# Discordant Phenotype in Siblings with X-Linked Agammaglobulinemia

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## Summary

**X-linked agammaglobulinemia (XLA) is a congenital humoral immunodeficiency caused by a defect in a B-cell-specific signaling molecule, Btk. There has been little concordance of phenotype with genotype in this disorder, and defects in *Btk* cause immunodeficiencies that range from mild impairment to complete inability to produce antibodies. The factors modifying the phenotype of XLA are not understood. The current study is the first description of two male siblings with identical $T^{134}\rightarrow C$ mutations in the translation initiation ATG of *Btk* who have different clinical phenotypes as well as different laboratory phenotypes. The proband lacks immunoglobulins and B cells and has recurrent infections, while the elder, affected brother has normal levels of IgG and IgM and very few infections. Both have undetectable levels of Btk kinase activity in circulating mononuclear cells. Complete sequencing of *Btk* gene transcripts in both brothers revealed no additional mutations to account for the discordant phenotypes. This description provides unequivocal evidence that the phenotype of XLA is influenced by factors additional to the *Btk* gene.**

## Introduction

Bruton type or X-linked agammaglobulinemia (XLA) was first described in 1952 (Bruton 1952) and is the prototypical humoral immune deficiency with normal cellular immunity. It occurs in ~1/50,000 live male births and is typically characterized by absent or extremely low numbers of circulating B lymphocytes, absence of B-dependent lymphoid tissue, reduced concentrations of immunoglobulins of all classes, and inability to make functional antibody responses. Patients with XLA are susceptible to recurrent bacterial infections with encapsulated organisms and are at risk of disseminated infections with enteroviruses (Wilfert et al. 1977; Lederman and Winkelstein 1985). The gene defect in XLA has been mapped to the long arm of the X chromosome at Xq22 by conventional linkage analysis (Kwan et al. 1986; Guiolo et al. 1989). The protein encoded by this gene has recently been identified as a cytoplasmic tyrosine kinase (Bruton agammaglobulinemia tyrosine kinase [Btk]; E.C.2.7.1.112) (Tsukada et al. 1993; Vetrie et al. 1993) that is activated through stimulation of surface immunoglobulin (de Weers et al. 1994*a*).

Not all XLA patients conform to the classic phenotype (Lederman and Winkelstein 1985; Leickly and Buckley 1986; Kornfeld et al. 1995). *Btk* gene analysis has facilitated the identification of various mutations in patients with XLA (Tsukada et al. 1993; Vetrie et al. 1993; Ohta et al. 1994; Saffran et al. 1994) expressing distinct phenotypes. Although variable phenotypes have been reported in these studies, the phenotype of all members of a family have been similar. We report the first case of XLA in which brothers with the same mutations in their *Btk* gene exhibit different phenotypic expression of the disease.

## Subjects and Methods

The proband (II1) is 7.5 years old and was well until 2 years of age, when he began developing recurrent pneumonia, sinusitis, and otitis media. His height and weight are in the 25th percentile for age. He has no palpable lymphoid tissue and scarred tympanic membranes. Laboratory evaluation revealed markedly reduced immunoglobulins and B cells, and failure to make specific antibodies, all of which were consistent with a diagnosis of XLA (tables 1–3). Informed consent was obtained for the use of peripheral blood from both brothers for the studies described below.

The elder brother (II2) of the proband was diagnosed with a clinical pneumonia at 2 years of age but has subsequently been well. He has never been hospitalized

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**Table 1**

**Serum Immunoglobins**

| Serum Immunoglobulins | Proband (II1) (5 years of age) | Affected Brother (II2) (12 years of age) |
|---|---|---|
| IgG | <.10 g/liter | 14.50 g/liter |
| IgA | <.08 g/liter | <.08 g/liter |
| IgM | .34 g/liter | .89 g/liter |
| IgE | 8 IU/ml | 13 IU/ml |
| IgG1 | None detected | 7.45 g/liter |
| IgG2 | None detected | .17 g/liter |
| IgG3 | None detected | 2.18 g/liter |
| IgG4 | None detected | .01 g/liter |

NOTE.—Laboratory values were obtained at the time of diagnosis, prior to IVIG therapy.

and has not required nor received frequent courses of antibiotics. His height and weight are in the 50th–75th percentile for age. He has absent tonsillar and lymphoid tissue and scarred tympanic membranes. Laboratory evaluation revealed normal IgG and IgM levels. IgA was not detectable, and levels of IgG2 and IgG4 subclasses were markedly reduced (tables 1–3). He failed to respond adequately to immunizations, although he had a minimal response to tetanus reimmunization.

