Supplementary Materials

Evaluation of suspected autosomal Alport Syndrome synonymous variants

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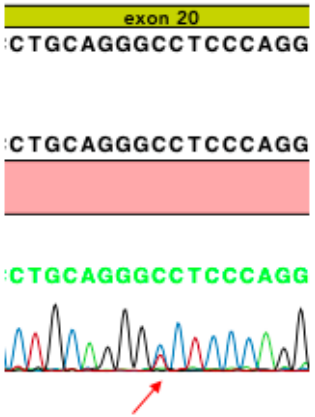
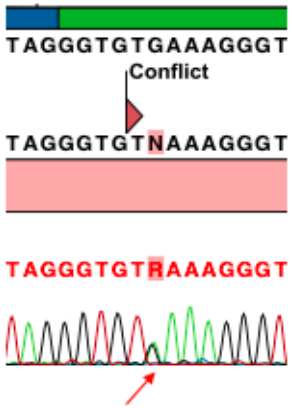
Supplementary Figure 1.

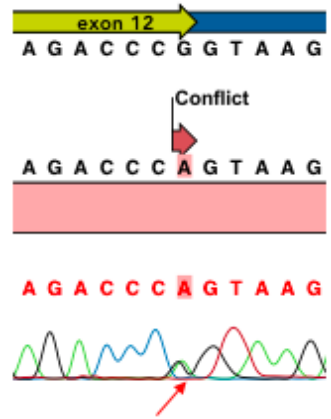
a. Patient No.1 (*COL4A4* c.1353 C>T, exon 20)



b. Patient No.2 (*COL4A3* c.693 G>A, exon 13)



c. Patient No.3 (*COL4A4* c.735 G>A exon 12)



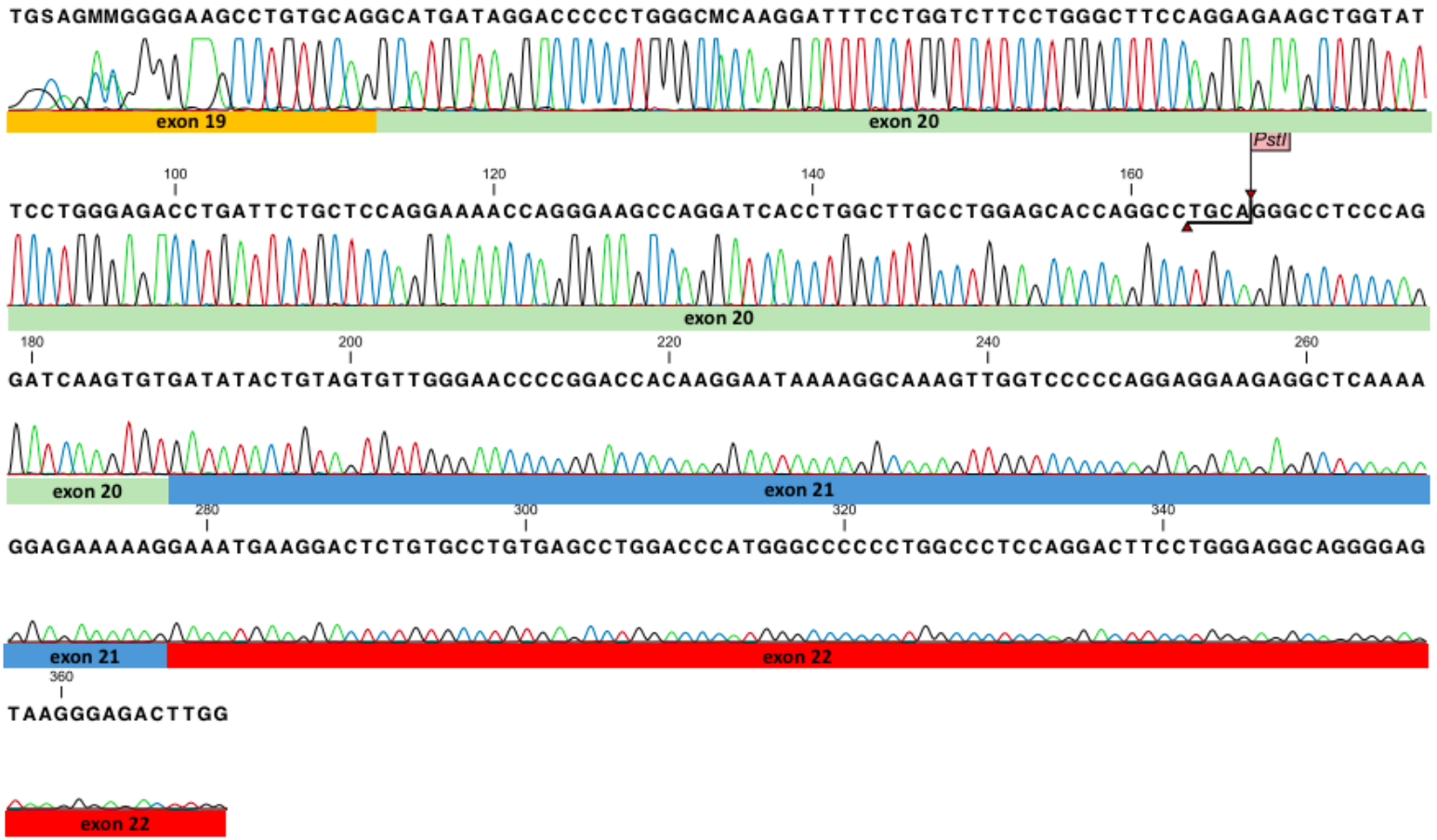
d. Patient No.4 (*COL4A4* c.870 G>A, exon 14)

Supplementary Figure 1. Sanger sequence of Alport synonymous cases. Patient no.3 and no.4 are harboring the synonymous mutation in the last nucleotide of the exon. With this variant, the splice site was broken, yield to exon skipping.

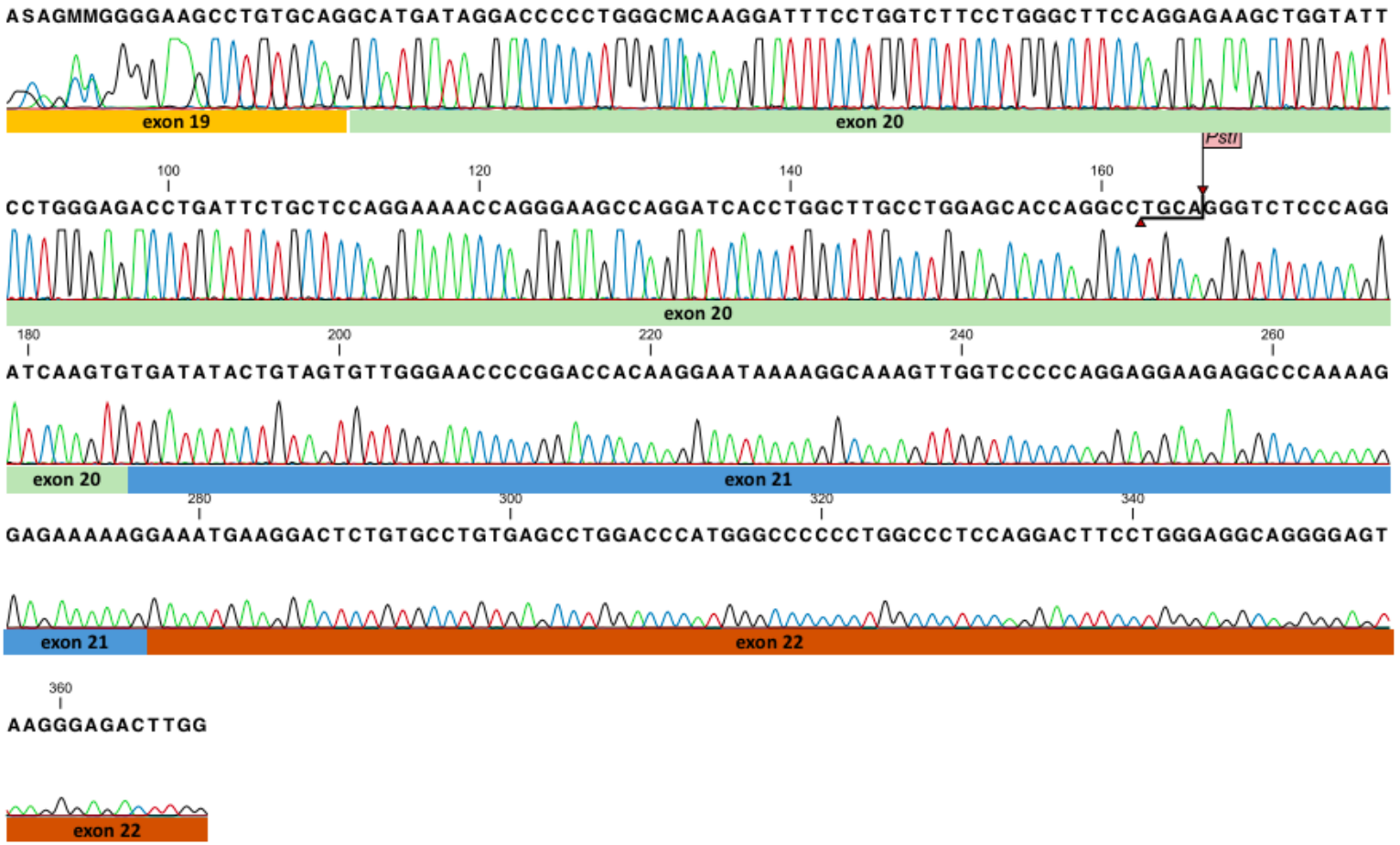
Supplementary Figure 2.

