ONLINE MUTATION REPORT

Spectrum of splicing errors caused by *CHRNE* mutations affecting introns and intron/exon boundaries

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Background: Mutations in *CHRNE*, the gene encoding the muscle nicotinic acetylcholine receptor ϵ subunit, cause congenital myasthenic syndromes. Only three of the eight intronic splice site mutations of *CHRNE* reported to date have had their splicing consequences characterised.
Methods: We analysed four previously reported and five novel splicing mutations in *CHRNE* by introducing the entire normal and mutant genomic *CHRNE*s into COS cells.
Results and conclusions: We found that short introns (82–109 nucleotides) favour intron retention, whereas medium to long introns (306–1210 nucleotides) flanking either or both sides of an exon favour exon skipping. Two mutations are of particular interest. Firstly, a G→T substitution at the 3′ end of exon 8 predicts an R286M missense mutation, but instead results in skipping of exon 8. In human genes, a mismatch of the last exonic nucleotide to U1 snRNP is frequently compensated by a matching nucleotide at intron position +6. *CHRNE* intron 8 has a mismatch at position +6, and accordingly fails to compensate for the exonic mutation at position −1. Secondly, a 16 bp duplication, giving rise to two 3′ splice sites (g.IVS10-9_c.1167dup16), results in silencing of the downstream 3′ splice site. This conforms to the scanning model of recognition of the 3′ splice site, which predicts that the first "ag" occurring after the branch point is selected for splicing.

Molecular defects of presynaptic, synaptic, or postsynaptic proteins at the motor endplate impair neuromuscular transmission and result in congenital myasthenic syndromes (CMS).[1] Mutations in the acetylcholine receptor (AChR) ϵ subunit gene (*CHRNE*; OMIM 100725) cause endplate AChR deficiency and/or kinetic abnormalities of AChR. *CHRNE* mutations causing endplate AChR deficiency include 13 missense, 27 frameshift, 6 nonsense, 8 splice site, 3 promoter region, and 1 chromosomal microdeletion mutations.[1] Only three of the eight splice site mutations have had their consequences characterised: IVS7-2A→G[2] and IVS9+1G→T[3] result in skipping of an adjacent exon, and IVS9-1G→C causes retention of intron 9.[4] Four of the five uncharacterised mutations were reported by us. Exclusive expression of *CHRNE* by subsynaptic nuclei has previously prevented analysis of splicing consequences of these mutations. We recently reported that the cloned entire *CHRNE* exhibits the same splicing properties as its pre-mRNA in the native state in transfected COS cells.[4] Therefore we used this method to analyse the splicing consequences of four previously reported and five novel splice site mutations in *CHRNE*.

MATERIALS AND METHODS

All human studies were in accord with the guidelines of the institutional review board of the Mayo Clinic.

Patients

Patients 1–5 (respectively a 59 year old woman, a 23 year old man, a 2.5 year old girl, a 6 year old boy, and a 44 year old man) have moderate to severe myasthenic symptoms that have been present since birth or infancy, decremental EMG responses, and no AChR antibodies. All respond partially to pyridostigmine. Patient 4 underwent an intercostal muscle biopsy for diagnosis, which showed severe endplate AChR deficiency (6% of normal) and compensatory expression of the fetal γ-AChR at the endplate.

Construction of *CHRNE* clones for splicing analysis

To examine the consequences of the identified splice site mutations, we used the previously constructed pRBG4-*CHRNE* plasmid, which carries a cytomegalovirus (CMV) promoter and the entire *CHRNE* spanning 12 exons and 11 introns.[4] For extended analysis of g.IVS10-9_c.1167dup16, we also constructed a pRBG4 minigene, spanning nucleotide 880 in exon 9 to nucleotide 1457 in exon 12, where position +1 represents the first nucleotide of the first codon of the mature peptide.

Mutations were engineered using the QuikChange Site Directed Mutagenesis kit (Stratagene). Presence of the expected mutation and absence of unwanted artefacts were confirmed by sequencing the entire insert.

Reverse transcription PCR analysis of cytoplasmic RNA of transfected COS cells

Wild type and mutant *CHRNE* clones were introduced into COS-7 cells, and cytoplasmic total RNA was isolated as described.[4] We used two or more sets of PCR primers for each construct to screen for skipping of an exon and retention of an intron.

