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Mutations in the *NDUFS4* gene of mitochondrial complex I alter stability of the splice variants

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Abstract The effect on the stability of alternative transcripts of different mutations of the *NDUFS4* gene in patients with Leigh syndrome with complex I deficiency is presented. Normally, two *NDUFS4* splice variants are degraded by nonsense mediated mRNA decay (NMD) while a third form does not trigger NMD degradation. In a patient with a premature termination codon in exon 1, all the three splice variants are up-regulated. The present is the first case of a nonsense mutation leading to the abrogation of NMD, which can represent an additional event to be considered in the evaluation of clinically relevant mutations.

Keywords: Respiratory complex I; Leigh syndrome; *NDUFS4* gene; Alternative splicing; Nonsense mediated decay

1. Introduction

An increasing amount of evidences indicate that nucleotide changes in the coding sequence can affect the pathway of mRNA splicing or mRNA stability [1]. A survey performed more than a decade ago found that approximately 15% of all point mutations responsible for human genetic diseases cause alternative RNA splicing [2], which is likely to be an underestimate because it is now known that aberrant splicing is also caused by mutations that modify exonic splicing regulatory elements (ESE) [1]. Faulty splicing, as well as nonsense or frameshift mutations can generate premature termination codons (PTCs) which usually trigger nonsense mediated mRNA decay (NMD) [3] or nonsense associated altered splicing (NAS) [4]. NMD is a highly conserved surveillance process leading to detection and selective reduction in the abundance of PTC-harbouring mRNAs to prevent the synthesis of abnormal proteins [5]. However, NMD has also been involved in degrading natural substrates, meaning that it can represent an additional mode of regulating gene expression [6,7].

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Abbreviations: *NDUFS4*, NADH-ubiquinone oxidoreductase Fe–S protein 4; PTC, premature termination codon; NMD, nonsense mediated decay; EJ, exon junction; ESE, exonic splicing enhancer; ESTs, expression sequence tags

The *NDUFS4* gene encodes for one of the 46 structural subunits of the mitochondrial NADH: ubiquinone oxidoreductase (complex I, E.C.1.6.5.3) [8,9]. This gene is a hotspot of mutations in an autosomal recessive form of a multisystemic fatal infantile disorder, the Leigh syndrome [10–13].

Here, we present a study on the effects of three different mutations on the splicing and stability of the *NDUFS4* transcripts. In particular, a nonsense mutation introducing a PTC, rather than eliciting nonsense mediated mRNA decay, stabilises and unmasks the existence of alternative *NDUFS4* transcripts.

2. Materials and methods

2.1. Cell culture and RT-PCR analysis

Fibroblasts from skin biopsies of three patients harbouring three different homozygous mutations, respectively, a nonsense mutation 44G > A (W15X) in the first exon [12], a 466–470Dupl^{AAGTC} in the exon 5 [10] and a 289delG introducing a stop codon in the exon 3 [11] of the *NDUFS4* gene and from a healthy individual as a control were grown as described [14]. Total RNA, extracted by the High Pure RNA isolation Kit (Roche) was reverse transcribed by the oligo-dT primer with AMV-Reverse Transcriptase-RNAseH minus (New England Biolabs). PCR amplification of the *NDUFS4* canonical entire open reading frame (ORF) was performed using 18k-F and 18k-R primers (Table 1). All the PCR amplifications were performed with Taq DNA Polymerase (Eppendorf) with primers and T_m as listed in Table 1. To specifically amplify the SV1 and SV2 isoforms, F-30 primer designed within exon 1 with R-crypto primer within the crypto-exon, were used after a first round of PCR with 18k-F and 18k-R primers (Table 1). The amplification products were gel extracted by QIAquick gel Extraction Kit (Qiagen), cloned by TAcloning Kit (InVitrogen) and sequenced on an automated ABI 310 sequencer (Applied Biosystems).