### *Sequence Analysis*

Mononuclear cells were purified from peripheral blood, and total RNA was isolated using RNazol B (Tel-Test). Single-stranded cDNA was prepared from total RNA (Haire et al. 1994). *Btk* cDNA was produced by PCR in two segments. The 5′ portion extended from nt 90 to nt 1623 by using primers 5′-TCAGGACTGAG-CACACAGG-3′ and 5′-ACCGCTTCCAGACTCAG-CAG-3′. The 3′ portion extended from nt 1326 to nt 2193 by using primers 5′-CCAAAGGACCTGACCTT-CTTG-3′ and 5′-GCTCGAAAATAAGCTTCTTGG-3′. The primers derived from the 5′ and 3′ UTR were *Bam*HI-linkered. The *Btk* DNA was cloned into M13 by *Eco*RI/*Bam*HI digestion by using the natural *Eco*RI site in the *Btk* at nt 1453. The complete nucleotide sequences of *Btk* transcripts from both brothers were determined, and the presence of a point mutation in exon 2 was confirmed by sequencing exon 2, primed from genomic DNA (Ohta et al. 1994). The primers used to amplify the second exon were upstream: 5′-GAGCATT-CCATTGCCTT-3′; and downstream: 5′-GCCAAGTC-CTTGATATC-3′. The mutation described in this report introduces an *Eag*I restriction site into exon 2 and serves as a basis for characterizing the mutation in other family members.

### *In Vitro Kinase Assays and Western Blots*

Purified monocytes, B cells, and T cells were obtained by fractionating peripheral blood mononuclear cells (PBMC). Monocytes were purified from PBMC by centrifugation in a continuous Percoll gradient (Sung et al. 1992), followed by magnetic bead depletion (Mini-MACS) of contaminating B, T, and NK cells by using biotinylated monoclonal antibodies against CD20 (KoB1), CD3 (A32), and CD56 (B159.5.2), and streptavidin-conjugated magnetic beads. Lymphocytes from PBMC depleted of monocytes and NK cells by leucylleucine methyl ester lysis (Thiele and Lipsky 1986) were separated into T and B cell fractions by E-rosetting. These fractions were subjected to additional purification by a magnetic depletion step using biotinylated monoclonal antibodies against CD14, CD56, and either CD20 or CD3, for T and B cell populations, respectively. The final cell purities by FACScan analysis were as follows: monocytes, 95%; T cells, 98%; and B cells, 90%.

Rabbit polyclonal and mouse monoclonal anti-Btk antibodies were generated against the PH domain (amino acids 1–175) of human Btk using a glutathione S-transferase (GST)–Btk fusion protein as the immunogen. The antibodies identify the 77-kD Btk in both western blot and immunoprecipitation experiments. The rab-

**Table 2**

**Specific Antibody Formation**

| | PROBAND (II1) | | AFFECTED BROTHER (II2) | |
|---|---|---|---|---|
| SPECIFIC ANTIBODY FORMATION[a] | Preimmune Titers | Postimmune Titers | Preimmune Titers | Postimmune Titers |
| Tetanus | <.1 IU/ml | <.1 IU/ml | <.1 IU/ml | 25 IU/ml |
| Diphtheria | <.01 IU/ml | <.01 IU/ml | <.01 IU/ml | <.01 IU/ml |
| H. influenza | <.55 mg/ml | <.55 mg/ml | <.55 mg/ml | <.55 mg/ml |
| Anti-A | None | | 1:8 | |
| Anti-B | None | | None | |

NOTE.—See note to table 1.

**Table 3**

**Lymphocyte Subsets**

| Lymphocyte Subsets | Proband (II1) | Affected Brother (II2) |
|---|---|---|
| CD3 | 2,360 cells/mm$^3$ | 1,857 cells/mm$^3$ |
| CD4 | 1,260 cells/mm$^3$ | 1,225 cells/mm$^3$ |
| CD8 | 1,060 cells/mm$^3$ | 494 cells/mm$^3$ |
| CD19 | 0 cells/mm$^3$ | 4 cells/mm$^3$ |
| CD20 | 8 cells/mm$^3$ | 6 cells/mm$^3$ |

NOTE.—See note to table 1.

bit anti-Btk antibody was depleted of anti-GST reactivities by passage of the antiserum over GST-Sepharose columns, followed by affinity column chromatography using GST-Btk-conjugated Sepharose beads. The rabbit anti-Syk polyclonal antibody was generated against the region of human Syk between the SH2 and kinase domains (amino acids 274–371) as a GST-Syk fusion protein and recognized the 72-kD Syk protein in western blots and immunoprecipitations. The rabbit polyclonal and mouse monoclonal anti-Lyn antibodies were from UBI and Transduction Laboratories, respectively.

