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Case report

# Reevaluating the pathogenicity of the mutation c.1194 +5 G>A in GAA gene by functional analysis of RNA in a 61-year-old woman diagnosed with Pompe disease by muscle biopsy

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**Abstract**

Glycogen storage disease type II, or Pompe disease, is an autosomal recessive disorder caused by deficiency of lysosomal acid alpha-glucosidase (GAA). We performed genetic analysis to confirm the diagnosis of Pompe disease in a 61-year-old patient with progressive weakness in extremities, severe Sleep Apnea-Hypopnea Syndrome, a significant reduction of alpha-glucosidase in liquid sample of peripheral blood and muscular biopsy diagnosis. *GAA* gene sequencing showed the patient is homozygous for the splice-site mutation c.1194+5G>A, considered as nonpathogenic in Pompe Center mutation database. Further molecular RNA characterization of GAA transcripts allowed us to identify abnormal processing of pre-mRNA, leading to aberrant transcripts and a significant reduction of GAA mRNA levels. Our results indicate that c.1194+5G>A is a pathogenic splice-site mutation and should be considered as such for diagnostic purposes. This study emphasizes the potential role of functional studies to determine the consequences of mutations with no evident pathogenicity.


*Keywords:* Pompe disease; Splicing mutation; Functional characterization.

## 1. Introduction

Pompe disease or glycogen storage disease type II, (OMIM: 232300), is an autosomal recessive inherited disorder caused by mutations in *GAA* gene. It is located in the long arm of chromosome 17 (Chromosome 17: 80,101,556-80,119,879 forward strand (GRCh38:CM000679.2)) and includes 20 exons [1]. *GAA* encodes the lysosomal acid alpha glucosidase (acid maltase; EC. 3.2.10.20), the enzyme responsible of the intra-lysosomal glycogen hydrolysis. GAA deficiency results in progressive accumulation of glycogen in the lysosomes. Pompe disease exhibits a wide clinical spectrum from the most fatal infantile-onset form to a less aggressive late-onset adult form. Patients with the infantile-onset form of the disease are carriers of very deleterious mutations. Patients with the adult form carry mutations that affect the functionality of the gene or protein in different ways. The vast majority of these are missense or splice site mutations [2].

Here we report the molecular and functional characterization, based on a combined application of different molecular techniques [3], of the potential splicing mutation c.1194+5G>A. The mutation was identified in a 61 year-old patient with diagnosis of late-onset Pompe disease. So far, this mutation has been considered non-pathogenic in the Pompe Center mutation database [2], based on *in silico* analysis [4]. However, we reveal its deleterious consequences based on functional analysis of mRNA.

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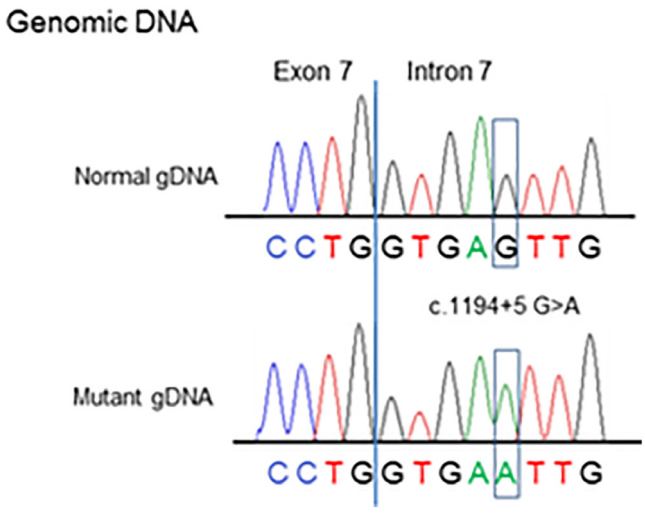


Fig. 1. Genomic sequence analysis. Sanger sequence of fragment depicting point mutation c.1194+5G>A in homozygosis, located in intron 7 of GAA gene. Vertical line indicates exon 7/intron 7 limit. The mutation is highlighted with a frame.