The quantitation of the *NDUFS4* isoforms, SV1, SV2, SV3 and of the canonical transcript normalized to GAPDH mRNA was performed by real-time-PCR with the iQ SYBRgreen Supermix (BioRad) on a BIORAD iCycler iQ instrument. The primers sequences and annealing temperature used are listed in Table 1. The PCR conditions were: 20″ at 94 °C, 30″ at 59 °C, 45″ at 72 °C for 45 cycles.

2.2. In vitro splicing of NDUFS4 minigene

Constructs including either the single *NDUFS4* exon1 or exons 1, 2, crypto and 3, were cloned in the pSPL3 vector [15]. Exons and flanking sequences were amplified from genomic DNA using restriction site-tagged primers. The 44G > A mutation was introduced in both constructs by cloning exon 1-containing region amplified from genome of patient with 44G > A mutation. The constructs were transiently transfected in Cos-7 cells using Metafectene (Biontex). After 48 h, total RNA was isolated with TRIzol (Invitrogen) and reverse transcribed primed by oligo-dT. The PCR amplification of pSPL3-containing exon1 was performed with EXPAND™ LongTemplate (Roche) using

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Table 1
Primer sets and annealing temperatures for PCR amplifications

Primer set and name	5′ → 3′ Sequence	Fragment size (bp)	Annealing temperature (°C)
Complete ORF			
18k-F	5′-ATGGCGGCGGTCTCAATGTC-3′	528	53°
18k-R	5′-TATTTTGTGGATACTCTTGTTC-3′		
SV1 and SV2			
F-30	5′-GTGGTACTGAGGCAGACGTTGT-3′	310 (SV1)	59°
R-crypto	5′-ATGGTGACCGGATGAAGTCCTC-3′	227 (SV2)	
Minigene Ex1			
F-SD6	5′-TCTGAGTCACCTGGACAACC-3′	355 (SD_V-Ex1-SA_V)	59°
R-SA2	5′-ATCTCAGTGGTATTTGTGAGC-3′	257 (SD_V-SA_V)	
Minigene Ex1-2-cry-3			
18k-F	5′-ATGGCGGCGGTCTCAATGTC-3′	442 (Ex1-2-cry-3)	58°
R-282	5′-TCCAGAACTGCATGTTATTGCG-3′	270 (Ex1-2-3)	
Untranscribed pSPL3			
pSPL3-F	5′-TCGCCGCATACACTATTCTC-3′	141	59°
pSPL3-R	5′-AGAAGTAAGTTGGCCGCAGT-3′		
Real time			
Canonical			
F-Ex2-Ex3-Real	5′-TGATGAAAAATTGGATATCACTAC-3′	111	59°
M18k2B	5′-TTTACTCCAGACTGCATGTTATTGC-3′		
SV1			
F840-Real	5′-CCAAGCCTGGAACTTGCTAC-3′	225	59°
M18k2B	5′-TTTACTCCAGACTGCATGTTATTGC-3′		
SV2			
F757nt-Real	5′-TGATGAAAAATTGGCATTTGGAG-3′	200	59°
M18k2B	5′-TTTACTCCAGACTGCATGTTATTGC-3′		
SV3			
F-Ex1-Ex3-Real	5′-GTTCCGACCAGGATATCACTAC-3′	109	59°
M18k2B	5′-TTTACTCCAGACTGCATGTTATTGC-3′		
Minigene Ex1			
F-30	5′-GTGGTACTGAGGCAGACGTTGT-3′	242	59°
R-SA2	5′-ATCTCAGTGGTATTTGTGAGC-3′		
GAPDH			
GLIC-1F	5′-GAAGGTGAAGGTCGGAGT-3′	110	59°
GLIC-4R	5′-CATGGGTGGAATCATATTGGAA-3′		

vector specific primers F-SD6 and R-SA2 (Table 1). Real-time quantification of the spliced product was performed with F-30 and R-SA2 and normalised to GAPDH mRNA. For the minigenes containing exons 1, 2, crypto and 3, the RT-PCR reactions were carried out for 18 cycles with F-30 and R-282 and the spliced products were normalised to *GAPDH* mRNA (Table 1). Transfection efficiency was evaluated by normalising the spliced products to an untranscribed region generated by pSPL3-F- and pSPL3-R (Table 1).

