

Genotype/phenotype correlations in X-linked agammaglobulinemia

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Abstract

No clear genotype/phenotype correlations have been established in patients with X-linked agammaglobulinemia (XLA). To determine if the specific mutation in Btk might be one of the factors that influences the severity of disease or if polymorphic variants in Tec, a cytoplasmic tyrosine kinase that might substitute for Btk, could contribute to the clinical phenotype, we examined the age at diagnosis, the percentage of peripheral blood B cells and the plasma IgM in a large group of patients with XLA. The results demonstrated that polymorphic variants in Tec were not correlated with phenotypic markers; however, the specific mutation in Btk did influence disease severity. Mutations that conceivably allow the production of some Btk, amino acid substitutions or splice defects that occur at conserved but not invariant sites in the splice consensus sequence were associated with older age at diagnosis, a higher percentage of B cells in the peripheral circulation and higher concentrations of plasma IgM. © 2005 Elsevier Inc. All rights reserved.

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Introduction

X-linked agammaglobulinemia (XLA) can be considered the prototype defect in early B cell development. Most patients have the early onset of recurrent bacterial infections, severe hypogammaglobulinemia and markedly reduced or absent B cells in the peripheral circulation [1–3]. However, the defect in B cell differentiation is leaky, such that the majority of patients have some serum IgG and/or IgM, and most have a small number of B cells in the blood [4]. Although the median age at diagnosis in patients with sporadic XLA is 26 months [5], some adults with XLA are not recognized to have immunodeficiency until a grandson or nephew is given the diagnosis [3,6,7]. Efforts to explain this clinical variability have been minimally rewarding.

Mutations in Btk, a hematopoietic specific cytoplasmic tyrosine kinase, are the cause of XLA [8,9]. Over 500 different mutations in Btk have been reported, and no single mutation

accounts for more than 3% of patients [3]. It is generally agreed that there is not a strong genotype/phenotype correlation in XLA [10–13]; but this does not rule out the possibility that the specific mutation in Btk may be one of the factors that contributes to the severity of disease.

Defining “severe” versus “mild” mutations in Btk is not straightforward. Most mutations, even many amino acid substitutions, result in the absence of Btk protein in platelets and monocytes [14–16]. However, some amino acid substitutions may result in proteins that are unstable but do have some function [17]. Splice defects that occur at sites in the splice consensus site that are conserved but not invariant may allow the production of a small amount of normal Btk [18,19]. In this paper, we have defined all amino acid substitutions and all splice defects that occur at conserved but not invariant sites as “mild” mutations. Splice defects at invariant sites within the splice consensus sequence (the first two and last two base pairs of an intron), premature stop codons and frameshift mutations have been defined as “severe” mutations.

Several factors can be used to measure the clinical severity of XLA. The age at diagnosis, the percentage of B cells in the peripheral circulation and the concentration of serum IgM are useful objective measures. Nevertheless, it is important to

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remember that these measures can be influenced by many variables. The age at diagnosis may be affected by the intensity of exposures from older children and the acuity of the primary physician. Techniques to measure serum immunoglobulins and B cell numbers may vary from laboratory to laboratory, and these parameters may be affected by the age of the patient, the presence of acute infection and treatment with gammaglobulin.

It has been proposed that modifying genetic factors contribute to the clinical severity of XLA [20,21]. Polymorphic variants in Tec, a cytoplasmic tyrosine kinase belonging to the same family as Btk, would be reasonable candidates for this effect. Tec is expressed in B cells, and it is activated by many of the same signal transduction pathways as Btk [22]. Furthermore, mice that are null for Tec have normal numbers of B cells and normal B cell function, but mice that are null for both Btk and Tec have a much more severe defect in B cell development than mice that are null for Btk alone [23]. It is reasonable to hypothesize that Tec can substitute for Btk and polymorphic variants of Tec may differ in their ability to perform this function.

To address the question whether subtle genotype/phenotype correlations can be seen in patients with XLA, we have examined the age at diagnosis, the amount of plasma IgM and/or the percentage of B cells in the blood of patients with mild or severe mutations in Btk. We have also determined whether polymorphic variants in Tec influence the severity of disease.

Materials and methods

Patients

The patients included in this study were seen in our clinic or were referred for mutation detection. Blood from the latter patients was sent by overnight courier and used to determine the percentage of B cells, the presence or absence of Btk in monocytes and the concentration of plasma IgM. A clinical survey that was completed by the referring physician included data about the age at diagnosis and the types of infections. Not all information was available on all patients.