Patient no.1, *COL4A4* c.1353 C>T exon 20

a. Wild type

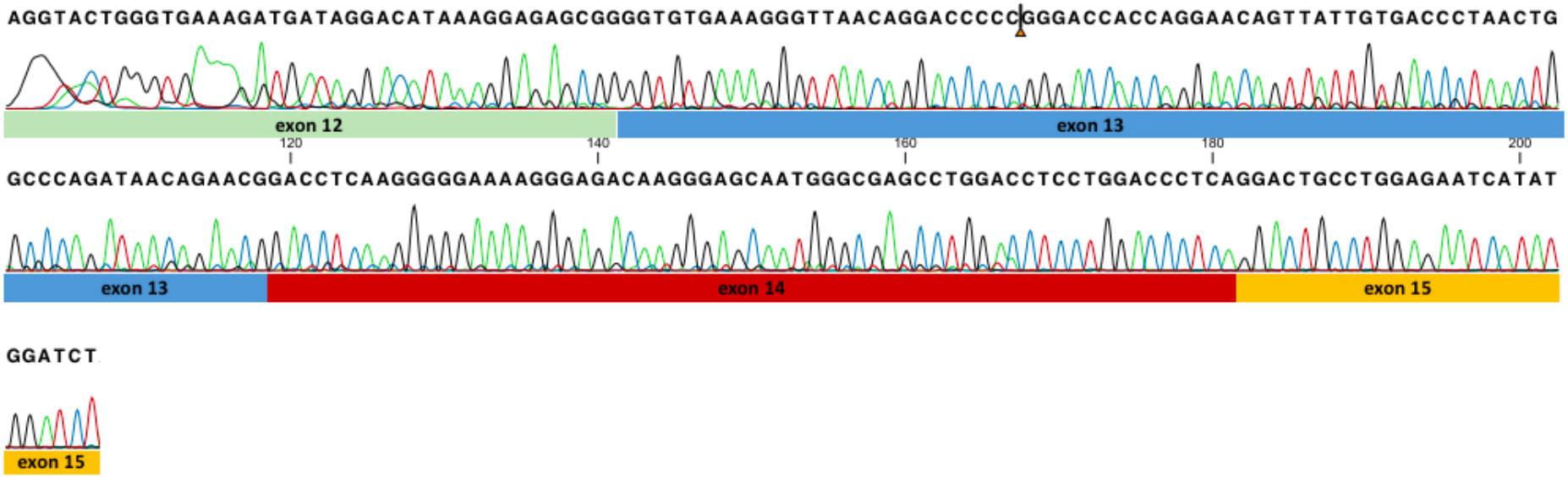


b. Patient

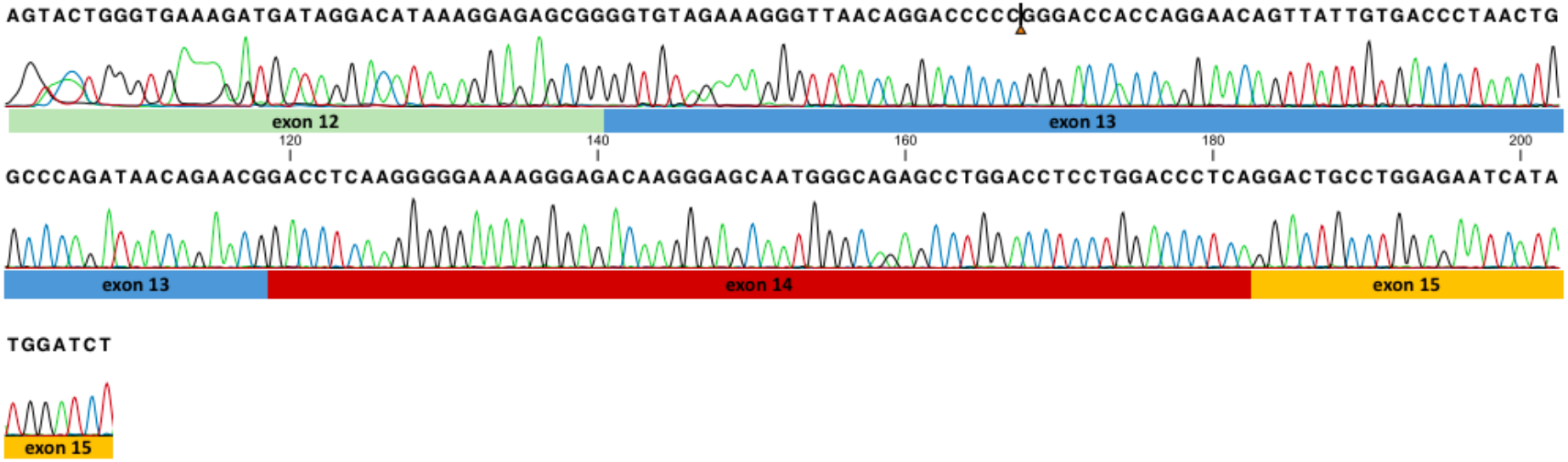

ASAGMMGGGGAAGCCTGTGCAGGCATGATAGGACCCCCTGGGCMCAAGGATTTCCTGGTCTTCCTGGGCTTCCAGGAGAAGCTGGTATT
exon 19
exon 20
PstI
100
120
140
160
CCTGGGAGACCTGATTCTGCTCCAGGAAAACCAGGGAAGCCAGGATCACCTGGCTTGCCTGGAGCACCAGGCCTGCAGGGTCTCCCAGG
exon 20
180
200
220
240
260
ATCAAGTGTGATATACTGTAGTGTTGGGAACCCCGGACCACAAGGAATAAAAGGCAAAGTTGGTCCCCCAGGAGGAAGAGGCCCAAAAG
exon 20
exon 21
280
300
320
340
GAGAAAAAGGAAATGAAGGACTCTGTGCCTGTGAGCCTGGACCCATGGGCCCCCCTGGCCCTCCAGGACTTCCTGGGAGGCAGGGGAGT
exon 21
exon 22
360
AAGGGAGACTTGG
exon 22