RESULTS

Each patient carries two mutant *CHRNE* alleles

We detected a total of seven *CHRNE* mutations in five patients (table 1). Patients 1, 2, and 3 carry homozygous splice site mutations. Five mutations affect pre-mRNA splicing. Analysis of family members reveals that affected siblings carry two mutant *CHRNE* alleles, whereas unaffected relatives harbour one or no mutant allele (data not shown), indicating that each mutation is recessive.

Splicing consequences of five novel and four previously reported mutations

Muscle mRNA was available only from patient 4. In this patient, RT-PCR analysis revealed that 1259del23 causes retention of intron 11 (fig 1, table 1). As no muscle specimens were available from the other patients for mRNA analysis, we introduced the four novel (table 1) and the four previously reported (table 2) splice site mutations into the cloned entire *CHRNE* and analysed the cytoplasmic RNA of the transfected COS cells by RT-PCR. Unlike minigenes, entire *CHRNE* clones mostly yielded a single splicing product. RT-PCR analysis revealed that IVS4-2A→C, IVS6+1G→T, IVS10+2T→G, and

Table 1 Five splicing, one frameshift, and one missense mutations in *CHRNE* in the five patients

No.	Mutation	Position on genomic DNA	Splicing consequence
1	g.IVS6-1G→C*	3′ splice site of intron 6	Active cryptic 3′ splice site†
2	g.IVS9-1G→A*	3′ splice site of intron 9	Retention of intron 9† Skipping of exon 10†
3	g.IVS10-9_c.1167dup16*	16 bp duplication comprising 8 bp at 3′ end of intron 10 and 8 bp at 5′ end of exon 11	Silencing of downstream 3′ splice site†
4	c.1259_g.IVS11+15del23	23 bp deletion comprising 8 bp at 3′ end of exon 11 and 15 bp at 5′ end of intron 11	Retention of intron 11‡
	c.1033delG	exon 10 (61st nucleotide)	NA
5	c.857G→T (p.R286M)	G→T substitution at 3′ end of exon 8	Skipping of exon 8†
	c.734C→T (p.P245L§)	exon 7 (193rd nucleotide)	NA

*Homozygous mutation. †Characterised using transfected COS cells. ‡Characterised using muscle mRNA. NA, not applicable. §We previously reported that P245L is a low expressor mutation that also prolongs channel opening events two fold.[18]

857G→T cause skipping of an adjacent exon; IVS7+2T→C and 1259del23 result in retention of the mutant intron; IVS9-1G→A causes both exon skipping and intron retention; IVS6-1G→C activates a cryptic 3′ splice site; and g.IVS10-9_c.1167dup16 silences the downstream copy of the 3′ splice site (fig 1, tables 1 and 2).

Why is the downstream copy of duplicated 3′ splice sites silent?

The 16 bp duplication (g.IVS10-9_c.1167dup16) generates two copies of 3′ splice sites, but only the upstream copy is used for splicing. To understand the underlying mechanism, we engineered a series of artificial mutations into a minigene spanning *CHRNE* exons 9 to 12 (fig 2A and B).

Firstly, we examined a role of the polypyrimidine tract of the upstream and downstream copies (fig 2C). The pyrimidine ratios in the polypyrimidine tract are the same for the two copies (18/24 = 75% for the upstream copy and 30/40 = 75% for the downstream copy), and are not likely to account for selection of the splice site. Substitution of ''ac'' for the invariant ''ag'' dinucleotide of the upstream copy activated the downstream copy (Mt-AC in fig 2D), indicating that the increased distance from the branch point to the ''ag'' dinucleotide does not hinder splicing. Because a stretch of t bases in the polypyrimidine tract is more efficient in splicing than c bases,[5] we mutated ''cctt'' to ''tctt'' (Mt-TC), ''cttt'' (Mt-CT), and ''tttt'' (Mt-TT), but none activated the downstream copy (fig 2D).

The role of the branch point sequence was then examined. Displacement of an invariant ''a'' nucleotide downstream (Mt-Br1 in fig 2E), disruption of the native branch point sequence (Mt-Br2 in fig 2E), or both (Mt-Br3 in fig 2E) had no effect on splicing. Preserved splicing even in the absence of the branch point consensus sequence CURAY in Mt-Br2 indicates that an ''a'' residue somewhere in intron 10 serves as a branch point, and confirms that the position and context of the branch point sequence is degenerative in mammals.