## 2. Case report

The patient, a 61-year-old woman, was diagnosed with an adult form of type-II glycogenosis (Pompe disease) by muscle biopsy. Quantification of GAA activity showed a significant reduction of alpha-glucosidase enzymatic activity: 0.72 μkat/kg protein, determined in lymphocytes in the presence of maltase-glucoamylase inhibitor acarbose, with a reference range: 4.8–13.3 μkat/kg protein. No antecedents of consanguinity have been identified in this patient. She started with the first symptoms at the age of 38, with progressive weakness in extremities, intolerance to exercise and myalgia with minimal efforts. In 2008, after 14 years of evolution, the patient required a wheelchair when leaving home. In October 2010, she was diagnosed with tetraparesis 3–4/5 with greater involvement at proximal and lower extremities. Since then, the progression of the disease has led to severe predominantly proximal, tetraparesis and incapacity to walk. As a result, the patient requires a wheelchair for any displacement. In addition, she developed severe Sleep Apnea-Hypopnea Syndrome (SAHS) (AHI: 33 / h) with acute nocturnal hypoventilation and needs Continuous Positive Airway Pressure (CPAP) during the night. However, serum measurements have always been normal, including CPK (<140 IU/L). Occasionally, pCO2 has shown levels of 45–50 mmHg, but with normal pO2.

Informed consent validated by Ethic Committee of our Hospital was obtained from the patient.

The genetic analysis was performed following a procedure described in 2007 [5]. Briefly, all exons and flanking introns were bidirectionally sequenced after PCR amplification. In addition, in order to exclude any potential deletion or duplication, a self-designed MLPA analysis was performed [6]. We identified the mutation c.1194+5G>A in homozygosis (NM_000152) (Fig. 1) and no additional variations were detected. The mutated nucleotide is in the 5′ splice site (5′ss) of the small intron 7, which only encompasses 89 bp. The genotype of this patient gave us an excellent opportunity to explore the functional consequences of this mutation.

To assess the potential splicing disruption, we completed a functional evaluation of the mutated allele. Total RNA obtained from patient's peripheral blood cells was used for the analysis. The approach is based on a combined application of RT-PCR, qPCR and sequence analysis of *GAA* transcripts [2]. We performed cDNA synthesis using random priming and systematically analyzed all coding exons, in order to explore every potential aberrant consequence of the mutated allele on GAA transcripts present in our patient.

The first stage in the approach involved the analysis by PCR amplification of each canonical exon, using oligonucleotides annealing to the flanking exons. Each amplicon was analyzed by agarose gel electrophoresis and all PCR products of exons showing aberrant size fragments were sequenced. Abnormal products were only observed in the analysis of exons 6, 7 and 8 (Fig. 2A). The analysis of exon 6 showed two products. An intense abnormal size fragment (amplicon 1), that was sequenced and resulted in the abnormal permanence of complete intron 6, and a low yield normal size fragment (amplicon 2), that represented the wild-type processing of intron 6 (Fig. 2B). Exon 7 study showed three different fragments. One product with abnormal size (amplicon 3) included the abnormal persistence of introns 6 and 7 in the mRNA. A second fragment (amplicon 4) was almost imperceptible and represented the wild-type splicing of introns 6 and 7, which might depict the functional fraction of GAA mRNA in this patient. Lastly, a very intense product (amplicon 5) that resulted from the skipping of exon 7 in the mRNA (Fig. 2B and Supplementary Fig. S1). The study of exon 8 showed two products: one intense abnormal size fragment (amplicon 6) indicating the abnormal permanence of intron 7, and a normal size fragment (amplicon 7) that represented the wild-type processing of intron 7 (Fig. 2B). The products observed in these three exons are likely to reflect the transcripts present in patient's sample as a consequence of the mutated allele. This is because exon 7 PCR amplification was performed using primers annealing to exon 6 (forward) and exon 8 (reverse) and, therefore, we amplified all possible transcript products of the mutated allele affecting splicing of intron 6 or intron 7. Furthermore, the same analysis on exon 6 and 8, which involved primers annealing to exon 7, showed that only transcripts maintaining exon 7 were amplified. In the study, it has been possible to identify three splicing variants. We consider the results obtained in the analysis of exon 7 were particularly relevant as the relative intensity of the three fragments may reflect the relative expression of the transcripts as a consequence of the abnormal mRNA processing. Firstly, the major splice variant leading to exon 7 skipping generated a mRNA with an altered reading frame and expected to be unstable, which suggests the implication of the nonsense mediated decay system (NMD). A second abnormal transcript was produced by keeping introns 6 and 7 in its structure. The

