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Clinical and mutational features of X-linked agammaglobulinemia in Mexico

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ABSTRACT

X-linked agammaglobulinemia (XLA) is caused by *BTK* mutations, patients typically show <2% of peripheral B cells and reduced levels of all immunoglobulins; they suffer from recurrent infections of bacterial origin; however, viral infections, autoimmune-like diseases, and an increased risk of developing gastric cancer are also reported. In this work, we report the *BTK* mutations and clinical features of 12 patients diagnosed with XLA. Furthermore, a clinical revision is also presented for an additional cohort of previously reported patients with XLA. Four novel mutations were identified, one of these located in the previously reported mutation refractory SH3 domain. Clinical data support previous reports accounting for frequent respiratory, gastrointestinal tract infections and other symptoms such as the occurrence of reactive arthritis in 19.2% of the patients. An equal proportion of patients developed septic arthritis; missense mutations and mutations in SH1, SH2 and PH domains predominated in patients who developed arthritis.

1. Introduction

X-linked agammaglobulinemia (XLA; OMIM# 300755) is, after common variable immune deficiency (CVID), the most frequent form of primary antibody deficiency [1]. Patients suffer from recurrent bacterial infections due to a block in early B cell development in the bone marrow [2]. The gene affected in XLA, *BTK*, was discovered in 1992 by two independent groups [3–5]. Btk has five different functional domains: the pleckstrin-homology (PH) domain is located proximal to the N-terminal, followed by the Tec- (TH), Src- (SH3), SH2 homology and Kinase domains (TK or SH1). While the SH1 domain is the catalytic domain for Tyr phosphorylation, the PH domain is important for Btk membrane localization. The TH domain is important for protein holding and activity regulation, and the SH3 and SH2 domains are involved in protein-protein interactions [6]. Since discovery of the gene, 1362 public entries for *BTK* mutations have been reported in different families worldwide. Such mutations are distributed in most domains of Btk, with the exception of the SH3 domain, in which no point mutations have been reported thus far [7,8].

Although Btk was, for a long time, believed to be important only for B cell development and function [9], there is growing evidence that it is also involved in TLR-mediated activation of innate immune cells such as monocytes and neutrophils. These cells show a proinflammatory phenotype revealed by increased proinflammatory cytokine secretion and exacerbated reactive oxygen species production [6]. Clinical data indicating altered function in such cells are not yet well represented in XLA patients, with the exception of a small number of patients reported with intestinal chronic inflammation of unknown origin [10]. Additionally, a web-based survey of 128 XLA patients indicated that a significant proportion of them showed autoimmune or inflammatory complications such as arthritis and inflammatory bowel disease [11]. The occurrence of arthritis in XLA have been reported to be variable depending on the genetic background, Caucasians show a frequency of 7–15%, while Asian populations reported arthritis in 29% of the patients [12–14]. Most cases of the arthritis reported in XLA patients are considered to be associated with active infections (septic) [14], but less frequently, rheumatoid arthritis and aseptic (reactive) arthritis, have also been described [15,16].

Abbreviations: BLNK, B cell linker protein; cDNA, complimentary DNA; gDNA, genomic DNA; MFI, median fluorescence intensity; PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; TLR, toll-like receptors.

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In this work, we identified *BTK* mutations in a cohort of 12 patients with XLA and revised the clinical symptoms from these patients and from an additional cohort of 14 previously reported patients [17]. We found four novel mutations (three affecting the mRNA splicing and one missense). Additionally, our results showed a high incidence of splice site mutations in the patients analyzed. Respiratory and gastrointestinal tract infections were commonly observed in this cohort. Patients also presented other complications comprised of, in order of frequency: meningitis, arthritis, conjunctivitis, dermatitis, pyodermitis, osteomyelitis, colitis, ecthyma gangrenosum, intestinal perforation, pericarditis, hepatitis, and mastoiditis. Occurrence of these complications was similar to that reported earlier, with arthritis occurring at a higher frequency to that reported by Hernandez-Trujillo et al. and other groups [11]. Analysis of mutations shows a higher frequency of missense mutations affecting SH1, SH2 and PH domains in those patients that develop septic or reactive (aseptic) arthritis.

2. Materials and methods

2.1. Patients

This study investigated 12 patients with a clinical diagnosis of XLA. All patients or patients' parents gave their consent to participation and were under an approved protocol from the National Institute of Pediatrics (# 39/2005). All patients analyzed had <2% of the normal number of peripheral B cells, and all immunoglobulin isotype levels were two standard deviations below the normal range. Additionally, in order to increase the number of patients with clinical data, the clinical records of the 12 patients with mutations reported here and those of 14 patients whose mutations we had reported previously were revised [17]. We specifically looked for history of infectious disease and occurrence of arthritis within these clinical records.

All patients were of Mexican mestizo origin and came from two main geographical regions: South-Central and Northeastern Mexico, and were enrolled in four main health centers in Mexico, National Institute of Pediatrics, National Medical Center “La Raza”, National Medical Center Siglo XXI and Northeastern Medical Center-IMSS.