The age at diagnosis of XLA was defined as the age at which the patient was recognized to have immunodeficiency and started receiving immunoglobulin replacement, not the age at which the diagnosis of XLA was confirmed by mutation detection. A patient was considered to have a family history of XLA when a relative had been diagnosed as having immunodeficiency but not when there was a family history of early childhood death because of infection.

Mutation detection

Genomic DNA from patients was screened for mutations in Btk using single-strand conformation polymorphism (SSCP) analysis as previously described [24,25]. Reactions resulting in the abnormal migration of a fragment were cloned and sequenced or the PCR product was directly sequenced. Mutations not detected by SSCP were found by Southern blot analysis [26].

B cell staining

Peripheral blood mononuclear cells were isolated from heparinized blood by Ficoll Hypaque centrifugation. Fc receptors were blocked using rabbit IgG, and cells were stained with phycoerythrin labeled anti-CD19 (BD Pharmingen) or an isotype control. Between 175,000 and 300,000 gated events were analyzed. The percentage of cells positive in the isotype control (usually 0.01% or less) was subtracted from the percentage of positive cells in the CD19 assay. Btk analysis was performed on permeabilized cells incubated with monoclonal antibody to Btk (48-2H, produced by S. Tsukada) followed by staining with FITC-labeled goat anti-mouse antibody. Cells that fell within the monocyte gate by forward and side scatter were evaluated for positive staining.

Quantitation of plasma IgM

Heparinized blood was diluted with an equal volume of phosphate-buffered saline for density gradient separation. After the separation, diluted plasma was retrieved and frozen in aliquots. ELISA plates were coated with polyclonal goat anti-IgM (Southern Biotech) and blocked with PBS containing 3% BSA. Plasma samples were analyzed at six 3-fold dilutions in parallel with an IgM standard, a plasma sample from a healthy individual and a plasma sample from a patient with μ heavy chain deficiency. The data were analyzed using the Softmax Pro. Serum samples from 3 patients with detectable serum IgM were analyzed in the clinical laboratory the same day that the diluted plasma was obtained. The results of the ELISA plasma assay were consistently 40% of the serum assay done the same day, allowing the use of a correction factor for all of the plasma samples.

Analysis of Tec

The sequence for Tec was obtained from the NCBI as published under NT_006238 at the time of the study. Single nucleotide polymorphisms (SNPs) were identified in the dbSNP database and analyzed by SSCP as previously described [24]. The primers used to analyze the SNPs or sequence Tec cDNA are shown in Table 1.

Statistical analysis

Wilcoxon rank sum test or Fisher's Exact Test was used to evaluate the correlation between the type of mutation in Btk, or the polymorphic variant in Tec, and the age at diagnosis, the percentage of circulating B cells and the plasma IgM. Spearman's correlation was used to evaluate the correlation between the percentage of circulating B cells, the plasma IgM and age at diagnosis.

Results

A total of 110 patients from 94 unrelated families were included in this study; all had proven mutations in Btk. Table 2 shows the types of mutations seen in these individuals. The 50 amino acid substitutions or splice defects that occurred at

Table 1
Primers used to evaluate Tec

	Forward primer	Reverse primer	Product size
<i>Analysis of polymorphic variants</i>			
5'UTR	AAGCTAAGGGTACTAGGAGG	GCTGGAGCAGATGCGTATTG	186
Intron 1	GATCCCATGGAATTTGAAGG	GTTGAGTCCACCAAATCAC	182
Intron 2	GTAAGAAAGTAACCTGGG	TAACCTTTCACCCACAG	230
Exon 4	AAGTAATGATCGACACTG	CATGTCACGTTCTTACCA	290
Intron 4	GTGTTAACTTCCACTTTTCCTT	CCAAAACCTTAAGACTTAAAGGC	204
Intron 10	GTGACCAAGCAGAATGATTA	CAAGGAAAATTAGCTCATGC	173
3'UTR	TTGAGCACTTGGACATGTAG	AACAACCTGTCACCTCAAGTGC	189
<i>Sequencing of Tec cDNA</i>			
	TCAGCCAGAATACTGGGATC	TCTCTTGCTCTCCACCAATG	715
	AGAGAGGCCAAGAGTATCTC	AATGACCTTGCTCTGTGCGG	780
	TTATGGTGTGTGCACCCAGC	AGGTGGACACCACTTCACAG	320
	TTGTCTAGTAAGTGAGGCGG	CTTCCTTGCTTGGGAATC	461

conserved but not invariant sites within the splice consensus sequence were considered mild mutations. The remaining 60 mutations were considered severe mutations.