Patient No.2, *COL4A3* c.693 G>A exon 13

a. Wild type

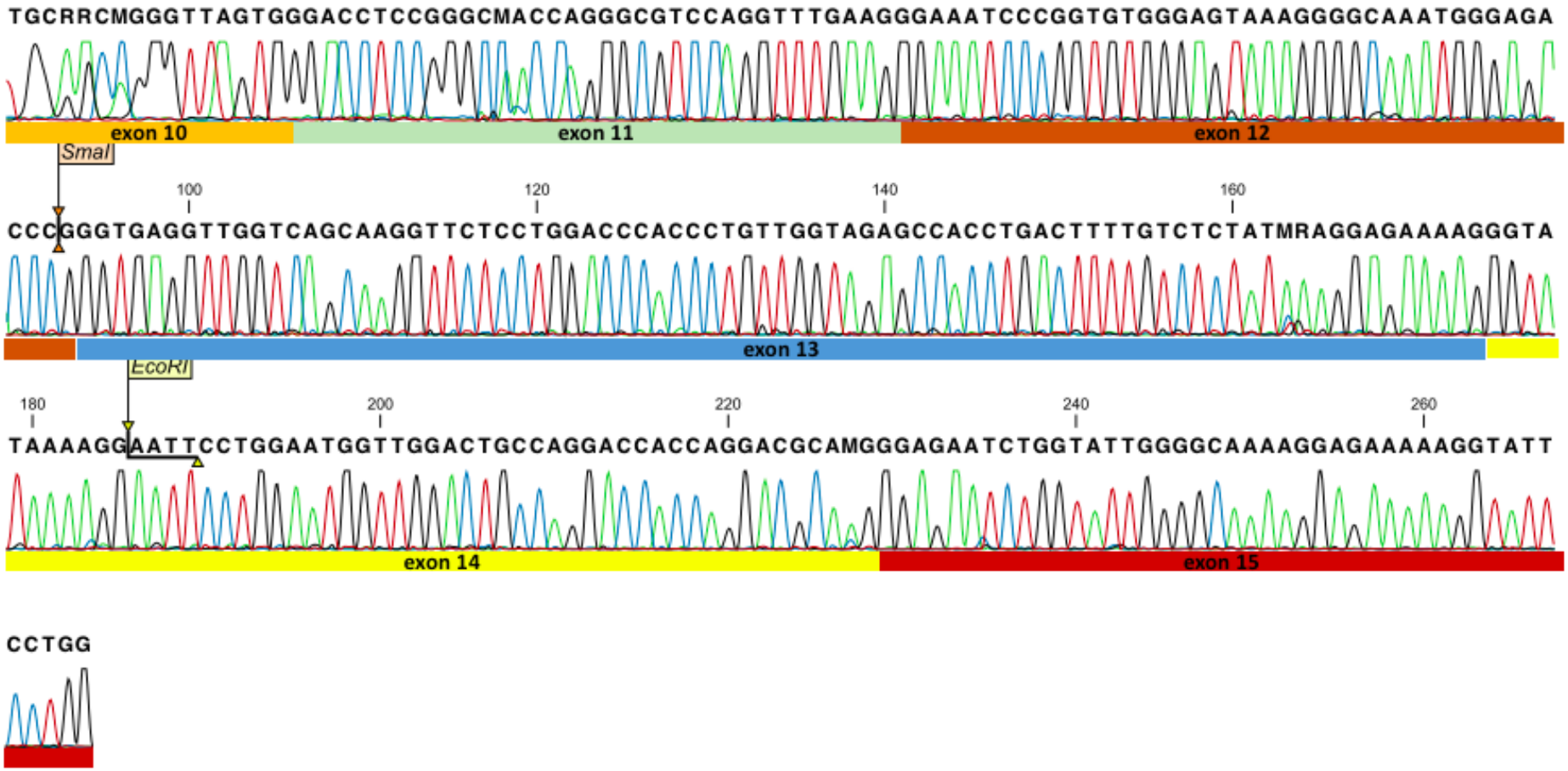


b. Patient

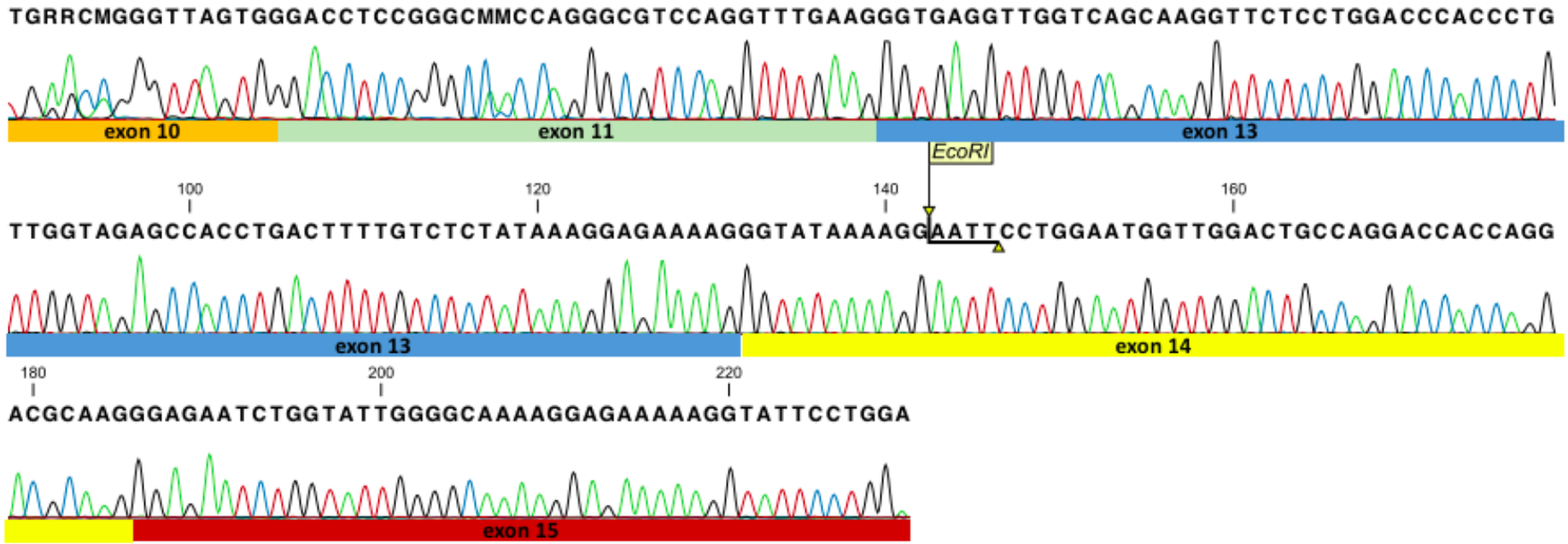

AGTACTGGGTGAAAGATGATAGGACATAAAGGAGAGCGGGGTGTAGAAAGGGTTAACAGGACCCCCGGGACCACCAGGAACAGTTATTGTGACCCTAACTG
exon 12
exon 13
120
140
160
180
200
GCCCAGATAACAGAACGGACCTCAAGGGGGAAAAGGGAGACAAGGGAGCAATGGGCAGAGCCTGGACCTCCTGGACCCTCAGGACTGCCTGGAGAATCATA
exon 13
exon 14
exon 15
TGGATCT
exon 15

