Deep Intrinsic \textit{GBE1} Mutation in Manifesting Heterozygous Patients With Adult Polyglucosan Body Disease

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\textbf{Importance} We describe a deep intronic mutation in adult polyglucosan body disease. Similar mechanisms can also explain manifesting heterozygous cases in other inborn metabolic diseases.

\textbf{Objective} To explain the genetic change consistently associated with manifesting heterozygous patients with adult polyglucosan body disease.

\textbf{Design, Setting, and Participants} This retrospective study took place from November 8, 2012, to November 7, 2014. We studied 35 typical patients with adult polyglucosan body disease, of whom 16 were heterozygous for the well-known c.986A>C mutation in the glycogen branching enzyme gene (\textit{GBE1}) but harbored no other known mutation in 16 exons.

\textbf{Main Outcomes and Measures} All 16 manifesting heterozygous patients had lower glycogen branching activity compared with homozygous patients, which showed inactivation of the apparently normal allele. We studied the messenger ribonucleic acid (mRNA) structure and the genetic change due to the elusive second mutation.

\textbf{Results} When we reverse transcribed and sequenced the mRNA of \textit{GBE1}, we found that all manifesting heterozygous patients had the c.986A>C mutant mRNA and complete lack of mRNA encoded by the second allele. We identified a deep intronic mutation in this allele, \textit{GBE1}-\textit{IVS15} + 5289_5297delGTGTGGTGGinsTGTTTTTTACATGACAGGT, which acts as a gene trap, creating an ectopic last exon. The mRNA transcript from this allele missed the exon 16 and 3’ UTR and encoded abnormal GBE causing further decrease of enzyme activity from 18% to 8%.

\textbf{Conclusions and Relevance} We identified the deep intronic mutation, which acts as a gene trap. This second-most common adult polyglucosan body disease mutation explains another founder effect in all Ashkenazi-Jewish cases.

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cosan accumulations. Cases with complete deficiency usually die prenatally. Patients with APBD have approximately 18% GBE activity and smaller PBs. Their livers and other nonnervous tissues, although containing PBs, remain clinically spared.13-16

Most patients with APBD are Ashkenazi-Jewish and have a homozygous GBE1 mutation, p.Y329S. Few are compound heterozygotes of p.Y329S with p.L224P. As expected, carrier parents are unaffected.1-4,16 A large fraction of patients (30%) carry the p.Y329S mutation in the heterozygous state with no other mutation in the 16 exons of the gene.2 There are no known Ashkenazi patients who do not carry at least 1 copy of the p.Y329S mutation. In the current study, we resolved this genetic conundrum and identified a deep intronic second mutation in the manifesting heterozygotes, which in the homozygous state, is likely lethal. To our knowledge, all known Ashkenazi patients with APBD are now explained.

Methods

Glycogen branching enzyme activity was measured in leukocytes, as previously described.12,13 The study was approved by the Columbia University Medical Center and Hadassah-Hebrew University Medical Center institutional review boards. Written informed consent was obtained from participants.

Haplotype Analysis

Two microsatellite markers flanking GBE1 were genotyped at chr3:81312404-81312683 (UCSC hg-19 build; http://genome.ucsc.edu/), with primer sequences F-GTGATGCTCTGGTGCA-CAT and R-TACCCCTCTAACAACATCCT and product sizes in our patients of 270 base pairs (bp), 278 bp, 280 bp, and 282 bp, as well as at chr3:81751119-81751368, with primers F-CTCAGGTCAGTGGACAAAA and R-GTGATGACAACCTAC-GTGAATA, sizes 242 bp, 246 bp, and 250 bp.

Mutation Identification

Messenger ribonucleic acid (mRNA) interruption was determined by a nested polymerase chain reaction (PCR) amplifying complementary DNA (cDNA) in 2 rounds: first, with GBE1-Ex13F primer (5′-ATATGGTTGGCTTGCAGCTC) and oligo dT(T)18, the resulting product was sequenced with a Sanger sequencing. Because of the nonspecificity of oligo-dT priming. To enrich for GBE1, we performed PCR amplified the cDNA using various primer pairs flanking GBE1 exon 7 and, with most pairs, obtained the homozygous c.986A>G sequence as in the gDNA. However, when we placed the reverse primer in exon 16, the PCR product was no longer heterozygous but homozygous for c.986A>C (Figure 1B). This suggested that the manifesting heterozygotes generate 1 transcript containing the exon 7 c.986A>C mutation and another transcript that lacks exon 16 or part thereof. We set out to identify and characterize the latter. This putative abnormal transcript had a poly-A tail because our starting cDNA was generated with an oligo-dT primer. We PCR amplified with forward primer in exon 13 and used oligo-dT as the reverse primer. The products were a mix with non-GBE1 sequences, which was unsurprising given the nonspecific nature of oligo-dT priming. To enrich for GBE1, we performed nested PCR, amplifying the exon 13–oligo-dT products with forward primer in exon 14 and oligo-dT reverse and then sequencing the PCR products with an exon 15 forward primer. The sequence obtained was the normal GBE1 coding sequence until the point where exon 16 should have started. At that point, the sequence became double: the normal exon 16 sequence and a second abnormal nonexon 16 sequence. We blasted this unknown sequence against the human genome and found that it was, in part, from GBE1 intron 15. We PCR amplified this intron 15 region in gDNA from manifesting heterozygotes and found that a 9-bp 5′-GTGGTGAGG sequence from intron 15 was replaced by a 20-bp 5′-GTGATGTCATTACAGGT new sequence (Figure 2A). This abnormal sequence contained a highly potent mRNA splice acceptor site (http://www.fruitfly.org

