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Original article

Molecular assay for an intronic variant in *NUP93* that causes steroid resistant nephrotic syndrome

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Conflict of Interest Statement

The authors have nothing to disclose.

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Abstract

Advances in molecular genetics have revealed that approximately 30% of cases with steroid resistant nephrotic syndrome (SRNS) are caused by single-gene mutations. More than 50 genes are responsible for SRNS. One such gene is the nucleoporin, 93-KD (*NUP93*). Thus far, few studies have reported mutations of *NUP93* in SRNS. Here, we describe an *NUP93* biallelic mutation in a 9-year-old boy with focal segmental glomerular sclerosis (FSGS). Notably, one mutation comprised an intronic variant; we conducted in vivo and in vitro analysis to characterize this variant. We found two heterozygous mutations in *NUP93*: c.2137-18G>A in intron 19 and a novel nonsense mutation c.727A>T (p.Lys243*) in exon 8. We conducted RNA sequencing and in vitro splicing assays by using minigene construction, combined with protein expression analysis to determine the pathogenicity of the intronic variant. Both RNA sequencing and in vitro splicing assay showed exon 20 skipping by the intronic variant. In protein expression analysis, aberrant subcellular localization with small punctate vesicles in the cytoplasm was observed for the intronic variant. Taken together, we concluded that c.2137-18G>A was linked to pathogenicity due to aberrant splicing. *NUP93* variants are quite rare; however, we have shown that even intronic variants in *NUP93* can cause SRNS. This study provides a fundamental approach to validate the intronic variant, as well as new insights regarding the clinical spectrum of SRNS caused by rare gene variants.

Keywords: minigene assay, pediatric steroid resistance nephrotic syndrome.

1 **Introduction**

2 The most common form of nephrotic syndrome in childhood is idiopathic nephrotic
3 syndrome (INS)¹, which is characterized by massive proteinuria, hypoalbuminemia,
4 edema, and hyperlipidemia². Most children with INS are steroid-responsive and
5 experience favorable outcomes. Approximately 10%–20% of children with INS do not
6 respond to steroid therapy; this disease presentation is known as steroid-resistant
7 nephrotic syndrome (SRNS), and is frequently accompanied by focal segmental
8 glomerulosclerosis (FSGS). Notably, 20%–40% of patients with SRNS progress to end-
9 stage renal disease (ESRD)^{3,4}.

10 Advances in molecular genetics have revealed that approximately 30% of SRNS
11 cases are caused by single-gene mutations, highly expressed in podocytes⁵. More than
12 50 genes are responsible for SRNS⁶. One such gene is the nucleoporin, 93-KD (*NUP93*).

13 The NUP93 protein is a critical component of large molecule assemblies
14 (approximately 125 MDa), so-called nuclear pore complexes (NPCs) embedded in the
15 nuclear envelope⁷. NPCs are large channels that cross the nuclear envelope, which
16 mediate nucleocytoplasmic transport. They consist of multiple copies of nucleoporin
17 (NUP) proteins.⁸ Most NUPs are symmetrically distributed in NPCs, building a
18 symmetric core that is decorated with asymmetric nucleoporins on the nuclear and
19 cytoplasmic ends.⁹ There are two major scaffolding modules in NUPs that compose a
20 symmetric structure, which span the inner and outer nuclear membranes to form a
21 central channel.¹⁰ Those scaffolding modules orchestrate the NUP93 complex by
22 directly or indirectly interacting with other NUPs (NUP53, NUP155, NUP188, and
23 NUP205) at the C-terminal alpha-helical domain of NUP93¹¹⁻¹³. It has been reported
24 that the coiled-coil domain of NUP93 at the N-terminus is essential for recruiting the

1 NUP62 complex, which comprises FG-NUPs rich in FG (Phe and Gly residues)-repeats;
2 this complex modulates the permeability barrier of the pore¹¹. Therefore, NUP93 has a
3 pivotal role in the NPC to organize nuclear pore architecture, the disruption of which
4 induces aberrant NPC assembly and subsequent dysfunction of nucleocytoplasmic
5 transport.

6 Thus far, only two studies have reported mutations of *NUP93* in SRNS^{14,15}. Herein,
7 we describe a *NUP93* biallelic mutation in a 9-year-old boy with FSGS: one mutation
8 was a novel heterozygous truncating variant, whereas the other was a heterozygous
9 intronic variant, the pathogenicity of which was unknown. To confirm the pathogenicity
10 of the intronic variant, we conducted in vivo and in vitro mRNA analysis, as well as in
11 vitro protein expression analysis.

12 **Methods**

14 **Ethical Considerations**

15 All procedures involving human participants in this study were performed in accordance
16 with the ethical standards of the Institutional Review Board of Kobe University
17 Graduate School of Medicine (IRB approval number 301) and with the 1964 Helsinki
18 Declaration and its later amendments or comparable ethical standards. Informed consent
19 was obtained from the parents of the patient.

21 **Patient**

22 The patient was a 9-year-old Russian boy. He exhibited proteinuria at 2 years old, and
23 was diagnosed with SRNS. Histological findings showed FSGS. At the onset,
24 hypoalbuminemia (2.6 g/dL), high serum creatinine (0.88 g/dL) with decreased renal

function (estimated glomerular filtration rate: 53.6 L/min/m² BSA), proteinuria (urine protein creatinine ratio: 2.37), and hematuria were found. The patient exhibited optic nerve atrophy as an extrarenal symptom. There was no family history of kidney diseases (Supplementary Figure 1).

Genomic DNA Analysis

DNA was isolated from a peripheral blood sample by using a QuickGene DNA whole blood kit (Kurabo, Kurashiki, Japan). Next-generation sequencing samples were prepared by using a HaloPlex target enrichment system kit (Agilent Technologies, Santa Clara, CA, USA), in accordance with the manufacturer's instructions. *NUP93* and other podocyte-related gene sequences were determined by targeted sequencing with a next generation sequencer (MiSeq; Illumina, San Diego, CA, USA); variant calling was performed by using a SureCall 4.0 software (Agilent Technologies). Primer pairs were constructed to amplify exons 8 and 20, as well as flanking introns including the variant sequence, which were identified by next generation sequencing. PCR and the direct sequencing method were used to confirm the DNA sequence by using an automated DNA sequencer (3,130 Genetic Analyzer; Thermo Fisher Scientific, Waltham, MA, USA).

RNA Sequence

Total RNA was extracted from peripheral blood leukocytes by using the RiboPure™ Kit (Thermo Fisher Scientific), followed by reverse transcription using RNA to cDNA EcoDry Premix (Double Primed) (Takara Bio, Shiga, Japan). cDNA was amplified with 40 reaction cycles by using a forward primer at exon 15 (5'-

GGAGAAAACATGTTTCTGCGCT-3') and a reverse primer at exon 22 (5'-CAGCAAAGGTAATCAGAGTGCG-3'); agarose gel analysis was then performed. Purified PCR products were subcloned into the pT7Blue T vector (Novagen, Darmstadt, Germany) and subjected to direct sequencing.

To confirm the compound heterozygosity, cDNA was amplified with 40 reaction cycles by using a forward primer at exon 5 (5'-CAGCGAATTCTGCACACAC-3') and a reverse primer at exon 21 (5'-GATGGACTTGTCCTTGAG-3'); agarose gel analysis was then performed. Purified PCR products were subjected to subcloning.

In Vitro Splicing Assay

A hybrid minigene was constructed by using the H492 vector, based on the pcDNA3 mammalian expression vector (Invitrogen, Carlsbad, CA, USA), to mimic *in vivo* splicing.^{16, 17} The vector was linearized by inverse PCR with the YH303 forward primer (5'-GGTACCACAGCTGGATTACTCGCT-3') and the YH304 reverse primer (5'-GTGAGAGACTTAACTGGCTGC-3'). We amplified genomic DNA from both patient and wild-type peripheral leukocytes to create hybrid minigene by using primers for *NUP93* intron 18 to intron 21, which were designed complementary to the ends of the linearized vector by the primer design tool for In-fusion (HD Cloning Kit, Takara Bio). This enabled cloning of the PCR products into the multiple cloning site of the vector, located within an intron between exons A and B. Linearized vector and amplified genomic DNA from both patient and wild-type leukocytes then underwent purification. We designed the in-fusion cloning reaction, then transformed competent *E. coli* HST08 premium competent cells (Takara Bio).

