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## Clinical and Genetic Study of X-linked Agammaglobulinemia Patients (The Benefit of Early Diagnosis)

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### ABSTRACT

X-linked agammaglobulinemia (XLA) is a primary immunodeficiency caused by genetic defects in the Bruton tyrosine kinase (*Btk*) gene. XLA is characterized as an antibody deficiency by recurrent bacterial infections, the absence of peripheral B cells, and profound reductions in all immunoglobulin isotypes. This study aims to report the clinical and genetic features of five Iranian patients with XLA.

Five male cases with recurrent bacterial infection entered this study based on clinical evaluation and Immunological screening tests. The levels of T-cell receptor excision circle (TREC) and kappa-deleting recombination excision circle (KREC) were also measured in dried blood spot (DBS) samples. Sanger sequencing was applied to PCR products of DNA samples of the patients for genetic studies.

All patients were from unrelated families with a mean age of 6.7 years (2.5-11) at the time of diagnosis with 4.8 mean years of delay in diagnosis. The most frequent clinical manifestations were recurrent respiratory infections and arthritis. In these patients, five previously reported mutations were found including four mutations (p.Q496X, p.Q497X, p.R520X, and p.R641H) in the Kinase domain besides one mutation (p.L37P) in the pleckstrin homology (PH) domain. Evaluations of KREC and TREC level in patients' DBS showed low-to-undetectable copies of KREC (0-2 copies/3.2mm DBS) with normal copies of TREC.

As patients with XLA have complete immunoglobulin defects and develop severe and recurrent infections, early diagnosis would be beneficial for the improvement of their quality of life. The study results may provide valuable information for the diagnosis, genetic counseling and prenatal diagnosis for the patients and their family members and emphasize performing KREC as an early diagnostic test in patients with XLA.

**Keywords:** Bruton tyrosine kinase; Early diagnosis; Mutation; X-linked agammaglobulinemia

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## INTRODUCTION

X-linked agammaglobulinemia (XLA) is a rare primary immunodeficiency (PID) in which affected patients are prone to recurrent bacterial infections and profound hypogammaglobulinemia and almost absent of B cells. The only gene associated with the disease is located on chromosome X (Xq21.3-q22) and encodes a cytoplasmic tyrosine kinase known as Btk. Btk is a key signaling molecule in B cell development whose deficiency can prevent pre-B cells from further maturation in the bone marrow. Also, Btk, as signaling molecule acting downstream of B cell receptor (BCR), provides mature B cells with essential signals for survival. Therefore, in Btk-deficient patients, the absence of circulating B cells and the absolute lack of antibodies in the serum are the immunological hallmark of the disease and result in defective antibody-mediated immunity. In addition to adaptive immunity, Btk is involved in innate immunity as well. In macrophages and dendritic cells Btk, as a signaling mediator downstream of toll-like receptors (TLRs), contributes to antimicrobial defense.<sup>1</sup> Also, Btk is crucial in neutrophils for both maturation and survival, but not for the function.<sup>2</sup>

Although, XLA patients have cell-mediated immunity the mortality risk among these patients has frequently been reported.<sup>3,4</sup> It emphasizes the importance of early diagnosis and treatment to prevent severe infections and adverse outcomes. This study aims to report the clinical and genetic features of five Iranian patients with XLA who were referred to Immunology, Asthma & Allergy Research Institute (IAARI).

## MATERIALS AND METHODS

### Patients

Five male patients who had recurrent bacterial infections from their early life and B cells less than 1% with humoral immunodeficiency were suspected to XLA referred to IAARI between 2008 and 2017 and subjected to further immunological investigations.

### Ethics

Ethics Committee of IAARI approved the study (code #: 1-21-85/1-137). All blood samples were obtained after taking informed written consent from the patients or their parents.

## Immunological and Genetic Studies

The primary and advanced PID screening tests performed for each patient were; complete blood count (CBC), measurement of serum immunoglobulin levels (IgG, IgA, and IgM), and lymphocyte subtypes (CD3, CD4, CD8, CD19, and CD16/56).