Cells lysates were prepared in 10 mM Tris HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 1% glycerol, 1 mM EDTA, 1 mM $Na_3VO_4$, 1 mM phenylmethylsulfonyfluoride, and 1 μg/ml each of aprotinin, trypsin inhibitor, and leupeptin. The supernatants (after centrifugation at 14,000 × *g*) were used in immunoprecipitations using rabbit Ab and protein A-Sepharose beads. The kinase reaction was performed at room temperature for 15 min by using 10 μCi of [$^{32}$P]-ATP (3,000 Ci/mmol; DuPont NEN). The samples were separated on 8% SDS-PAGE and transferred to nitrocellulose membrane. The labeled proteins were visualized by autoradiography. Proteins were detected on the same blots by using western blots and enhanced chemiluminescence (Amersham). Band intensities were quantitated by scanning densitometry using a flatbed scanner (Color One Scanner; Apple) and a Biosoft analysis program (Scan Analysis).

## Results

The proband (II1) had clinical and laboratory findings compatible with XLA. In contrast, his elder brother (II2) had clinical and laboratory features most compatible with common variable immunodeficiency or IgA deficiency with IgG2 and IgG4 subclass deficiencies. The elder brother not only had normal levels of IgG and IgM, he was also able to produce isohemagglutinins and antitetanus responses (tables 1 and 2). In an effort to establish the diagnosis more clearly in these patients, *Btk* transcripts were amplified from PBMC mRNA. A $T^{134}$→C mutation in the translation initiation ATG (Vetrie et al. 1993) located in the second exon (Ohta et al. 1994) of the *Btk* gene was identified in both brothers (fig. 1). This mutation, which creates a site for the restriction endonuclease *Eag*I, would be expected to impair translation of the *Btk* mRNA. In order to rule out the unlikely possibility that an additional mutation(s) was present, the complete nucleotide sequence of the cDNA was resolved in both patients. No additional mutations were identified.

Family studies were performed to determine whether other male family members had inherited the *Btk* mutation but were asymptomatic. DNA was prepared from each available family member; exon 2 was amplified by PCR and digested with *Eag*I to detect the mutation (fig. 2). The $T^{134}$→C mutation introduces an *Eag*I site, which allows for restriction digest detection of the mutation. The nephew (III1) of II1 and II2 was found to have inherited the same *Btk* mutation as his uncles and had a phenotype at 3 mo of age that resembled that of the proband. He had 0.4% B cells and immunoglobulin levels which were markedly low: IgG 197 mg/dl, IgA <7 mg/dl, and IgM 8 mg/dl. An 18-year-old male sibling

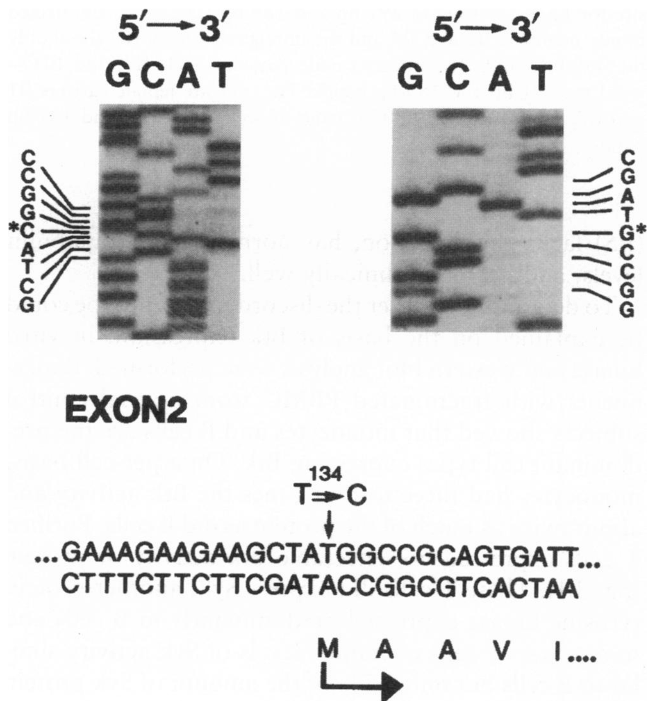


**Figure 1** Alterations in the translation initiation site, caused by the Btk mutation. The *Btk* genes from both affected brothers were sequenced completely in both directions in both cDNA and genomic DNA, revealing a T→C mutation in the translation initiation codon in exon 2. The relevant sequence from the proband is shown in both directions. This mutation introduces a cleavage site for *Eag*I into exon two.