2.2. Btk protein expression

Heparinized peripheral blood samples were obtained from patients and healthy controls. Peripheral mononuclear cells were obtained by gradient centrifugation, and 0.5×10^6 cells were used for Btk detection by flow cytometry, using a previously described protocol [17]. Briefly, cells were fixed with 1% paraformaldehyde and permeabilized with 0.1% saponin in PBS. After permeabilization, anti-Btk (Becton Dickinson, Franklin Lakes, NJ, USA) was added to the cells and incubated for 30 min. Cells were washed once with 0.01% saponin in PBS, and anti-IgG2a-PE (eBioscience, San Diego, CA, USA) was added to the cells and incubated for 30 additional minutes. After washing, anti-CD14 APC (Becton Dickinson) was added to the cells and incubated for an additional 15 min, at which point, the cells were washed a final time and fixed. An FACSAria cytometer (Becton Dickinson) was used to acquire 20,000 events, and Btk expression was analyzed in CD14 positive cells using Flowjo software (Treestar, Ashland, OR, USA). Median fluorescence intensity (MFI) was calculated for all patients and controls. Statistical analysis was performed using Mann–Whitney *U* test. Differences between patients and controls were considered significant when $p < 0.05$. Data were analyzed using GraphPad prism software v4.00 for Macintosh (GraphPad software, San Diego, CA, USA).

2.3. Samples and nucleic acid extraction, RNA extraction, and Btk amplification

Peripheral blood samples from patients and healthy controls were obtained using Vacutainer™ tubes containing acid citrate dextrose (Becton Dickinson). Peripheral blood mononuclear cells (PBMCs) were purified using a gradient of Ficoll-Hypaque (Axis-Shield, Oslo, Norway), and RNA was extracted from 3×10^6 cells lysed in Trizol® (Invitrogen, Carlsbad, CA, USA), using previously described procedures [17]. Complementary cDNA was synthesized from RNA using reverse transcriptase (Thermo-Scientific, Waltham, MA, USA). Genomic DNA were obtained from total blood using the Gentra Puregene Blood kit (Qiagen, Hilden, GE). *BTK* cDNA was amplified using four oligonucleotide pairs: *BTK*_p1, *BTK*_p2, *BTK*_p3, and *BTK*_p4; while primers were designed for each of exons and surrounding introns sequences. Primers sequences and melting temperatures used in this work are available under request. Polymerase chain reaction (PCR) was performed using Taq DNA polymerase (New England Biolabs, Ipswich, MA, USA).

2.4. Cloning and sequencing of PCR products

PCR products obtained from amplification with either oligonucleotide pair were cloned into the pJet vector using the clonJET PCR cloning kit (Thermo Scientific). To screen for positive *Escherichia coli*-transformed colonies, PCR was carried out using the pJET primers. Positive clones were grown in Luria-Bertani medium with 150 μg/mL ampicillin, and plasmid extractions were performed using the Nucleospin® plasmid kit (Macherey-Nagel, Düren, Germany).

For sequencing, reactions were set up with 150 ng plasmid DNA and either pJET forward or pJET reverse primers using the Big Dye sequencing kit (Applied Biosystems, Foster City, CA, USA). When mutations were detected, we sequenced in total three clones to confirm the mutation. When multiple alternative spliced transcripts were detected, shorter transcripts were chosen for sequencing.

2.5. Btk sequence analysis

Sequence files were analyzed using Chromas Lite (http://technelysium.com.au/) and BLAST (http://blast.ncbi.nlm.nih.gov/). The ENST00000308731 and U78027 reference sequences for cDNA and gDNA were used for all analyses and were obtained from the ENSEMBL and GenBank databases, respectively.

3. Results

3.1. Btk expression analysis

Btk expression was assessed using total PBMCs, as described above. As expected, we found a lack of protein expression in most patients. In patients, Btk MFI was below the levels detected in healthy controls (Fig. 1b), with the exception of P54, who showed a comparable MFI (40.76) with that observed in healthy controls (Fig. 1c). P54 carried a previously reported missense mutation (R288Q). The R288Q mutation was also found in P25; however, the Btk MFI was much lower (15.94) than that observed in P54, such differences are discussed below.

3.2. BTK amplification and mutation analysis

The patients analyzed in this report showed aberrant transcripts in which intron insertions or exon deletions were detected (Table 1, Fig. 1a). Missense mutations were detected in four patients with the following changes: R288Q (P25 and P54), P597L (P33), and L222P (P56). Splice site mutations were detected in P3, P15, P23, P26, P27, P28, and P30. P29 showed a single base pair deletion (c.delC1877; Table 1). With the exception of P29, all splice site mutations were first observed because of the varying lengths of Btk PCR products, as shown in Fig. 1a. All deletions and insertions in Btk cDNA are predicted to encode an abnormal protein. As shown in Table 1, P3 showed a 7-bp deletion in cDNA (c.delA2073–G2079) at the 5′ end of exon 19. Such a deletion would encode an aberrant protein with a frameshift starting at K637 and a premature stop codon at position 646 (FsdK637*646). Two