

Diagnosis and evaluation of primary panhypogammaglobulinemia: A molecular and genetic challenge

Maaz S. Mohiuddin, MD,^a Jordan K. Abbott, MD,^a Nicholas Hubbard, BS,^b Troy R. Torgerson, MD, PhD,^b Hans D. Ochs, MD,^b and Erwin W. Gelfand, MD^a *Denver, Colo, and Seattle, Wash*

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Activity Objectives

1. To be able to provide a differential diagnosis for hypogammaglobulinemia.
2. To discuss the initial work-up for hypogammaglobulinemia.
3. To describe the extended evaluation for establishing a definitive molecular diagnosis of X-linked agammaglobulinemia (XLA).

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CLINICAL VIGNETTE

The patient, a 2-year-old boy, presented to the Immunology Clinic at National Jewish Health for evaluation of frequent respiratory illnesses and nasal congestion. His history included 3 episodes of croup necessitating oral steroid therapy, 2 episodes of chest radiography–documented pneumonia requiring antibiotics, an episode of sinusitis treated with antibiotics, and 10 episodes of otitis media requiring antibiotics and

placement of ventilation tubes. He continued to have intermittent purulent otorrhea after ventilation tube placement. Results of physical examination were remarkable for a paucity of tonsillar and lymphoid tissues. There was no family history of immunodeficiency.

Initial laboratory investigations revealed a normal white blood cell count with low absolute numbers of neutrophils (1000/ μ L). Initial immunoglobulin quantitation at 2 years of age was as follows: IgG, less than 75 mg/dL; IgA, less than 0.7 mg/dL; IgM, 29 mg/dL; and IgE, 22 IU/mL. Lymphocyte enumeration revealed 4326 CD3⁺ cells/ μ L, 3019 CD4⁺ cells/ μ L, 1306 CD8⁺ cells/ μ L, and 23 CD19⁺ cells/ μ L, representing 0.5% of total lymphocytes. As predicted, responses to vaccination with DTaP, Hib, and pneumococcal conjugate were absent. The patient was started on intravenous immunoglobulin replacement therapy.

Bruton's tyrosine kinase (B-cell tyrosine kinase; BTK) protein expression in platelets^{E1} was markedly reduced with a mean fluorescence intensity of 16.2 compared with a mean fluorescence

From ^athe Division of Allergy and Immunology, Department of Pediatrics, National Jewish Health, Denver, and ^bthe Department of Pediatrics, University of Washington School of Medicine and Seattle Children's Research Institute, Seattle.

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Corresponding author: Erwin W. Gelfand, MD, National Jewish Health, 1400 Jackson St, Denver, CO 80206. E-mail: gelfande@njhealth.org.

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intensity of 96.9 in a healthy control subject (see Fig E1 in this article's Online Repository at www.jacionline.org). The proband's mother was also evaluated and found to have 2 distinct platelet populations, one with normal and one with reduced protein expression, indicating carrier status (see Fig E1). Although the severely reduced level of BTK protein in the patient's platelets suggested X-linked agammaglobulinemia (XLA), sequence

analysis of the patient's genomic DNA (gDNA) did not identify a mutation in the coding region and exon/intron junctions of the *BTK* gene.

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REVIEW

Congenital X-linked panhypogammaglobulinemia occurs in approximately 1 in 200,000 male subjects and is characterized by profound decreases in all classes of immunoglobulin with an absence of circulating B lymphocytes.^{E2,E3} BTK has multiple roles in the development, survival, signaling, and proliferative responses of B cells. In the absence of BTK, there is a block in B-cell maturation between the pro- and pre-B-cell stages, leading to severe reduction in mature B-lymphocyte numbers.