Hybrid minigenes were checked by sequencing, then transfected into HEK293T and HeLa cells by using Lipofectamine[®] 2000 (Thermo Fisher Scientific). Twenty-four hours later, total RNA was extracted from cells by using the RNeasy[®] Plus Mini Kit (QIAGEN, GmbH, Hilden, Germany). One microgram of total RNA was subjected to reverse transcription by using the RNA to cDNA EcoDry Premix (Double Primed) (Takara Bio), and PCR was performed with a forward primer corresponding to a segment upstream of exon A (YH307: 5'-ATTACTCGCTCAGAAGCTGTGTTGC-3') and a reverse primer complementary to a segment downstream of exon B (Y308:5'-CTGCCAGTTGCTAAGTGAGAGACTT). PCR products were analyzed by electrophoresis on a 1.5% agarose gel, followed by Sanger sequencing.

In Vitro Protein Expression Analysis

Protein Expression Analysis

NUP93 cDNAs (NM_001242795.1, isoform 2) from patient and wild-type leukocytes were subcloned into the pcDNA3.1 TOPO vector (Thermo Fisher Scientific). *NUP93* isoform 2 (NM_001242795.1; 696aa, a shorter N-terminus) has an in-frame start codon at exon 5 of *NUP93* isoform 1 (NM_014669.4; 819aa); both isoforms contain the necessary and sufficient C-terminal domain to form NPCs.¹¹ N-terminally V5-tagged *NUP93* isoform 2 was obtained through PCR amplification by using the following primers (forward with V5-tag (underlined sequence): 5'-GCCATGGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACGTCTAGAGCTGAGGAGTACCATCGGGAGTCAA-3', reverse: 5'-GCACTTAATTCATGAGGACCTCCA-3'). Consequently, we obtained three types of plasmids in the pcDNA3.1 TOPO vector: wild-type and variants of truncating

(c.727A>T) and exon 20-skipping (c.2137_2220del). In addition, because only full-length cDNA of isoform 2 (but not of isoform 1) could be cloned from affected individual leukocytes, full-length *NUP93* cDNA (NM_014669.4) from human kidney cDNA (Clontech Laboratories, Mountain View, CA, USA) was cloned into the pAcGFP-1-C1 vector (Clontech Laboratories). We obtained three types of plasmids: wild-type and mutagenesis variants of truncating (c.727A>T) and exon 20-skipping (c.2137_2220del). All plasmids were transfected into HeLa cells and mouse podocytes¹⁸ (immortalized mouse podocytes from Katsuhiko Asanuma, Chiba University, Chiba, Japan) with Lipofectamine[®] 3000. Cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS, followed by blocking with 5% BSA in PBS. The cells were incubated with primary antibodies¹⁴ (rabbit anti-V5 tag Ab, 1:2 000, Abcam, Cambridge, UK; rabbit anti-green fluorescent protein (GFP) polyclonal antibody, 1:200, Abcam; mouse anti-NPC Proteins Ab (MAb414 clone to recognize FG-repeats), 1:5 000, BioLegend, San Diego, CA, USA) and visualized with secondary Abs (AlexaFluor488-conjugated donkey anti-rabbit IgG and AlexaFluor555-conjugated donkey anti-mouse IgG Abs, Thermo Fisher Scientific). Nuclei were counter-stained with Hoechst33342 (Thermo Fisher Scientific) and cell images were obtained by using a LSM710 confocal microscope (Carl Zeiss, Dublin, CA, USA) or a BZ-X700 series microscope (Keyence, Osaka, Japan).

Protein Extraction and Western Blotting

Cell proteins were extracted by addition of a lysis buffer containing RIPA buffer (Wako Diagnostics, Mountain View, CA, USA), protease inhibitor, phosphatase inhibitor (Thermo Scientific) and PMSF solution (Santa Cruz, Dallas, TX, USA). The suspension

was centrifuged at $17\,900 \times g$ and the supernatant containing cellular protein was collected.

For western blotting, Mini-PROTEAN TGX Precast Gels (Bio-Rad, Hercules, CA, USA) were run under standard conditions, with 40 μg loaded in each lane. The gel was placed in transfer buffer and transferred to a polyvinylidene fluoride membrane at 200 mA for 30 minutes. The membrane was rinsed in Tris-buffered saline, followed by rinsing in blocking buffer (block ACE, Bio-Rad) overnight. After rinsing with wash buffer, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit antibody (Cell Signaling Technology, Danvers, MA, USA) for 90 minutes at 1:50 000. After final washing, the membrane was developed using ECL chemiluminescence reagent (Amersham Biotech, Amersham, UK).

In Silico Splicing Assay

We predicted splicing domain strength in the intron 19 variant by using the Human Splicing Finder (HSF) (<http://www.umd.be/HSF3/HSF.shtml>) and SVM-BP Finder (http://regulatorygenomics.upf.edu/Software/SVM_BP/).

Results

Genomic DNA and mRNA Analysis

Next generation sequencing analysis of the patient revealed two heterozygous mutations on *NUP93* (NM_014669.4;**16q13**): in exon 8, c.727A>T, p.Lys243*, and in intron 19, c.2137-18G>A. The same results were obtained from Sanger sequencing analysis (Figure 1). The variant in intron 19 has been reported as rs376379631, with allele frequencies of 0.0001391 (13/9348) in Jewish individuals and 0.0003688 (46/124718)

in non-Finnish European individuals (gnomAD). To confirm splicing abnormality in the intron 19 variant, we conducted transcript analysis and detected exon 20 skipping between *NUP93* exon 19 and exon 21 (Figure 2). This *in vivo* finding suggested pathogenicity of the intron 19 variant. A segregation study could not be performed because the patient's father's DNA sample was not obtained; however, it was confirmed that the intronic variant (c.2137-18G>A), but not the nonsense variant (c.727A>C), was present in the patient's mother (Supplementary Figure 1). In addition, compound heterozygosity was revealed by subcloning of two types of transcripts in the patient's cDNA, which consisted of either nonsense mutation or exon-20 skipping bands (Supplementary Figure 2).

In Vitro Splicing Assay

For further *in vitro* investigation of c.2137-18G>A, hybrid minigenes were constructed by inserting the genomic DNA sequence from exon 19 to 21, with the up- and downstream flanking introns of the *NUP93* gene, into the dedicated exon-trapping vector. The mature mRNA obtained from *in vitro* splicing in HEK293T or HeLa cells was reverse-transcribed into cDNA and amplified with specific primers. PCR products were analyzed in an agarose gel, indicating only one band in the control in both HEK293T and HeLa cells; the size of this band was equivalent to the cDNA sequence yielded from putative *in vitro* splicing. Sequencing analysis of the single band proved that the predicted exons exclusively proceeded to form mature mRNA by splicing of all introns. In comparison, one additional shorter band was detected in the patient in both cell lines; subsequent sequencing analysis demonstrated that in-frame exon 20-skipping was present in the shorter cDNA (Figure 3). Note that much higher content of exon 20-

1 skipping cDNA was observed in HEK293T cells, compared with HeLa cells; this may
2 be because regulatory factors related to splicing differ between tissues or cell types.
3 These results were concordant with the analysis of mRNA extracted from peripheral
4 blood leukocytes, indicating that c.2137-18G>A was the causative variant for exon-20
5 skipping in *NUP93* mRNA.

6 7 **In Vitro Protein Expression Analysis**

8 To address the subcellular localization of NUP93 variants, wild-type and two variants of
9 p.Lys243* and p.Ile713_Ile740del (exon 20-deletion) were overexpressed in HELA
10 cells (Figure 4 for isoform 2, **Supplementary Figure 3** for isoform 1) and mouse
11 podocytes (**Supplementary Figure 4** for isoform 1). As expected, expression of
12 truncating variant pLys243* was scarcely observed, proving obvious pathogenicity.
13 Subcellular localization of the exon 20-deleted variant was observed in a punctate
14 pattern, represented as small vesicles scattered throughout the cytoplasm, whereas the
15 wild-type protein was primarily restricted to the nuclear membrane. These results
16 suggested that the aberrant expression and subcellular localization of NUP93 were
17 induced by the loss of exon 20, despite its small size with respect to the in-frame
18 deletion. Western blotting revealed that mutant cDNAs of *NUP93* showed reduced
19 expression of NUP93 protein level in HELA cells (**Supplementary Figure 5**). These data
20 implied that the compound heterozygous mutation of c.727A>T (p.Lys243*) and
21 c.2137-18G>A in the *NUP93* gene could be involved in the onset of SRNS in the
22 affected individual.