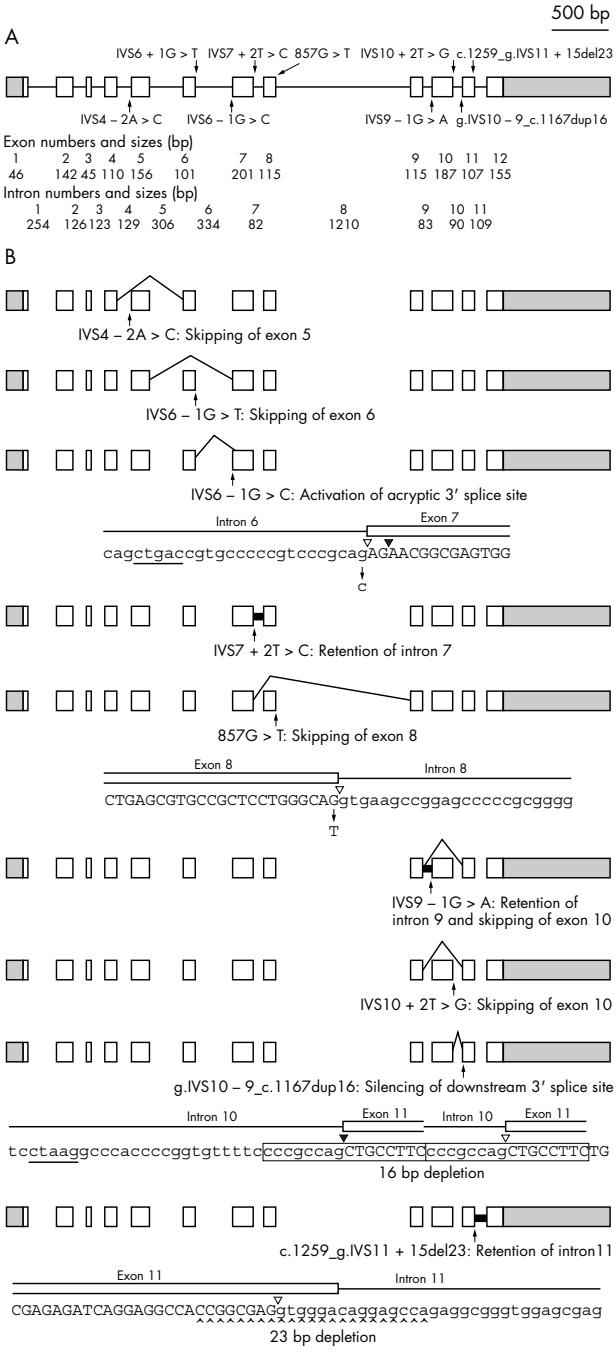

Figure 1 Nine analysed *CHRNE* mutations affecting pre-mRNA splicing. (A) The *CHRNE* gene structure is drawn to scale. Shaded areas indicate untranslated regions. Sizes of exons 1 and 12 represent those of the coding regions. (B) Schematic presentation of identified splicing consequences. Exon skipping and activation of a cryptic splice site are represented by thin oblique lines connecting two remote points. Intron retention is represented by a thick horizontal line. Four splicing mutations are shown in detail, with partial *CHRNE* sequence below each scheme. Open and closed arrowheads indicate inactive and active splice sites, respectively. Putative branch point sequences are underlined.

We next swapped the seven residue segments and placed the native branch point sequence 16 residues downstream (Mt-Br4 in fig 2E). Mt-Br4 activated both the upstream and downstream copies of the 3′ splice sites, probably because the shortened polypyrimidine tract rendered the upstream copy of the splice acceptor site less competitive than the downstream copy.

Table 2 Four previously reported splice site mutations in *CHRNE*

Mutation	Position on genomic DNA	Splicing consequence
g.IVS4-2A→C[4]	3′ splice site of intron 4	Skipping of exon 5
g.IVS6+1G→T[19]	5′ splice site of intron 6	Skipping of exon 6
g.IVS7+2T→C[20]	5′ splice site of intron 7	Retention of intron 7
g.IVS10+2T→G[21]	5′ splice site of intron 10	Skipping of exon 10

