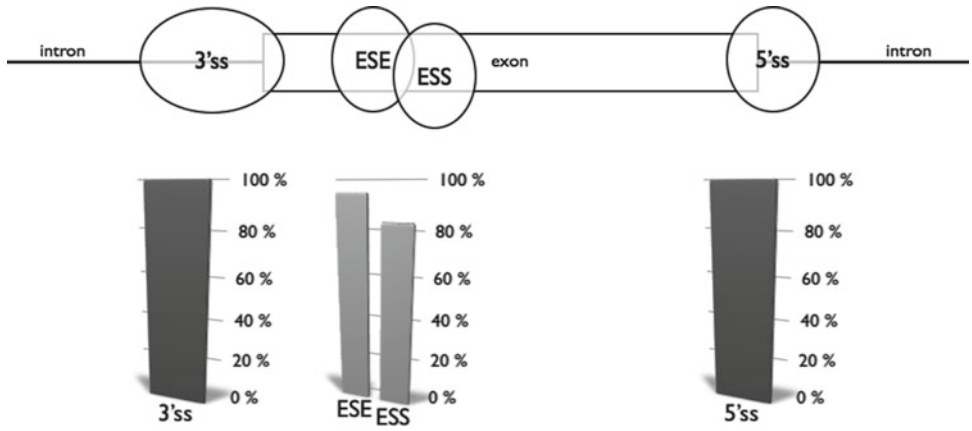

Fig. 1. Bioinformatics predictions of splice site signals disrupted or created by mutations leading to exon skipping. For ESE and ESS, the values indicate the percentage of mutations predicted to disrupt an ESE and/or to create an ESS.

In 1988, Monaco demonstrated that the phenotype of patients harboring a mutation of the *DMD* gene could be predicted thanks to the reading frame rule: mutations leading to out-of-frame transcripts will lead to Duchenne muscular dystrophy (severe form), while mutations conserving the reading frame lead to the Becker muscular dystrophy (mild form) (45). This discovery has opened the way to the exon skipping strategy induced by various approaches (see Chapters 6 and 7 for overviews). One recurrent question is now "which genes are eligible for the therapeutic exon-skipping approach?" One way to answer would be to identify genes harboring mutations leading to exon skipping and associated with a mild phenotype. As shown here, it is quite difficult to find such information in core database and only the most advanced LSDBs known as Knowledge bases could harbor it. The situation is simpler if we take into account large rearrangements detected by various techniques such as the multiplex ligation dependent probe amplification (MLPA) that is routinely applied to many genes (46, 47) or the Comparative Genomic Hybridization array technology (CGH-array) (48). They allow the rapid characterization of the deletion or the duplication of one or more exons. The knowledge of the exon phasing can directly predict if the exon skipping or duplication will result in an in-frame or an out-of-frame transcript taking into account the impact at the junctional codon (49).

A second candidate gene for the therapeutic exon skipping approach has been recently identified. It corresponds to the *DYSF* gene whose mutations are responsible for dysferlinopathies. Various large deletions have been described in this gene but they target key regions of the protein and therefore are associated with the severe form of the disease. A lariat branch point mutation was reported associated with a mild dysferlinopathy (50). This mutation was predicted by the HSF-BPS algorithm to disrupt the BPS of intron

31 leading to an in-frame skipping of exon 32, suggesting small insertion/deletions and nonsense mutations localized in this exon could be rescued by exon skipping. This has been demonstrated experimentally on myoblasts from the patient (51).

In conclusion, many mutations leading to exon skipping are present in the pool of disease-causing mutations discovered yearly. If large rearrangements are now identified with a higher frequency thanks to new technologies (MLPA, CGH-array), the majority of mutations leading to exon skipping correspond to small molecular events altering core or auxiliary splicing signals. Mutations altering 5′ss and 3′ss are efficiently predicted to disrupt the key splicing signals (19) and therefore predicted to lead to exon skipping even if the activation of cryptic splice sites cannot be ruled out. The knowledge of the impact of all types of exonic mutations (nonsense, missense, synonymous) on auxiliary splicing sequences is very complex, and making bioinformatics predictions to discriminate true from false-positive signals remains challenging. Only the experimental demonstration of the impact of mutations affecting core or auxiliary splicing signals can formally demonstrate the presence of exon skipping (see Chapters 3–5). In the context of new therapeutic approaches to induce exon skipping, the knowledge of such mutations associated to mild phenotypes is of major importance to identify candidate genes for this new promising therapeutic approach. We thus encourage LSDB curators as well as biologists and researchers to annotate the consequence of such mutations on transcripts. This will also help in the validation of new sophisticated algorithms to predict the consequence of mutations impacting core splicing signals in the near future and auxiliary sequences in the long term.

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