2.3. Inhibition of protein synthesis

Primary fibroblasts from the two patients and a control individual were grown to 80% confluence. To assess the sensitivity to NMD, puromycin (100 μg/ml) was added to the colture medium. Puromycin was then removed after 12 h by medium replacement, the cells were let to recover in puromycin-free medium and total RNA was extracted at different times (4, 8 and 12 h) after puromycin removal.

Table 2
Prediction of NMD sensitivity of *NDUFS4* transcripts according to the "50–55nt rule"

NDUFS4 isoforms	Ctrl	44G > A	466-470Dupl
SV1	(126nt EJ4)	(54nt EJ2)	(126nt EJ4)
	+	−/+	+
SV2	(44nt EJ3)	(54nt EJ2)	(44nt EJ3)
	−	−/+	−
Canonical	(STOP last exon)	(54nt EJ2)	(STOP last exon)
	−	−/+	−
SV3	(157nt EJ3)	(54nt EJ2)	(157nt EJ3)
	+	−/+	+

In parenthesis the distances in nucleotides (nt) between the PTC and the closest exonic junction (EJ) is reported. +, predicted to undergo to NMD; −, predicted to escape NMD. +/−, NMD commitment not easily predictable.

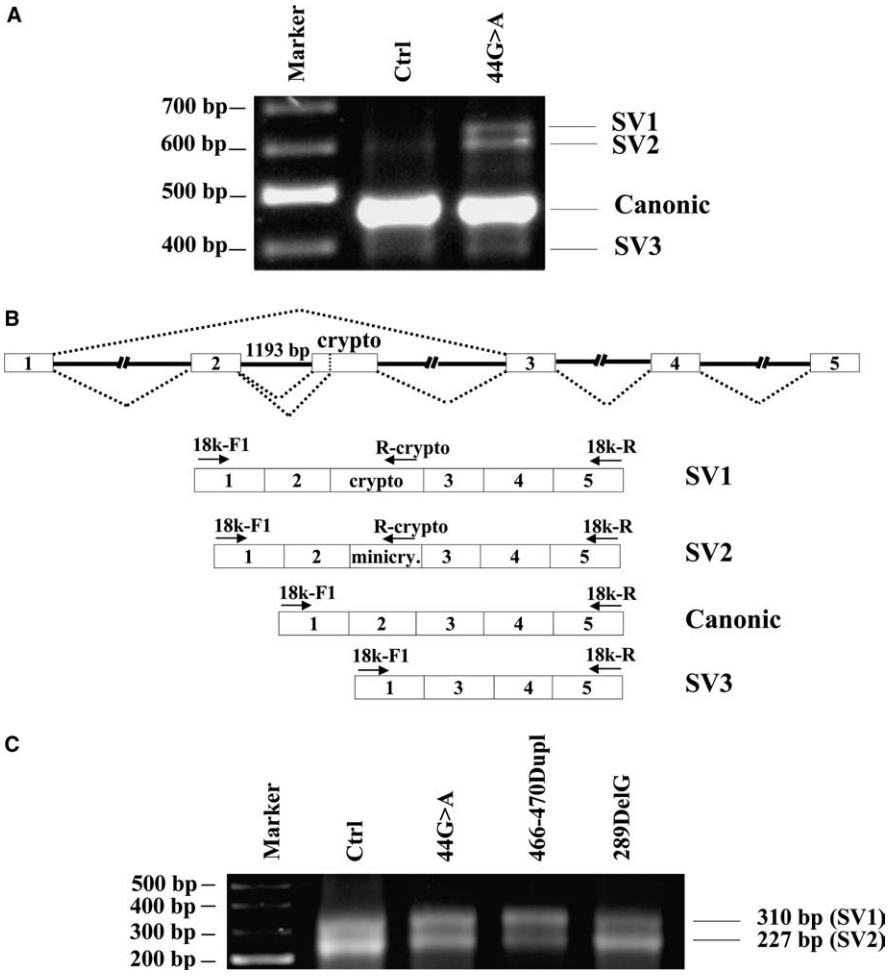

Fig. 1. Identification of *NDUFS4* splice variant SV1, SV2 and SV3. (A) RT-PCR of the entire *NDUFS4* ORF from control (Ctrl) and the 44G > A patient. (B) Representation of alternative transcripts generated by the *NDUFS4* gene indicated as connecting lines. (C) RT-PCR specific for *crypto*-containing transcripts in fibroblasts of a healthy child (Ctrl) and of the patients harbouring, respectively, the 44G > A, 466–470Dupl and 289DelG mutations. Localization and names of used PCR primers are indicated in (B).