DISCUSSION

Sizes of flanking introns predict exon skipping or intron retention

We analysed splicing consequences of nine mutations in *CHRNE*. Three other splicing mutations had been previously characterised.[2–4] To summarise, exons 5, 6, 8, 9, and 10 are skipped, and introns 7, 9, and 11 are retained. The three retained introns are all short (intron 7, 82 bp; intron 9, 83 bp; and intron 11, 109 bp), whereas four of five skipped exons flank medium to long introns on either or both sides (exon 5 is flanked by 129 and 306 bp introns; exon 6 by 306 and 334 bp introns; exon 8 by 82 and 1210 bp introns; and exon 9 by 1210 and 83 bp introns). This is in accordance with collation of splicing mutations,[6] and is consistent with the model of exon recognition in vertebrate splicing.[7] Exon 10, however, is flanked by 83 and 90 bp introns, and is skipped by two distinct mutations, indicating that the size of the flanking introns is only one of several factors that determine the splicing consequence. Indeed, even when we eliminated 1127 bp in the middle of intron 8 (IVS8+17 to IVS8-59) to reduce its size to 83 bp, both IVS7-2A→G and 857G→T still resulted in skipping of exon 7 (data not shown).

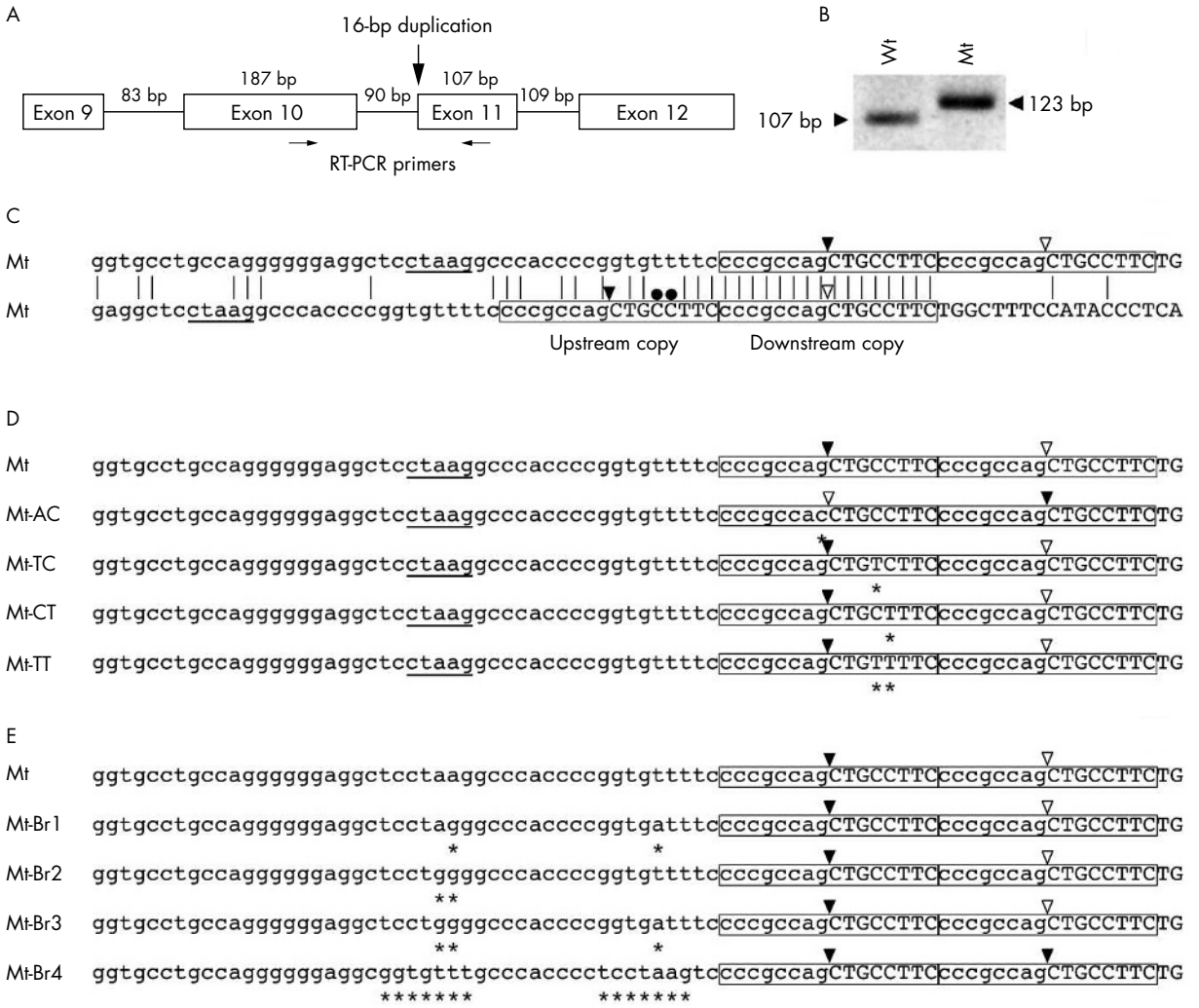

Figure 2 A 16 bp duplication of the 3′ splice site of *CHRNE* intron 10/exon 11 boundary results in silencing of the downstream copy of the 3′ splice sites by competition against the upstream copy. (A) A minigene spanning exons 9–12, which is inserted into a CMV based expression vector. (B) RT-PCR analysis of cytoplasmic RNA of transfected COS cells shows that only the upstream copy of the splice acceptor site is active. (C) Alignment of active (closed arrowhead) and inactive (open arrowhead) 3′ splice sites of the mutant (Mt) intron 10/exon 11 boundary. The two sequences are identical, but are shifted by 16 bp. Vertical lines indicate identical nucleotides. Duplicated 3′ splice sites are enclosed by boxes. Putative branch point sequence (CTRAY with an invariant A) is