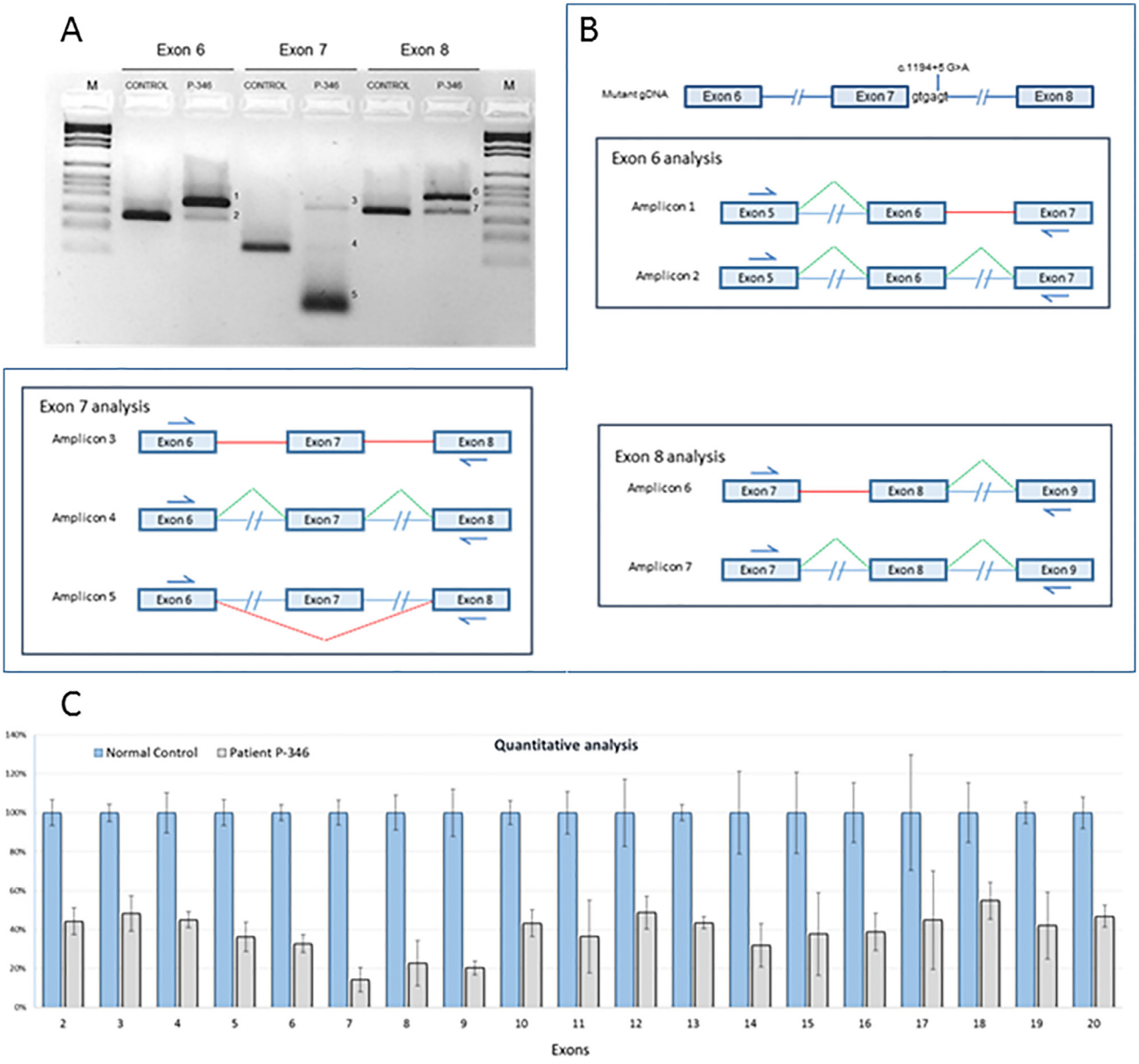


Fig. 2. RNA splicing analysis. (A) Agarose gel electrophoresis of RT-PCR fragments obtained during splicing analysis of Control sample and patient P-346. Exon numbers are marked above the lanes. Healthy sample is indicated as Control and the patient's sample is P-346. Each exon has been amplified with primers annealing to flanking exons. The analysis reveals the presence of several aberrant products (amplicons numbered 1–7). (B) Graphic representation of genomic GAA fragment, showing the splice mutation c.1194+5G>A and the splicing variants (boxed) identified by RNA analysis. Exons are represented as boxes and introns as lines. Double slash indicates no proportional representation of introns. A green broken line indicates normal processing of intron, red broken line shows aberrant splicing, continuous red line indicates non spliced intron. (C) Exon RT-qPCR analysis using internal primers. Beta -2 – microglobilin was used for normalization. Expression levels from control sample were considered and set to 100%. Error bars show Standard Deviation. ($n = 3$).

persistence of both introns in this aberrant transcript does not change the reading frame and the intronic sequences do not introduce themselves any premature stop signal, hence the transcript is expected to be stable. Finally, a minor wild-type mRNA was also observed. However, we cannot exclude the presence of additional minor transcripts that did not reach the detection threshold of the technique.