23 24 **In Silico Analysis of Intron 19 Variant**

To predict the influence of the intronic variant (c.2137-18G>A) on consensus splice sites and enhancer elements, we conducted in silico analysis. The Human Splicing Finder showed that c.2137-18G>A led to no significant alterations on the consensus 5' and 3' splice sites (reference score: 7.92; mutation score: 7.18; variation: -9.34%, **Supplementary Figure 6**). With regard to the potential branch point algorithm, no branch point motif was found in intron 19. The intronic enhancer motifs extracted by ESE finder matrices and predicted ESE octamers demonstrated that the intronic splicing enhancer¹² motif in intron 19 was predicted to be broken (variation -100%) (**Supplementary Figure 7**). The SVM-BP Finder showed no difference in polypyrimidine tract length and score between affected individual and control (**Supplementary Figure 8**). These analyses indicated that the splicing alteration might occur as a result of ISE aberration, but not aberrations of splice sites, branch points, or the polypyrimidine site, which highlights the need to clarify the pathogenicity of the variant by further biological investigation (**Supplementary Figure 9**).

Discussion

In this study, we found two heterozygous mutations on *NUP93* in a patient with SRNS. One mutation was a nonsense variant, and its pathogenicity was obvious. To verify the pathogenicity of the intronic variant (c.2137-18G>A), it was necessary to define the aberrant splicing by using mRNA from the patient's sample. This analysis revealed that the intronic variant caused exon skipping. The intronic variant was not localized on an apparent splice site, such as a splicing consensus site or branch point. Moreover, it did not change polypyrimidine tract sequences. Therefore, we conducted an in vitro splicing assay to confirm aberrant splicing by the intronic variant. As was expected, the result

1 showed exon 20-skipping that was identical to the RNA sequencing result obtained with
2 the patient's sample.

3 The reverse transcript product obtained from mature mRNA in HELA cells by
4 minigene assay revealed a lower amount of the exon-20 skipping band than that in
5 HEK293T cells; this might have been because of differences in splicing regulatory
6 factors between the cell lines. This is a notable limitation of the in vitro splicing assay in
7 our study. To form mRNA, introns must be removed by splicing; then, exons must be
8 ligated together by the molecular "tailor" of the cell, the spliceosome¹. During splicing,
9 the spliceosome must manage a number of challenges, including matching of correct
10 conserved sequence elements of the pre-mRNA. The consensus sequences are the 5'
11 splice site (n/GUAUGU), branch point sequence, polypyrimidine tract, and 3' splice site
12 (YAG/n)¹⁹. Mutations involving variant GT and AG nucleotides in the 5' and 3' splice
13 sites most frequently yield human diseases. These dinucleotides are fundamental for
14 exon definition and appropriate splicing. However, mutations occurring at positions
15 distal to the 5' and 3' splice sites can also lead to aberrant splicing, and typically lead to
16 exon skipping. In this study, we found that a single base substitution between 5' and 3'
17 splice site boundaries (c.2137-18G>A) led to exon skipping²⁰⁻²².

18 By in silico analysis, the intronic variant did not affect the splicing consensus site,
19 branch site, or polypyrimidine tract; however, it disrupted the SF2/ASF binding site, in
20 which the ISE protein works. This may be the cause of exon skipping by the intronic
21 variant, c.2137-18G>A (Supplementary Figure 7, 9). When we evaluated the
22 pathogenicity of the two variants in accordance with the American College of Medical
23 Genetics and Genomics guideline, both variants demonstrated very strong evidence of

1 pathogenicity (intron 19 variant: PSV1+PS3, PS4+PM2; exon 8 variant: PSV1+PS3),
2 justifying classification as pathogenic variants.²⁴

3 One study reported six individuals with *NUP93* mutations who developed SRNS at
4 onset ages between 1 and 6 years old, and who progressed to ESRD between the ages of
5 1 and 11 years old with FSGS as the primary histological finding¹⁴. Compared with our
6 patient, the phenotypes are almost identical. To confirm the pathogenicity of the
7 intronic variant in the relevant cell type (i.e., kidney cells), we repeatedly attempted to
8 transfect hybrid minigene constructs into podocytes. However, we were unsuccessful
9 because of the low transfection efficacy of podocytes²³; this was another important
10 limitation of our study.

11 In conclusion, by the use of some simple molecular techniques, we defined the
12 pathogenicity of a very rare intronic variant in *NUP93*. In both clinical and laboratory
13 settings, this assay can be used to clarify the pathogenicity and disruption of complex
14 splicing mechanisms, as well as to yield clues regarding the underlying causes of rare
15 diseases.

16

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3

4 **Conflict of Interest**

5 The authors have nothing to disclose.

6

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- 23

24

Titles and Legends to Figures

Figure 1

Nucleotide changes in *NUP93*. Two heterozygous single-base substitutions of A to T at exon 8 (c.727A>T) (**A**) and G to A at intron 19 (c.2137-18G>A) (**B**) were detected. With this intronic variant, the splice site was broken, yielding exon 20-skipping.

Figure 2

Transcript abnormality in *NUP93*. Left panel. Electrophoresis results using mRNAs from control and patient peripheral blood leukocytes. PCR products of the patient's mRNA showed double bands, whereas the control showed a single band. Right panel. Direct sequences after subcloning. The patient's sample showed both normal and exon 20-skipping sequences. The control sample showed only the normal sequence.

Figure 3

RT-PCR-amplified products of hybrid minigene transcripts. Electrophoresis results. The PCR product of the control sample showed one band. The patient's sample from both cell lines showed two bands: one was the full length of the minigene construct and the other was shorter, with exon 20-skipping (lower panel).

Figure 4

In vitro protein expression analysis in the *NUP93* variant. Subcellular localization of overexpressed *NUP93* isoform 2 in HELA cells. Wild type nucleoporin 93-KD was clearly expressed surrounding the nuclear membrane. However, for p.I714_741 deletion, the nucleoporin protein was expressed in the cytoplasm in a particulate manner,

1 indicating abnormal protein localization. For p.Lys243*, no expression was observed
2 because of the truncating mutation. NPC: nuclear pore complex protein
3
4
5