Quantitative analysis of *NDUFS4* isoforms by real-time PCR was performed on fibroblasts cultures from the two patients and from a normal individual after 12 h incubation with puromycin (100 μg/ml).

3. Results

3.1. Identification of NDUFS4 splice variants

In the patient harboring the 44G > A mutation, RT-PCR amplification of the entire *NDUFS4* ORF (528nt) showed, in addition to the expected one, three products (Fig. 1A). Sequencing analysis of these products revealed that they were alternative transcripts originating from the *NDUFS4* gene. The first product of 700 bp presented an insertion of 172 bp at position +186 of the coding sequence that we named *crypto*-exon. This corresponds to a splice variant SV1 of 840nt (Fig. 1B). PCR-sequencing analysis of the corresponding genomic region revealed that the *crypto*-exon was localized at 1193nt of intron 2 (Fig. 1B). The *Splice Site Prediction* analysis tool (http://www.fruitfly.org/seq_tools/splice.html) predicted a score of 0.99 for the exon 2 donor site and a score of 1.00 for exon 3 acceptor site. The prediction for the acceptor and the donor sites of the *crypto*-exon gave a score of 0.96 and 0.90, respectively, implying that, in vivo, the efficiency of the junction formation between exon 2 and *crypto* can compete efficiently with the canonical junction between exons 2 and 3. The second isoform, SV_2, (Fig. 1B) corresponds to a 758nt transcript containing a shortened crypto-exon, lacking the first 83nt, that we named *mini-crypto*. It represents the product of an alternative 3′ splice site. In all RT-PCR reactions performed with specific primers for exons 1 and 5, both in normal and patients samples, a third form, named SV3, was always present. Sequencing analysis revealed that it corresponds to a transcript lacking the entire exon 2 (Fig. 1). To check for the presence of SV_1 and SV2 in normal subjects as well as in the patient harboring the 466–470Dupl^{AAGTC} and 289delG, nested RT-PCR reactions were performed. Sequencing analysis revealed that the 310 bp and 227 bp fragments corresponded, respectively, to SV1 and SV2, and confirmed that they were present in all the samples analyzed (Fig. 1C). A search in the human and non-human EST database (ESTdb) showed that the SV1 and SV3 are present only in humans – clone BM471270 and BU677366.1, respectively, according to recent data supporting the low evolutionary conservation of alternative splicing patterns [16] while SV2 was not represented in human ESTdb.

3.2. In vitro splicing assay

The algorithm ESEfinder 2.0©identified a newly introduced consensus motif AGCGGA – mutation 44G > A is underlined – (score 3.069, Threshold 2.67) for the splicing factor SRp55 [17]. We set up an in vitro splicing assay based on the pSPL3 plasmid [15] introducing mutated or wild-type exon 1 with adjacent intronic sequences. The results obtained did not reveal any difference in size or in quantity of the spliced product (Fig. 2A), as also confirmed by Real-time assay (not shown). We also generated a "minigene" containing exons 1, 2, crypto and exon 3 to more faithfully resemble the in vivo *NDUFS4* gene organization that was used to transfect Cos 7 cells. RT-PCR analysis in conditions chosen to avoid saturation of the reaction was performed by amplifying the mutant and wild-type spliced products and normalizing to GAPDH mRNA (Fig. 2B). The results obtained, again did not show any qualitative or quantitative (not shown) difference comparing the expression between the wild-type and mutated exon 1-containing constructs (Fig. 2B).