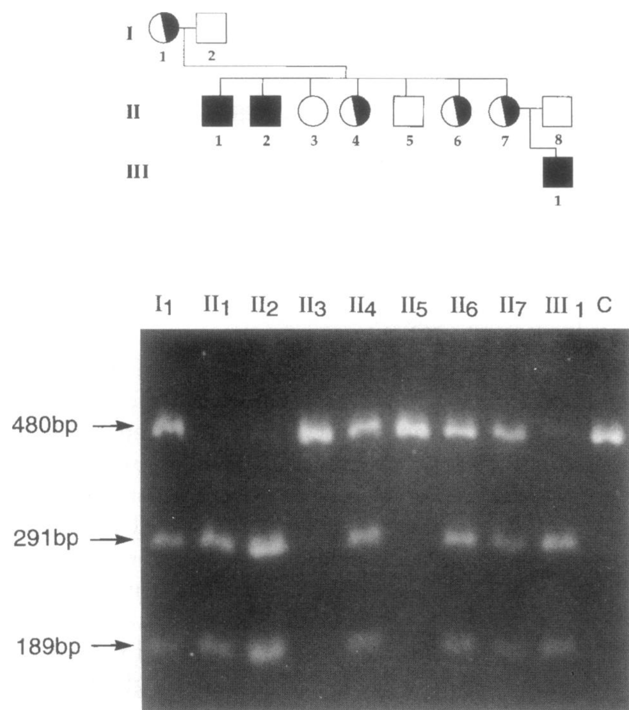


**Figure 2** *Eag*I restriction digest detection of the *Btk* mutation in family members. PCR amplification of exon 2 in *Btk* results in a 480-bp band. The mutation T→C in *Btk* exon 2 introduces a cleavage site for *Eag*I, resulting in 291-bp and 189-bp fragments. Unaffected family members II3 and II5 and the unrelated control (C) show only the 480-bp band. The affected male patients—II1, II2, and III1—yield the 291-bp and 189-bp bands. The obligate female carriers, I1 and II7, yield a normal 480-bp band as well as 291-bp and 189-bp bands.

(II5) lacks the mutation, has normal immunoglobulin levels, and has been clinically well.

To determine whether the discordant phenotype could be explained on the basis of Btk expression, in vitro kinase and western blot analysis were performed. Experiments with fractionated PBMC from normal control subjects showed that monocytes and B cells are the predominant cell types expressing Btk. On a per-cell basis, monocytes had three to four times the Btk activity and about twice as much of the protein as did B cells. Purified T cells expressed no detectable Btk activity or protein (fig. 3*A*). These T cells did express Syk, another protein tyrosine kinase expressed predominantly in B cells and monocytes. T cells contained levels of Syk activity similar to B cells but only 25% of the amount of Syk protein as B cells.

In vitro kinase analysis of total PBMC lysates from the two brothers showed no detectable Btk activity, whereas Btk activity was readily detected in 1/16 the number of normal cells (fig. 3*B;* compare lane 5 with lanes 7 and 9). Western blots showed that a faint protein band, <10% of that of control, was detected in II1, but no Btk could be detected in II2 PBMC (fig. 3*B*, lanes 7 and 9). Because Btk activity and protein expression in PBMC were primarily due to their presence in monocytes (fig. 3*A*) and the PBMC of II1 and II2 contained 17% and 8% of monocytes, respectively, these results showed that Btk expression was severely depressed in the patients, and this depression is not due solely to the absence of circulating B cells. There is a cross-reactive kinase activity with different mobility in the Btk lanes (marked by an asterisk [*] in fig. 3*A* and *B*). This activity has also been observed by other investigators (Tsukada et al. 1993; Aoki et al. 1994). This band is of the same intensity in the two brothers as in normals. The functional significance of this kinase is uncertain. To examine whether the phenotypic differences in the two brothers were due to differences in the expression of B cell and monocyte kinases other than Btk, the activities and protein levels of two major tyrosine kinases (Syk and Lyn) responsible for signal transduction in these cells were examined (Perlmutter et al. 1993). Syk activity in II1 and II2 was 70% and 30%, respectively, of that in control cells, although PBMC Syk protein content was near normal (fig. 3*B*). These results suggest that Syk is less activated in patient cells. The kinase activity and protein level of Lyn were also found to be normal in the patient PBMC (fig. 3*B*).