Figure 1

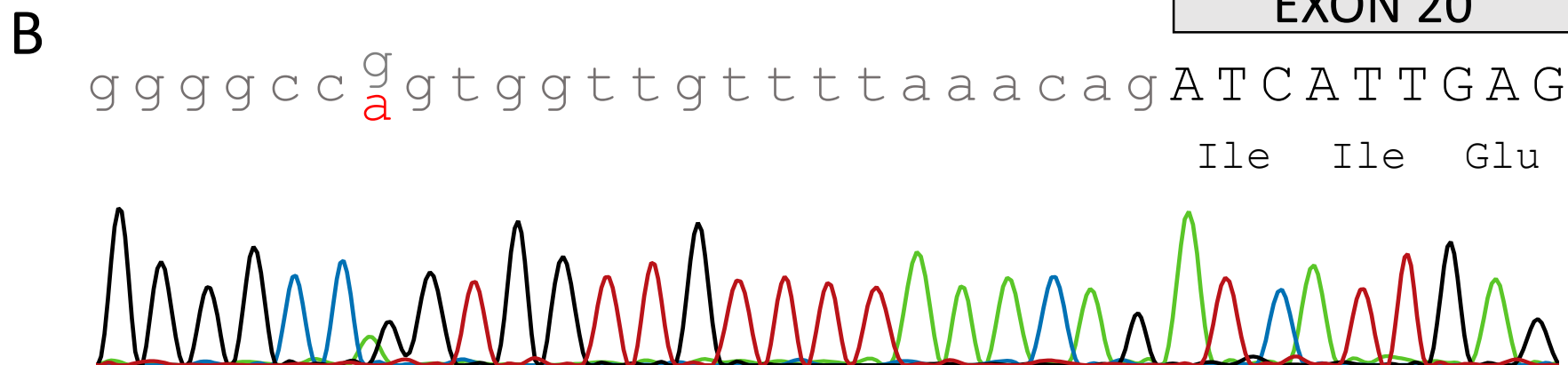
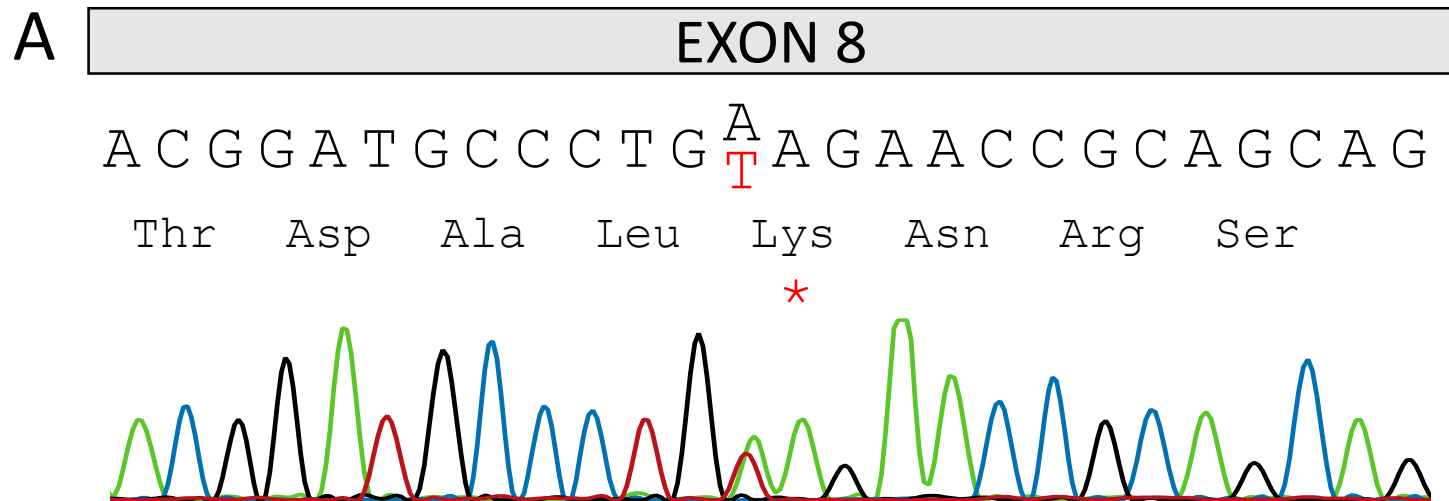


