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Short communication

*POLG* exon 22 skipping induced by different mechanisms in two unrelated cases of Alpers syndrome

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# a b s t r a c t

The *POLG* genes were sequenced in two unrelated patients presenting with Alpers syndrome. The novel c.3626\_3629dupGATA and the c.3643+2TN C alleles were associated in *trans* with p.A467T and p.[W748S; E1143G], respectively. *POLG* transcripts from skin ﬁbroblasts showed complete exon 22 skipping for patient 2, but surprisingly partial exon 22 skipping from the c.3626\_3629dupGATA for patient 1. The creation of a putative exonic splicing silencer could be responsible for the splicing anomaly observed in patient 1. Both c.3643+2TNC and c.3626\_3629dupGATA create a premature termination codon and a low polymerase γ activity in skin ﬁbroblasts is responsible for the severe phenotype in these patients.

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1. Introduction

Alpers-Huttenlocher syndrome (AHS) (OMIM 203700) is a rare autosomal recessive disease associated with mitochondrial (mt) DNA depletion and mutations in the *POLG* gene. AHS is usually characterized by a clinical tetrad of psychomotor regression, intractable epilepsy, cortical blindness and liver disease in infants and young children ([Harding, 1990; Naviaux and Nguyen, 2004; Kurt et al., 2010](#_bookmark17)). Replication of mtDNA involves a complex machinery called mtDNA replisome including the mtDNA polymerase γ (pol γ) and the Twinkle helicase ([Farge et al., 2007](#_bookmark17)). It was established that the holoenzyme pol γ is a heterotrimer consisting of one catalytic POLG1 subunit and a homodimer of the POLG2 accessory subunit ([Yakubovskaya et al., 2007](#_bookmark17)). Recently, the crystal structure of the human holoenzyme pol γ has been reported, providing a structural basis for understanding interactions between catalytic and accessory subunits ([Lee et al., 2009](#_bookmark17)).

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The human *POLG* (also known as *POLG1* or pol γA) is localized on chromosome band 15q25 and spans nearly 18.5 kb with 23 exons. The 4464 bp transcript (NM\_002693) encodes the 1239 aminoacid POLG1 protein. The mature protein is a 135-kDa polypeptide composed of three functional domains: an amino-terminal 3′-5′ exonuclease (*exo*) domain responsible for the ﬁdelity of mtDNA replication ([Longley et](#_bookmark17) [al., 2001](#_bookmark17)), a linker domain and a carboxy-terminal polymerase (*pol*)

domain ([Ropp and Copeland, 1996; Lecrenier et al., 1997](#_bookmark17)). The 50-kDa POLG2 (or pol γB) subunit encoded by the *POLG2* gene (chr 17q21) acts as a processivity factor accelerating polymerisation rate, in addition to increasing afﬁnity of pol γ for DNA ([Lim et al., 1999; Farge](#_bookmark17) [et al., 2007](#_bookmark17)).

Human DNA pol γ is known to be an effective *in vitro* reverse transcriptase (RNA-directed DNA polymerase) and a possible physio- logical role for this reverse transcriptase activity has been suggested ([Murakami et al., 2003](#_bookmark17)). The most sensitive assays developed to date for the measurement of polg γ activity used homopolymeric RNA templates. Clinical assays were performed on mitochondria isolated from skeletal muscle ([Naviaux et al., 1999](#_bookmark17)) or cultured ﬁbroblasts ([Taanman et al., 2009](#_bookmark17)). Characterization of the enzyme has been assessed *in vitro* with puriﬁed or recombinant pol γ ([Carrodeguas et al.,](#_bookmark10) [1999; Farr et al., 1999; Lee and Johnson, 2007](#_bookmark10)) or *in vivo* by expressing a POLG1 fusion protein in human cultured cells ([Spelbrink et al., 2000](#_bookmark17)).

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To date, more than 150 different *POLG* variations have been reported in a broad range of clinical phenotypes in children and adults (<http://tools.niehs.nih.gov/polg/>). AHS is one of the six major groups of POLG disease deﬁned by [Wong et al., 2008](#_bookmark17).

In this study, we investigated *POLG* in two unrelated patients presenting with infantile-onset AHS. A novel GATA duplication in exon 22 and a yet reported donor splice site alteration in intron 22 were identiﬁed in a compound heterozygoty with the most common variation, p.A467T, and the p.[W748S;E1143G], respectively. For both patients, cDNA study showed that both the GATA duplication and the donor splice site alteration cause a splicing defect leading to partial or complete skipping of exon 22. The mechanisms inducing exon 22 skipping in these patients are discussed.