Results

Manifesting Heterozygote Patients Share 2 Haplotypes

In a cohort of 35 patients with APBD, 16 were homozygous and 19 heterozygous for the c.986A>C (p.Y329S) mutation. This mutation segregated with 2 haplotypes; 3 heterozygous patients harbored a third haplotype associated with a second missense exon 16 mutation, c.671T>C (p.L224P). However, in the remaining 16 heterozygous patients for the c.986A>C mutation, extensive searches, including exonic and cDNA sequencing, multiplex ligation-dependent probe amplification, and whole-genome sequencing (Complete Genomics; see further on), failed to reveal a second mutation. The possibility arose that in these patients with c.986A>C (p.Y329S), 1 allele alone was somehow sufficient to cause the disease, ie, that these cases were manifesting heterozygotes. However, haplotype analysis showed that all these patients shared a common haplotype separate from their c.986A>C-associated haplotypes, suggesting that they actually did harbor a second mutation, which we were simply failing to detect despite the whole-genome sequencing.

Identification and Characterization of the Missing Mutation in the Manifesting Heterozygote Patients

By definition, all manifesting heterozygote patients were heterozygous for the exon 7 c.986A>C mutation in gDNA (Figure 1A). We synthesized cDNA from leukocyte mRNA from these patients using oligo-dT priming (oligo-dT is complementary to the poly-A tail at ends of mRNA). We PCR amplified the cDNA using various primer pairs flanking GBE1 exon 7 and, with most pairs, obtained the homozygous c.986A>C sequence as in the gDNA. However, when we placed the reverse primer in exon 16, the PCR product was no longer heterozygous but homozygous for c.986A>C (Figure 1B). This suggested that the manifesting heterozygotes generate 1 transcript containing the exon 7 c.986A>C mutation and another transcript that lacks exon 16 or part thereof. We set out to identify and characterize the latter. This putative abnormal transcript had a poly-A tail because our starting cDNA was generated with an oligo-dT primer. We PCR amplified with forward primer in exon 13 and used oligo-dT as the reverse primer. The products were a mix with non-GBE1 sequences, which was unsurprising given the nonspecific nature of oligo-dT priming. To enrich for GBE1, we performed nested PCR, amplifying the exon 13–oligo-dT products with forward primer in exon 14 and oligo-dT reverse and then sequencing the PCR products with an exon 15 forward primer. The sequence obtained was the normal GBE1 coding sequence until the point where exon 16 should have started. At that point, the sequence became double: the normal exon 16 sequence and a second abnormal nonexon 16 sequence. We blasted this unknown sequence against the human genome and found that it was, in part, from GBE1 intron 15. We PCR amplified this intron 15 region in gDNA from manifesting heterozygotes and found that a 9-bp 5′-GTGGTGAGG sequence from intron 15 was replaced by a 20-bp 5′-GTGATGTCATTACAGGT new sequence (Figure 2A). This abnormal sequence contained a highly potent mRNA splice acceptor site (http://www.fruitfly.org
Exon 15 spliced into this ectopic splice site rather than into exon 16. Reverse transcription PCR using forward primer from exon 7 and reverse primer from the new abnormal putative exon generated precisely this product, i.e., exon 15 spliced into the ectopic splice acceptor, creating a new abnormal exon 16 with a new stop codon (Figure 2B). Therefore, the missing mutation in the manifesting heterozygotes was IVS15 + 5289_5297delGTGTGGTGGinsTGTTTTTTACATGACAGGT, generating an mRNA that encoded a truncated protein (Figure 2B).