3.3. Inhibition of protein synthesis

Fibroblasts from the 3 *NDUFS4* mutated patients and a control individual were incubated with puromycin which has been shown to inhibit NMD [18]. A 12 h incubation of normal and patients fibroblasts with puromycin (100 μg/ml) fully restored the SV1 and SV2 in normal and in both the 466–470Dupl and 289DelG patients, whereas the transcripts remained unchanged in the patient harbouring the 44G > A (Fig. 3A). Since SV3 was present at very low levels changes of its expression have been evaluated only by real time PCR (see below). RT-PCR analysis after removal of puromycin at different times showed a progressive restoration of the sensitivity of SV1 and SV2 transcripts to NMD only in the normal fibroblasts but not in the fibroblasts harboring the 44G > A mutation (Fig. 3B).

3.4. Real-time PCR of NDUFS4 transcripts

Real-time PCR analysis was carried out to quantify the expression level of *NDUFS4* transcripts in normal and patients fibroblasts. It was not possible to perform the analysis in the fibroblasts harbouring the 289DelG since they lost viability. Fig. 4A reports the ratios of SV1, SV2, SV3 and canonical mRNA normalized with respect to the GAPDH mRNA in the patients relative to the control individual as obtained by real-time-PCR. The results indicated that in the case of 44G > A mutation, there was an up-regulation of ~8 fold for SV1 and SV2 and ~6 fold for SV3 isoform whilst the canonical form remained unaltered. In the case of the 466–470Dupl mutation, all the *NDUFS4* isoforms were expressed approximately at the same level as in the normal control sample. When the protein synthesis inhibitor puromycin was added to the colture medium (Fig. 4B), an up-regulation of SV1 and SV2 of 7 and ~5 fold, respectively, was evident in the control sample. This up-regulation was present, although to a lesser extent probably owing to the poor growth of these cells, also in 466–470Dupl fibroblasts but not in 44G > A harboring fibroblasts where all the transcripts were unaffected by the inhibition of protein synthesis. The level of SV3 was on the other hand unaffected by puromycin.

4. Discussion

In the present work, we show the effects of different mutations on the expression and the stability of alternative mRNA isoforms of the *NDUFS4* gene (Fig. 5B). Mutations in the *NDUFS4* gene have been described to prevent the synthesis of the protein [14]. This was ascribed to inability to produce a full-length protein in the patient harbouring the 44G > A substitution, NMD of the canonical transcript caused by a

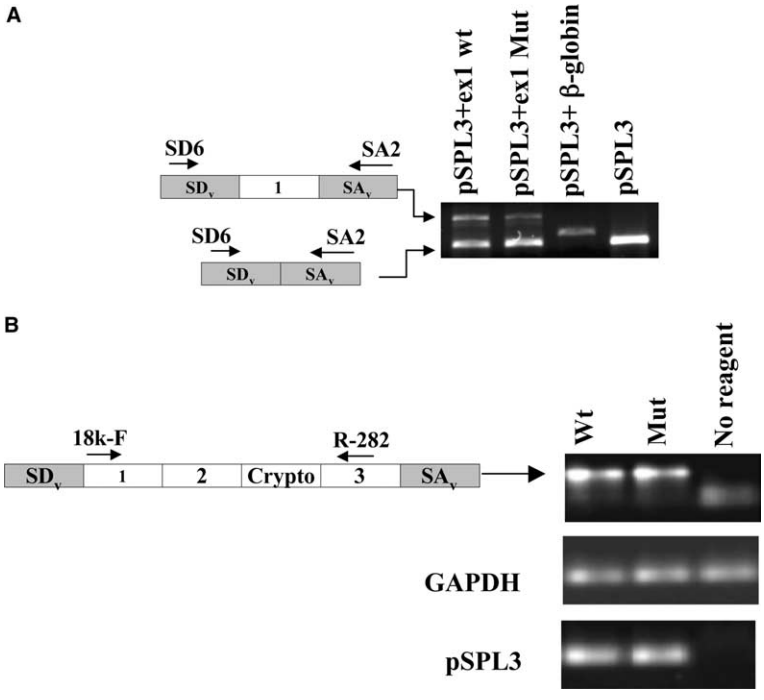

Fig. 2. RT-PCR of Cos-7 cells transfected with the wild-type (Wt) and mutant (Mut) *NDUFS4* exon 1. (A) Minigene containing only exon 1. Control reaction using a beta-globin exon cloned in pSPL3. (B) Minigene containing Exons 1, 2, *crypto* and 3. *No reagent* lane shows the endogenous canonical *NDUFS4* transcript, the only isoform present in Cos7 cells.