To ﬁnd out if these *POLG* mutants could be responsible for the AHS phenotype, we performed assays of pol γ activity in mitochondria from ﬁbroblasts. We showed a signiﬁcant decrease of polymerase activity *in organello* for both patients, providing evidence that these *POLG* variations affect enzyme catalysis and play a major role in the AHS phenotype.

1. Subjects and methods
   1. *Case reports*
      1. *Patient 1*

This girl was the second child of non-consanguineous French parents with no clinical relevant family history. Height and weight growth decreased at 6 months of age. At 1 year of age, she presented a right- sided clonic status epilepticus resistant to multiple antiepileptic drugs. Carbamazepine worsened myoclonic jerks. A ketogenic diet was well tolerated but was ineffective. Occipital frontal circumference was normal. EEG showed subcontinuous discharges of rythmic slow spike waves on the left hemisphere, related to right hand and foot clonic jerks. Brain magnetic resonance imaging (MRI) at 12 months of age was normal. At the same time, a dramatic psychomotor regression was observed with poor interaction, major global hypotonia and no eye contact due to cortical blindness. Mild lactic acidosis and elevated lactate/pyruvate ratio were documented in blood (lactate: 2.8 mmol/l;

normal: 0.5–2.0) (lactate/pyruvate ratio: 31; normal b 20). Elevation of

cerebrospinal ﬂuid (CSF) lactate was noticed twice (2.8 and 2.2 mmol/l; normal 1.4–2.0). CSF protein content was normal. Content of mtDNA was slightly reduced in muscle (62% of age-matched control mean) with no major abnormalities of the mitochondrial respiratory chain activities. A diagnosis of Alpers syndrome was suggested. In the following months, her neurological condition evolved into epilepsia partialis continua

reinforced by recurrent episodes of tonic clonic seizures. At 16 months of age, liver enlargement was noticed associated with mild cytolysis (ASAT 90 IU/L, normal b 50) and increased level of gamma-glutamyl- transferase (GGT) (138 IU/L; normal: 5–25). The prothrombine time and the clotting factors were decreased (II 36%, V 39%, VII 59%, and prothrombine time 48%). The patient died at 27 months of age of

respiratory failure. Informed parental consent was obtained in agree- ment with the French recommendations for genetic analysis.

* + 1. *Patient 2*

This boy was the ﬁrst child of healthy young unrelated French parents without relevant family history. At 16 months of age, he started a severe status epilepticus. CSF protein content was elevated (0.78 g/l; normal: 0.15–0.35). Cerebral MRI showed brain cortical atrophy with symmetrical thalamic T2 and Flair hyperintense signals associated with cytotoxic oedema within basal ganglia on diffusion sequences. Then, he developed intractable epileptic encephalopathy associated with gener-

alized hypotonia and cortical blindness. At 24 months of age, a new cerebral MRI conﬁrmed brain cortical atrophy associated with marked T2 hyperintense signals of the cerebellar dentate nuclei and of posterior periventricular white matter at a less extent. Proton magnetic resonance spectrometry revealed an accumulation of lactic acid in CSF and

periventricular regions. One month later, a new acute episode occurred with diffuse hypotonia, lethargy and vomiting. Lactate was mild elevated in plasma (2.8 mmol/l; normal: 0.5–2.0 mmol/l) and in CSF (3.2 mmol/l; normal 1.4–2.0 mmol/l). CSF protein content was elevated

at 0.59 g/l (normal 0.15–0.35 g/l). Enzymatic measurements of the

respiratory chain complexes of a muscle biopsy revealed a partial defect of the activities of the complexes I, II+III and IV (44, 52 and 63% of the mean control values, respectively). During the two following years, epilepsy was always very active including spasms and myoclonic jerks. At 46 months, valproate therapy was started because of refractory seizures. At the beginning, valproate was clinically well tolerated but no clear improvement of seizure frequency was noticed leading to the introduction of a ketogenic diet. This last therapy was stopped after 6 weeks because of intestinal intolerance. Despite stopping valproate, a rapidly progressive hepatic failure with increasing levels of transami- nases and ammoniemia, hypoalbuminaemia and decrease of clotting factors led to the death of the child at 50 months of age. The diagnosis of Alpers syndrome was proposed and then conﬁrmed by the following analysis. Depletion of mtDNA in liver (86%) was demonstrated, associated with the defects of mtDNA-related respiratory chain complexes (17, 65% and 25% of the mean control values for the complexes I, III and IV, respectively). Informed parental consent was obtained for this study.

