The deleterious effect of missense mutations on pre-mRNA splicing

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Running title: Missense mutations causing exon skipping

ABSTRACT

The presence of missense mutations detected during genetic testing makes it difficult to classify their pathogenic effect. It is possible that the predicted amino acid change affects protein function; however, it is also possible that a missense mutation does not act at the protein level but rather at the nucleotide level by interfering with the correct assembly of the pre-mRNA splicing machinery. In this chapter we describe that short 6 to 9 nucleotides-containing sequence motifs act as exonic splicing regulatory elements. They are specifically recognized by corresponding splicing factors, which then assist in the recognition of the conserved splice site motifs by the spliceosome. Many examples show that a point mutation in these exonic splicing regulatory elements is sufficient to change splicing factor binding, which impairs inclusion of an exon during the splicing reaction. Thus, the molecular consequence of a missense mutation can be exon skipping and thus cause a frameshift in the messenger RNA that results in a premature stop codon and loss of function of the affected allele. Although several bioinformatic tools exist to predict splicing factor binding to mRNA, this effect of a missense mutation on splicing cannot yet be accurately predicted by sequence analysis alone. In order to determine whether a missense mutation has a deleterious effect on splicing of the corresponding mRNA, experimental analysis with either patient RNA or splicing reporter minigenes is required.

INTRODUCTION

Genetic diseases are characterized by the presence of mutations that inactivate single genes. The sequence analysis of the corresponding candidate disease genes allows confirmation of diagnosis or realization of genetic testing in family members. Whenever sequence variants are found during routine genetic testing, they are classified as pathogenic mutations if they either (a) result in the introduction of a stop codon that truncates the protein (for example, an amino acid alteration to a stop codon or as consequence of a frameshift), or (b) affect an invariant splice junction consensus sequence, or (c) were previously reported as pathogenic in the literature based on supporting functional data (Cotton and Scriver 1999).

These criteria are not fulfilled if a missense mutation is detected. It is possible that the predicted amino acid change affects protein function, but this requires experimental confirmation using biochemical or cellular activity assays. Increasing evidence over the last decade has, however, demonstrated that many missense mutations rather act at the nucleotide level. This is due to the complexity of the pre-mRNA splicing process, which separates the coding information of the exons from the more abundant non-coding sequences in the introns. This process not only relies on consensus sequences at the intron/exon junction but also requires additional splicing regulatory elements in their vicinity, including the exon sequences. Many examples have shown that a single nucleotide change in an exon can interfere with the correct assembly of the splicing machinery and lead to either complete skipping of the exon, retention of an intron, or the introduction of a new splice site within an exon or intron. In consequence, even translationally silent sequence variations now need to be evaluated for their pathogenic effect in the patient. For many of the studied disease genes, up to 50% of point mutations within exons affect splicing, and more than half of all known disease-causing mutations are now estimated to disrupt splicing (Lopez-Bigas et al. 2005).

A GENERAL OVERVIEW ON THE PRE-mRNA SPLICING REACTION

Genes are transcribed into a pre-mRNA from which intron sequences are removed and exons joined together to generate the mature protein-coding mRNA transcript. The chemistry of the splicing reaction is mediated by the spliceosome, an RNA-based machine containing five snRNAs and numerous associated proteins (Jurica and Moore 2003). Both the snRNA and protein components of the spliceosome interact with defined core splicing signal sequences at the exon/intron boundaries to direct intron excision and exon ligation (Wang and Burge 2008) (see Figure 1). At the 5' splice site only the first two bases of the intron (GU) are generally conserved. The second consensus sequence at the 3' splice site is defined by three separate elements: the branch site, the polypyrimidine tract, and the 3' splice site dinucleotide AG.