The next stage of the approach involved the amplification of each individual exon with internal primers followed by a standard qPCR analysis to evaluate the extent of splice defects and determine the expression level of each exon. The relative amount of exons 6, 8 and 9 was 20–40% compared to a healthy control, while exon 7 showed an additional reduction with an expression of ~15% of healthy control. The other exons were expressed between 40 and 60% of normal levels (Fig. 2C). These results suggest that an expression rate of ~15% of residual wild-type mRNA could allow the production of functional GAA protein, which is consistent with the GAA enzyme level of 0.72 μkat/kg protein found in this patient.

In addition to the functional characterization, we performed several analyses with a number of bioinformatic tools to

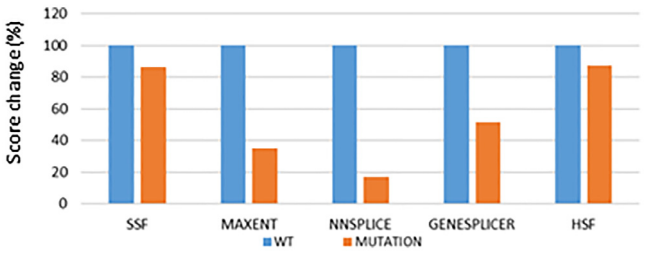


Fig. 3. Splice site prediction programs analysis. We have used Max Entropy Scan (MES), Splice Site Finder (SSF), NNSPLICE, Genesplicer and Human Splicing Finder (HSF), included in Alamut Software Suite version v.2.7.2. The score range of every program is different. To facilitate comparison, the scores obtained with wild-type 5′ ss sequence were set to 100%. Results obtained with mutation c.1194+5G>A represent the adjusted residual % of healthy control.

evaluate their prediction on the probability of mutation c.1194+5G>A on disrupting the normal splicing of *GAA*. We applied the splice prediction programs Max Entropy Scan (MES), Splice Site Finder (SSF), NNSPLICE, Genesplicer and Human Splicing Finder (HSF), which are integrated in Alamut Software Suite version v.2.7.2. The primary analysis was carried out using the default settings and it was modified when the predicted score for the mutated allele was under the threshold.