* 1. *Cell cultures and mitochondria-enriched preparations*

Dermal ﬁbroblast and amniotic ﬂuid cells were obtained from CBC Biotec, CRB-HCL. Shortly, ﬁbroblasts from forearm skin explants obtained from patients, family members and controls were cultured in Ham F10 medium supplemented with 12% of fetal calf serum, with penicillin, streptomycin and amphotericin B and 50 mg/L of uridine. Cells were routinely cultured in plastic ﬂasks (T175) at 37 °C in a humidiﬁed atmosphere of 5% CO2 in air. Cultures were checked for mycoplasma infection prior to all experiments. Mitochondria- enriched preparations from three T175 ﬂasks of cell lines were obtained as described previously ([Mousson de Camaret et al., 2007](#_bookmark17)). The pelleted mitochondria were resuspended to an approximate concentration of 10 g/L and kept in aliquots at −80 °C. Protein concentration was measured by the bicinchoninic acid method.

* 1. *Molecular investigations*

For mtDNA quantiﬁcation, continuous real-time quantitative PCR, with the ﬂuorescent SYBR green I double strand DNA-speciﬁc dye, was used to determine the copy number for three mitochondrial genes (ND2, ND5 and 16 S) and a nuclear gene (ATPsynβ), as described previously ([Chabi et al., 2002](#_bookmark13)).

*POLG* exons 2–23 and intron/exon boundaries were ampliﬁed and

sequenced from the genomic DNA of both patients and their parents. Total RNA was isolated from cultured skin ﬁbroblasts using the RNeasy Mini Kit (Qiagen). RNA samples were free of any contami- nating DNA by treatment with the DNA-freeTM Kit (Ambion Inc.). Reverse transcription of RNA was performed with the GeneAmp® RNA PCR Core Kit (Applied Biosystems) as recommended by the supplier. Ampliﬁcation and sequencing of *POLG* cDNA were per- formed. To assess exon 22 skipping, PCR products with exon 21 and 23 primers were puriﬁed by agarose gel electrophoresis and extracted by the DNA and Gel Band Puriﬁcation Kit (GE Healthcare) and sequenced. Results were compared with the GeneBank reference sequences

NC\_000015.8 (range 87679030….87660554, complement) and

NM\_002693.2.

* 1. *Enzymatic measurements*

The DNA pol γ activity was measured in mitochondria-enriched preparations from cultured ﬁbroblasts using the RNA-directed DNA

polymerase assay with a procedure based on that described previously ([Naviaux et al., 1999](#_bookmark17)). Thawed mitochondria were lysed as reported ([Mousson de Camaret et al., 2007](#_bookmark17)). Serial dilutions of lysed mitochondria were prepared to obtain ﬁnal concentration of 0.15, 0.25, 0.5, 1.0 and 1.5 μg per assay. The standard reaction mixture

contained 50 mM Tris–HCl (pH 8.0), 50 mM NaCl, 5 mM KCl, 5 mM

MgCl2 and 0.5 mM MnCl2, 10 mM DTT, 10 mg/L poly(rA) and 5 mg/L oligodT12–18 (Amersham Biosciences), 5 μM dTTP and 50 μCi/ml of [α-32P] dTTP (Perkin Elmer; 10 μCi/μl, 3000 Ci/mmol; 0.017 μM ﬁnal concentration) in a ﬁnal volume of 20 μl. Assays were initiated by addition of 0.15 to 1.5 μg of mitochondrial protein per assay and conducted at 37 °C during 30 min. The reaction was stopped at +4 °C

and the amount of the reaction products was determined by spotting aliquots of 4 μl on Whatman DE-81 anion-exchange papers (Fischer Bioblock). The papers were then washed as described ([Naviaux et al.,](#_bookmark17)

[1999](#_bookmark17)). For each dilution of mitochondria, a parallel assay was performed with 5 μM 2′,3′-dideoxythymidine 5′-triphosphate (ddTTP). Data obtained from each dilution were used to establish a regression line for each sample. Regression analysis was used to calculate speciﬁc activity according to [Naviaux et al., 1999](#_bookmark17). DNA pol γ speciﬁc activity (ddTTP-sensitive) was obtained as the difference

between the two parallel assays measured with and without ddTTP. One unit of speciﬁc activity (U) is given as the incorporation of one picomole of dTTP per min per mg of protein.