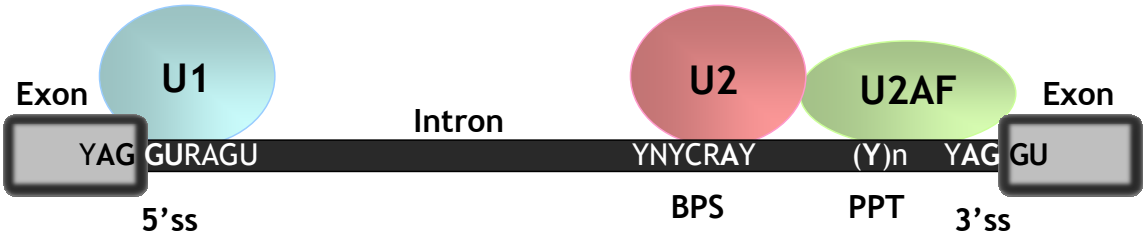

Figure 1: Classical consensus sequences targeted by mutation in human disease. The consensus sequences define exon/intron boundaries, in particular; *ss*: splice site, *BPS*: branch point sequence, *PPT*: polypyrimidine tract, Y=U or C; R=G or A. The 5' ss is recognized by the U1 snRNP, whereas U2AF and U2 snRNP recognize the 3'ss elements (adapted from House and Lynch 2008).

Although the consensus splice sites function to direct the splicing machinery, these sequence elements are short so that additional sequence elements outside of the splice sites contribute to the control of pre-mRNA splicing. Recognition of most exons during splicing is now believed to be under the combinatorial control of multiple regulatory RNA elements that increase the overall fidelity of the splicing reaction. These elements are recognized by specific splicing protein factors, which then recruit spliceosomal components through protein-protein interactions (Smith and Valcarcel 2000; Singh and Valcarcel 2005; Hertel 2008; House and

Lynch 2008). Importantly, these regulatory RNA sequences are targets for pathogenic mutation (Pagani and Baralle 2004).

ENHANCERS AND SILENCERS OF THE PRE-mRNA SPLICING REACTION

Additional splicing regulatory sequences are c*is*-acting, can occur within either exonic or intronic regions and function by recruiting *trans*-acting splicing factors. They can either promote recruitment of the spliceosome and exon inclusion (splicing enhancers) or disrupt assembly of the splicing complex and cause exon skipping (splicing silencers) (Pagani and Baralle 2004). The best characterized regulatory elements, exonic splicing enhancers (ESEs), are usually recognized by a family of proteins known as SR proteins, which contain an RNA-binding domain and a region rich in arginine-serine dipeptides (RS domain). It is likely that SR proteins are required for the correct splicing of most exons. However, regulation of pre-mRNA splicing is much more complex than the simple ESE recruitment model. Intronic splicing enhancers (ISEs) and splicing silencers, either exonic (ESS) or intronic (ISS), occur frequently and influence splice site selection (Figure 2) (Black 2003; Pagani and Baralle 2004). ESS- or ISS-mediated splicing repression involves their recognition by heterogeneous nuclear RNP (hnRNP) proteins and several mechanisms have been proposed. hnRNPs can oligomerize along splicing silencers and repress spliceosomal assembly (Zhu et al. 2001), or block the recruitment of snRNPs (Tange et al. 2001), or act by looping out exons (Martinez-Contreras et al. 2006). ESS sequences have higher content of T (38%) and G (36%) and reduced levels of A (17%) and C (9%) (Wang et al. 2004), while ESEs include purine-rich and AC-rich elements (Graveley 2000; Zheng 2004).