Figure 2

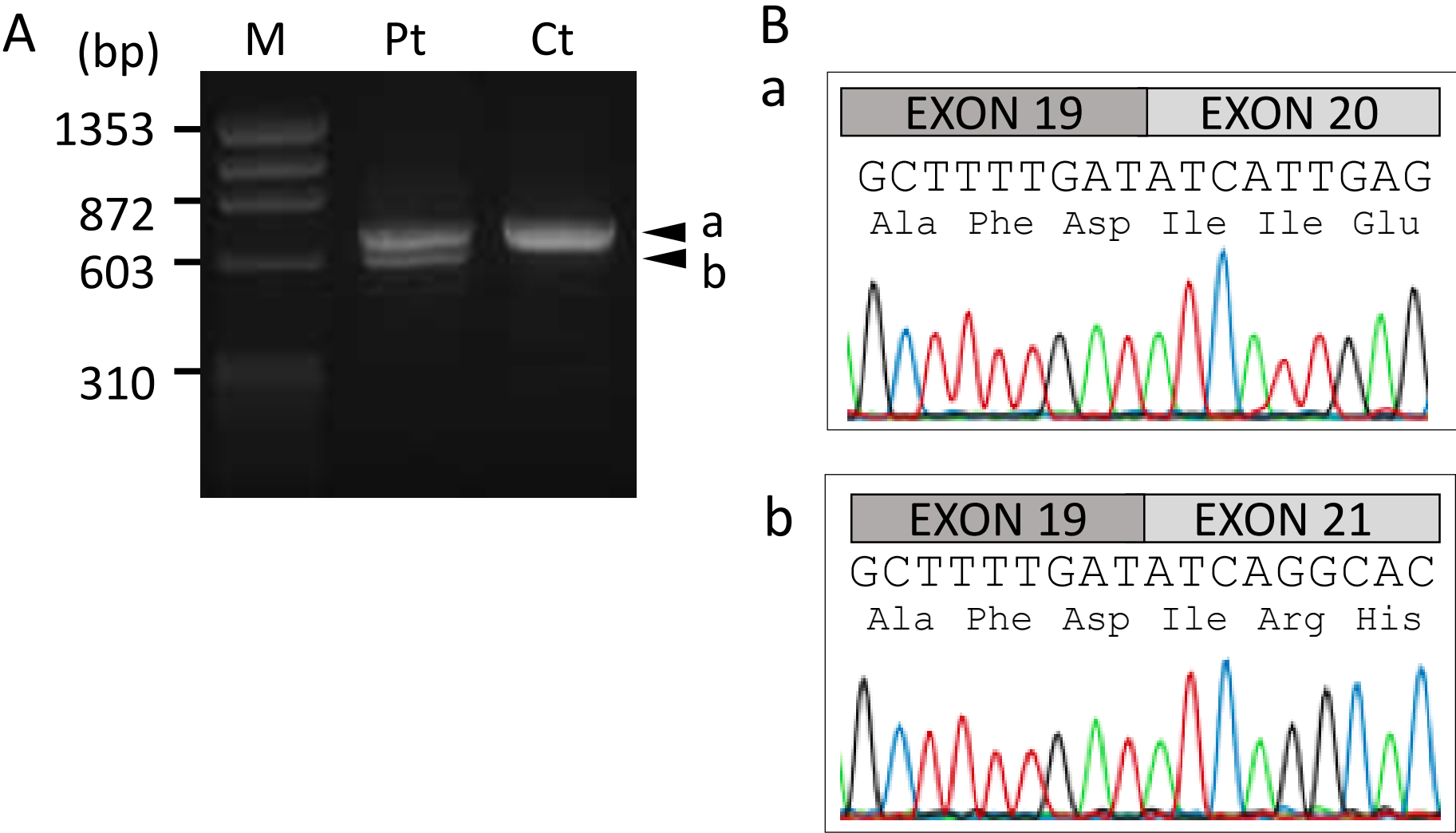


Figure 3

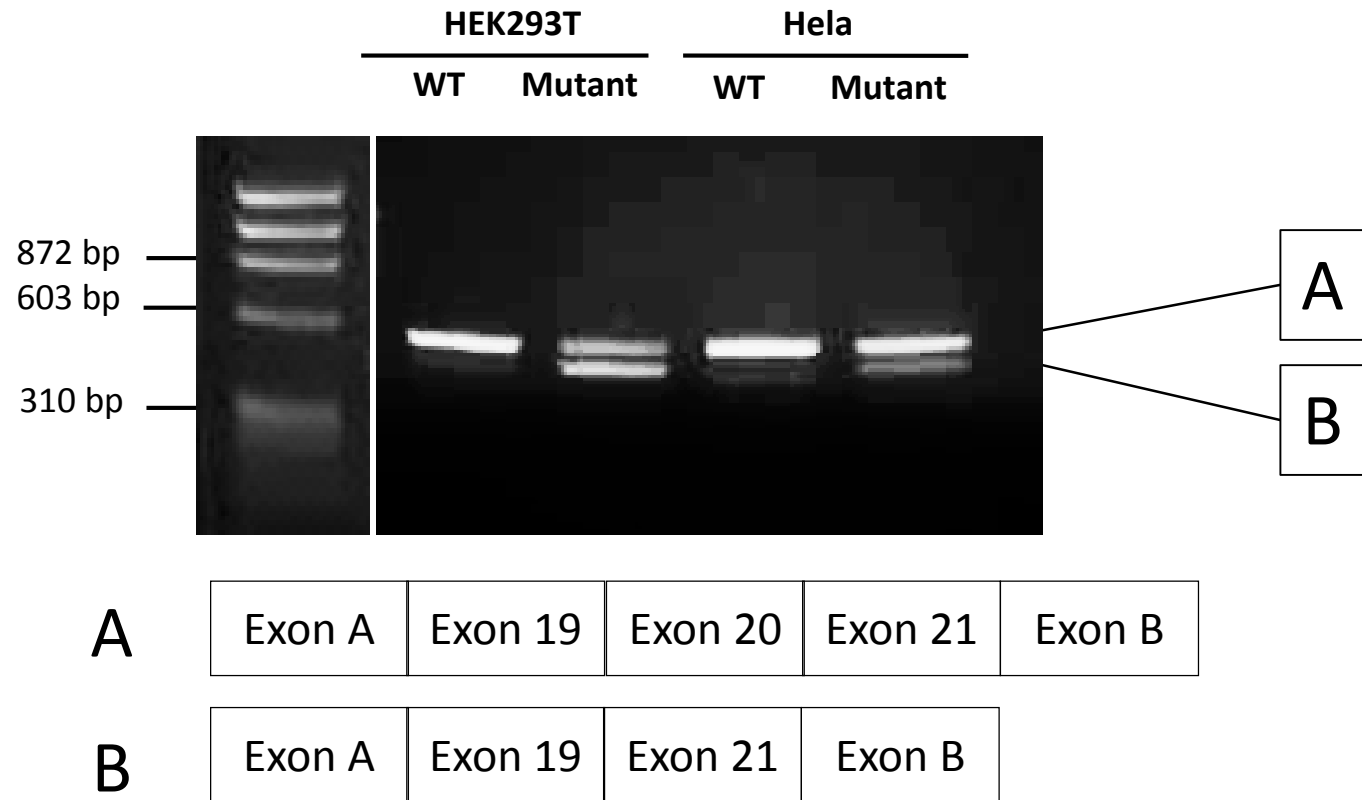
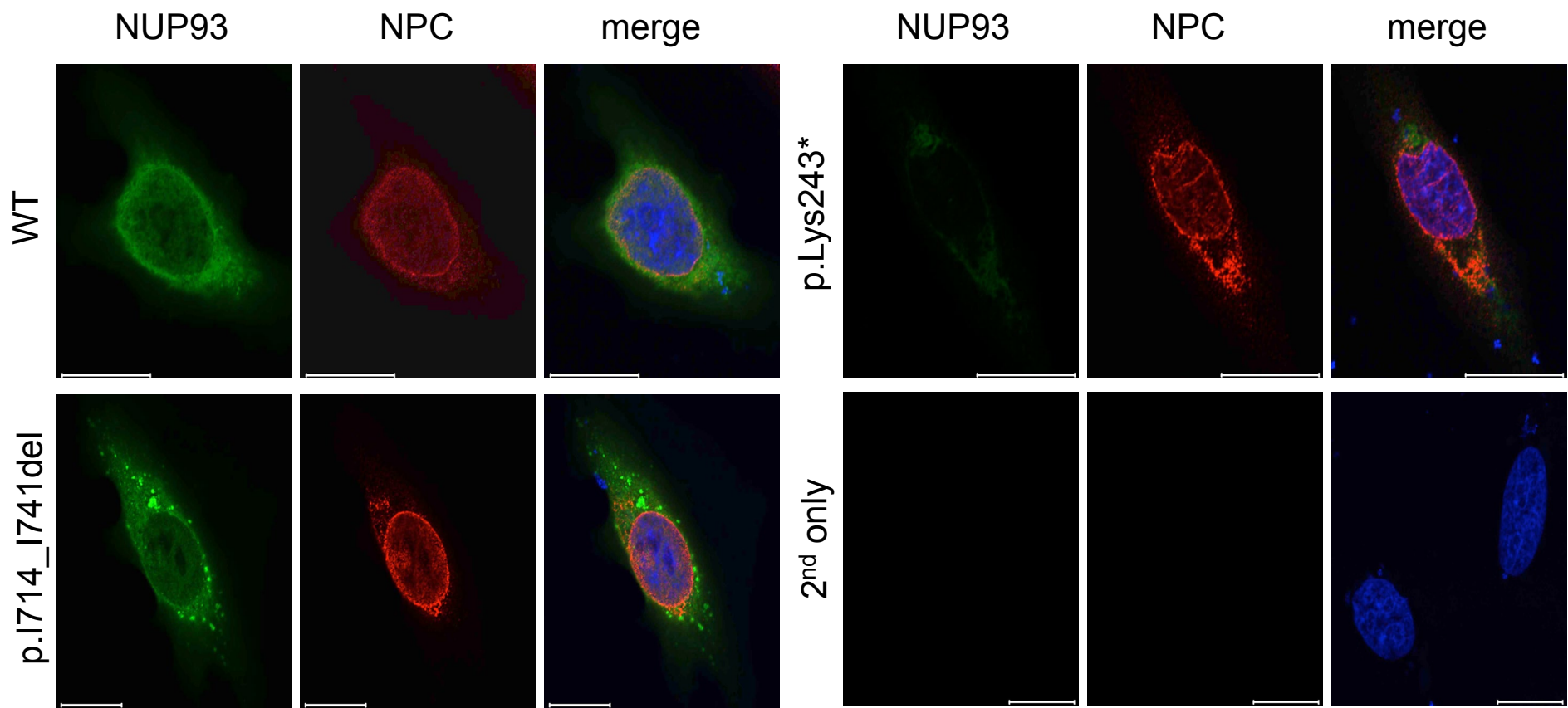
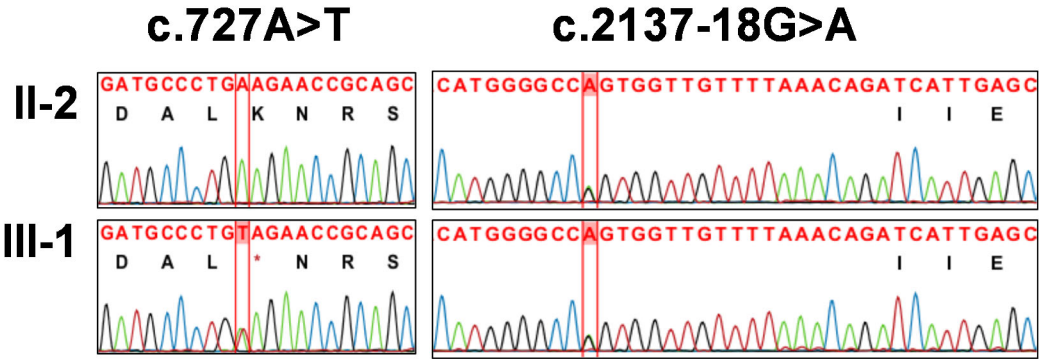
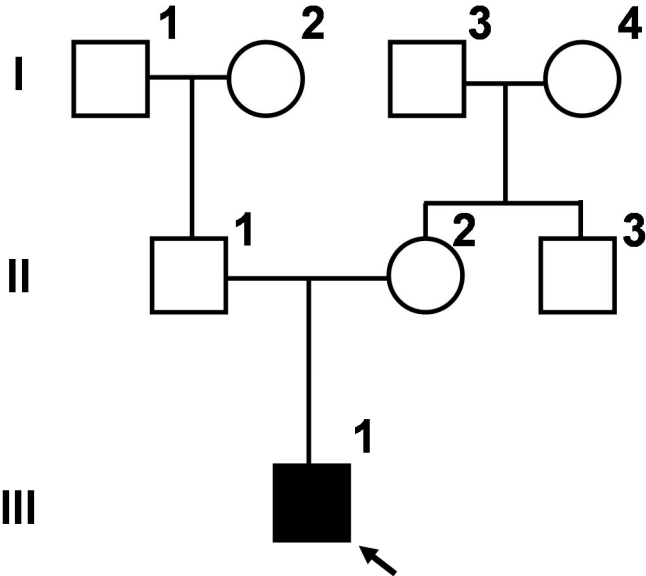