Patient No.3, *COL4A4* c.735 G>A exon 12

a. Wild type

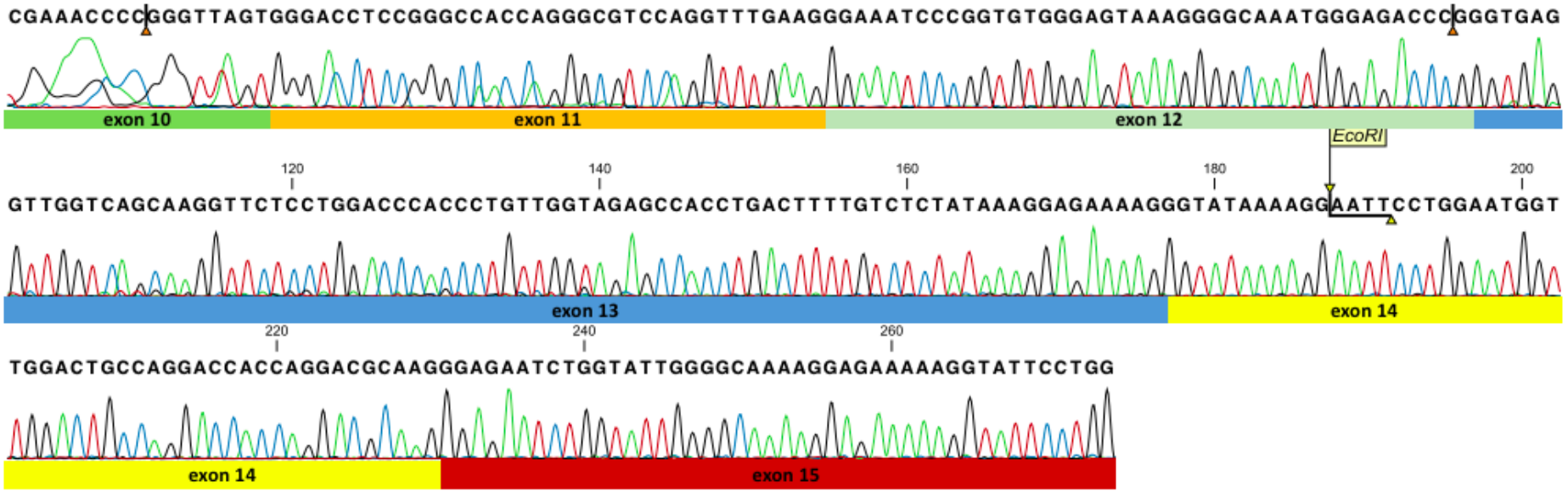


b. Patient

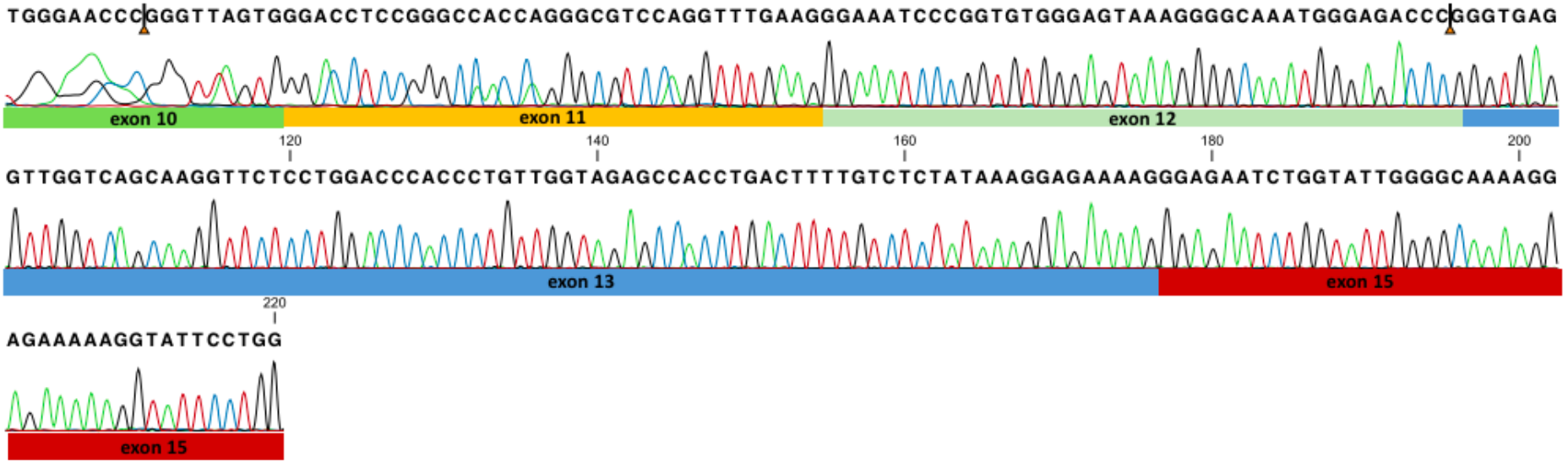

TGRRCMGGGTTAGTGGGACCTCCGGGCMMCCAGGGCGTCCAGGTTTGAAGGGTGAGGTTGGTCAGCAAGGTTCTCCTGGACCCACCCTG
exon 10
exon 11
exon 13
EcoRI
100
120
140
160
TTGGTAGAGCCACCTGACTTTTGTCTCTATAAAGGAGAAAAGGGTATAAAAGGAATTCCTGGAATGGTTGGACTGCCAGGACCACCAGG
exon 13
exon 14
180
200
220
ACGCAAGGGAGAATCTGGTATTGGGGCAAAAGGAGAAAAAGGTATTCCTGGA
exon 15

Patient No.4, *COL4A4* c.870 G>A exon 14

a. Wild type



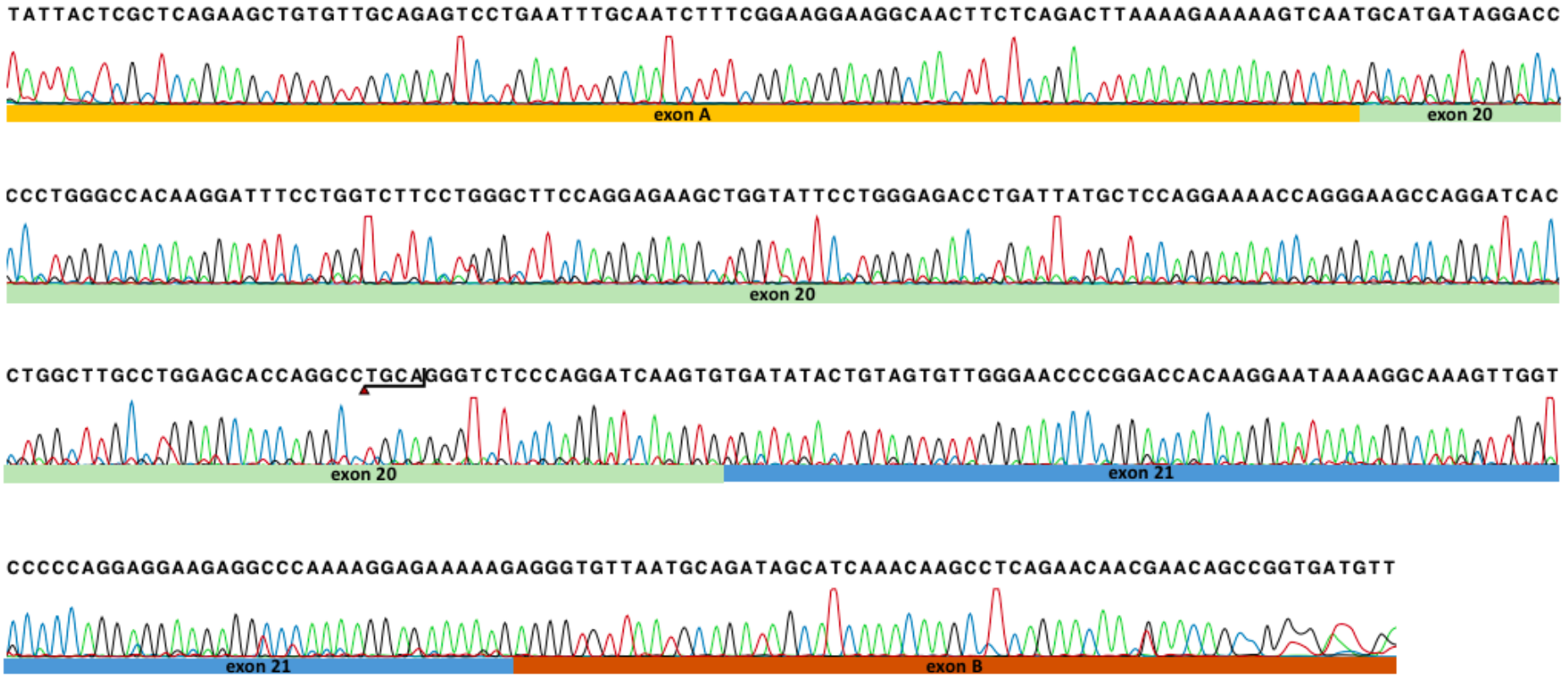
b. Patient


TGGGAACCCGGGTTAGTGGGACCTCCGGGCCACCAGGGCGTCCAGGTTTGAAGGGAAATCCCGGTGTGGGAGTAAAGGGGCAAATGGGAGACCCGGGTGAG
exon 10
exon 11
exon 12
120
140
160
180
200
GTTGGTCAGCAAGGTTCTCCTGGACCCACCCTGTTGGTAGAGCCACCTGACTTTTGTCTCTATAAAGGAGAAAAGGGAGAATCTGGTATTGGGGCAAAAGG
exon 13
exon 15
220
AGAAAAAGGTATTCCTGG
exon 15