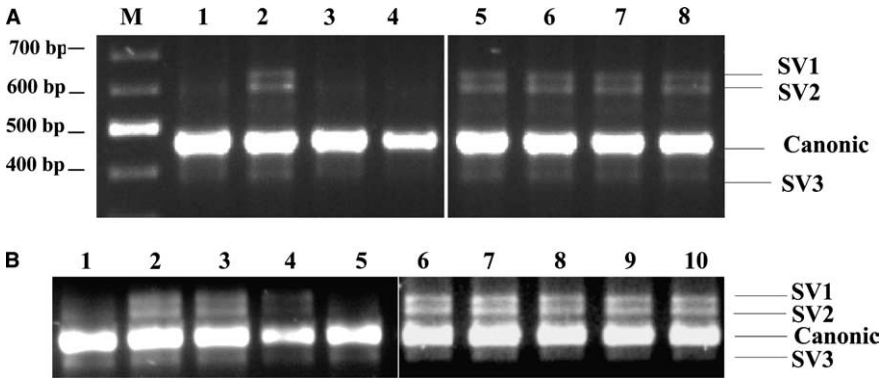

Fig. 3. RT-PCR analysis of the effect of puromycin on the stability of the *NDUFS4* transcripts. (A) Control and patients harbouring, respectively, the 44G > A, 466–470Dupl and 289DelG mutations either untreated (1–4) or treated (5–8). (B) Fibroblasts from Control (1–5) and 44G > A patient (6–10). After 12 h of puromycin incubation (lanes 2, 7) puromycin was removed from the medium and the RNA samples were analysed after 4 h (3, 8), 8 h (4, 8), 12 h (5, 10), (1, 6, untreated).