Firstly, we analyzed the reference sequence of the boundary between exon 7 and intron 7 to evaluate the ability of each predictor to detect the wild-type 5′ splice site. The scores corresponding to the wild-type 5′ splice site with each program were calculated and considered as 100% splicing efficiency. The sensitivity of each program was defined as the ability to predict a reduction (%) in the splice score in the presence of the pathogenic variant compared to the wild-type [7].

The highest sensitivity (as % of score change) was shown by NNSPLICE with −83.1% (Fig. 3). This severe reduction of predictive score supports the potential pathogenicity of the mutated allele. In contrast, the lowest reduction was shown by HSF, −12.9%. The web-based HSF [8] interpreted the effect of the mutation as: “No significant splicing motif alteration detected. This mutation has probably no impact on splicing”. The discordant results given by the predictive bioinformatic tools may lead to ambiguous conclusions about the potential pathogenicity of the mutation, however, our functional analysis shows unequivocal results supporting the pathogenic role of c.1194+5G>A.

## 3. Discussion

Here, we report the functional characterization of c.1194+5G>A, a splice mutation found in homozygosity in a patient affected with a late-onset form of Pompe disease, with no other molecular alterations in the structure of *GAA* gene. The mutation is located in the consensus sequence of the 5′ss, complementary to the U1 snRNA sequence AUACUUACCUG [9] and affects one of the three key nucleotides that interact with U6 snRNA [10]. According to these data, we hypothesized that the mutated allele could compromise the interaction with any of the mentioned snRNAs. Some comparative analysis of dependencies of 5′ss in humans have shown that nucleotides −1 and +5 play a major role in intron / exon processing [11]. These data reinforce the potential major role of the +5 nucleotide during the initial steps of splicing. Based on these antecedents, we considered the potential pathogenicity of the mutation, however, it had previously been described and considered as non-pathogenic based on *in silico* approaches [4]. Somehow, this certain degree of discrepancy boosted us to carry out the functional analysis.

The analysis was carried out using total RNA from peripheral blood cells, an easy source of RNA where *GAA* gene is expressed [12].

We used a generic procedure [2] to evaluate the potential effects of the mutation c.1194+5G>A on the pre-mRNA of the own patient instead of using alternative techniques as minigenes-based assays [13].

The results of our functional study strongly suggest that the mutation c.1194+5G>A disrupts the use of the constitutive 5′ splice site of intron 7. The main consequence is the skipping of exon 7 in a major proportion of transcripts. This phenotype is the most frequent one found in mutations affecting the 5′ constitutive splice sequence according to previous studies [14]. In addition, we have identified a significant proportion of transcripts retaining introns 6 and 7. The process of exon definition can be altered by mutations affecting the 5′ consensus sequence and might reduce the splicing of two introns, the intron where the mutation is located and the intron previous to the exon located in between [15]. In vitro assays have confirmed that mutation of 5′ splice site reduces the removal of the upstream intron by 20-fold [16]. We have identified a significant reduction of mRNA level which suggests the potential degradation of some aberrant transcripts via the NMD. We have not found the use of any alternative splice site in the mutated allele.

Finally, predictive analysis using five different scores integrated in Alamut Software Suite version v.2.7.2 has shown different capacity to estimate the potential pathogenicity of the mutation. Hence, our functional analysis can be used to validate the performance of the splice predictive programs included in Alamut Suite for this particular mutation. While NNPLICE and MAXENT showed good ability to predict the potential pathogenic role of the mutation, HSF and SSF did not anticipate any potential pathogenic role.

In summary, our results show that the c.1194+5G>A mutation generates different amounts of aberrant splice products with potential pathogenic implications on *GAA* expression. We conclude that the mutation is pathogenic and that it should be considered as such in a diagnostic setting. Our study highlights that, in certain circumstances, the inclusion of functional studies can help to adequately characterize the possible pathogenicity of variants that, otherwise, could be misclassified.

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## Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.nmd.2018.12.003.

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