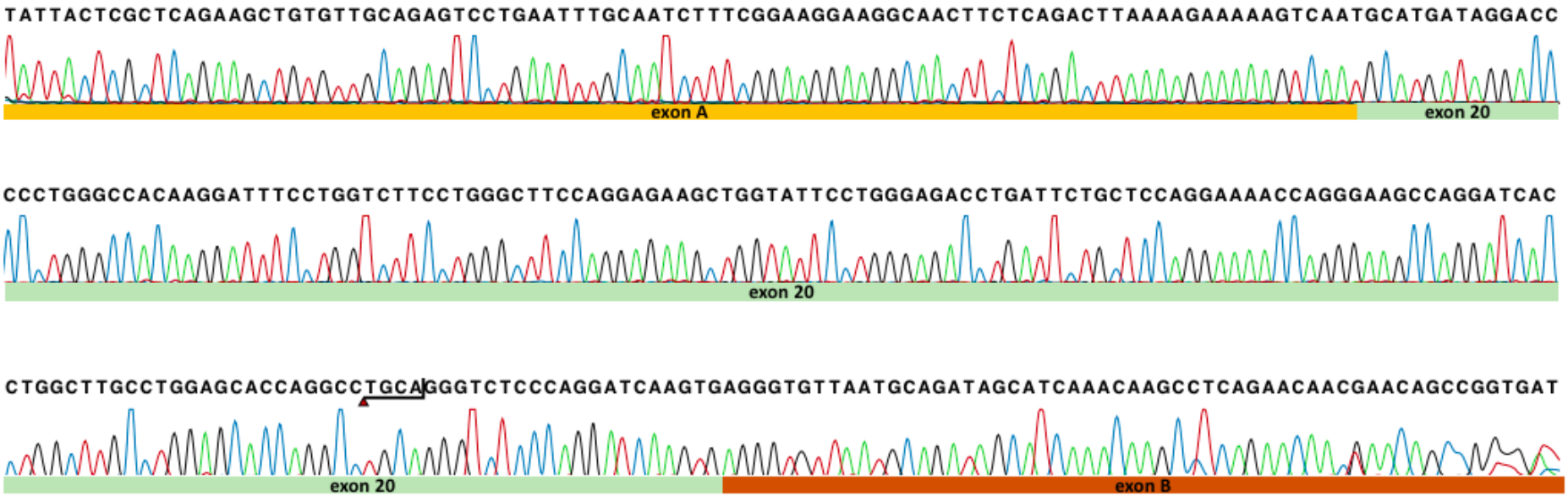
Supplementary Figure 2. *In-vivo* Transcript Sequence of 4 Variants Suspected ADAS. Exon skipping was yield from Patient no.3 and no.4, showed that some of synonymous variants can probably cause disease due to aberrant splicing.

Supplementary Figure 3.