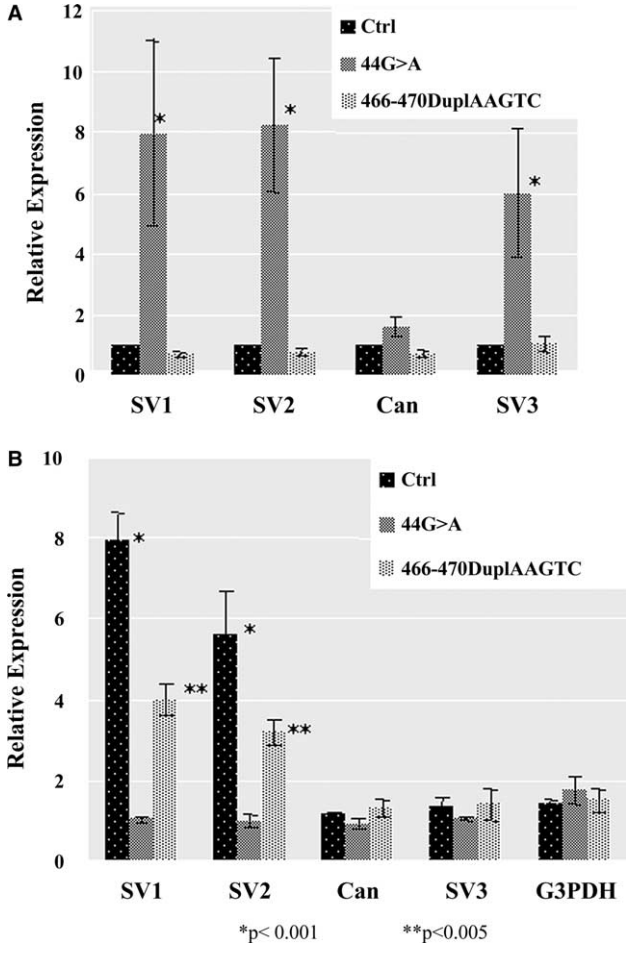

Fig. 4. Real-time PCR assays for *NDUFS4* transcripts in control and patients fibroblasts. (A) Results are reported as the relative expression of the four *NDUFS4* transcripts with respect to *GAPDH* mRNA in all the patients, with the level in control referred to as 1, and shown as means ± s.d. (B) Results are reported as relative expression of the four *NDUFS4* and *GAPDH* transcripts in all the puromycin treated samples with respect to the untreated ones and referred to a standard curve derived from a dilution series of *GAPDH* cDNA containing plasmid, and shown as means ± s.d. All the experiments were run in triplicate and the values are the mean results from six independent experiments. The data were analysed using Student's *t* test, and $^{*}P < 0.01$ and $^{**}P < 0.05$ were considered to indicate significant differences.

289delG and to possible translation defect or protein degradation caused by 466–470Dupl^{AAGTC} [14]. In the present study, we have detected two novel splice variants, SV1 and SV2, resulting from the insertion of a *crypto*-exon which uses two alternative acceptor sites, at an abnormally high level in the presence of the 44G > A substitution. Nested RT-PCR assays showed that SV1 and SV2 are present even in Dupl466–470 and 289delG patients and in the normal control subject. A third splice variant, SV3, detected in all the subjects analysed, derived from the skipping of exon 2. Quantitation of the different splice variants by real-time PCR showed that the relative expression of SV1, SV2 and SV3 was highly increased – from 6 (SV3) to 8 fold (SV1 and SV2) – with respect to control housekeeping gene when the 44G > A mutation is present, whilst the canonical transcript was normally represented. As the 44G > A mutation introduces a putative ESE we set up an in vitro splicing system to evaluate the effect of this regulatory splicing element on *NDUFS4* gene splicing pathway. This system showed, however, that the newly introduced regulatory element did not per se activate aberrant splicing and suggested instead the existence of a nonsense codon-specific up-regulatory mechanism acting independently of ESE introduction. Experiments with the translation inhibitor puromycin, commonly used to stabilise transcripts undergoing degradation by NMD, showed that SV1 and SV2 are conveyed to NMD whereas SV3 is insensitive to puromycin. Several papers suggest that when PTCs are followed by an intron that is located more than 50–55nt downstream, this mRNA is degraded by NMD [3,19]. In wild-type gene, SV1, SV2 and SV3 mRNAs carry stop codons located in exon 3, *mini-crypto* and exon 3, respectively (Fig. 5B). According to the 50–55nt rule (see Table 2), in both normal and Dupl466–470 samples, SV1 and SV3 both are bona fide candidates of NMD having stop codons 126nt and 157nt upstream exonic-junction (EJ) 4, respectively. SV2 should be normally stable having a stop codon 44nt upstream from EJ3 (Fig. 5B). In the patient harbouring the 44G > A mutation, all the *NDUFS4* isoforms should be insensitive to NMD degradation having a PTC 54nt upstream the first EJ. However, the NMD commitment in the presence of the 44G > A mutation is not easily predictable since the PTC falls just in the 50–55nt boundary where no safe prediction on NMD sensitivity can be made. Our data show in fact, that in the patient with 44G > A mutation, SV1, SV2 and SV3 were all up-regulated. In experiments with puromycin (Fig. 4B) in