Age at diagnosis

To determine if the age at diagnosis was influenced by the type of mutation in Btk, the patients were divided into four groups based on age at diagnosis: (1) 1 year or less; (2) 13–26 months of age; (3) 27–60 months of age; and (4) greater than 60 months of age. Because patients with a family history of disease may have been evaluated early because of the known history, rather than the severity of their symptoms, ten patients with a positive family history of XLA who were diagnosed at 26 months of age or less (median age at diagnosis in patients with sporadic XLA) were excluded. However, the four patients with a positive family history of disease who were diagnosed at greater than 26 months of age were included. As shown in Fig. 1, the percentage of patients with mild mutations increased as a function of age at diagnosis ($P = 0.04$). In the youngest group, 29.4% of the patients had mild mutations, whereas, in the oldest group, 56.2% had mild mutations.

B cells

The percentage of peripheral blood lymphocytes expressing the B cell marker CD19 was evaluated in 76 patients. The

Table 2
Mutations in Btk in patients included in this study

Mutation	Number of patients (110)
Amino acid substitutions	40 (36.4%)
Frameshift	11 (10%)
Premature stop codons	17 (15.5%)
Splice defects	28 (25.4%) ^a
Gross deletions	6 (5.4%)
Duplication/inversions	3 (2.7%)
Complex	4 (3.6%)
Retroposon insertion	1 (0.9%)

^a There were 18 patients with splice site defects at invariant sites and 10 patients with defects at conserved but not invariant sites.

threshold of sensitivity of this assay was 0.01%. Seventeen patients had fewer than 0.01% B cells. Patients were divided into those with less than 0.10% B cells and those with 0.10 to 1.5% B cells. Patients who were examined on multiple occasions consistently fell into either the low or high B cell group. Mild mutations were found in 18/51 (35.2%) of patients with less than 0.10% B cells and 14/25 (58.3%) of the patients with higher B cell percentages ($P = 0.09$).

Previous studies have suggested that percentage of B cells detected in patients with XLA decreases as the patients get older [4]. The median age of the 17 patients with less than 0.01% B cells was 17 years at the time of the analysis. By contrast, the median age of the patients with greater than 0.01% B cells was 5 years ($P = 0.014$). It is important to point out that the age at which B cell assessment was performed was not significantly different in those patients who had mild mutations, median age 6.1 years versus those who had severe mutations, median age 5.3 years.

Plasma IgM

A total of 89 plasma samples from patients with XLA were analyzed for IgM. There was a highly significant correlation

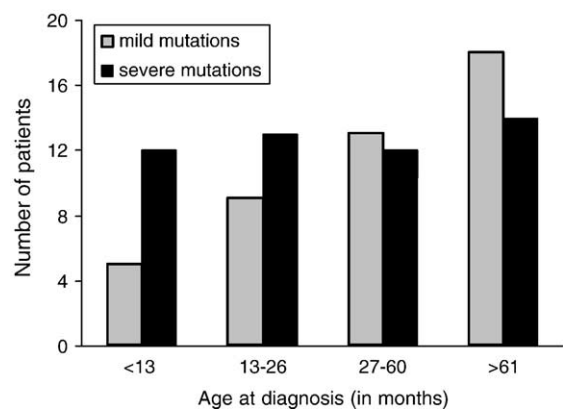


Fig. 1. The relationship between age at diagnosis and the type of mutation in Btk. The patients were divided into four groups based on the age (in months) at the time of diagnosis. The number of patients with mild and severe mutations in Btk in each age group is shown.