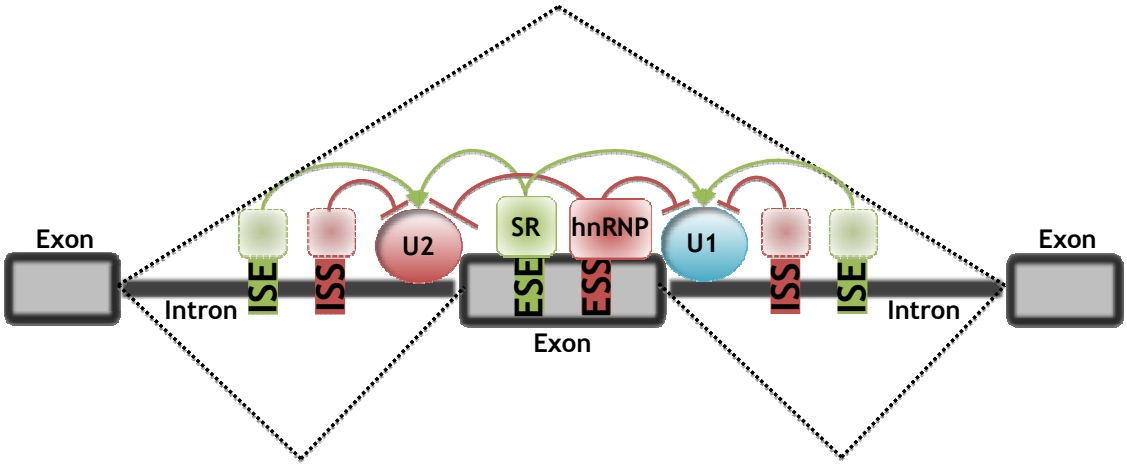

Figure 2: Schematic representation of splicing regulation. Correct splicing depends on *cis*-elements including exonic splicing enhancers (ESE) or silencers (ESS) as well as intronic splicing enhancers (ISE) or silencers (ISS) recognized by *trans*-acting splicing factors (SR proteins, hnRNPs, other factors). This combinatorial regulation mode is also at the origin of alternative splicing events naturally observed in many genes (dashed lines, with the middle exon either included or excluded) (adapted from Wang and Burge 2008).

Typically, silencers and enhancers are present within the vicinity of potential exon/intron junctions, suggesting that the interplay between activating and repressing *cis*-acting elements modulates the probability of exon inclusion (Hertel 2008). Current knowledge indicates that the recognition of a typical exon is influenced by multiple distinct *cis*-acting elements, a notion strongly supported by computational analyses (Zhang and Chasin 2004; Wang et al. 2004). As a consequence of this concept, the distinct *cis*-acting elements are targets for intronic or exonic point mutations that disrupt normal splicing of the affected gene.

EXAMPLES OF MUTATIONS THAT DISRUPT ENHANCERS OR CREATE SILENCERS

Table 1 shows representative examples from 10 human disease genes in which missense mutations or silent nucleotide changes were experimentally reported to interfere with the correct mRNA splicing. Many of these mutations were mis-classified in the past as missense mutations or silent variants whenever the analysis was limited to genomic sequence analysis alone.

Table 1: Examples of missense or silent mutations that cause aberrant splicing. Representative mutations from ten disease genes were selected and designated with regard to both the nucleotide in the coding sequence (c.) and the corresponding amino acid in the protein sequence (p.), according to the international nomenclature convention available at http://www.hgvs.org/mutnomen/recs-DNA.html.

Gene	Disease	Mutation	Exon	Effect on splicing	Reference
APC	Familial adenomatous polyposis (FAP)	c.1918C>G, p.Arg640Gly	14	Exon skipping by disruption of an ASF/SF2 ESE motif	Gonçalves et al., 2009
hMSH2	Hereditary Non Polyposis Colorectal Cancer (HNPCC)	c.806C>T, p.Ser268Leu	5	Exon skipping by disruption of a SRp55 ESE motif	Lastella et al., 2006
hMLH1	Hereditary Non Polyposis Colorectal Cancer (HNPCC)	c.842C>T, p.Ala281Val	10	Exon skipping by disruption of ASF/SF2 and SC35 ESE motifs	Lastella et al., 2006
BRCA1	Breast and ovarian cancers	c.5242C>A, p.Ala1708Glu	18	Exon skipping by creating an ESS for hnRNP A1 and H/F	Millevoi et al., 2009
NF1	Neurofibromatosis	c.945G>A, p.Gln315Gln	7	Exon skipping by disruption of an ASF/SF2 ESE motif	Bottillo et al., 2007
ATM	Ataxia-telangiectasia	c.6154G>A, p.Glu2032Lys	44	Exon skipping	Teraoka et al., 1999
POMGNT1	Congenital muscular dystrophy	c.636C>T, p.Asp179Val	7	Exon skipping by creating an ESS for hnRNP H	Oliveira et al., 2008
RPGR	Retinitis pigmentosa	c.213G>A, p.Gly52Arg	2	Exon skipping	Demirci et al., 2004
ATP6AP2	X-linked mental retardation and epilepsy	c.321C>T, p.Asp107Asp	4	Exon skipping by disruption of an ESE motif	Ramser et al., 2005
CFTR	Cystic fibrosis	c.1826A>G, p.Asp565Gly	12	Exon skipping by disruption of a composite exonic regulatory element of splicing (CERES)	Pagani et al., 2003