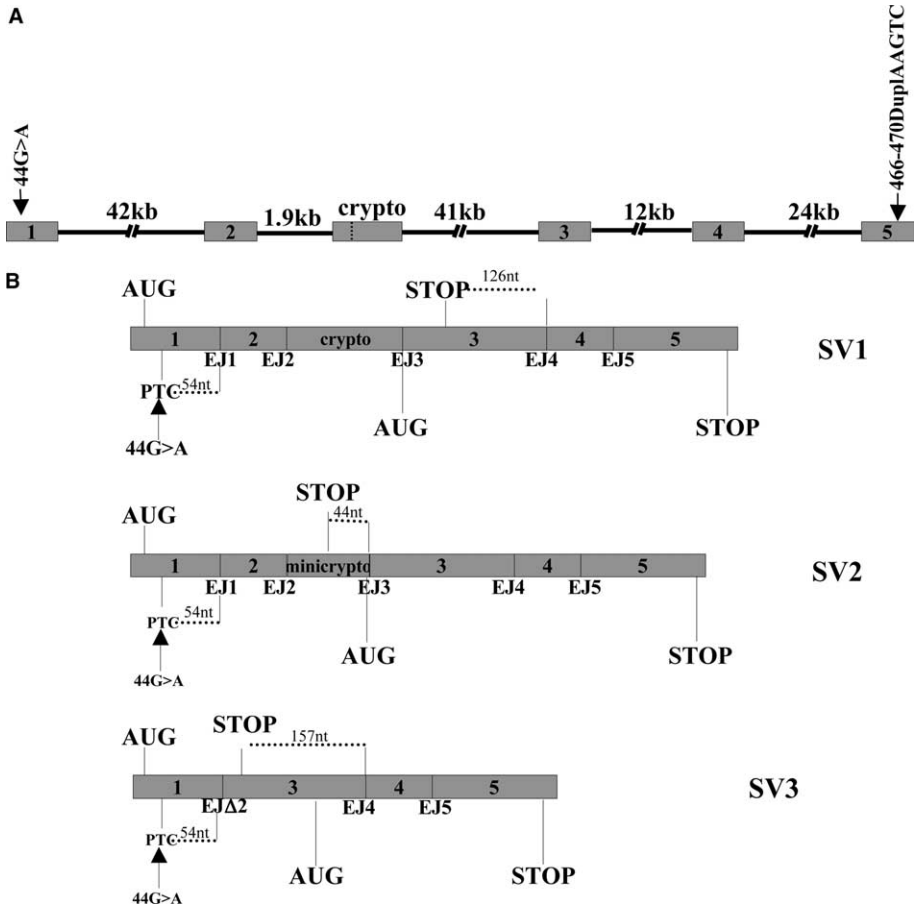

Fig. 5. (A) Organisation of *NDUFS4* gene. (B) Schematic representation of the PTCs positions in SV1, SV2, SV3 and their relative distances to the next exonic junctions, EJs.

normal and 466–470Dupl samples, SV1 responded as predicted but, on the contrary, SV2 was upregulated and SV3 was insensitive. These data indicate that the determinants of NMD are more complex than it would be predicted simply on the basis of "50–55nt boundary rule". This has been reported to happen for several other genes such as TPI [20], β-globin [21], BRCA1 [22], TCR-b [23], fibrinogen Aα-chain and ALG3 transcripts [24]. Our results add evidences of additional transcripts that do not obey to the "50–55nt boundary rule".

Our findings suggest that SV3 degradation is independent from the cytoplasmic translation and the existence of additional unidentified determinants that function to modulate the NMD sensitivity for SV2. The fate of the SV2 in Dupl466–470 and in normal samples is probably the result of a more complex regulation. In SV2 there is an additional not-overlapping ORF of 117aa starting from an AUG located at the last three nucleotides of *mini-crypto*. This ORF, which is not far from the canonical stop codon could confer altered response to NMD commitment. Translation reinitiation was previously found to stabilize PTC-mutated transcripts [20,25]. When the 44G > A mutation is present, for all the *NDUFS4* isoforms, it determines an apparent return to the 50–55nt rule in the NMD commitment. In addition, the same mutation creates a PTC in close proximity to the canonical AUG which can represent a further determinant in NMD resistance [26]. This is consistent with the findings that puromycin failed to enhance the SV1 and SV2 levels in the case of 44G > A fibroblasts whilst it induced an up-regulation of these transcripts both in Dupl466–470 and in 289DelG patients (Fig. 4B) (see also [14]). In conclusion, in evaluating pathogenicity or the clinical relevance of specific mutations the possibility of interference with normal commitment to NMD of splice variants can be considered as an additional mechanism of mutations in hereditary diseases.

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