Screening of the Bruton Tyrosine Kinase (BTK) Gene Mutations in 13 Iranian Patients with Presumed X-Linked Agammaglobulinemia

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ABSTRACT

X-linked agammaglobulinemia (XLA) is an immunodeficiency caused by mutations in the Bruton tyrosine kinase (Btk) gene. In order to identify the mutations in Btk gene in Iranian patients with antibody deficiency, 13 male patients with an XLA phenotype from 11 unrelated families were enrolled as the subjects of investigation for Btk mutation analysis using PCR-SSCP followed by sequencing. Five different mutations were identified in 5 patients from 5 unrelated families. Three mutations had been reported previously including TTTG deletion in intron 15 (4 bps upstream of exon 16 boundary), nonsense point mutation (1896G>A) that resulted in a premature stop codon (W588X) in kinase domain, and nucleotide alteration in invariant splice donor site of exon12 (IVS12+1G>A). While 2 novel missense mutations (2084A>G, 1783T>C) were identified leading to amino acid changes (I651T, Y551H). The results of this study further support the notion that molecular genetic testing represents an important tool for definitive and early diagnosis of XLA and may allow accurate carrier detection and prenatal diagnosis.

Keywords: Agammaglobulinaemia Tyrosine Kinase; Bruton's Tyrosine Kinase; Iran; X-linked Genetic Disease

INTRODUCTION

X-linked agammaglobulinemia (XLA), was first described by Bruton in 1952[1] clinically characterized by a profound deficiency of all immunoglobulins, marked reduction of B lymphocytes in the peripheral circulation and early onset of recurrent bacterial infections.[2-5] In 1993, the defective gene in XLA was identified by two independent groups and termed *Btk* (Brutons tyrosine kinase).[6,7]

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The *Btk* gene is expressed in B-cells throughout their differentiation (except plasma cells) and in myeloid cells but not in T-cells. Mutations in the Btk gene affect the early stages of B-cell differentiation.[8]

The *Btk* gene which is localized at the long arm of chromosome X in the region of Xq21.3-q22,[2,3,9] encompasses 37.5 kb containing 19 exons. The protein consists of five structural domains: pleckstrin homology (PH) domain (amino acids 1 to 138), Tec homology (TH) domain (amino acids 139 to 215), Src homology 3 (SH3) domain (amino 216 to 280), Src homology 2 (SH2) domain (amino acids 281 to 377) and kinase (SH1) domain (amino acids 375 to 659).

In order to determine the range of mutations that has been identified in patients with XLA worldwide

XLA mutation registry named BTKbase (http://www.uta.fi/imt/bioinfo/BTKbase/) was established in 1994.[10] At present over 400 unique *Btk* mutations have been found in patients with XLA.[11] These include missense, nonsense, insertion, deletion and splice site mutations as well as frameshift and inframe insertions and deletions. These mutations are found in both exons and introns throughout the gene and may result in complete absence of protein, or presence of truncated or full-length non-functional proteins. Many studies in different countries have demonstrated that about 85% of males with presumed XLA have mutations in the *Btk* gene.[12] In this report, we describe the first identification of *Btk* mutations in Iranian male patients with XLA phenotype.

MATERIALS AND METHODS

Patients and Study Design

Iranian Primary Immunodeficiency Registry (IPIDR) was established in 1999 and 440 patients with primary immunodeficiency were registered in our registry, who had been observed over a period of 20 years. Among our patients, the antibody deficiencies were the most common type of diagnosed immunodefciencies (n=202).[13] Thirty-three male patients with an immunological phenotype compatible with XLA have been registered in IPIDR. In order to identify the mutation in Bt*k* gene in Iranian patients with presumed XLA, 13 male patients (from 11 families) aged 3 to 31 years with XLA phenotype who had been diagnosed during 1995-2002 were enrolled as the subjects of our investigation in this study (Table1). The phenotypes of the patients were characterized by increased susceptibility to bacterial infections, decreased serum immunoglobulin levels (IgG levels lower than 300 mg/dl), and reduced B-cell numbers (less than 2%) assessed by flowcytometric analysis of peripheral blood lymphocytes (Table1). T cell number and function were normal in all patients.

