A Novel Mutation ($Cys^{145 \rightarrow Stop}$) in Bruton's Tyrosine Kinase Is Associated with Newly Diagnosed X-Linked Agammaglobulinemia in a 51-Year-Old Male

Stephen J. Kornfeld,* Robert N. Haire,* Scott J. Strong,* Huayang Tang,† Sun-Sang J. Sung,† Shu Man Fu,† and Gary W. Litman*

*Department of Pediatrics, University of South Florida College of Medicine, All Children's Hospital, St. Petersburg, Florida, U.S.A.
†Department of Internal Medicine, University of Virginia School of Medicine, Charlottesville, Virginia, U.S.A.

ABSTRACT

Background: X-linked agammaglobulinemia (XLA) is a severe, life-threatening disease characterized by failure of B cell differentiation and antibody production and is associated with mutations in Bruton's tyrosine kinase (*Btk*). The proband in this study is a 51-year-old male presenting with chronic nasal congestion, recurrent sinusitis, sporadic pneumonia, and pronounced B cell deficiency. A family history suggestive of an X-linked immunodeficiency disease was noted.

Materials and Methods: cDNA was synthesized from mRNA prepared from peripheral blood mononuclear leukocytes. *Btk* cDNA amplified by polymerase chain reaction (PCR) was subjected to both manual and automated DNA sequencing. A DNA sequence corresponding to exons 6 and 7 of *Btk* was amplified from genomic DNA. Western blot analysis employed both polyclonal and monoclonal antibodies to *Btk* and reaction patterns were obtained both by chemiluminescence and an in vitro kinase assay.

Results: A mutation ($Cys^{145 \rightarrow Stop}$) was identified in *Btk* cDNA and was confirmed in amplified exon 6 of genomic DNA from both the proband and an affected nephew. Neither *Btk* nor a truncated peptide was detected in Western blot analyses of peripheral blood mononuclear cell lysates.

Conclusions: The C145A mutation reported here is novel. This family study is extraordinary in that affected male members who did not undergo aggressive medical management either succumbed to complications in early life or survived into later life. The proband is the oldest de novo diagnosed patient with XLA reported to date.

INTRODUCTION

X-linked agammaglobulinemia (XLA) is an X-linked recessive severe, life-threatening disease characterized by the absence of B lymphocytes, total absence or severe deficiency of all immunoglobulin classes, and recurrent bacterial infection with pyogenic pathogens (1). The disease occurs in ~1/50,000 males and typically presents during infancy or early childhood and, unless treated aggressively with intravenous γ-globulin and/or antibiotics, results in significant morbidity and, in most cases, premature death secondary to overwhelming infection or pulmonary insufficiency (2,3). XLA is associated with mutations and deletions in the gene encoding Bruton's tyrosine kinase (*Btk*) (4,5). *Btk* is expressed in a number of different cell types in addition to B lymphocytes, making mutation analyses possible in cases with profound B lymphocyte deficits.

Address correspondence and reprint requests to: Gary W. Litman, Department of Pediatrics, University of South Florida College of Medicine, All Children's Hospital, 801 Sixth Street South, St. Petersburg, FL 33701, U.S.A.

Over 150 different mutations, which appear almost uniformly throughout the gene, have been identified (Ref. 6 and Haire, unpublished data) and are associated with variable disease phenotypes.

MATERIALS AND METHODS

Peripheral blood mononuclear cells (PBMC), total RNA extraction, synthesis of single-strand cDNA from RNA, polymerase chain reaction (PCR) amplification with Taq polymerase, and cloning in M13 were carried out as described previously (7). Amplification with Pfu polymerase (Stratagene, La Jolla, CA, U.S.A.) was carried out in independent reactions. Automated (LI-COR, Lincoln, NE, U.S.A.) and conventional ^{35}SdATP dideoxynucleotide chain termination DNA sequencing were carried out on three cDNA clones representing both strands using two different dideoxynucleotide termination-based methods as well as on genomic DNA. In the latter case, exon 6 and 7 were amplified directly using primers complementing intron 5 (3′) and intron 7 (5′): 5′-TCCATGTCAGATGTGATCTC-3′ and 5′-TTTAACAGTGGCAGCACCCA-3′. The genomic sequence was determined using automated DNA sequencing. Cell fractionation, production of monoclonal and polyclonal antibodies, cell lysis, immunoprecipitation, Western blotting, in vitro kinase assay, and chemiluminescent detection of proteins were carried out as described (7).