a. Full transcript

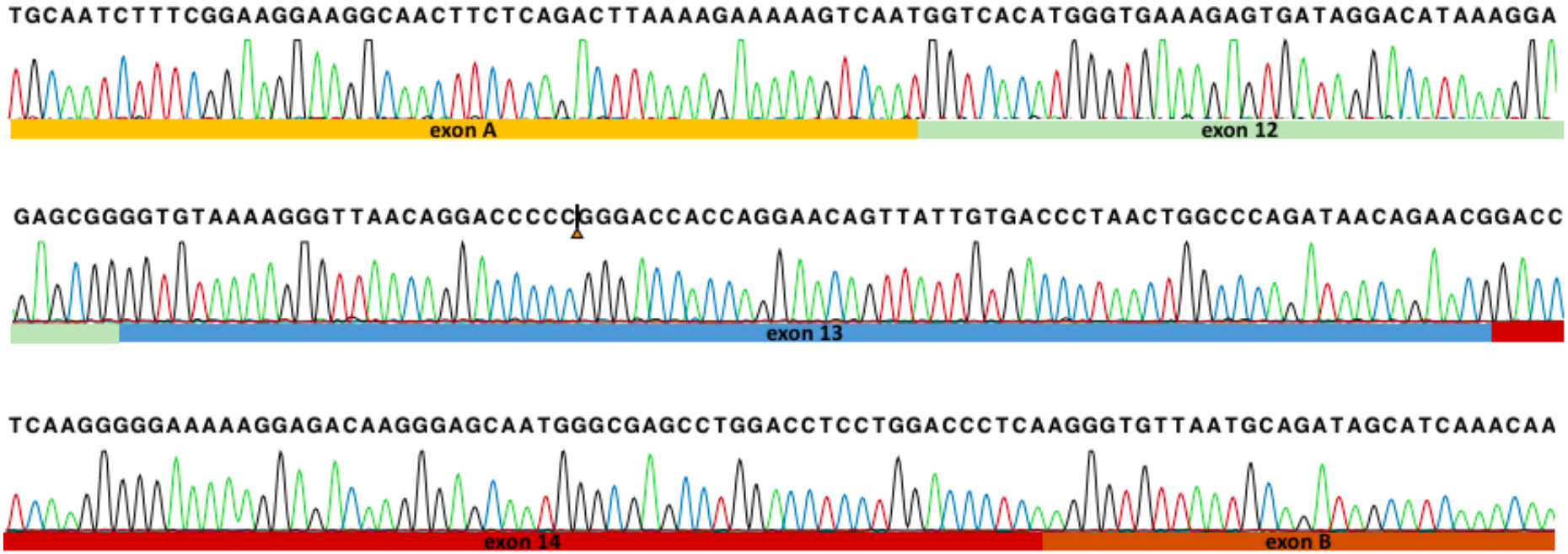


b. Exon 21 skipping



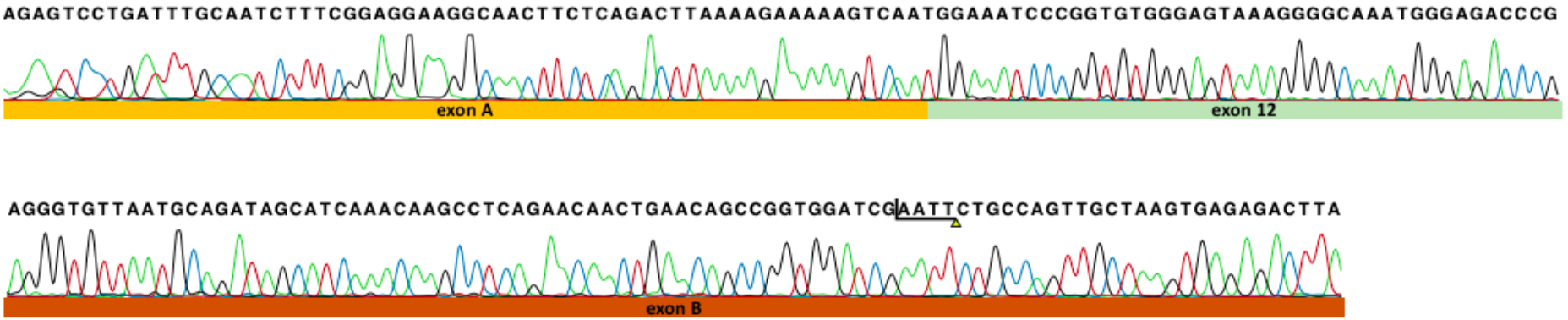
Patient no.2, *COL4A3* c.693G>A

a. Full transcript

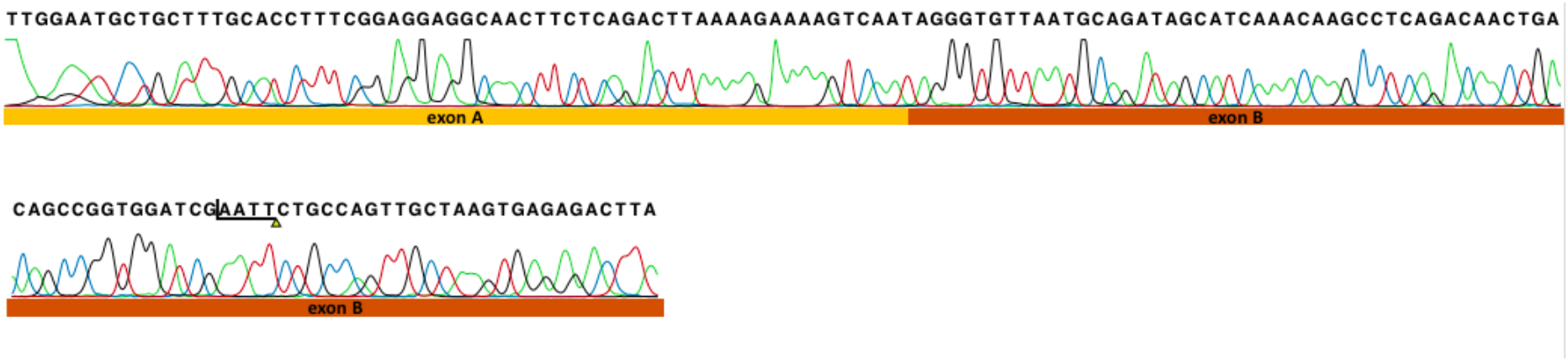


Patient no.3, *COL4A4* c.735 G>A

a. Full transcript

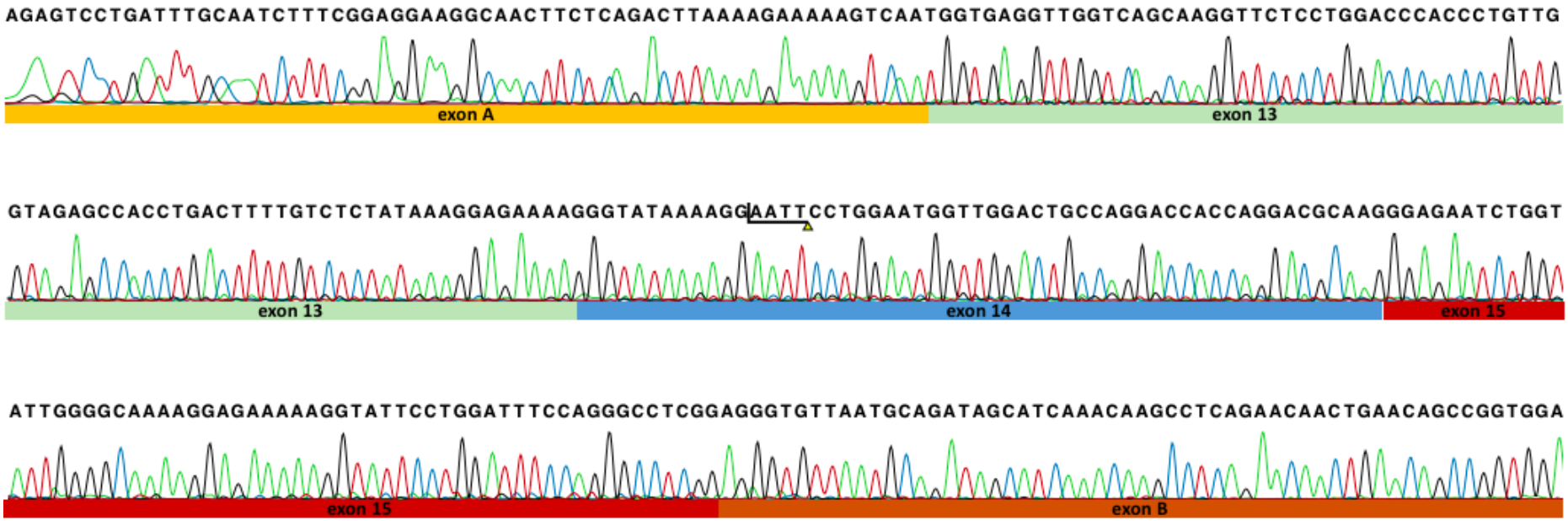


b. Exon 12 skipping

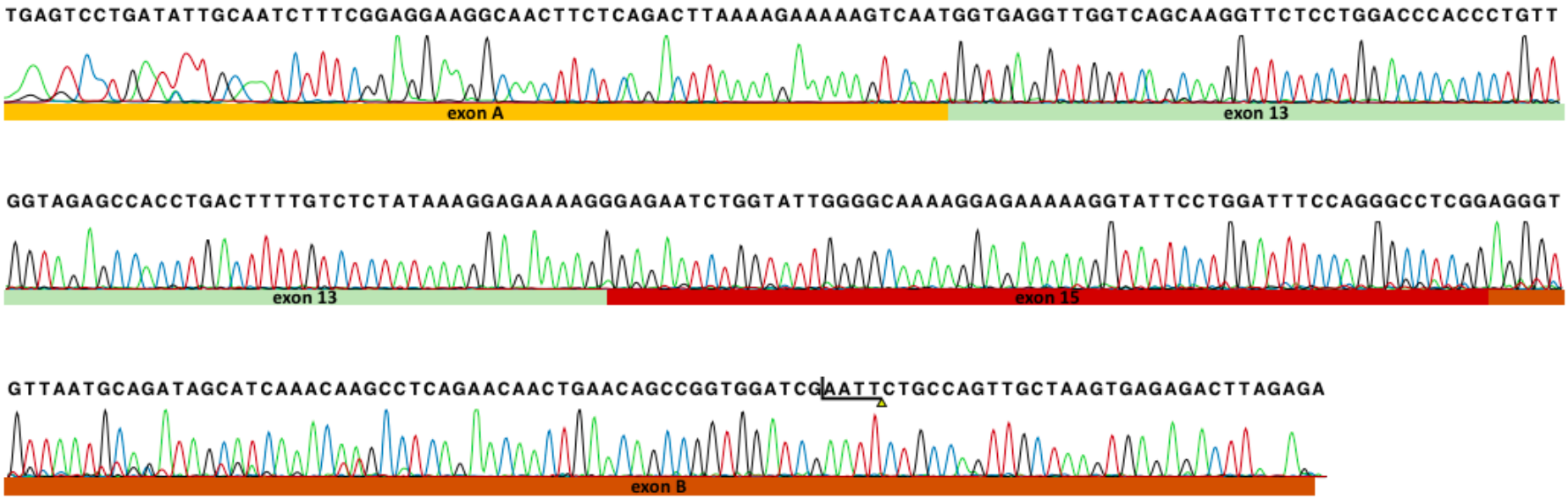


Patient no.4, *COL4A4* c.870 G>A exon 14

a. Full Transcript

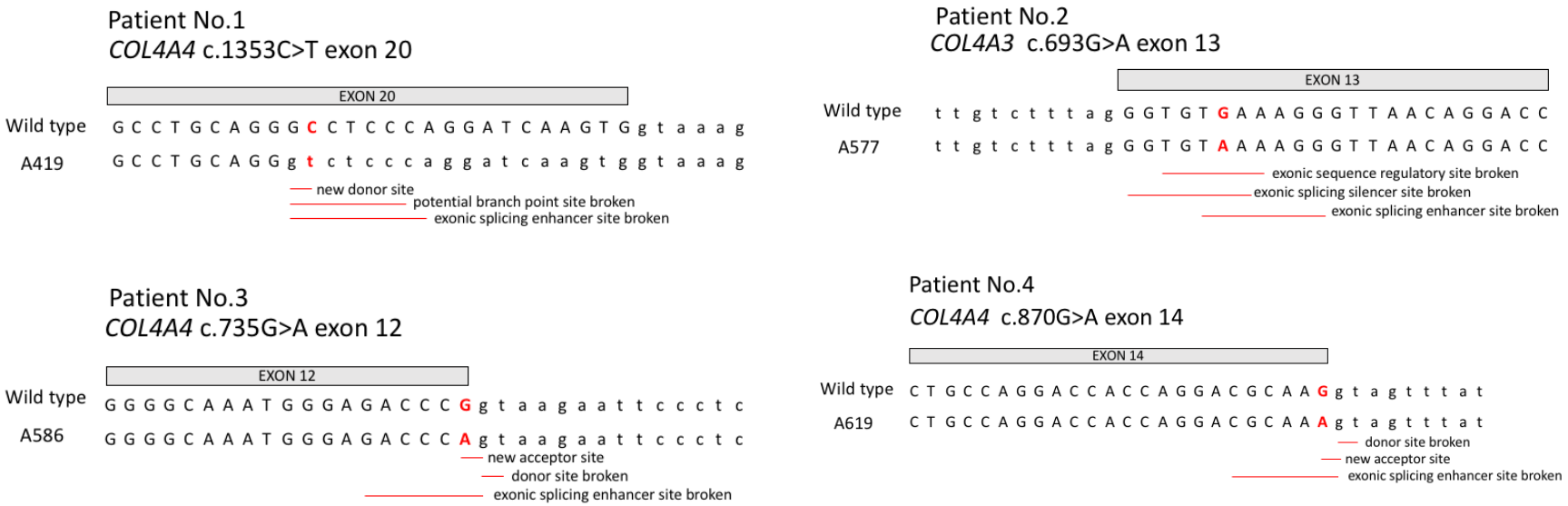


b. Exon 14 skipping



Supplementary Figure 3. Minigene transcript sequence of 4 cases with suspected ADAS. Patient nos.3 and 4 yield aberrant splicing in the form of exon skipping, which was concordance with transcript analysis derived from patient's peripheral blood leukocytes. Aberrant splicing by minigene assay was not confirmed in the remaining case (Patient no.1, and no.2). Exon skipping band was observed in the minigene transcript of Patient nos.1 and 2.

Supplementary Figure 4.



Supplementary Figure 4. Mutations' effect prediction on splicing motifs by Human Splicing Finder (HSF). Prediction of 3 cases (patient nos.1, 3 and 4) may cause aberrant splicing via the disruption of potential splice site (donor, acceptor and potential branch point site). All of synonymous variant in this study broke the exonic splicing enhancer (ESE) site.

Supplementary Figure 5.

A. Patient no.1, *COL4A4* c.1353C>T exon 20

EX-SKIP - Results for submitted sequences

Seq	PESS (count)	FAS-ESS hex2 (count)	FAS-ESS hex3 (count)	IIE (count)	IIE (sum)	NI-ESS trusted (count)	NI-ESS all (sum)	PESE (count)	RESCUE -ESE (count)	EIE (count)	EIE (sum)	NI-ESE trusted (count)	NI-ESE all (sum)	ESS (total)	ESE (total)	ESS/ESE (ratio)
WT	0	0	0	5	65.1889	0	-0.7167	4	3	12	168.1980	18	24.6419	5	37	0.14