Figure 4

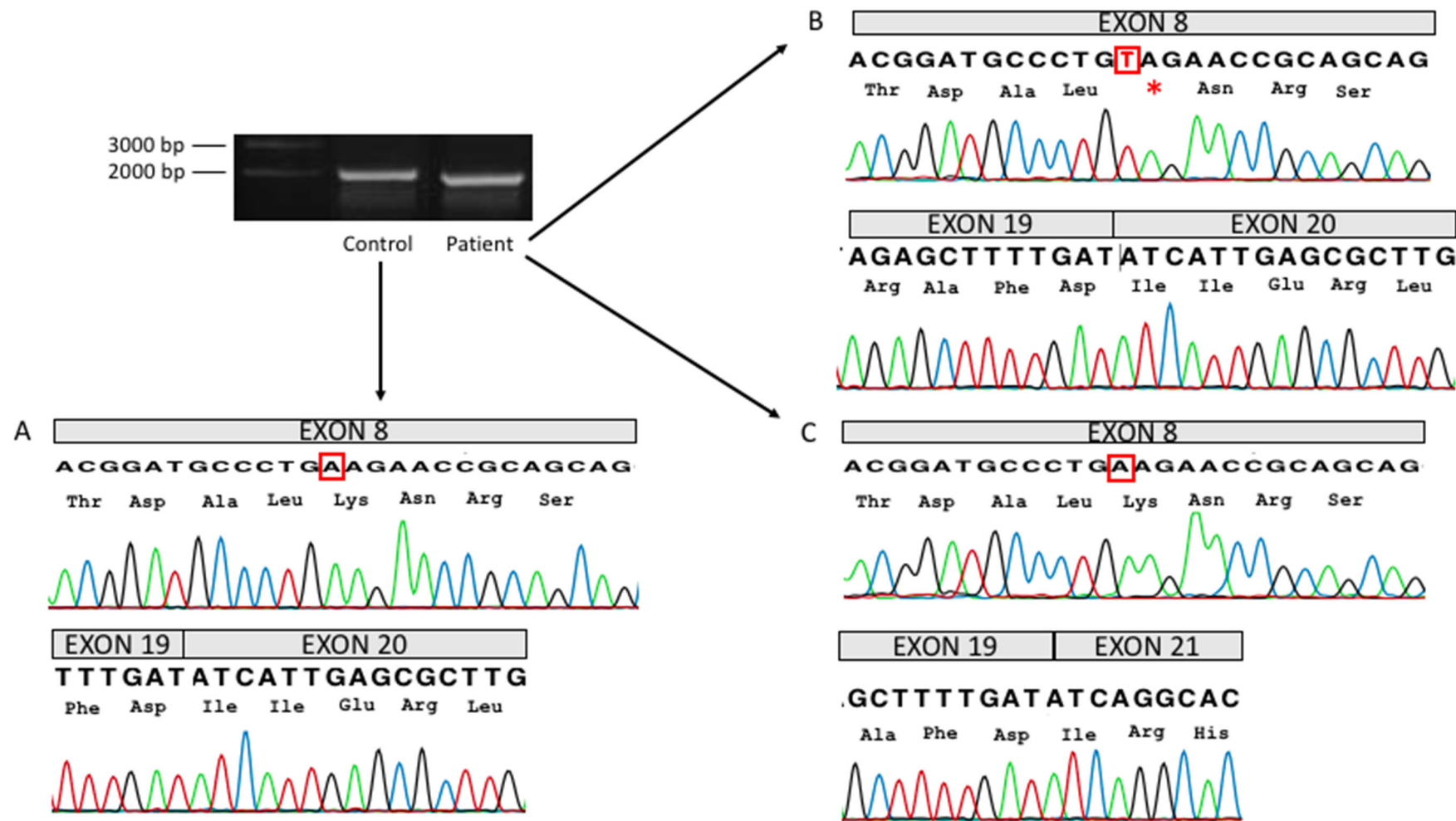


Supplementary Data

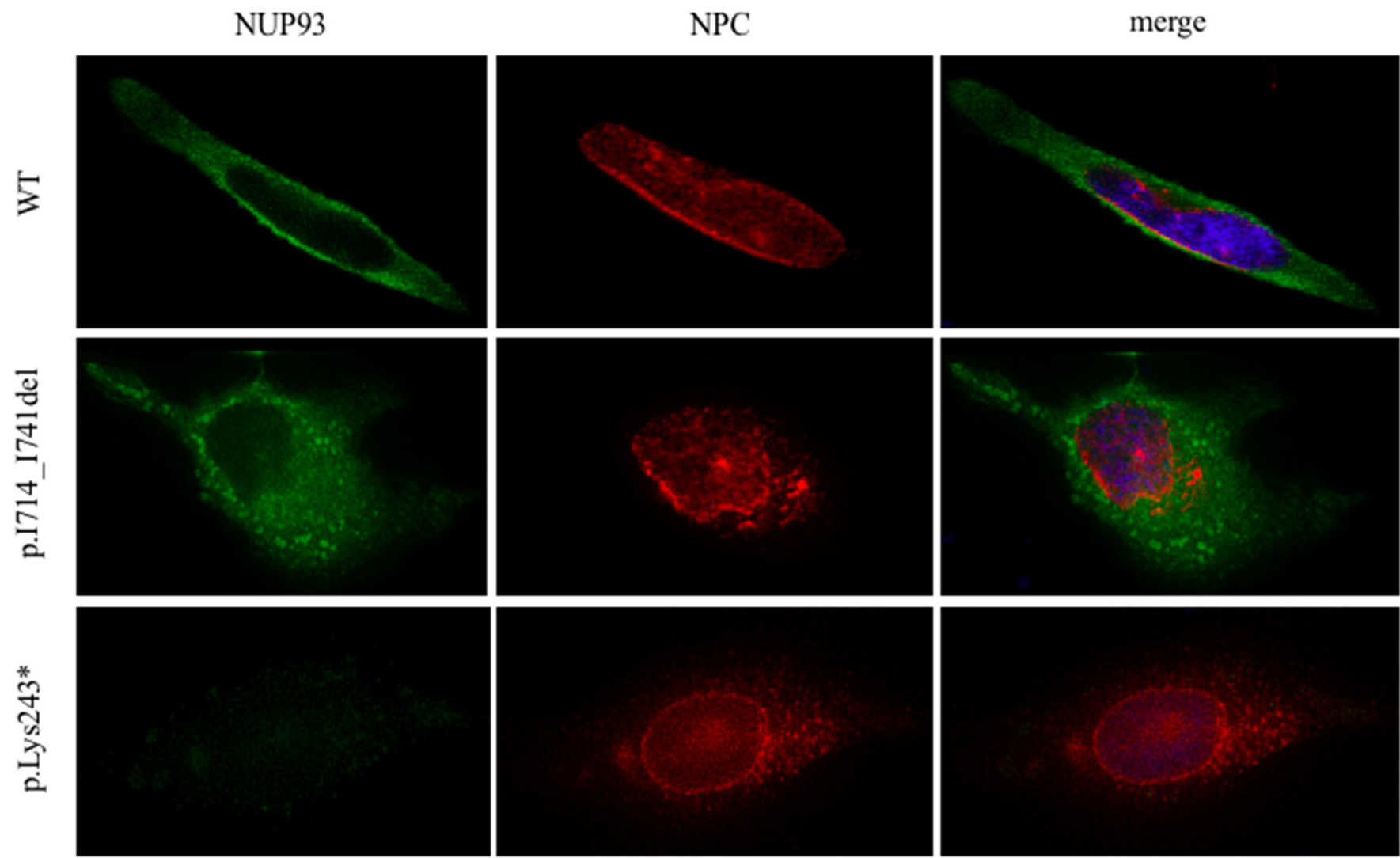
Supplementary Figure 1



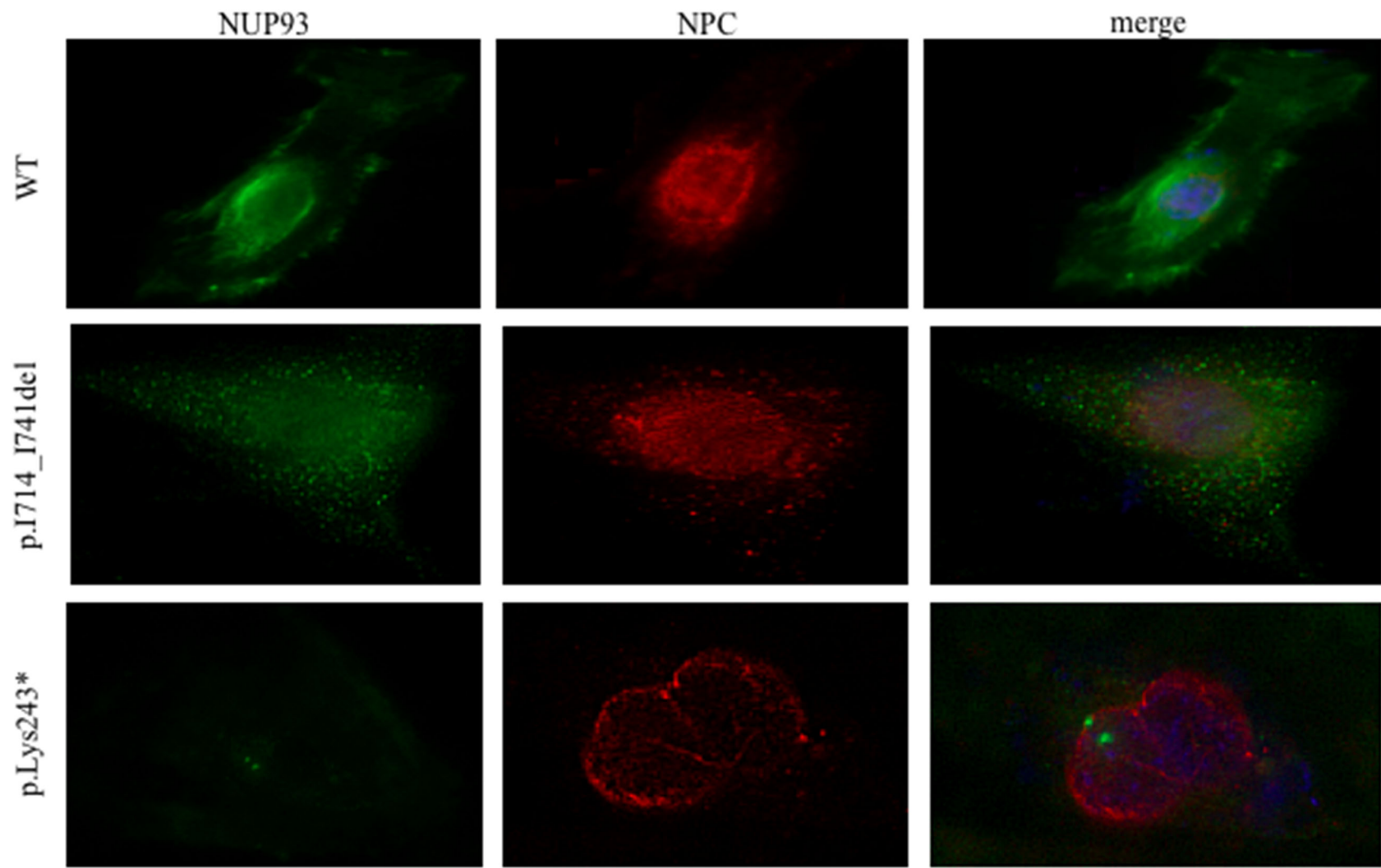
Supplementary Figure 2



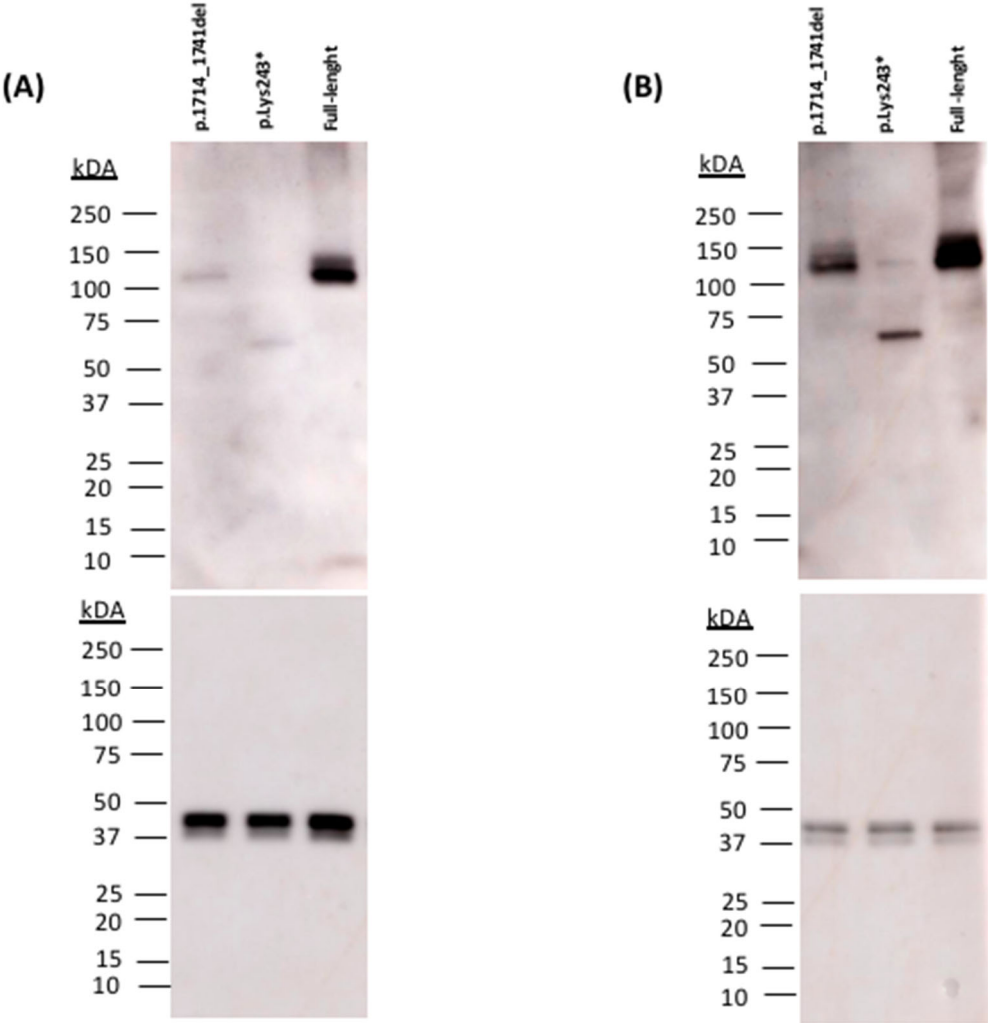
Supplementary Figure 3



Supplementary Figure 4



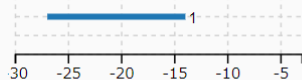
Supplementary Figure 5



Supplementary Figure 6

This table shows only relevant results related to the mutation position and context.

The mutation occurs in the late intronic positions, the following table show results of acceptor splice sites that could be affected by the mutation

Predicted signal	Prediction algorithm	cDNA Position	Interpretation
New Acceptor Site	1 - HSF Matrices		Activation of an intronic cryptic acceptor site. Potential alteration of splicing.

▼ Raw Data Tables

Potential splice sites	Potential Branch Points	Enhancer motifs	Silencer motifs	Other splicing motifs							
▼ HSF Matrices											