For the genetic study, genomic DNA was extracted from whole blood samples using the High Pure PCR Template Preparation Kit (Roche, Germany) according to the manufacturer's instructions. 19 pairs of primers were designed by Gene Runner software for the promoter, all coding exons and the exon-intron boundaries of the Btk (NM\_001287344). Polymerase chain reaction (PCR) was performed for each patient using designed primers. Subsequently, all PCR products were purified and sequenced. Sequencing results were viewed and analyzed using Finch TV software and Basic local alignment search tool (BLAST), respectively. Upon identification of a disease-causing mutation in the patients, DNA samples of their mothers were evaluated for carrier status. Also, Btk expression was assessed by Western blotting for two patients (4 and 5) whose samples were available.

Along with the genetic study, the levels of T-cell receptor excision circle (TREC) and kappa-deleting recombination excision circle (KREC) as circular DNA fragments were also assessed by multiplex Real-time PCR in dried blood spot (DBS) samples obtained from patients, as previously described.<sup>5</sup> We considered TRECs<11mm DBS and KRECs<6mm DBS as cutoff values according to a study from Iran.<sup>6</sup>

## RESULTS

The patients regularly received IVIG upon disease diagnosis; demographic data and clinical manifestation of the patients are summarized in Table 1. All patients had recurrent bacterial infections and upon receiving IVIG, they experienced better quality of life with diminished bacterial infections and disappearance of arthritis. The diagnosis delay was 4.8 years in this study. Immunoglobulin isotypes including IgM, IgG, and IgA were extremely low in the patients, while patient 5 had a normal IgA. All patients had less than 1% B cell. No expression of Btk was found in patients 4 and 5 by Western blotting (samples from patient 1, 2 and 3 were not available). Molecular analysis of the patients revealed five previously reported point mutations including three nonsense mutations

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(p.Q496X, p.Q497X, and p.R520X) besides two missense mutations (p.R641H and p.L37P) (Table 2). The mothers of patients 1, 2, 3, and 4 were carriers; the blood sample of patient 5's mother was not available.

Also, all patients of this study showed normal TREC levels, but low or undetectable copies of KREC (0-2 copies/3.2mm DBS) as shown in Table 2.

**Table 1. Demographic data and clinical manifestations of XLA patients**

Patients	1	2	3	4	5
Onset age (y)	0.4	3.0	1.0	3.0	2.0
Diagnosis age (y)	2.5	7.0	11.0	9.0	4.0
Diagnosis delay (y)	2.1	4	10	6	2
Pneumonia	+	-	-	+	+
Sinusitis	+	-	-	-	+
Recurrent otitis media	-	+	-	-	-
Pharyngitis	-	+	-	-	-
Meningitis	-	-	-	+	-
Septic arthritis	-	-	+	+	-
Osteomyelitis	-	-	+	-	-
Diarrhea	+	-	-	-	-
Lymphadenitis	+	-	-	-	-

**Table 2. Laboratory findings and genetic results of XLA patients**

P.	IgM (mg/dL)	IgG (mg/dL)	IgA (mg/dL)	B cells (%)	**KREC (copies/ $\mu$ L)	***TREC (copies/ $\mu$ L)	cDNA mutations	Protein alteration	Mother
1	0 (48-168)	*670 (424-1051)	0 (14-123)	<1%	0	74	c.1650C>T	p.Q496X	Carrier
2	11 (48-207)	*1183 (633-1280)	<5 (33-202)	<1%	1	177	c.1722C>T	p.R520X	Carrier
3	3 (52-242)	342 (608-1572)	0 (45-236)	<1%	1	317	c.2086G>A	p.R641H	Carrier
4	NA	406 (608-1572)	NA	<1%	1	96	c.274T>C	p.L37P	Carrier
5	20 (43-196)	0 (463-1236)	133 (25-154)	<1%	2	38	c.1653C>T	p.Q497X	NA

P: Patient, Age: age at the time of diagnosis, NA: Not available, \*Data is after IVIG replacement therapy, \*\*Normal range of KRECs<6mm, \*\*\*Normal range of TRECs<11mm, \*\*\*\*Genetic evaluation of the patients' mothers