MUT	0	0	0	5	64.9103	0	-1.3467	5	3	12	168.1980	18	24.0159	5	38	0.13

Allele WT has a higher chance of exon skipping than allele MUT.

B. Patient no.2, *COL4A3* c.693G>A exon 13

EX-SKIP - Results for submitted sequences

Seq	PESS (count)	FAS-ESS hex2 (count)	FAS-ESS hex3 (count)	IIE (count)	IIE (sum)	NI-ESS trusted (count)	NI-ESS all (sum)	PESE (count)	RESCUE -ESE (count)	EIE (count)	EIE (sum)	NI-ESE trusted (count)	NI-ESE all (sum)	ESS (total)	ESE (total)	ESS/ESE (ratio)
WT	0	3	2	4	52.9747	5	-6.2414	1	1	14	174.3746	13	16.8549	14	29	0.48
MUT	0	2	2	4	35.9131	6	-8.3214	1	0	10	123.5251	10	14.4226	14	21	0.67

Allele MUT has a higher chance of exon skipping than allele WT.

C. Patient no.3, *COL4A4* C.735G>A exon 12

EX-SKIP - Results for submitted sequences

Seq	PESS (count)	FAS-ESS hex2 (count)	FAS-ESS hex3 (count)	IIE (count)	IIE (sum)	NI-ESS trusted (count)	NI-ESS all (sum)	PESE (count)	RESCUE -ESE (count)	EIE (count)	EIE (sum)	NI-ESE trusted (count)	NI-ESE all (sum)	ESS (total)	ESE (total)	ESS/ESE (ratio)
WT	0	4	0	5	94.6212	6	-7.9414	0	0	9	100.9873	5	7.0815	15	14	1.07
MUT	0	4	0	5	94.6212	6	-7.9414	0	0	9	107.2081	5	7.1347	15	14	1.07

Both alleles have a comparable chance of exon skipping.

D. Patient no.4, *COL4A4* c.870 G>A exon 14

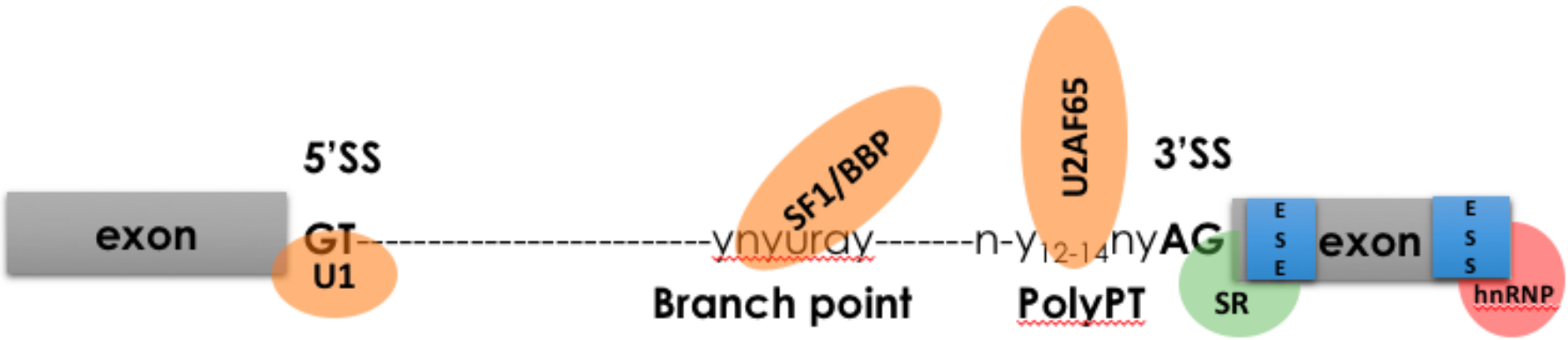
EX-SKIP - Results for submitted sequences

Seq	PESS (count)	FAS-ESS hex2 (count)	FAS-ESS hex3 (count)	IIE (count)	IIE (sum)	NI-ESS trusted (count)	NI-ESS all (sum)	PESE (count)	RESCUE -ESE (count)	EIE (count)	EIE (sum)	NI-ESE trusted (count)	NI-ESE all (sum)	ESS (total)	ESE (total)	ESS/ESE (ratio)
WT	0	1	0	2	34.1477	1	-0.9999	2	2	9	70.6878	18	21.5294	4	31	0.13
MUT	0	1	0	2	34.1477	1	-0.9999	2	2	8	61.3320	18	21.2097	4	30	0.13

Both alleles have a comparable chance of exon skipping.