Sequence Position	cDNA Position	Splice site type	Motif	New splice site	Wild Type	Mutant	If cryptic site use, exon length variation	Variation (%)			
73	-28	Acceptor	acatggggccggtg	acatggggccagTG	45.49	74.44	+16	New site +63.64			
81	-20	Donor	cgggtggtt	CCAgtggtt	73.43	62.86	+8	Site broken -14.38			
▼ MaxEnt											
Threshold values:											
5' Motif: 3 3' Motif: 3											
Sequence Position	cDNA Position	5' Motif					3' Motif				
		Ref Motif	Ref Score	Mut Motif	Mut Score	Variation (%)	Ref Motif	Ref Score	Mut Motif	Mut Score	Variation (%)
81	-20						ccggtggttgttttaaacagATC	7.92	ccagtggttgttttaaacagATC	7.18	-9.34

Supplementary Figure 7

Potential splice sites

Potential Branch Points

Enhancer motifs

Silencer motifs

Other splicing motifs

▼ ESE Finder matrices for SRp40, SC35, SF2/ASF and SRp55 proteins

Threshold values:

SF2/ASF: 72.98 SF2/ASF (IgM-BRCA1): 70.51 SRp40: 78.08 SC35: 75.05 SRp55: 73.86

Variation expresses the difference between reference and mutant values. Wild Type value is taken as reference.