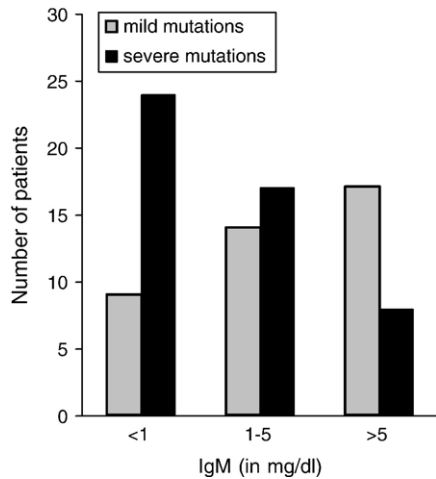


Fig. 2. The relationship between the plasma IgM and the type of mutation in Btk. The patients were divided into 3 groups based on the plasma IgM, those with plasma IgM of less than 1 mg/dl, those with plasma IgM between 1 and 5 mg/dl and those with plasma IgM greater than 5 mg/dl. The number of patients with mild and severe mutations in Btk in each group is shown.

between the type of mutation in Btk and the plasma IgM ($P < 0.001$). Of the 33 patients with less than 1 mg/dl IgM, 9 (27.2%) had mild mutations (Fig. 2). Fourteen of the 31 patients with 1–5 mg/dl IgM (45.1%) had mild mutations, and 17 of the 25 patients (68%) with greater than 5 mg/dl IgM had mild mutations.

Correlation between age at diagnosis, percentage of B cells and plasma IgM

In 65 patients, the age at diagnosis, the percentage of circulating B cells and the total IgM were known. Although the age at diagnosis did not correlate with either the percentage of circulating B cells or the plasma IgM, the percentage of B cells and the IgM correlated with each other ($P < 0.001$). Patients with less than 0.01% B cells had a median IgM of 0.64 mg/dl; patients with between 0.01 and 0.09% B cells had a median IgM of 2.56 mg/dl; and those with 0.10% or greater circulating B cells had a median IgM of 6.0 mg/dl.

Btk expression

Immunofluorescence staining, to detect the presence of Btk in monocytes, was done in a total of 68 patients with 57 different mutations. Positive staining for Btk was seen in 9/26 (35%) of patients with 7 different amino acid substitutions. The specific amino acid substitutions that allowed expression of Btk were R28C, R28H, R288Q, H364D, R520Q, D521G and F574L. Monocytes from patients with any other type of mutation, including splice site defects at conserved but not invariant sites, were negative for Btk. To determine if the milder symptoms in the patients with amino acid substitutions could be explained entirely by the mutations that allowed production of Btk protein, patients with amino acid substitutions were divided into those who were Btk+ and those who were Btk–.

The Btk+ patients were slightly older at diagnosis (mean age 86 months) and had slightly higher mean plasma IgM (7.4 mg/dl) compared to the Btk– patients with amino acid substitutions (mean age at diagnosis 77 months and mean plasma IgM 6.7 mg/dl), but these two groups were not statistically different from each other. Both groups had statistically higher plasma IgM than the patients with severe mutations ($P = 0.02$ for Btk+ and $P = 0.04$ for Btk–). Both groups were older than the patients with severe mutations at the time of diagnosis, but the difference was statistically significant only when the Btk+ and Btk– patients with amino acid substitutions were combined. Only the patients who were Btk+ had statistically higher percentages of B cells ($P < 0.003$).

Polymorphisms in Tec

To determine if polymorphisms in Tec contribute to the clinical variability in patients with XLA, we analyzed seven single nucleotide polymorphisms (SNPs) spanning the entire gene (Table 3). None of these SNPs demonstrated a significant correlation with the age at diagnosis or the percentage of B cells in the peripheral circulation; however, there was a trend toward higher incidence of the C at the intron 1 SNP in patients diagnosed at an older age and in patients with more than 0.1% B cells. The frequency of this allele was 33/98 (33.6%) in patients with less than 0.1% B cells and 19/46 (41.3%) in the patients with more than 0.1% B cells. Of note, the frequency of the C allele was higher in the African Americans included in this study 10/16 (62.5%) versus 43/128 (33.5%) in the non-African American patients. If the African American patients were excluded, the frequency of the C allele at intron 1 was 29/88 (32.9%) in the patients with low B cells and 14/40 (32.5%) in the patients with higher B cell percentages.