For further examples and details, the reader is referred to a number of excellent review articles (Cartegni et al. 2002; Faustino and Cooper 2003; Pagani and Baralle 2004; Baralle and Baralle 2005).

BIOINFORMATIC TOOLS TO PREDICT THE EFFECT OF POINT MUTATIONS

Computational methods have recently been developed to predict sequence motifs for enhancers and silencers, ESEs are short, frequently purine-rich sequences and are recognized by members of the SR protein family. Attempts to elucidate the RNA binding specificities of each SR protein have shown that they bind a vast array of RNA sequences which are highly degenerate. The factors that bind to ESS and ISS have not been characterized to a similar extent, however, hnRNPs have been generally implicated in interactions with these elements.

Table 2: List of selected bioinformatic tools to identify splicing regulatory RNA elements

Program	URL	Reference
ESEfinder	http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home	Cartegni et al., 2003
PESX: Putative Exonic Splicing Enhancers/Silencers	http://cubweb.biology.columbia.edu/pesx/	Zhang and Chasin, 2004; Zhang et al., 2005
ESR search	http://ast.bioinfo.tau.ac.il/ESR.htm	Goren et al., 2006
Splicing Rainbow	http://www.ebi.ac.uk/asd-srv/wb.cgi?method=8	Stamm et al., 2006
Human Splicing Finder	http://www.umd.be/HSF/	Desmet et al., 2009

Using these software tools, normal and mutant sequences can be submitted and the differences in splicing factor binding predicted. Unfortunately, enhancers and silencers lack a well defined consensus sequence, are not always unequivocally defined and may overlap in

their functions. This currently precludes a reliable prediction of the effect of a genomic mutation on the splicing process (Baralle and Baralle 2005; Houdayer et al. 2008; Gonçalves et al. 2009).

EXPERIMENTAL CONFIRMATION OF THE MUTATION EFFECT ON SPLICING

Although a variety of bioinformatic tools have been developed in recent years that predict splicing regulatory elements, they are at present insufficient to decide whether a genomic point mutation affects splicing. For this, experimental confirmation is still required, as detailed in the following.

Whether a suspected disease-causing mutation affects splicing should ideally be determined in RNA from the affected tissue because *cis*-acting splicing mutations can have tissue-specific effects. Unfortunately, the appropriate tissues are often not available. Frequently, the study of RNA extracted from peripheral blood lymphocytes of individual patients is sufficient to confirm splicing defects. In this case, the transcript pattern in RNA samples from patient and healthy controls should be compared. For example, a novel missense mutation in exon 14 of the APC gene was identified in a patient with familial adenomatous polyposis syndrome (FAP) (Gonçalves et al. 2009). To characterize whether the resulting p.Arg640Gly mutation could affect splicing of the APC transcript, RNA was isolated from the proband or from healthy individuals. The APC transcript between exons 13 and 15 was amplified by RT-PCR and the obtained product bands were isolated by agarose gel electrophoresis and sequenced. All healthy individuals were found to express the expected wild-type product containing exons 13, 14 and 15. In the patient, however, a second transcript lacking exon 14 became the predominant product. Densitometric estimation of the band intensities revealed that in normal individuals the full length APC transcript accounted for roughly 85% whereas in the patient,

the expression of this product dropped to 40%. These experimental data demonstrated a splicing defect in the patient (see Figure 3).