## Discussion

Btk is a cytoplasmic protein tyrosine kinase that is expressed in most hematopoietic tissues (Aoki et al. 1994). Although direct demonstration of protein-protein interactions with B cell surface receptors has not been shown, it has been inferred that Btk functions in a manner analogous to the other protein tyrosine kinases, on the basis of structural homologies. The Tec homology region of the molecule, conserved in all Btk family members (Smith et al. 1994), interacts with the SH3 region of the Src family proteins Fyn, Lyn, and Hck (Cheng et al. 1994). A second domain with homology to pleckstrin may be responsible for recruiting Btk to the cell membrane (Musacchio et al. 1993). The requirement of Btk for the normal developmental program of B cells has been demonstrated in vivo in XLA patients who lack normal numbers of mature B cells (Grey et al. 1971). XLA patients can have normal levels of pre-B cells in their marrow (Pearl et al. 1978) implying that the critical requirement for Btk occurs during the transition from pre-B cell to B cell. The demonstration of rare mature B cells in the periphery (Mensink et al. 1986) suggests that the maturational signals may be redundant, allowing occasional cells to mature through a separate pathway.

It has become increasingly evident that different mutations within the same gene in single-gene-defect diseases

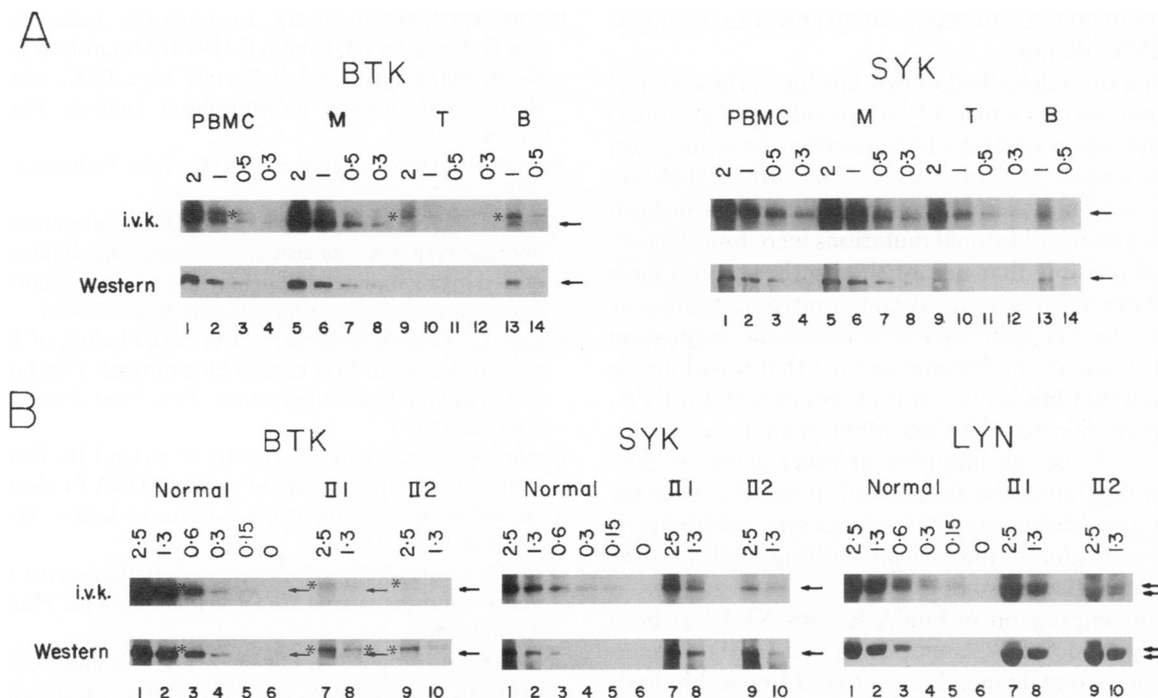


**Figure 3** Expression of Btk in normal and patient PBMC. *A,* The kinase activities and protein levels of Btk and Syk in total PBMC and fractionated cell populations were examined by in vitro kinase and western blotting. PBMC and purified monocytes (M), T cells (T), and B cells (B) from the same buffy coat were used. *B,* Total PBMC from a normal control individual and from the patients II1 and II2 were used in studying the expression of Btk, Syk, and Lyn. The numbers above the lanes indicate the number of cells $\times 10^6$ used in each assay. Arrows indicate the position of the tyrosine kinases. The asterisk (*) in the Btk lanes indicate the position of a cross-reactive kinase.