1. Results
   1. *Molecular investigations*
      1. *POLG sequence variations*

- Patient 1 is a compound heterozygote for two sequence variations:

(i) the common c.1399GNA in exon 7 which predicts a substitution of Ala with Thr at codon 467 (p.Ala467Thr) (A467T) in the thumb subdomain of the protein *pol* domain ([Naviaux and Nguyen, 2004; Lee et](#_bookmark17)

[al., 2009](#_bookmark17)), (ii) a novel GATA duplication (c.3626\_3629dupGATA) located 14 nt before the 3′-end of exon 22. This duplication creates a premature termination codon (PTC) located 17 nt upstream from the intron 22. The mother was the carrier of the A467T and the father of the c.3626\_3629dupGATA (not shown).

- Patient 2: *POLG* of patient 2 harbored two heterozygous base changes: (i) the previously reported combination in *cis* p.[W748S; E1143G] ([Van Goethem et al., 2004; Ferrari et al., 2005](#_bookmark17)) (ii) the recently reported T to C substitution (c.3643+2TNC) abolishing the invariable consensus GT splice donor site of intron 22 ([Roels et al.,](#_bookmark17) [2009](#_bookmark17)). Patient 2 inherited the p.[W748S;E1143G] from his mother and the c.3643 + 2 TNC from his father (not shown).

* + 1. *RT-PCR analysis*

To identify the impact of these variations on splicing, cDNAs from both patients and a control were ampliﬁed with primers located in exons 21 and 23. As expected for patient 2, two different ampliﬁcation products were revealed: an approx. 496 bp band of expected size and a 336 bp shorter one ([Fig. 1](#_bookmark8), lane 3). Sequencing of these puriﬁed

products demonstrated that the 496 bp fragment corresponded to exons 21–22–23 from the p.[W748S;E1143G] allele while the shorter one corresponded to exon 21 directly linked to exon 23 ([Fig. 2](#_bookmark9)). Thus, the c.3643+2TNC variation in patient 2 causes complete skipping of exon 22. Furthermore, exon 22 skipping introduces a PTC located one codon into the last exon 23, resulting in a C-terminal 78-amino-acid

truncation of the protein.

For patient 1 surprisingly, the same pattern of ampliﬁcation products was obtained ([Fig. 1](#_bookmark8), lane 1) but the intensity of the 336 bp product was fainter than for patient 2. Sequencing of the normal size 496 bp product showed two different species: one bearing the GATA duplication and the other bearing the wild-type GATA sequence, and sequencing of the 336 bp shorter product showed exon 22 skipping

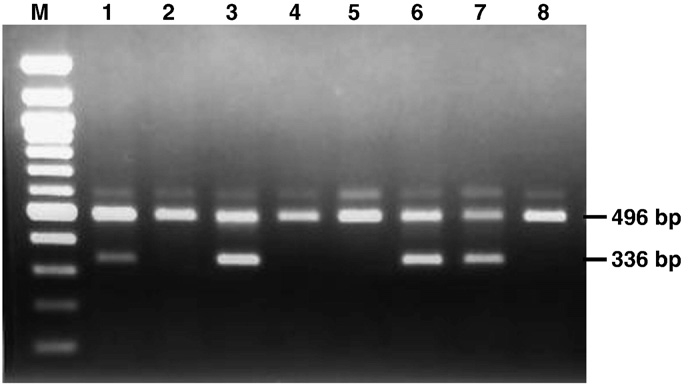


Fig. 1. Ampliﬁcation of exons 21–23 from cDNAs of patients. Exons 21–23 of cDNAs were ampliﬁed using the exon 21 (forward) primer (nt 3356-3375) and the exon 23 (reverse) primer (nt 3851-3832). Nucleotide numbering uses the A of the ﬁrst ATG translation initiation start codon as nucleotide +1. Lanes 1 and 2: cDNA from ﬁbroblasts of patient 1 and a control, respectively. Lanes 3, 4, 5 and 6: cDNA from ﬁbroblasts of patient 2, a control, his mother and his father, respectively. Lanes 7 and 8: cDNA from amniotic cells (patient 2 family) and a control, respectively. M is a 100 bp ladder.

([Fig. 2](#_bookmark9)). This cDNA study showed an unexpected splicing defect for patient 1 as two kinds of transcripts were produced from the GATA duplicated allele: one containing exon 22 with the GATA duplication and one with exon 22 skipping.