Presentation

Most patients with this form of panhypogammaglobulinemia present with recurrent infections, often beginning after 3 months of age. They demonstrate increased susceptibility to bacterial infections, particularly encapsulated pyogenic bacteria. The most common infections before diagnosis include recurrent otitis media, followed by pneumonia and sinusitis, but can include recurrent episodes of bacterial sepsis, osteomyelitis, septic arthritis, and bacterial meningitis.^{E4} Susceptibility to enteroviruses in the form of meningoencephalitis, dermatomyositis/fasciitis, and hepatitis is also seen in the untreated population.^{E4} Septic arthritis from atypical bacteria, such as *Mycoplasma/Ureaplasma* species, has been reported,^{E5} and its frequency is likely underestimated. *Salmonella*, *Campylobacter*, and *Giardia* species are often implicated in those with gastroenteritis.^{E4} *Flexispiral/Helicobacter* species can lead to chronic bacteremia and lymphatic infections.^{E6} The incidence of these infections is markedly reduced with regular immunoglobulin replacement therapy.

Differential diagnosis

The differential diagnosis for panhypogammaglobulinemia can vary depending on age of onset or presentation and includes XLA, transient hypogammaglobulinemia of infancy, common variable immunodeficiency, autosomal recessive B-cell deficiency, X-linked lymphoproliferative syndrome, and combined T- and B-cell immunodeficiency with hypogammaglobulinemia.^{E7} As a general rule, lack of B lymphocytes excludes transient hypogammaglobulinemia of infancy and other causes of primary hypogammaglobulinemia, such as X-linked hyper-IgM, X-linked lymphoproliferative disease, or secondary causes, such as drug-induced hypercatabolism, protein loss (nephrotic syndrome, protein-losing enteropathy, and lymphangiectasia), malignancy, and HIV infection. Absence of T cells directs the work-up toward defects associated with severe combined immunodeficiency. The isolated congenital deficiency of B cells supports the diagnosis of XLA in a male subject, but the clinical phenotype can be mimicked by rare mutations in genes encoding the μ heavy chain, Ig α , Ig β , λ 5, B-cell linker (*BLNK*), leucine-rich repeat-containing 8 (*LRRC8*), or the p85 α subunit of phosphoinositide 3-kinase (PI3K).^{E8,E9}

Diagnostic approach

Testing should begin with measurement of quantitative serum immunoglobulin levels and antibody titers to vaccine antigens, along with lymphocyte subset analysis. If the clinical phenotype suggests XLA, molecular diagnostic studies are indicated, which includes assessment of BTK protein levels in platelets or monocytes by means of flow cytometry and sequence analysis of the *BTK* gene.^{E1,E10} If sequencing of gDNA does not detect a mutation in *BTK* in a patient with absent BTK protein,

deletion/duplication analysis should be performed along with sequencing of cDNA to identify mutations occurring outside the coding regions. Mutations in the promoter region that interfere with binding of the transcription factor PU.1, leading to the defective transcription of *BTK*, have been described and illustrate how mutations occurring outside of the coding region of *BTK* affect BTK expression.^{E11} Because a small subset of patients with XLA have normal protein expression, a definite diagnosis can only be made by identifying a *BTK* mutation in those without a family history of disease.^{E1,E10} The extended sequence analysis might be limited by availability and costs but has important implications for the genetic counseling offered to the family. Extended molecular analysis is offered by academic institutions that can measure BTK expression and sequence *BTK*.

If B-cell deficiency occurs in a female subject or if BTK expression in platelets or monocytes is normal in the setting of B-lymphocyte deficiency, a less frequent autosomal recessive mutation in genes encoding the μ heavy chain, Ig α , Ig β , λ 5, *BLNK*, *LRRC8*, or the p85 α subunit of PI3K should be considered.