Italic numbers between parentheses represent the range in the age-adjusted normal range from "The HARRIET LANE HANDBOOK, Jason W. Custer and Rachel E. Rau, Eighteenth edition"

### DISCUSSION

Herein, we described the clinical and genetic features of five patients with X-linked agammaglobulinemia. XLA is a disease of humoral immunity characterized by recurrent and severe bacterial infections, a profound reduction in B cells

(<1%) and the absolute lack of all immunoglobulins. Patients with XLA usually start developing symptoms from age six-months when maternal immunoglobulins vanish. Despite clear clinical and laboratory descriptions of XLA provided by large cohort studies from United State,<sup>3</sup> Europe,<sup>4</sup> and Asia,<sup>7</sup> significant delays in diagnosis (4.8 years) have still been noticed in

this study and previous studies (4.2 and 5.5 respectively)<sup>8</sup> which seems to be due to lack of awareness among primary care physicians. Early diagnosis in patients 1 and 5 may be due to their family history. Unexplained early death and family history could lead physicians to early diagnosis.

Consistent with the other studies, respiratory tract infections are the most prominent clinical manifestations in the patients.<sup>4,8</sup> Arthritis was the next common manifestation in this study. The occurrence of arthritis which could completely disappear after IVIG replacement therapy has been frequently reported in XLA patients.<sup>4,8</sup> Otitis media, which combined with undetectable tonsils or lymph nodes, could lead physicians to XLA diagnosis, occurred with less frequency in these patients compared with other studies.<sup>3,8</sup>

All immunoglobulin isotypes are significantly decreased in the patients with defective Btk, however, one or two isotypes may be detectable in some patients defined as having "leaky" XLA.<sup>8</sup> Patient 5 of this study could be taken as an example of "leaky" XLA since his IgA level was normal.

The quantification of both TREC and KREC in DBS samples and tandem mass spectrometry are currently available for early identification of T and B cell-mediated diseases in newborn screening programs. These DNA circles persist in the cells and do not replicate during cell division. Therefore, patients with T and/or B-cell lymphopenia showed low levels of TREC and KREC, regardless of the molecular etiology of underlying PID. KREC levels have recently been analyzed in patients with XLA and non-XLA as well.<sup>5,9</sup> In a study by Nakagawa et al. the measurements of KREC copies in DBS samples of these two diseases showed no KRECs in 30 XLA and five non-XLA patients.<sup>9</sup> Some previous studies showed that the levels of TREC and/or KREC might not agree with T and B numbers respectively in patients with CID even in cases with a severe form of the disease.<sup>6</sup> Consistent with the other studies, all patients in this study had zero or low copies of KREC with normal levels of TREC, which were in accordance with the immune-phenotype of these patients. Therefore, KREC value would be beneficial for early diagnosis as the previous study by Esenboga et al suggested that early diagnosis and receiving timely and appropriate treatment would improve the quality of life in the patients with XLA.<sup>10</sup>

Btk consists of five essential structural domains

including the pleckstrin homology (PH) domain, Tec homology (TH) domain, Src homology3 (SH3) domain, Src homology2 (SH2) domain, and the catalytic kinase (Tk) domain. The most common type of mutations have been found in Btk are missense followed by nonsense mutations. In addition, according to previous studies mutations usually occur in all domains of Btk with a preference for the TK domain.<sup>11</sup> Five point mutations found in this study were previously described as disease-causing; four of which occurred in the TK domain besides one mutation in the PH domain.<sup>7,12-15</sup>

XLA is considered as a familial disorder in which mutations in Btk pass down through the generations however sporadic cases have also been reported with a prevalence of 20% to 60% of all cases.<sup>3</sup>

Increasing awareness among physicians seems to be critical for reducing diagnostic delay and for definitive diagnosis of XLA genetic study is essential. The study results may provide information for the diagnosis, genetic counseling and indicating KREC as an early valuable diagnosis test especially for those who have a history of immunodeficiency in their family and can also be used for prenatal diagnosis.

#### CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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