Sequence Position	cDNA Position	Linked SR protein	Reference Motif (value 0-100)	Linked SR protein	Mutant Motif (value 0-100)	Variation
78	-23	SF2/ASF	gggccgg (76.70)			Site broken -100
79	-22	SC35	ggccgggtg (88.94)	SC35	ggccAgtg (91.52)	+2.9 %
79	-22	SF2/ASF (IgM-BRCA1)	ggccgggt (71.15)	SC35	ggccAgtg (91.52)	+28.62 %
79	-22	SF2/ASF	ggccgggt (76.82)	SC35	ggccAgtg (91.52)	+19.13 %
81	-20			SRp40	ccAgtgg (86.05)	New site
82	-19	SF2/ASF (IgM-BRCA1)	cggtggt (80.92)	SF2/ASF (IgM-BRCA1)	cAgtggt (78.69)	-2.76 %
82	-19	SF2/ASF (IgM-BRCA1)	cggtggt (80.92)	SF2/ASF	cAgtggt (78.68)	-2.77 %
82	-19	SF2/ASF	cggtggt (76.06)	SF2/ASF (IgM-BRCA1)	cAgtggt (78.69)	+3.46 %
82	-19	SF2/ASF	cggtggt (76.06)	SF2/ASF	cAgtggt (78.68)	+3.45 %

▼ RESCUE ESE hexamers

No difference between mutant and reference sequence was found with this matrice.

▼ Predicted PESE Octamers from Zhang & Chasin

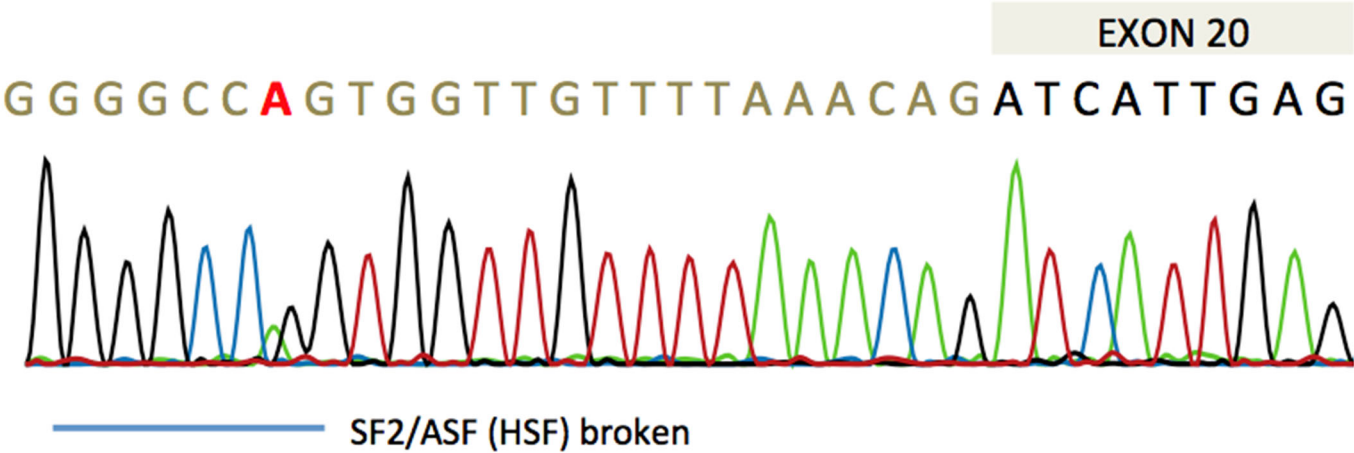
Sequence Position	cDNA Position	Reference motif	Motif value (0-100) reference sequence	Mutant motif	Motif value (0-100) mutant sequence	Variation
77	-24	ggggccggg	3.40			Site broken -100

Supplementary Figure 8

seq_id	agez	ss_dist	bp_seq	bp_scr	y_cont	ppt_off	ppt_len	ppt_scr	svm_scr
intron Wt	53	78	atgtgaggt	-1.84176430138	0.438356164384	43	7	11	-2.7971372
intron Wt	53	70	tgctgactt	2.98414993368	0.446153846154	35	7	11	-0.3986587
intron Wt	53	65	actttacat	-3.16252856316	0.416666666667	30	7	11	-2.4984057
intron Wt	53	50	agatgaaat	-2.2113639793	0.466666666667	15	7	11	-1.1603565
intron Wt	53	45	aaatgactg	1.10723640726	0.5	10	7	11	0.46628968
intron Wt	53	39	ctgtgatgg	0.906555136144	0.5	4	7	11	0.7675034
intron Wt	53	28	gtgttacat	-1.65921638913	0.478260869565	15	7	16	-0.8938408
intron Pt	16	78	atgtgaggt	-1.84176430138	0.438356164384	43	7	11	-2.7971372
intron Pt	16	70	tgctgactt	2.98414993368	0.446153846154	35	7	11	-0.3986587
intron Pt	16	65	actttacat	-3.16252856316	0.416666666667	30	7	11	-2.4984057
intron Pt	16	50	agatgaaat	-2.2113639793	0.466666666667	15	7	11	-1.1603565
intron Pt	16	45	aaatgactg	1.10723640726	0.5	10	7	11	0.46628968
intron Pt	16	39	ctgtgatgg	0.906555136144	0.5	4	7	11	0.7675034
intron Pt	16	28	gtgttacat	-1.65921638913	0.478260869565	15	7	16	-0.89384082

The PPT length and PPT score is same between Wt and Pt.

Supplementary Figure 9



Supplementary Figure Legends

Supplementary Figure 1. Pedigree from individual with *NUP93* variant. The proband is indicated by an arrow with a filled symbol and unaffected individuals are shown with open symbols. Of note, no other family members exhibited any features of kidney diseases.

Supplementary Figure 2. Compound heterozygosity was observed in the patient's transcripts. Subcloning band of the patient's cDNA shows two types of transcripts, which consist of nonsense mutation and exon-20 skipping bands. A. Normal transcripts from wild-type sample without truncating variant or exon-20 skipping bands. In contrast, transcripts from the patient's sample showed truncation (B) or exon skipping (C), which indicated that the patient has compound heterozygous mutations for these two variants.

Supplementary Figure 3. In vitro protein expression analysis in the *NUP93* variant in HELA cells.

Subcellular localization of overexpressed *NUP93* isoform 1 in HELA cells. cDNAs were cloned from the kidney cDNA library and variants were inserted by mutagenesis. The expression patterns were identical to those shown in Figure 4 (isoform 2 cloned from the patient's sample).

Supplementary Figure 4. In vitro protein expression analysis in the *NUP93* variant in podocytes.

Subcellular localization of overexpressed *NUP93* in mouse podocytes. cDNAs were cloned from the kidney cDNA library and variants were inserted by mutagenesis. The expression patterns were identical to those shown in Figure 4 (isoform 2 cloned from the patient's sample) and Supplementary Figure 7 (isoform 1 transfected into HELA cells).

Supplementary Figure 5. Western blotting analysis of NUP93 using total protein extract in HELA cells (A) and HEK293T cells (B)

The GFP-NUP93 p.1714_1741del construct showed reduced expression of *NUP93* protein with a nearly normal size (116 kDa), while the GFP-NUP93 p. Lys243* construct showed expression at 55 kDa, which is the fusion protein of NUP93 truncated at Lys243 and green fluorescent protein (GFP).

Supplementary Figure 6. *In silico* analysis of the influence on splice sites by Human Splicing Finder. The intronic variant c.2137-18G>A showed no remarkable difference in recognition of the 5' and 3' splice sites (Ref score:7.92; Mutation Score: 7.18; Variation: -9.34%).

Supplementary Figure 7. *In silico* analysis of splicing regulation elements (intronic splicing enhancer) by Human Splicing Finder. *In silico* analysis of the intronic enhancer motifs by both ESE finder matrices and predicted PESE octamers revealed the intronic splicing enhancer (ISE) site to be broken (variation -100%) by the intronic variant c.2137-18G>A.

Supplementary Figure 8. *In silico* analysis of the polypyrimidine tract and the branch point by SVM-BP Finder. Neither the polypyrimidine tract score and length nor the branchpoint score were different between the reference and intronic variant c.2137-18G>A.

Supplementary Figure 9. Disruption of SF2/ASF binding site (the intronic splicing enhancer) by c.2137-18G>A. Analysis of enhancer motifs by Human Splicing finder

showed that the putative SF2/ASF binding site lied in the upstream position, -24 or -23 to -17 in intron 19 from the nucleotide position 2137 of *NUP93* cDNA, where the intronic variant was located, resulting in a broken site.