A definitive diagnosis for XLA can be assumed in any male subject with less than 2% CD19⁺ cells and one of the following: (1) a mutation in *BTK*; (2) absent *BTK* mRNA on Northern blot analysis of PBMCs, neutrophils, or monocytes; (3) absent BTK protein in monocytes or platelets; and (4) maternal male cousins, uncles, or nephews with less than 2% CD19⁺ cells (Table E1).^{E12} Patients meeting these criteria account for approximately 85% of cases of panhypogammaglobulinemia and extremely low circulating B-cell numbers, whereas patients with mutations in genes associated with the autosomal recessive forms (μ heavy chain, Ig α , Ig β , λ 5, *BLNK*, *LRRC8* or the p85 α subunit of PI3K) account for 5% to 10% of patients with panhypogammaglobulinemia and low or absent B cells.^{E9,E13} In the remaining 5% of cases, an undefined defect is likely because normal levels of BTK protein were detected in the majority.^{E8,E9}

Diagnosis of XLA in adulthood has been reported in the literature, including a now 69-year-old patient who survived with only antibiotic therapy and no immunoglobulin replacement therapy.^{E14} The most common misdiagnosis of XLA in adults is common variable immunodeficiency (CVID); however, less than 5% of patients with CVID have less than 1% CD19⁺ cells, and therefore XLA should be ruled out in adult male patients presenting with profound hypogammaglobulinemia, decreased B-cell numbers, neutropenia, recurrent sinopulmonary infections, meningitis, absent tonsils or palpable lymphoid tissue, or any other typical XLA findings. Mutations in *BTK* have been described in atypical presentations of XLA, including an adult originally given a diagnosis of CVID; this patient had normal serum IgG levels and vaccine responses, decreasing CD19⁺ cell numbers, and normal BTK expression, which leads to questions of whether all mutations are pathogenic.^{E15,E16}

Mutations

More than 600 patients with mutations in *BTK* involving all functional domains have been registered in an extensive database (BTKbase),^{E17} and additional mutations have been described in the literature, indicating that new disease-causing variants continue to be discovered. Insertions and deletions of less than 5 bp and single-base-pair substitutions are responsible for more than 90% of the documented mutations.^{E8} Relevant to the patient reported here, up to 3% of sequence

abnormalities are not identified by using conventional gDNA sequencing.^{E2}

Management

Immunoglobulin replacement remains the mainstay for therapy. The majority of patients are treated with intravenous immunoglobulin at a dose of 400 to 600 mg/kg per month generally administered every 3 to 4 weeks. Subcutaneous injections are an alternative delivery method. Aggressive antibiotic therapy for suspected or documented infections with prolonged courses might be necessary. The use of specific antibiotics can be indicated to cover atypical infections seen in these patients, such as infections with *Mycoplasma* species.^{E5} Live virus vaccinations are contraindicated. Theoretically, vaccination (with inactivated virus) might provide protection by generating T cell–mediated responses, but such data are not available. Because of the risk of chronic lung disease in patients with XLA, especially if lung disease antedated immunoglobulin replacement therapy, patients should be monitored on a regular basis with comprehensive pulmonary function testing. Computed tomographic scans of the chest and sinuses at baseline are indicated, with follow-up based on symptoms and lung function.

Genetic counseling

XLA is inherited in an X-linked manner, whereas mutations in $Ig\alpha$, $Ig\beta$, $\lambda 5$, *BLNK*, *LRRC8*, or the $p85\alpha$ subunit of PI3K are inherited in an autosomal recessive manner. All female offspring of a proband will inherit the *BTK* mutation but will be asymptomatic carriers. Female carriers have a 50% chance of transmitting the disease-causing mutation to their offspring, with all affected male subjects becoming symptomatic and female subjects becoming carriers; 50% of patients with XLA do not have a family history.^{E2} In these cases, either the affected male has a *de novo* pathogenic mutation (15% to 20%) or the mother is a carrier of the pathogenic mutation (80% to 85%).^{E2} Carrier testing should be offered to at-risk female subjects if the mutation is known. Assessment of BTK protein expression by means of flow cytometry is dependent on the detecting antibody used, the epitope it recognizes on the protein, and the cell population studied. Therefore one cannot rely solely on flow cytometry, and identifying the mutation has significant implications for genetic counseling.^{E1} If the mutation is known in a given family, the mutation analysis in additional family members, including potential carriers, will be less costly. Prenatal testing is available, although differences in opinion exist regarding the utility and risks of prenatal testing for a condition that is treatable and in which early diagnosis can be made postnatally without placing the newborn at risk. Preimplantation genetic diagnosis is also available and should be discussed with at-risk female subjects. The immunologist, in conjunction with a geneticist, can discuss these aspects with families.