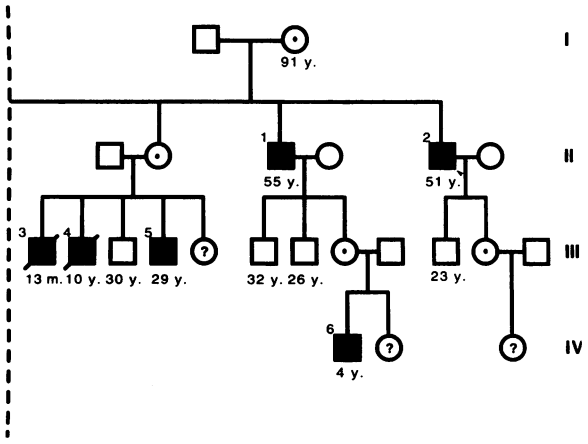


FIG. 1. Family pedigree

Blackened squares, affected males; diagonal slash, deceased; open symbols, unaffected; circle with a black central point, obligate carriers; circle with a question mark, a female of unknown carrier status. Individual identification numbers are indicated at the upper left of the symbol(s). Age is in years (y.) or months (m.), at present or at time of death. Generation is indicated to the right with a Roman numeral. Dashed vertical line indicate that there are additional family members who are not informative. Proband (II-2) is designated by an arrow.

RESULTS

The Proband

The proband is a relatively healthy 51-year-old male who has been treated only intermittently with antibiotics and has never been treated with intravenous gammaglobulin (IVIG). He had been well until 9 years of age when, due to recurrent upper respiratory infections, he underwent a tonsillectomy and adenoidectomy. During his twelfth year he suffered four episodes of pneumonia, which responded to treatment with oral antibiotics, and since has experienced eight additional scattered episodes of pneumonia as well as three to four episodes of sinusitis per year. An immunologic evaluation in 1980 revealed the following immunoglobulin levels (age-matched normal values are shown in parentheses): IgG 501 mg/dl (639–1349), IgA <7 mg/dl (90–410), IgM 17 mg/dl (55–300). His current values were equivalent: IgG 401 mg/dl (723–1685), IgA <7 mg/dl (69–382), IgM 15 mg/dl (63–277). T and B lymphocyte enumeration showed 87% CD3 (62–87), 70% CD4 (32–62), 17% CD8 (17–44), and 1% CD19 (7–22). The patient's anti-B isoagglutinin levels were within the normal range, but his responses to vaccinations were greatly diminished.

Family Study

The proband's 55-year-old brother (Fig. 1, II-1) had a history of recurrent pneumonia, otitis media, and mastoiditis, and first received intravenous gammaglobulin (IVIG) at 41 years of age. The patient has three affected nephews (III-3, III-4, and III-5). One nephew (III-3) died of septicemia at 13 months of age, and his brother (III-4) died of encephalitis at 10 years of age. Another brother (III-5) was treated with intramuscular γ-globulin for recurrent infections between the ages of 3 and 15 years and subsequently has been maintained on IVIG. The proband's brother's grandson (IV-5) is also receiving IVIG for recurrent infections. No female relatives were noted to have a history of recurrent infections.