The truncated protein was unstable. Western blotting of leukocyte extracts from the manifesting heterozygote patients using an antibody against the middle unaffected portion of the GBE protein revealed more reduced total protein compared with p.Y329S homozygote patients (Figure 2C). The mean (SD) GBE activity in the manifesting heterozygote patients was 8% (6%) of normal (n = 3) compared with 18% (4%) (n = 6) in simultaneously measured p.Y329S homozygotes. The new mutation was present in all manifesting heterozygotes and was not found in 120 anonymous Ashkenazi individuals.

In light of having identified the missing mutation, we reanalyzed our whole-genome sequence data. The computerized genome reassembly failed to resolve the repetitive sequences in this region and failed to detect the mutation.

Discussion

We resolved the genotype of manifesting heterozygous Ashkenazi patients with APBD. They were compound heterozygotes for 2 mutations: c.986A>C (p.Y329S) and IVS15 + 5289_5297delGTGTGGTGGinsTGTTTTTTACATGA-CAGGT. Both are founder mutations. No patient was homozygous for the new mutation. This fact, combined with the greater severity of the new mutation (as determined by Western blot analysis) and the absence of any patients with classic GSD-IV within our families with APBD, indicated that homozygosity for the new mutation is likely prenatal lethal. Compound heterozygosity for the 2 mutations did not appear to be more severe than homozygosity for p.Y329S, suggesting that 8% residual GBE activity was not significantly worse than 18%. However, careful clinical reassessment is warranted to confirm this point.

While the mutation identified here is not the first intronic mutation causing neurological disease, it is unusual in its last intron location, which both complicated and simplified its discovery. It was complicated because there was no exon beyond the mutation on which to place a primer and amplify the aberrant transcript. It was simplified because premature termination involving last exons did not cause nonsense-mediated mRNA decay and the aberrant transcript was therefore present and eventually specifically amplifiable.

A, Genomic DNA in manifesting heterozygotes is heterozygous for the c.986A>C mutation. B, Complementary DNA amplified with forward primers upstream of c.986 (i.e., forward primer placed in exons 1 through 6) and reverse primers in exons 8 through 15 identify the c.986A>C mutation in its expected heterozygous state; an example electropherogram from a product with primers in exons 6 and 15 is shown. When the reverse primer is in exon 16, c.986A>C appears in the homozygous state. Horizontal arrows above the electropherograms indicate exon boundaries within the segments of complementary DNA sequence shown. The gels at the bottom of panel B show example polymerase chain reacting products with their respected size in base pairs (bp) indicated by 1 kilobase pair (kbp) plus the DNA ladder. Corresponding amino acids coded by DNA and messenger ribonucleic acid are indicated by single letters.
Conclusions

Whole-genome sequencing shears gDNA, sequences billions of fragments, and bioinformatically reassembles their sequences into a genome. Output files catalog the millions of variants as well as innumerable regions where the program fails, usually in noncoding regions where DNA is repetitive, to generate correct reassembly. Our mutation was in such a region and highlighted present-day shortfalls of whole-genome sequencing in that a mutation in a known locus, ie, in a known single gene, could not be detected. How many such mutations are missed in projects where the locus is unknown?

Our study predicted that therapy might be harder to achieve in compound heterozygous cases (8% residual GBE activity) compared with p.Y329S homozygotes (18% activity) compared with p.Y329S homozygotes (18% activ-

Figure 2. Deletion/Insertion in Genomic DNA of Manifesting Heterozygote Patients, Inserting a Pseudo Exon That Encodes an Unstable Protein

A, The deletion/insertion sequence was resolved by subtracting the control sequence from the patient sequence. B, Manifesting heterozygotes have 2 different glycogen branching enzyme (GBE) messenger ribonucleic acids. When a forward primer is placed in exon 7 and a reverse primer is placed in the normal 3' untranslated region (UTR), the c.986A>C mutation in exon 7 and the normal exon 16 are seen (top panel); when the reverse primer is in the new abnormal 3' UTR, the c.986A>C mutation and the normal exon 16 are not seen.

Instead, the abnormal pseudo exon 16 is seen (lower panel). C, Glycogen branching enzyme protein is very reduced in manifesting heterozygous patients compared with p.Y329S homozygous cases (leukocyte Western blot using 30-μg protein and a polyclonal GBE antibody raised against a synthetic peptide from the central region of human glycogen branching enzyme; Pierce Biotechnology). GAPDH indicates glyceraldehyde-3-phosphate dehydrogenase.
ity). However, new therapies exploiting antisense oligonucleotides may be used to neutralize the cryptic splice site in the former and convert these patients into true unaffected heterozygotes, as are being tested in Duchenne dystrophy.20 To our knowledge, our study is the first that allows specific diagnosis in most Ashkenazi patients with APBD, as well as precise genetic and prenatal diagnosis and counseling.

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