* 1. *DNA pol* γ *measurements*

To assess the pathogenicity of the *POLG* gene variations in both patients, we used a RNA-directed DNA polymerase assay for mitochondria-enriched preparations from cultured ﬁbroblasts. The

ddTTP-sensitive incorporation of dTTP was used to calculate DNA polymerase γ activity. A 85–95% ddTTP sensitivity of the total dTTP incorporation was obtained in control and patients mitochondria. Similar results of ddTTP sensitivity were reported elsewhere ([Naviaux](#_bookmark17) [et al., 1999; Spelbrink et al., 2000; Taanman et al., 2009](#_bookmark17)).

A low level of enzyme activity was measured in the mitochondria of both patients ([Table 1](#_bookmark11)): 18.8 U for patient 1 and 15.8 U for patient 2, compared to the controls (56.6 U± 17.8; n= 8) (range: 36.6–91.0). Residual activities were 33% and 28% of the mean control value for patients 1 and 2, respectively.

1. Discussion

We investigated *POLG* in two unrelated children presenting with Alpers syndrome. Patient 1 harbored the novel c.3626\_3629dupGATA in exon 22 *in trans* with the common p.A467T and patient 2, the recently reported c.3643+2TNC *in trans* with the p.[W748S;E1143G]. In order to evaluate the consequences of the mutant alleles on POLG1 biochemical function, catalytic activity of DNA pol γ was assessed *in organello* on mitochondria-enriched preparations from cultured ﬁbroblasts. The A467T/c.3626\_3629dupGATA and the [W748S;E1143G]/c.3643+2TNC enzymes showed a decreased cata- lytic rate (33% and 28% of the mean control activity for patients 1 and 2,

respectively).

As both the c.3626\_3629dupGATA and the c.3643+2TNC create a PTC and probably inactive proteins, the level of residual activity for each patient is to be likely the reﬂect of the A467T or the W748S; E1143G mutant proteins. It has been established that either the A467T or the [W748S;E1143G] recombinant proteins harbored decreased activity ([Chan et al., 2005a, 2006](#_bookmark14)). Recently, disease-associated pol γ variations have been divided into three classes ([Lee et al., 2009](#_bookmark17)). W748S and A467T are included into class II and class III, respectively. The class II variations are located in the DNA binding channel, leading to reduced DNA binding and lower polymerase activity. The most

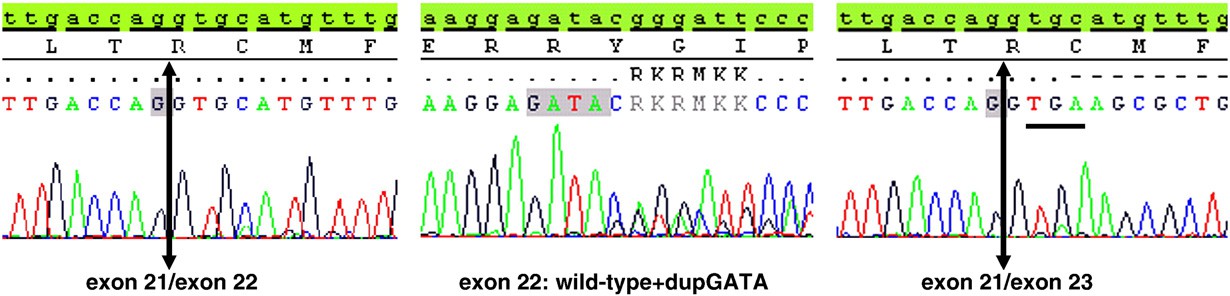
[](image%20of%20Fig. 2)

Fig. 2. Sequence analysis of cDNA fragments. *Left panel*. Sequences from the approx. 496 bp band found in lanes 2–8 showing only the wild-type exon 21/22 junction. *Middle panel*. Sequences from the approx. 496 bp band found in lane 1 corresponding to both the c.3626\_3629 dupGATA and non duplicated sequences. *Right panel*. Sequences from the approx. 336 bp band found in lanes 1, 3, 6 and 7 showing the exon 21/23 junction and the skipping of exon 22. Exon 22 skipping produces a TGA termination codon, at the ﬁrst coding position of exon 23 (underlined).

common substitution, A467T, falls into class III containing mutants that are located along the subunit interface between POLG1 and POLG2, reducing both processivity of pol γ and DNA binding.