We considered the possibility that rare polymorphisms that change the amino acid sequence of Tec might be found in patients with unusually mild disease. Therefore, we sequenced the cDNA for Tec from an African American patient who was recognized to have XLA at 20 years of age, after his nephew was given the diagnosis. This man had had several pneumonias, treated as an out-patient, and he had chronic sinusitis. He had a serum IgG of 698 mg/dl, IgM of 46 mg/dl and IgA of 311 mg/dl. In addition, he had positive antibody titers to tetanus toxoid and several pneumococcal serotypes and 0.16% CD19+ B cells in the peripheral circulation. His mutation in Btk

Table 3
Polymorphic variants in Tec

Area of polymorphism	Position of polymorphism relative to the start codon	Sequence of polymorphism
5' untranslated rs3792618	–143	A/T
Intron 1 rs2664019	+470	T/C
Intron 2 rs7669393	+57,129	T/C
Exon 4 rs2271173	+58,410	C/T
Intron 4 rs2243840	+60,007	C/T
Intron 10 rs3749503	+82,241	C/T
3' untranslated rs3062021	+91,361–91,365	ATAA/–

resulted in the substitution of proline for the wild type leucine at codon 452. His monocytes were negative for Btk expression. The Tec cDNA sequence from this individual-matched known sequences in the NCBI database; however, it differed from the published sequence [27] in that it had a homozygous T to G alteration at codon 514, resulting in the replacement of phenylalanine with valine. Analysis of this region of Tec in DNA from 16 additional patients with mild or severe XLA showed that all were homozygous for G at this site, suggesting that the published sequence represents a rare polymorphic variant or a sequencing error. The lack of unusual polymorphisms in Tec in the patient with mild disease makes it less likely that rare variants in Tec that compensate for Btk are the typical cause of less severe disease.

Discussion

This study shows that the specific mutation in Btk is one of the factors that influences the severity of XLA. Patients with amino acid substitutions or base pair substitutions at sites within the splice consensus site that are conserved, but not invariant, are more likely to have a later diagnosis, higher percentages of B cells and more plasma IgM. It has been well recognized that patients with severe mutations, for example, premature stop codons, or defects in the start codon, may have mild disease [6,24,28]; and, patients from the same family, with the same mutation, may have either mild or severe disease [6,7,17,28]. However, the possibility that patients with mild mutations are more likely to have mild disease has not previously been addressed.

Our division of mutations into the mild and severe categories is somewhat arbitrary. One would expect that some amino acid substitutions in Btk would completely ablate the function of the molecule or even possibly have a dominant negative effect, impairing the ability of a redundant protein to substitute for Btk or another protein to bypass Btk. Some splice defects at invariant positions in the splice consensus site may result in the use of cryptic splice sites that allow the production of some in-frame protein with a small number of added or subtracted amino acids [18]. Furthermore, factors that affect the efficiency of splicing or protein stability may vary from person to person. A more accurate assessment of the severity of mutations would require a combination of quantitative cDNA analysis, protein turn-over studies and protein function studies.

Two factors influenced our ability to demonstrate a genotype/phenotype correlation in this study. First, the number of patients included in the study was large; and, second, the quantitation of the percentages of B cells and the plasma IgM was done in a single laboratory using a single technique. The percentage of B cells and the plasma IgM correlated with each other, but neither correlated with the age at diagnosis. The age at diagnosis is the phenotypic marker that is most influenced by environmental variables. In addition, genetic variants in proteins in the innate immune system may affect the frequency and severity of infections and therefore the age at diagnosis.

Other investigators have described patients with XLA who had a less severe block in B cell differentiation or who had

higher concentrations of serum immunoglobulins than expected. Most but not all of those patients have had either amino acid substitutions in Btk or a splice defect that did not involve the invariant first or last two base pairs in an intron [21,29,30]. Generally, a late diagnosis in a patient with a severe mutation has been ascribed to environmental factors or polymorphic variants in other components of the immune system.

We examined 7 common polymorphisms in the gene for Tec, a cytoplasmic tyrosine kinase related to Btk, to determine if genetic variants in this gene could help explain the clinical variability in XLA. None of the polymorphic variants was statistically associated with the age at diagnosis or the percentage of B cells. We cannot rule out the possibility that rare polymorphic variants of Tec influence the course of disease in some patients with XLA; but attempts to find such a variant in an African American patient with nearly normal concentrations of serum immunoglobulins, who was recognized to have XLA at 20 years of age, were unrevealing.

The correlation between the specific mutation in Btk and the severity of disease is not sufficiently strong to allow one to predict the clinical course in a single patient with XLA based on the mutation. However, the data do indicate that some mutations in Btk, particularly mutations that allow production of normal amounts of Btk in monocytes, are more likely to be associated with milder disease.

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