One patient had parental consanguinity. Seven patients from 5 families had a family history of immunodeficiency in their male family members (P2 is uncle of P12, and P4 is brother of P5). Six patients (from 6 families) had no obvious family histories in favor of immunodeficiency.

BTK Mutation Detection

Genomic DNA was obtained from whole blood in 13 patients by the conventional phenol/chloroform method. A polymerase chain reaction (PCR) was carried out by using primers encompassing each exon/intron boundary of the *Btk* gene as described. [14] The following PCR conditions with AmpliTaq Gold polymerase (PE Applied Biosystems, Foster City, CA) were used for all reactions: 95°C for 10 minutes followed by 40 cycles of 95°C for 30 seconds; 51°C for 30 seconds; 72°C for 40 seconds with a final extension of 7 minutes at 72°C.

Table 1. Clinical data and Btk mutations of patients.

Patients Number	Age at Diagnosis	IgG (mg/dl)	IgA (mg/dl)	IgM (mg/dl)	CD19+ B Cells	Family History	Nucleotide Change	BTK Domain	Consequence
P1	7 years	152	12	13	<1%	-	IVS15-13delTTTG	SH1	Exon 16skipping
P2	11 years	0	0	0	<1%	+		-	-
P3	3 years	14	0	0	<1%	-		-	-
P4	6 years	0	0	0	<1%	+		-	-
P5	7 years	0	0	0	<1%	+		-	-
P6	2 years	0	0	0	<1%	+		-	-
P7	5 years	50	5	10	<1%	-	IVS12+1G>A	SH2	Splice donor defect
P8	5 years	290	10	23	<1%	-	2084A>G	SH1	1651T
P9	14 years	50	0	0	<1%	-	1896G>A	SH1	W588X
P10	5 years	50	10	25	<1%	+		-	-
P11	5 years	100	10	20	<1%	-		-	-
P12	10 years	50	0	0	1.23%	+		-	-
P13	4 years	50	5	10	1%	-	1783C>T	SH1	Y551H

The mutated allele was detected by single-strand conformation polymorphism (SSCP) analysis. The amplified DNA was mixed with the denaturing solution in a 1:1 ratio, denatured for 5 minutes at 95°C, and chilled on ice immediately. The sample was loaded onto Gene Excel 12.5/24 polyacrylamide gel (Amersham Pharmacia Biotech, Buckinghamshire, England), electrophoresed at 10°C for 80 min, and then visualized by silver staining. When the abnormally mobilized band was detected, the PCR product was purified and sequenced with the corresponding forward and reverse primers using the BigDye terminator cycle sequencing kit (PE Applied Biosystems) with an automated ABI PRISM 310 genetic analyzer (PE Applied Biosystems).[15] When an abnormal mobility was not found on a gel, all the exons were sequenced.

RESULTS

BTK Mutations

In this study we performed BTK mutation analysis in 13 male patients with XLA phenotype from 11 unrelated families.

Five different mutations were identified in 5 patients from 5 unrelated families (Table 1). In P1, TTTG deletion in intron 15 (4 bps upstream of exon 16 boundary) was identified. This mutation has already been shown to result in exon 16 skipping. In P7, we found a nucleotide alteration in invariant splice donor site of exon12 (IVS12+1G>A), which might cause the skipping of exon 12. In P8, the mutation 2084A>G was identified leading to amino acid change (I651T). P9 displayed a nonsense point mutation (1896G>A) that resulted in a premature stop codon (W588X) in kinase domain. In P13, a missense mutation (1783T>C) was identified, which led to amino acid change (Y551H). The mutations in three patients (P1, P7 and P9) had been reported previously. The remaining 2 mutations (P8 and P13) are novel mutations.