The c.3643+2TNC identiﬁed in the *POLG* gene from patient 2 induces complete exon 22 skipping from the transcript. Substitution affecting the invariant GT dinucleotide at the donor splice results either in the use of a preexisting but weaker cryptic donor site or in exon skipping ([Krawczak et al., 1992](#_bookmark17)). The transcript lacking exon 22 has a PTC in exon 23 and is not destroyed by the nonsense-mediated mRNA decay (NMD) pathway as this PTC is in the last exon. The truncated protein encoded by this abnormal transcript would have only 46% of the C-terminal *pol* subdomain ([Lee et al., 2009](#_bookmark17)) and could be misfolded and sent for degradation.

The c.3626\_3629dupGATA allele has two different transcripts both containing a PTC and both escaping NMD: 1) a full length carrying the duplication resulting in a PTC in exon 22 and 2) a transcript with a skipped exon 22. The PTC created by the duplication 17 nt upstream from the last exon junction complex (EJC) is not located at least 50–55 nt upstream from at least one EJC as described previously in the 873-X allele ([Chan et al., 2005b](#_bookmark15)). This PTC results in a truncated protein

lacking the last 31-aminoacids and removing 21% of the C-terminal *pol* subdomain ([Lee et al., 2009](#_bookmark17)) and probably sent for degradation. These two abnormal transcripts arising from the duplicated allele are not targets for NMD as described for other *POLG* transcripts containing PTC ([Chan et al., 2005b, 2009](#_bookmark15)). Whatever the relative amount of these two abnormal transcripts, they lead obviously to truncated proteins and haploinsufﬁciency.

Splicing is a highly regulated mechanism and variations affecting splicing regulatory elements (SRE) can disrupt splicing ([Cartegni et al.,](#_bookmark12) [2002](#_bookmark12)). Exonic SRE promoting exon inclusion are referred to as exonic splicing enhancers (ESE) and those inhibiting exon inclusion are exonic

Table 1

DNA polymerase γ activity of human wild-type and mutant enzymes. Samples were measured under standard conditions (see [Subjects and methods](#_bookmark7)). For patients and controls, two different aliquots of mitochondria were submitted to serial dilutions (0.15 to 1.5 μg of proteins per assay). Each dilution was measured twice and data are averaged. Speciﬁc activity (U) was calculated as described in [Subjects and methods](#_bookmark7). For patients, U values are the mean of two independent measurements with each value in parentheses. For controls, U value is the mean for eight control lines within the age range of patients. SD: standard deviation. (n): number of samples. (%): percentage of the mean control value.

|  |  |
| --- | --- |
| Speciﬁc activity (U) (ddTTP-sensitive) | |
| *Controls* |  |
| mean±SD | 56.6 ± 17.8 |
| range (n) | 36.6–91.0 (8) |
| *Patients* |  |
| patient 1 (A467T/c.3626\_3629dupGATA) | 18.8 (16.9, 20.7) (33%) |
| patient 2 (W748S;E1143G/c.3643+2TN C) | 15.8 (14.1, 17.5) (28%) |

splicing silencers (ESS). Exon inclusion/skipping is a result of a ﬁne balance between enhancers and silencers both in constitutive and alternative splicing. These sequences are bound by serine/arginine-rich (SR) proteins for many ESE and heterogeneous nuclear ribonucleopro- teins (hnRNP) for ESS ([Jurica and Moore, 2003](#_bookmark17)). To test the hypothesis that the c.3626\_3629dupGATA could involve a SRE, the normal and duplicated sequences were submitted to search for ESE/ESS motifs with Human Splicing Finder (HSF) website ([Desmet et al., 2009](#_bookmark16)). No signiﬁcant change of the motif-scores for four SR proteins (SF2/ASF, SC35, SRp40 and SRp55) was observed in the GATA duplicated sequence compared with the wild-type sequence. But, when screening for ESS motifs, it appears that the GATA duplication creates a new binding site (TAGATA) for hnRNP A1 with a signiﬁcant strength. Binding of hnRNP A1 on a fraction of the transcript with the duplicated sequence could be responsible for the partial exon 22 skipping observed in cDNA from patient 1. Although this should be experimentally demonstrated, a recent paper ([Woolfe et al., 2010](#_bookmark17)) showed a clear enrichment for silencer creation in splice affecting genome variants.

In conclusion, two different mechanisms are involved in the haploinsufﬁciency in these patients, *i.e.* PTC and partial exon 22 skipping for patient 1, complete exon 22 skipping for patient 2, and the severe AHS phenotype in these patients was a consequence of a single-copy gene dose of the p.A467T or the p.[W748S;E1143G] allele.

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