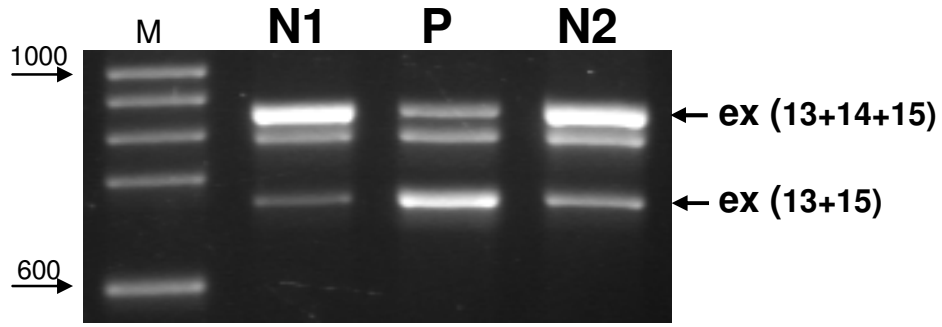

Figure 3: Skipping of exon 14 in the patient with APC mutation p.Arg640Gly. RT-PCR analysis of APC expression in peripheral blood lymphocytes isolated from the patient (P) or two healthy individuals (N1, N2). APC transcripts were amplified between exons 13 and 15 and the identity of the indicated bands verified by direct sequencing (the third unlabelled band corresponds to a heteroduplex product formed under non-denaturing gel conditions; (M) = 100 base pair molecular weight marker).

In other cases, the necessary samples may be difficult to obtain or the gene of interest is not expressed in lymphocytes. An alternative measure to demonstrate that a missense mutation affects the splicing fidelity is their subcloning into minigenes that serve as splicing reporters (Cooper 2005; Baralle and Baralle 2005). In order to determine whether the above mentioned APC p.Arg640Gly point mutation was sufficient to cause the observed exon skipping, such a splicing reporter vector was constructed (see Figure 4). The wild type and mutant exon 14 sequences were amplified from normal or patient genomic DNA together with flanking intron sequences of 118 bp upstream and 245 bp downstream. Both fragments were cloned between constitutively spliced exons of the chimeric pTB reporter, as previously published (Pagani et al. 2003).

Following transfection into appropriate cells (in this case colorectal cell lines), the minigenes are transcribed and spliced in vivo. Then the mRNA derived from the hybrid minigene can be analysed using reverse transcriptase PCR (RT-PCR) with primers that amplify specifically the minigene-derived products. Finally, the spliced products are visualised on an agarose gel.

This analysis demonstrated that the APC missense mutation p.Arg640Gly is sufficient to cause exon 14 skipping.