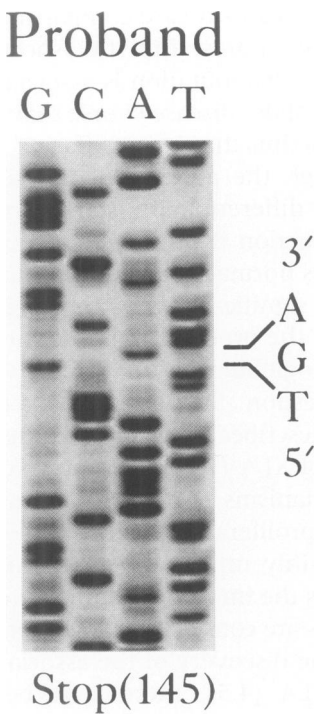


FIG. 2. Sequence analysis of the mutation C567A

DNA sequencing is based on the dideoxynucleotide chain termination method. Automated image obtained using on-line near infrared fluorescence detection (LI-COR). In the analysis shown, template is genomic DNA spanning exons 6 and 7 of *Btk*. Mutation was confirmed in cDNA, amplified in separate reactions using different polymerases and identified in separate reaction chemistry using conventional ^{35}S dATP technology, in both directions.

Mutation Analysis

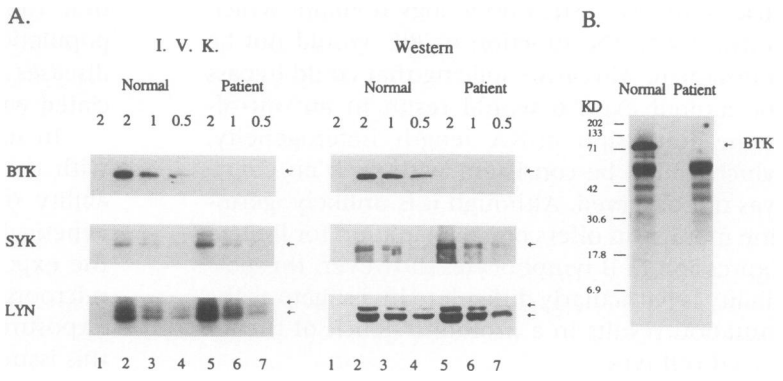
The complete sequence of the *Btk* cDNA was determined and a single nucleotide difference from normal at $C^{567}\rightarrow A$, which introduces a $Cys^{145}\rightarrow$Stop codon in exon 6 of *Btk* was detected in the proband (5,8). No additional differences, with the exception of a recognized polymorphism, were noted in the *Btk* gene. The mutation was confirmed by sequencing through the corresponding region of *Btk* exon 6 amplified from genomic DNA (Fig. 2). In addition, the same mutation was confirmed at the genomic DNA level in the proband's nephew (III-5), whose brothers had died in childhood from infectious diseases.

Btk Peptide Studies

In order to determine whether the truncated peptide spanning the first 144 N-terminal amino acid residues of *Btk*, which would result from a $Cys^{145}\rightarrow$Stop codon mutation, was synthesized by PBMC of the patient, the cell lysate was subjected to immunoprecipitation with rabbit anti-*Btk* polyclonal antibodies which recognize the pleckstrin homology domain of *Btk* (amino acids 1–175) (7,9). The resulting immunoprecipitate was subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Western blot analyses using a pool of seven monoclonal antibodies detected neither *Btk*, the predicted ~16-kD *Btk* peptide, nor its corresponding autocatalytic kinase activity from the patient's cells (Fig. 3). Western blot analyses using polyclonal rabbit antibody also failed to detect *Btk* or the

FIG. 3. Expression of *Btk*, *Syk*, and *Lyn* in normal and patient PBMC

(A) I.V.K. (in vitro kinase assay) and conventional Western blot (peptide detection) analyses. The number of cells × 10^6 in each assay is indicated above the lanes; lane numbers are indicated below. Lane 1 shows the immunoprecipitation of normal control cell lysate with normal rabbit serum. Arrows correspond to the predicted molecular size(s) of the corresponding protein tyrosine kinases. (B) Western blot analyses (peptide detection). *Btk* was immunoprecipitated with rabbit polyclonal antibody, separated by 13% SDS-polyacrylamide gel electrophoresis, and visualized by Western blotting using a pool of the seven different monoclonal antibodies against *Btk* as described (7).



truncated component (data not shown). In contrast, both pooled monoclonal and polyclonal antibodies to *Btk* detected the 77-kD *Btk* band and kinase activities in the normal control. These observations are in contrast with analyses that have been carried out in another case of atypical XLA, associated with a point mutation and significant IgG production, in which reduced autocatalytic kinase activity could be demonstrated (10).