result in a range of phenotypes (Dean and Santis 1994; Jabs et al. 1994; Muenke et al. 1994; Park et al. 1995; Rutland et al. 1995; Wilkie et al. 1995). Accordingly, mutations within *Btk* have been associated with a range of XLA phenotypes, although the phenotype associated with each individual mutation is usually similar. The mutations have included single-base-pair substitutions, insertions or deletions (Vetrie et al. 1993; de Weers et al. 1994*b*) resulting in amino acid substitutions, deletions, aberrant stop codons, frameshifts, abnormal splicing, or combinations of these events (Ohta et al. 1994). The patients exhibit a wide variety of phenotypes, including delayed or mild presentation with normal antibody responses to immunizations. The phenotypes in these patients were not predictable on the basis of the mutation, although all members of one particular kindred were affected equally.

Mutations involving the initiation codon of the *Btk* gene have been described elsewhere. In one study (Bradley et al. 1994) a patient was found to have the same $T^{134}\rightarrow C$ mutation reported here. A second patient in that study and one reported in another study (Conley et al. 1994) had a different initiation codon mutation, $A^{133}\rightarrow G$. All three patients appeared to have the classic XLA phenotype. The ACG codon has been reported to have some translation initiation function in eukaryotes (Kozak 1991). Two alternative ATG codons at −23 and +89 do not allow for translation of full-length Btk product, suggesting that if any functional Btk is produced, the mutant initiation site must be functional.

Variable phenotypic expression in patients with identical mutations is difficult to explain. This is the first description of brothers with identical *Btk* mutations and discordant phenotypes. The younger brother was first seen at 5 years of age with recurrent sinopulmonary infections, including five episodes of pneumonia, and laboratory values consistent with XLA, including absent immunoglobulins, absent functional antibodies, and markedly diminished peripheral B lymphocytes. His elder brother had few infections and was initially diagnosed with IgA and $IgG_2/IgG_4$ subclass deficiency. The elder brother routinely had IgG levels in the 1,200–1,400 mg/dl range, prior to initiation of intravenous immunoglobulin (IVIG) therapy, although he produced little functional antibody in response to immunization. A reservoir of mature B lymphocytes in his spleen, lymph nodes, or bone marrow may have produced the immunoglobulins. Serum immunoelectrophoresis did not demonstrate oligoclonal bands, suggesting that the basis for the persistently elevated serum IgG level is not an oligoclonal gammopathy. This is further supported by the fact that he produced both IgM and measurable

quantities of all four subclasses of IgG prior to the initiation of IVIG therapy.

The mutation described in this kinship is the result of a single nucleotide change $T^{134} \rightarrow C$, resulting in an amino acid substitution of Met→Thr affecting the translation initiation codon (Kozak 1991). The *Btk* cDNA sequences were fully resolved in both directions in both brothers, and no additional mutations were found; however, it is possible that one of the brothers could have a second mutation in an upstream or intronic regulatory region of the *Btk* gene that is affecting the expression of the first mutation. Arguing against that possibility is the finding that Btk activity was absent in both brothers. One explanation for the discordant phenotypes in this kindred could be the influence of other genes or gene products regulating the translation of the *Btk* message (Romeo and McKusick 1994). A second possibility is that different kinases may be substituting for Btk in the less severely affected sibling.

Variable expression in kindreds with XLA has been previously reported. "Classic" XLA and CVID were diagnosed in two male members in two different kindreds (Wedgewood and Ochs 1980). Since XLA and CVID are not commonly seen within a family, variable expression of the X-linked trait was postulated. XLA associated with dysgammaglobulinemia in the same kindred has been reported (Buckley and Sidbury 1968; Goldblum et al. 1974), although a definitive molecular diagnosis of XLA was not made. This is the first report of discordant XLA phenotype within the same kindred where sequencing has confirmed the inheritance of the identical mutant *Btk* gene.

It has become increasingly clear that the phenotype in XLA cannot be predicted by the site or type of the mutation within the *Btk* gene (Kornfeld et al. 1994). The establishment of a Btk database (Vihinen et al. 1995) will facilitate attempts to predict natural history on the basis of genotype, but it is now evident that other factors are involved that may affect the phenotype expressed in patients with identical *Btk* mutations.

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