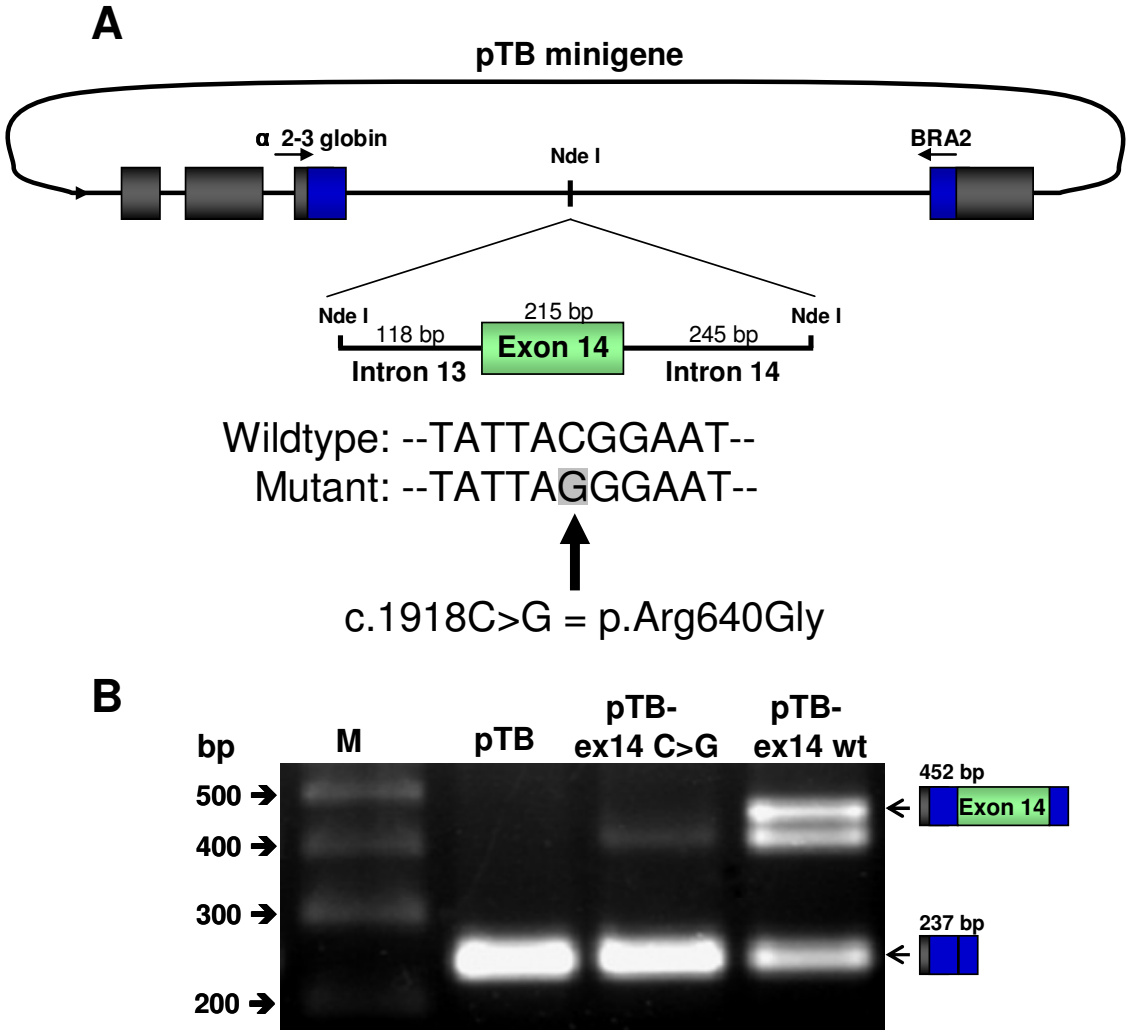

Figure 4: Splicing minigene reporter assay to confirm the effect of APC mutation p.Arg640Gly. (A) Schematic representation of the pTB reporter minigene used (Pagani et al. 2003) and the subcloning of a genomic APC fragment isolated from either wild type or mutant alleles. (B). RT-PCR analysis with primers 'α2-3 globin' and 'BRA2' of the transcripts derived from the indicated reporter minigenes following their expression in colorectal DLD-1 cells. Note that exon 14 is completely skipped in the pTB reporter containing the p.Arg640Gly (c.1918C>G) mutation (pTB-ex14 C>G), whereas exon 14 is included in the construct containing the wild type sequence (pTB-ex 14 wt).

Such minigene reporter assays not only help to clarify the mutation effect but also represent an important opportunity to validate bioinformatic prediction tools and clarify the basic molecular mechanisms that underlie the pre-mRNA splicing process. For example, in

the above example of APC exon 14, the mutant sequence was examined with several bioinformatic search tools, which predicted loss of recognition motifs for SRp55 and for ASF/SF2, or the creation of a splicing silencing hnRNP A1 motif. Upon further experimental analysis only ASF/SF2 showed convincing evidence for a role in this splicing event.

CONCLUSION

The pathogenic effect of missense mutations or silent nucleotide substitutions, which are encountered during genetic testing in human disease genes, is frequently due to interference with essential splicing regulatory RNA elements. Because bioinformatic prediction is currently insufficient, the pathogenic potential of missense mutations can only be decided upon experimental analysis of the splicing pattern in either patient RNA or reporter minigenes.

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