DISCUSSION

In terms of disease course and survival, this mutation is associated with the most extreme phenotypic variant of XLA yet reported. From a clinical standpoint, this kindred is unique in that only minimal (uncharacteristic) morbidity has been suffered by the proband and his brother, both of whom were born prior to the advent of antibiotic therapy and survived in generally good health until early in their sixth (proband) and fifth (sib) decades without treatment with intravenous gammaglobulin and with only intermittent antibiotic therapy. Typically, XLA patients of this era presented with severe recurrent infections by 2.5 years of age (3) and suffered infection-related demise early in childhood (3,9, 11,12).

Quantitative analysis of *Btk* production relative to other B and hematopoietic cell tyrosine kinases, *Syk* and *Lyn*, is consistent with the selective absence of this protein and corresponding kinase activities. Furthermore, the nature of the novel mutation (premature stop codon) and a failure to detect the putative prematurely terminated protein makes it particularly unlikely (relative to a replacement change) that the genetic defect in the proband is leaky and somehow allows the production of some *Btk*. Furthermore, the possibility that an alternative initiation codon is being used is unlikely as a product that is lacking the pleckstrin homology domain, which is integral to the function of *Btk*, would not be transcribed. Alterative splicing that could bypass the altered exon 6 would result in an out-of-frame transcript; cDNA length heterogeneity, which would be consistent with such an effect, was not observed. Although it is unlikely, germline mosaicism offers one explanation for limited expression of B lymphocytes; however, this possibility is particularly difficult to investigate as the mutation results in a profound deficit of the affected cell type.

Although case-to-case comparisons are difficult, it is of interest that the short transcript predicted for this mutation is associated with an apparently milder disease course than are certain longer transcripts that possess (far) 3′ frameshifts (8). Although the absence of *Btk* is known to affect B cell differentiation, some functional antibody production is preserved, as evidenced by this patient's normal isoagglutinin titers and that presence of significant amounts of IgG were diminished in the proband. Whether or not any of the immunoglobulin is of protective value is open to question. Significant levels of IgG also have been described by us in two other relatively mild cases of XLA (7,8). The nature of compensatory mechanisms that could account for limited B cell proliferation are unknown and if a factor, certainly must vary on a patient-to-patient basis as the immunoglobulin levels in other XLA patients are considerably lower or absent (9).

Since the discovery of the associated genetic defect in XLA (4,5), there have been several reports of phenotypic variability (7,8,13). In one study reported from our laboratory, mutations in three different individuals were found to affect the kinase domain; however, the predicted disruptions in *Btk* were not correlated with either the age of onset or the relative severity of the disease course. More recently we reported a case in which marked variation in disease presentation occurred in 5- and 12-year-old brothers with an identical mutation (7). None of the reports documents the extreme magnitude of clinical presentation and progression of disease that are seen in the family described in this paper in which the youngest affected male (III-3) died at 13 months of age secondary to sepsis and his presumably similarly affected uncles (II-1 and II-2) survive into middle adult life. As such, this study is instructive in increasing awareness of the wide variation in immune competence associated with a disease in which the sites and nature of mutations are highly variable. In addition, these findings direct attention in the adult population to a group of genetically inherited diseases that typically are considered to be associated with early childhood.