underlined. Upper and lower case letters represent exonic and intronic nucleotides, respectively. Dots point to mismatches that are corrected in Mt-TC, Mt-CT, and Mt-TT in panel D. (D) Disruption of an "ag" dinucleotide in the upstream copy activates the downstream copy (Mt-AC), whereas partial (Mt-TC and Mt-CT) or complete (Mt-TT) restoration of a "tttt" stretch in the polypyrimidine tract has no effect. Closed and open arrowheads point to active and inactive 3′ splice sites, respectively. Asterisks indicate artificially mutated nucleotides. (E) Partial displacement of the native branch point (Mt-Br1), disruption of the branch point (Mt-Br2), or both (Mt-Br3) fails to activate the downstream copy of the duplicated 3′ splice sites, whereas swapping of the seven residue segments (Mt-Br4) activates both the upstream and downstream copies.

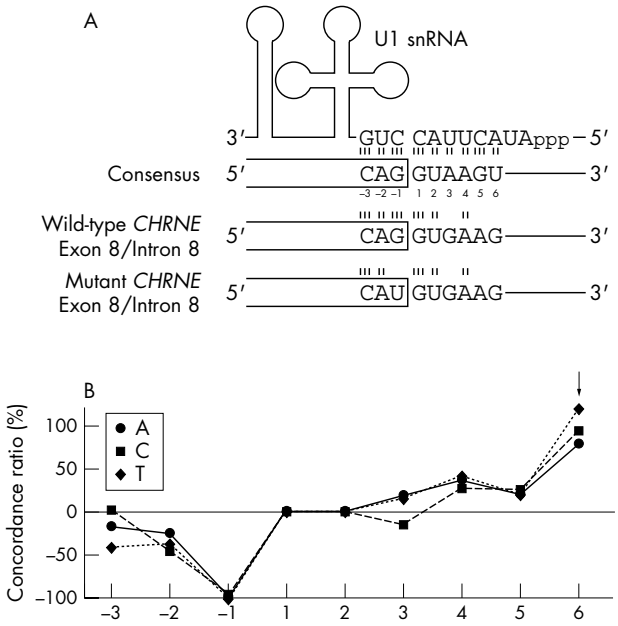

Figure 3 (A) U1 snRNA recognises three nucleotides at the 3′ end of an exon and six nucleotides at the 5′ end of an intron. The complementary nucleotides to U1 snRNA constitute the consensus sequence. Wild type *CHRNE* exon 8/intron 8 has mismatched nucleotides at positions +3, +5, and +6. The 857G→T mutation introduces another mismatch at position −1. (B) A mismatch at the last nucleotide of an exon (position −1) to U1 snRNA is mostly compensated for by a match at position +6 in 1801 human exons. The ratios of A, C, G, and T at position −1 are 8.8%, 3.3%, 80.3%, and 7.5%, respectively, in 1801 human exons.[17] When a concordant G is used at position −1, the ratio of concordant T at position +6 is 38.9%. In contrast, when a discordant T is used at position −1, the ratio of concordant T at position +6 is 85.9%. The concordance ratio was calculated by (85.9–38.9)/38.9 = 121% (arrow). A positive concordance ratio at a specific position indicates that a nucleotide complementary to U1 snRNA is preferentially used to compensate for a mismatch at position −1.

Why does a mutation at the 3′ end of an exon affect pre-mRNA splicing?