In the remaining 6 families, mutations in the *Btk* gene were not identified, even though the patients showed clinical and immunological profiles that were very similar to those of XLA.

DISCUSSION

The observations of low immunoglobulin serum levels and absence of circulating B cells in male patients can allow to a definite diagnosis of XLA only in the case of the recurrence of affected males in different generations.[16] However, approximately one third to one half of XLA are sporadic cases, making a diagnosis solely based on clinical and immunological criteria more difficult. Mutation analysis using PCR-SSCP followed by direct sequencing can provide an unambiguous molecular diagnosis and also offers the possibility of carrier assignment and prenatal diagnosis.

In this study, 13 Iranian male patients (from 11 families) with XLA phenotype were examined for presence of *Btk* gene mutation. Five patients (P1, P7, P8, P9, and P13) from 5 families were diagnosed to have mutations in *Btk* gene. Three (P7, P8, and P13) out of five patients with *Btk* mutation presented with recurrent infections since their infancies. By contrast P1 and P9 were well until 9 and 7 years of age, respectively. Some patients with XLA have a milder phenotype regarding clinical manifestations and /or may have higher concentrations of serum immunoglobulins than expected.[17,18] Therefore they may not be recognized to have immunodeficiency in early life and even a few patients with XLA were recognized only in adulthood.[19]

This heterogeneity does not reflect the consequence of different mutations. Mutations in *Btk* gene are highly variable and as reported previously, there is no correlation with the clinical phenotype and the site of the mutation,[20-23] indicating that other factors, which are yet to be determined, are contributing to the phenotype and affecting the severity of the disease.

We suspect that modifier genes and possibly environmental factors could influence the clinical expression pattern of B-cell deficiency. Approximately 65% of mutations are premature stop codons, frameshift mutations, splice site defects or gross deletions.[24,25]

The majority of the remaining mutations are amino acid substitutions. Most of these mutations lead to a marked decrease or absence of *Btk* protein.[26-28]

It has been documented that approximately 85% of patients with presumed XLA have mutations in B*tk*.[27] In our study, 8 patients (from 6 families), despite very low number of B cells and highly decreased immunoglobulin levels and positive family history in 4 and 2 out of the 4 patients belonging to

the same sibship, had no mutations.

It has been reported that a few XLA patients who lacked the Btk protein, had no mutations in the coding regions of the *Btk* gene.[28,29] Although mutations in the Btk gene were not observed in 4 patients from 2 families (P2, P4, P5 and P12) the presence of positive family history in these 4 patients raised the possibility of mutations in other genes involved in their early B-cell development. Studying Btk expression and/or analysis of cDNA could help us to confirm results of SSCP analysis before we focus to other genes, because some mutations may escape SSCP analysis.[30]

Mutations in *Btk* do not account for all the patients with defects in early B-cell development. Some patients may have defects in pre-B cell receptor complex genes and other genes that have not yet been identified. Therefore, the role of additional molecules is likely to be discovered in the future. In conclusion, this report has described our study to identify *Btk* mutation in Iranian patients with presumed X-Linked Agammaglobulinemia. Although, profound deficiency of all serum immunoglobulin levels, severe reduction of B lymphocytes in the peripheral circulation and presence of positive family history make a clinical diagnosis of XLA very likely, however to achieve a definite diagnosis solely based on clinical and immunological criteria is difficult in the sporadic cases. Our results further demonstrate that mutation analysis using PCR-SSCP followed by direct sequencing, can provide an unambiguous molecular diagnosis of X-Linked Agammaglobulinemia.

ACKNOWLEDGEMENT

We are grateful to the families of patients for their participation in this study and also to Mrs. Khosravi, Batol Moradi, and Bita Ansari for excellent assistance in the DNA extraction in Department of Immunogenetics of Tehran University of Medical Sciences. This study was supported by a grant from the Tehran University of Medical Sciences, Grand-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and grant from the Ministry of Health, Labour and Welfare, Japan.

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