Future

Newborn screening for B-lymphocyte deficiency might become available in the future because detection of immunoglobulin kappa-deleting recombination excision circles has been validated. Quality of life and long-term prognosis have significantly improved since the institution of regular immunoglobulin replacement therapy, although delays in diagnosis persist, with evidence for target organ damage if treatment is delayed. Appropriate immunoglobulin dosing is also important because

significant variations in patient-to-patient requirements are becoming evident.^{E18} Gene therapy in the murine model of XLA has led to sustained B-cell recovery, but further studies are required before it becomes applicable in human subjects.^{E19}

THE CASE REVISITED

The patient reported here had classic features and laboratory findings of XLA, including undetectable IgG and IgA levels and a marked paucity of circulating B lymphocytes in the absence of a family history. Evaluation revealed the absence of BTK expression in platelets, but sequence analysis of the coding region and exon/intron boundaries with gDNA did not identify a mutation.

Sequence analysis of cDNA generated from mRNA derived from the patient's buffy coat revealed an additional 153-bp fragment derived from intron 1 originating 147 bp downstream of the end of exon 1 (Fig E2). Although the inserted fragment was “in frame,” it contained a stop codon within the first 4 codons of the insert. Sequencing of intron 1 identified an A>G substitution occurring 300 bp downstream of exon 1 (c.141+300 A>G), generating a new donor splice site that, by using a cryptic splice acceptor site (C141+147), generated an in-frame “pseudoexon” of 153 bp that contained the stop codon. The same point mutation was demonstrated in one of the maternal *BTK* alleles. This alteration was not reported as a single nucleotide polymorphism in either the data sets available from 1000 Genomes or dbSNP, supporting the conclusion that the patient's A>G mutation 300 nucleotides downstream of exon 2 generated a novel splice donor site that altered the normal splicing by inserting an intron 1–derived fragment containing a stop codon.

Review of the family history revealed a paucity of male subjects on the maternal side without a history of early childhood deaths. There is a healthy maternal grandfather and uncle with no clinical evidence of disease. Molecular analysis confirmed that the mother had the same mutation. Whether this is a *de novo* mutation in the patient's mother is not known at this time.

In summary, we report a novel intronic single nucleotide substitution that disrupts splicing and results in a nonfunctional truncated transcription product that was not detected by using conventional gDNA sequencing. This discovery highlights the importance of BTK protein expression analysis as a complement to sequencing studies, especially when gDNA sequencing limited to exons and exon/intron boundaries in a suspected patient does not identify a mutation. As shown here, the absence of BTK protein is not always caused by mutations in the coding region or exon/intron boundaries. The absence of BTK can also occur in association with a splice-site defect, a mutation in the regulatory elements, the poly-A region, or a large deletion involving the entire *BTK* gene (and possibly neighboring genes).^{E2,E20,E21} Searching for mutations beyond normal sequencing approaches might be required. Establishing the precise genetic basis for the disease not only is important for guiding therapy, discussing prognosis, and genetic counseling but also is a prerequisite for carrier detection, prenatal diagnosis, and future potential genetic interventions. The present case highlights the importance of an extended genetic evaluation in rare cases in which conventional molecular approaches do not provide a definitive diagnosis of XLA or another genetically determined primary immunodeficiency.