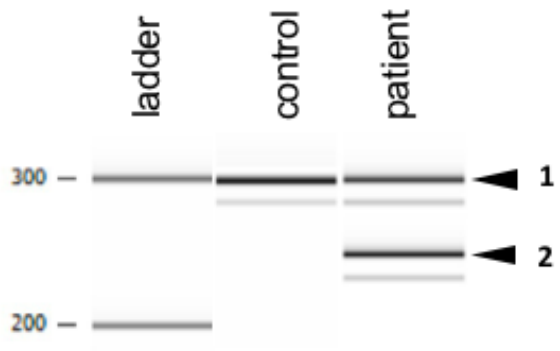
Supplementary Figure 5. *In silico* prediction analysis by the Alamut. Upper panel of each mutations showed splice site prediction modul, integrating a number of predicting algorithms and splicing prediction data with score computed by the corresponding algorithm (SpliceFinder-like, MaxEntScan, NNSPLICE, GeneSplicer, NNSPLICE, known constitutive signals and Mercer et al. high-confidence branchpoints). Higher score indicates a higher probability of a site being a true splice site. The window displays the reference (wild-type) and mutated sequences. Hits from each prediction algorithm are displayed as blue vartical bars for 5' (donor) sites and as green vertical bars for 3' (acceptor sites). The lower panel showed Exonic Splicing Enhancer (ESE) binding site detection by EX-SKIP, quickly determine which exonic variant has the highest chance to skip the exon, by calculating the total number of some ESE signals detection methods. By the Alamut *in silico* prediction, all mutations disrupt the original splice site, and has a probability to yield exon skipping in the mRNA product.

Supplementary Figure 6.



Supplementary Figure 6. Molecular interactions in the early phase of spliceosome assembly. The assembly begins with the binding of U1 to 5'SS of the intron. This interaction is stabilized by the members of SR protein. In addition, this phase also involve s the binding of SF1/BBP to branch point and U2AF65 to the polypyrimidine tract. Together these molecular interactions yield the spliceosomal E complex.

Supplementary Figure 7.



Supplementary Figure 7. *In vivo* mRNA analysis from patient No.4. using High Sensitivity DNA Assay. PCR products of patient's mRNA showed double band, both normal (band No.1) and exon 14 skipping band (band No.2), whereas the control showed single band. (Band No.1 size = 297 bps, band No.2 size = 243 bps, *COL4A4* exon 14 = 54 bps).

Supplementary Table 1. Primer Set for In-vivo transcript PCR analysis

Case No.	Mutation	First PCR primer set	Second PCR primer set
1	*COLA4*	Forward: ACTGGTTGGAGATCCTGGGC	Forward: CTGGTCCCCCAGGTCTCTTG
	c.1353C>T	Reverse: CAGGACCTCTTTCTCCTTTG	Reverse: CCAAGTCTCCCTTACTCCCC
2	*COL4A3*	Forward: TGATGCAAAAGGCGACCCCG	Forward: ATGGGACCTAGAGGACCTAA
	c.693G>A	Reverse: AATGCCATCTTCACCCATTA	Reverse: AGATCCATATGATTCTCCAG
3	*COLA4*	Forward: CATCCTGGGGAAAAGGGAGA	Forward: GGCAGGTCCCACAGGATATC
	c.735G>A	Reverse: ACCAACTTTGCCTTTTATTC	Reverse: CCAGGAATACCTTTTTCTCC
4	*COLA4*	Forward: CATCCTGGGGAAAAGGGAGA	Forward: GGCAGGTCCCACAGGATATC
	c.870G>A	Reverse: ACCAACTTTGCCTTTTATTC	Reverse: CCAGGAATACCTTTTTCTCC

Supplementary Table 2. ACMG[*] classification for synonymous *COL4A3* and *COL4A3* variants

Patient No.	Mutation	Exon	Gene	Criteria	Sequence variant classification
1.	c.1353C>T	20	*COL4A4*	PM1+PM2 PP3+ PP4	Likely pathogenic
2.	c.693G>A	13	*COL4A3*	BS3+BS4	Benign
3.	c.735G>A	12	*COL4A4*	PSV1 PM1+PM2 PP3+ PP4	Pathogenic
4.	c.870G>A	14	*COL4A4*	PSV1 PM1+PM2 PP3+ PP4	Pathogenic

*)Richards, *et al*. *Genet. in Med*. 2015

ACMG guideline provided 2 sets of criteria: pathogenic or likely-pathogenic and benign or likely benign variants. Each pathogenic criterion is weight as very strong (PSV1), strong (PS1–4), moderate (PM1–6), or supporting (PP1–5). Each benign criterion is weighted as stand alone (BA1), strong (BS1–4), or supporting (BP1–6)

Supplementary Table 3. Summary of input, output and interpretation of prediction scores for 5' and 3' splice site prediction*

Tool	Interpretation	Input	Output
Human Splicing Finder	Higher score implies greater potential for splice site	Single sequence ≤5,000 bp	S & S score (0–100)
MaxEntScan	Higher score implies a higher probability of the sequence being a true splice site	Single/multiple sequences (52: 9 bp (−3 to +6); 32: 23 bp (−20 to +3))	Maximum entropy score (log odds ratio)
Splice-Site Analyzer Tool	Higher score implies a more similar ss sequence with the consensus sequence	Single/multiple sequences (52: 9 bp (−3 to +6); 32: 15 bp (−14 to +1))	S & S score (0–100)
NatGene2	Higher score implies a higher confidence of true site	Single sequence (200 bp < length < 80,000 bp)	Confidence score (0–1)
NNSplice	Higher score implies greater potential for splice site	Single/multiple sequences	Score (0–1)
GENSCAN	Higher score implies a higher probability of correct exon	Single sequence ≤1 million bp	Probability score (0–1)
SpliceView	Higher score implies a more similar ss sequence with the consensus sequence	Single sequence ≤31,000 bp	S & S score (0–100)
Hbond	Higher score implies a stronger capability of forming H-bonds with U1 small nuclear RNA	Single/multiple 11 bp sequences (−3 to +8) containing GT in +1/+2 or one genomic sequence	Hbond score
SplicePredictor	Higher value implies greater reliability of the predicted splice site	Single/multiple sequences	*-Value (3–15) determined by *P*, ⟩, and γ values
Automated splice site analyses	Color coded by direction and type of change in Ri	Mutation to be analyzed and the reference sequence	Information contents Ri
SplicePort	Higher score implies a more precise prediction of splice site	Single/multiple sequences ≤30,000 bp	Feature generation algorithm score
CRYP-SKIP	Higher value implies a higher probability of cryptic ss activation as opposed to exon skipping	Single/multiple sequences ≤4,000 bp containing one exon in upper case and flanking intronic sequence ≥4 bp in lower case	Probability of cryptic ss activation (0–1)
SROOGLE	Higher percentile score implies a higher ranking of the ss within precalculated distributions	Target exon along with two flanking introns	Different scores with their percentile scores (0–1)
AASsites	Probable, likely, or unlikely	Single sequence containing the SNP(s) and the Ensembl gene ID to which the SNP(s) belong(s)	Classification of the probability for a change in splicing
Spliceman	Higher percentile rank implies a higher likelihood the point mutation is to disrupt splicing	Single/multiple sequences with one mutation and ≥5 bp in each side of the mutation	L1 distance and percentile rank

SNP, single-nucleotide polymorphism; ss, splice site

*)Jian, Boerwinkle, Liu. *Genet in Med*. 2014