Analysis of 1801 human 5′ splice sites[17] revealed that a mismatch at position −1 to U1 snRNA is mostly compensated for by a match at position +6 (fig 3). As *CHRNE* intron 8 has a mismatch at position +6 (fig 1), 857G→T at position −1 probably prevents U1 snRNA from recognising the 5′ splice site of intron 8, and hence causes skipping of exon 8.

To date, 23 splicing mutations of the last nucleotide of an exon have been reported in human to date (table 3). In 17 mutations, the nucleotide at position +6 does not match to U1 snRNA (underlined nucleotides in table 3). In six other mutations that have a matched T nucleotide at position +6, 12 out of 18 nucleotides at positions +3 to +5 are mismatched, whereas in 836 human 5′ splice sites that carry a matched T nucleotide at position +6, only 766 out of 2508 nucleotides are mismatched[17] (Fisher's exact test, $p = 0.003$). These observations also support the idea that a mismatch at position −1 is compensated for by matches at positions +3 to +6, especially at position +6.

Duplication of the 3′ splice site

We constructed and analysed a series of artificial mutants to understand the mechanism by which the duplicated 3′ splice sites resulting from g.IVS10-9_c.1167dup16 silence the downstream copy. Scanning model of recognition of the 3′ splice site indicates that the first "ag" dinucleotide after the branch point is used for splicing catalysis.[8 9] Three exceptions have been reported:[10–12] (*a*) an "ag" dinucleotide less than 13 nucleotides downstream of the branch point is not recognised, probably due to steric effects of *trans* acting elements; (*b*) the first "ag" dinucleotide is hidden in a stable secondary structure; and (*c*) two "ag" dinucleotides that are <12 nucleotides apart compete for being recognised by the splicesome. As the naturally occurring duplication mutant and all artificial mutants except for Mt-Br4 conform to none of the exceptions, they followed the scanning model that favours the first "ag" after the branch point. On the other hand, displacement of a branch point sequence 16 residues downstream (Mt-Br4 in fig 2) placed the "ag" dinucleotide <13 nucleotides downstream of the branch point, and made the upstream copy less competitive than the downstream copy.

Pathogenic duplication of the 3′ splice site has been reported in two other human genes. Both follow the scanning model of recognition of the 3′ splice site. An 18 nucleotide duplication comprising 16 intronic and 2 exonic residues of *HEXB* encoding the β subunit of β-hexosaminidase results in an active upstream copy of the 3′ splice sites.[13] A 69 nucleotide duplication comprising 7 intronic and 62 exonic residues of *SLC4A1* encoding anion exchanger member 1 also results in an active upstream copy of the 3′ splice sites.[14]

Thus, the scanning model of recognition of the 3′ splice site applies to most physiological and pathological duplications of the 3′ splice sites, though exceptions do occur and await explanation.[10 15 16]

Table 3 The 23 previously published splicing mutations at the last nucleotide of an exon

Gene	Exon	Wild type sequence	Mutant nucleotide at −1	Wild type nucleotide at +6
ATM[22]	1	AAGgtagga	A	a
CFTR[23]	2	CAGgtacta	C	a
CPS1[24]	8	AAGgtgcaa	C	a
CYP27[25]	6	GCGgtagga	A	a
FAH[26]	2	CAGgtagga	T	a
HEXA[27]	3	GAGgtaaca	A	a
IL2RG[28]	6	ACGgtgaga	A	a
PKLR[29]	9	GCGgtagga	A	a
PROC[30]	7	CAGgtggga	C	a
COL1A1[31]	6	ATGgtgagc	A	c
COL1A2[32]	6	ATGgtatgc	A	c
COL3A1[33]	3	AAGgtaacc	A	c
CYP27A1[34]	3	AAGgtaccc	C	c
LIPA[35]	8	CAGgtaggc	A	c
LIPA[36]	8	CAGgtaggc	A	c
CDKN2A[37]	2	CAGgtgagg	T	g
UROS[38]	4	AAGgtgagg	T	g
ATM[22]	2	AAGgtatat	A	t
HBB[39]	1	CAGgttggt	C	t
SERPINC1[40]	3	AAGgtgagt	A	t
XPA[41]	3	CAGgtactt	A	t
XPA[41]	4	CAGgtctct	C	t
XPA[42]	5	AAGgtagat	C	t

Nucleotides that do not match to U1 snRNA are underlined, where the matched optimal sequence is "CAGgtaagt". Exonic and intronic nucleotides are indicated by upper and lower case letters, respectively.

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