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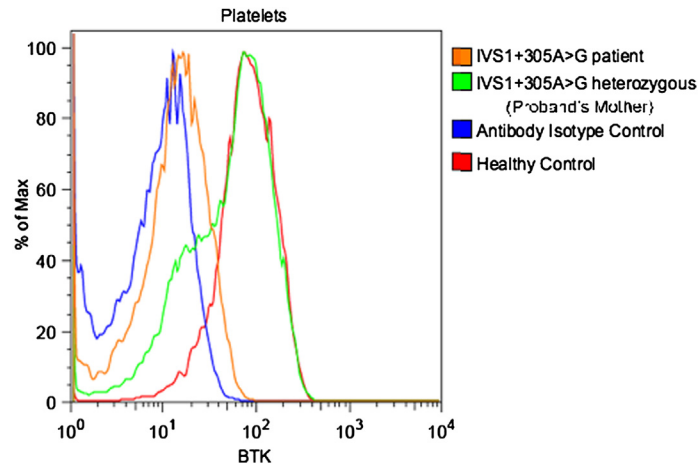


FIG E1. Flow cytometry showing absent BTK expression in the patient and carrier status in the mother of the proband.

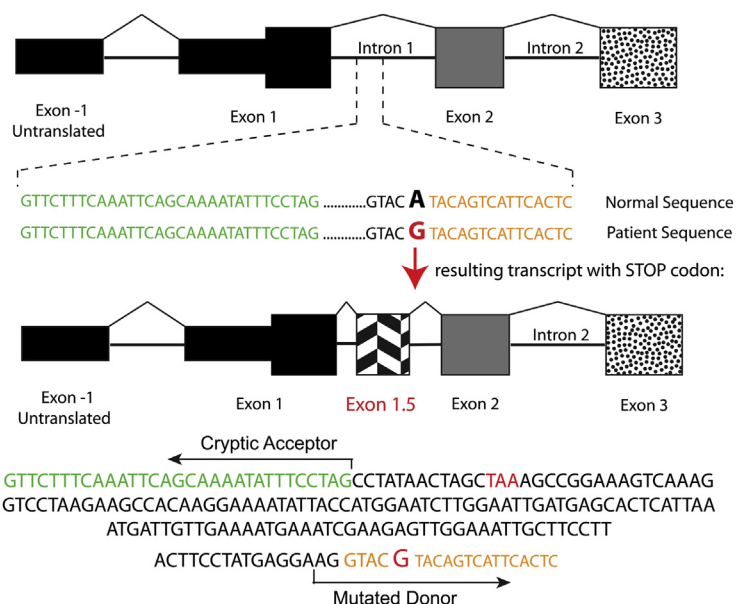


FIG E2. Intronic mutation of *BTK* creates a new splice donor site, which, when paired with an upstream cryptic splice acceptor, causes a fragment of the intron to be spliced into the mRNA between exons 1 and 2 (noted here as *Exon 1.5*). This leads to a termination signal (TAA) at the fifth codon within the inserted fragment. *Black print* denotes the inserted intronic fragment, and *red print* denotes the patient's mutation and new stop codon.

TABLE E1. Diagnostic criteria for XLA

Definitive diagnosis
Male patient with <2% CD19 ⁺ B cells and ≥1 of the following:
1. Mutation in <i>BTK</i>
2. Absent <i>BTK</i> mRNA on Northern blot analysis of neutrophils or monocytes
3. Absent BTK protein in monocytes or platelets
4. Maternal cousins, uncles, or nephews with <2% CD19 ⁺ B cells
Probable diagnosis
Male patient with <2% CD19 ⁺ B cells in whom all of the following are true:
1. Onset of recurrent bacterial infections in the first 5 years of life
2. Serum IgG, IgM, and IgA levels >2 SDs less than normal for age
3. Absent isohemagglutinins, poor response to vaccines, or both
4. Other causes of hypogammaglobulinemia have been excluded
Possible diagnosis
Male patient with <2% CD19 ⁺ B cells in whom other causes of hypogammaglobulinemia have been excluded and ≥1 of the following are true:
1. Onset of recurrent bacterial infections in the first 5 years of life
2. Serum IgG, IgM, and IgA levels >2 SDs less than normal for age
3. Absent isohemagglutinins

Patients with a definitive or probable diagnosis are assumed to have a greater than 98% and 85% probability, respectively, that in 20 years they will still have the same diagnosis. Patients with a possible diagnosis are those with some but not all of the characteristic clinical or laboratory findings of XLA.^{E12}