In interpreting these findings, one is faced with the complex issue of explaining the variability of longevity in patients with the same genetic disease. One commonly held view is that the exposure history of a patient to pathogenic microorganisms dictates the severity of disease. Exposure history certainly is a factor, and it is this issue that makes the report of this mutation

particularly significant. The age of the proband exceeds by decades that of the next oldest cases in which individuals with genetically documented XLA have not received extensive γ-globulin replacement (9). We can infer that the other affected members of his family have the same mutation, and it is unlikely that over five decades the patient reported here has not been threatened with potentially lethal infections. It is reasonable to assume that other protective mechanisms outside of potential for limited B cell proliferation are influencing the overall health of the affected individuals. If such influences have a genetic component, it would seem that this family is of potential interest for further studies along such lines.

ACKNOWLEDGMENTS

We would like to thank Barbara Pryor for her editorial assistance and Drs. M. Berger and D. Friedman for sharing clinical information and assistance in obtaining peripheral blood samples from family members. This work was supported by grants from the Eleanor Naylor Dana Charitable Trust and the Valerie Fund to GWL, and National Institutes of Health Grant CA34546 to SMF.

REFERENCES

1. Bruton OC. (1952) Agammaglobulinemia. *Pediatrics* **9:** 722–728.
2. Rosen FS, Cooper MD, Wedgwood RJP. (1984) The primary immunodeficiencies. *N. Engl. J. Med.* **311:** 235–242.
3. Lederman HM, Winkelstein JA. (1985) X-linked agammaglobulinemia: An analysis of 96 patients. *Medicine* **64:** 145–156.
4. Tsukada S, Saffran DC, Rawlings DJ, et al. (1993) Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. *Cell* **72:** 279–290.
5. Vetrie D, Vorechovsky I, Sideras P, et al. (1993) The gene involved in X-linked agammaglobulinaemia is a member of the *src* family of protein-tyrosine kinases. *Nature* **361:** 226–233.
6. Vihinen M, Iwata T, Kinnon C, et al. (1996) BTKbase, mutation database for X-linked agammaglobulinemia (XLA). *Nucleic Acids Res.* **24:** 160–165.
7. Bykowsky MJ, Haire RN, Ohta Y, et al. (1996) Discordant phenotype in siblings with X-linked agammaglobulinemia. *Am. J. Hum. Genet.* **10:** 477–483.
8. Ohta Y, Haire RN, Litman RT, et al. (1994) Genomic organization and structure of the Bruton agammaglobulinemia tyrosine kinase: Localization of mutations associated with varied clinical presentations and course in X chromosome-linked agammaglobulinemia. *Proc. Natl. Acad. Sci. U.S.A.* **91:** 9062–9066.
9. Sideras P, Smith CIE. (1995) Molecular and cellular aspects of X-linked agammaglobulinemia. *Adv. Immunol.* **59:** 135–223.
10. Saffran DC, Parolini O, Fitch-Hilgenberg ME, et al. (1994) Brief report: A point mutation in the SH2 domain of Bruton's tyrosine kinase in atypical X-linked agammaglobulinemia. *N. Engl. J. Med.* **330:** 1488–1491.
11. Leickly FE, Buckley R. (1986) Variability in B cell maturation and differentiation in X-linked agammaglobulinemia. *Clin. Exp. Immunol.* **65:** 90–99.
12. Wedgwood RJ, Ochs HD. (1980) Variability in the expression of X-linked agammaglobulinemia: The co-existence of classic XLA (Bruton type) and "common variable immunodeficiency" in the same families. In: Seligmann M, Hitzig WH (eds). *Primary Immunodeficiencies.* INSERM Symposium No. 16. Elsevier/North-Holland Biomedical Press, The Netherlands, pp. 69–78.
13. Kornfeld SJ, Kratz J, Haire RN, et al. (1995) X-linked agammaglobulinemia presenting as transient hypogammaglobulinemia of infancy. *J. Allergy Clin. Immunol.* **95:** 915–917.

Contributed by S. H. Orkin on May 24, 1996.