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Original Articles

Correlation Analysis of Mutation Severity and BTK-expression and Clinical Manifestations in the Patients with X-linked

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Abstract

Backgrounds/Objectives X-linked agammaglobulinemia (XLA) is a primary immunodeficiency disorder caused by mutations in the Bruton tyrosine kinase (*BTK*) gene. It is characterized by severely reduced numbers of peripheral B cells and a significant deficiency in all serum immunoglobulins. In the present study, the impact of mutation severity on the clinical and immunological characteristics of XLA patients was evaluated.

Methods Mutation analysis was performed in 19 XLA patients by PCR assay to identify variations in the *BTK* gene. Subsequently, the western blotting technique was applied for measuring *BTK* expression and function. A genotype-phenotype correlation was investigated regarding the impact of mutation severity on clinical and immunological parameters.

Results Mutation detection in the *BTK* gene revealed missense mutations in 9 patients, nonsense mutations in 3 cases, splicing site defects in 5 patients, and small in-frame deletions in 2 patients; 31% of patients displayed normal *BTK* expression. A significant correlation was found between types of *BTK* mutation and *BTK* expression.

Discussion Generally, genotype-phenotype correlation studies on XLA disease seem to be very controversial. The results of the correlation analysis in the present study could indicate that evolution of the disorder is not completely similar in all cases, even with the same mutation.

Keywords XLA, Correlation analysis, Mutation severity, Clinical and immunological characteristics

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Introduction

X-linked agammaglobulinemia (XLA) is one of the commonest primary immunodeficiencies (PIDs) characterized by strongly reduced numbers of peripheral B-cells, significant hypogammaglobulinemia, and recurrent bacterial infections, particularly in the respiratory and gastrointestinal tracts and the central nervous system (CNS) (1, 2). The prognosis of XLA has been profoundly improved by earlier diagnoses and more significantly by immunoglobulin replacement therapy which increases normal concentrations of immunoglobulin (3).

Bruton's tyrosine kinase (*BTK*), a responsible gene for XLA, is a member of the Tec family of non-receptor tyrosine kinases (nRTKs). It is involved in the pre–B-cell immunoglobulin receptor signaling pathway and composed of five distinct domains, the pleckstrin homology (PH), Tec homology (TH), Src homology 3 (SH3), SH2, and the kinase domain SH1 (4). The *BTK* gene is located on the X chromosome at Xq21.3–Xq22 (1, 5, 6). Up to now, over 1100 unique mutations in the *BTK* gene (BTKbase) have been reported (7, 8), resulting in the defective expression and function of the *BTK* protein. A lack of *BTK* protein, or nonfunctional protein, has been considered as the most reliable evidence confirming XLA disorder (9, 10). However, affected patients show heterogeneity in the expression of the *BTK* protein as well as clinical manifestations (8, 11). Various investigations have shown the absence of any significant genotype/phenotype correlation in XLA disease (12-15). Nevertheless, some have indicated that well-defined mutations may profoundly influence *BTK* expression as well as the clinical outcome of this disorder (11, 16, 17).

In the present study, efforts were made to determine whether the type of variation in the *BTK* gene is associated with the expression of the *BTK* protein and if the mutation severity is associated with the clinical outcome of XLA patients.

Materials and Methods

Patients

Nineteen male patients with defined X-linked agammaglobulinemia were enrolled in the present study. The diagnosis criteria were those of the European Society of Immune Deficiencies (ESID, (https://esid.org/Working-Parties/Registry/Diagnosis-criteria)) and included circulating B cells lower than 2%, normal T cell counts, and decreased serum IgG level (according to age) with evidence of recurrent infections before the age of 5 years. Blood samples were collected with the ethical approval of Tehran University of Medical Sciences (TUMS). Demographic, clinical, and laboratory data was obtained from patients' hospital records. Written informed consent was obtained from all participants and their parents.

BTK gene mutations were grouped into "severe" or "mild" (11, 16, 18). Premature stop codons, gross deletions, frameshifts, and splicing-site mutations that affect the splice consensus invariant sequences were considered as ''severe''. Mild mutations

considered the missense mutations in non-conserved subdomains and those in non-invariant splicing sites. Disease severity was defined as the age of onset (≤6 month) and the presence of severe recurrent infections.

DNA extraction and polymerase chain reaction

Genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) using the conventional phenol-chloroform method and was quantified using a NanoDrop 1000TMS spectrophotometer (OD 260 nm/OD 280 nm). PCR amplification of the *BTK* gene was performed as previously described (19). Amplicons were sent for direct sequencing in accordance with the manufacturer's instructions. Analysis of sequences was carried out using chromas software and NCBI-Blast.

Western immunoblotting assay

PBMCs were lysed and used for western blot analysis. Western immunoblotting was performed with primary antibodies against *BTK* and β-actin proteins (Sigma). Immunoreactivity was detected using an enhanced chemiluminescence western blotting detection kit (Amersham, Piscataway, NJ). Anti β-actin antibody (Sigma) was recruited as the internal control.

Statistical analysis

Statistical analysis was performed using the SPSS software version 21.0. The diagnostic delay was defined as the time between onset of clinical symptoms and diagnosis. The time between diagnosis time and the last patient visit was considered as the follow-up time. The correlation between the mutation type and *BTK* expression was determined using the Fisher exact test. The association between the clinical/immunological findings and affected domains and the mutation severity was examined using the chi-square and Mann-Whitney tests. A p-value of <0.05 was considered to be statistically significant in all tests.

Results

Demographic and clinical characteristics

A total of 19 Iranian male XLA patients from 13 unrelated families were evaluated in the present study. The main demographic and biological features of all patients at the time of diagnosis are summarized in **Table 1**. The median age at the time of diagnosis was 5 years. The median age of the cases at disease onset was 1 year. All patients are alive and were followed up for a median period of 17 years. Six out of 19 (13%) cases were from a consanguineous marriage, and 8 (42%) patients had a family history of XLA. P10 is the uncle of P13, and (P8 and P12) were brothers as well as (P6 and P7) and (P15 and P19). At the time of diagnosis, upper and lower respiratory tract infections were the commonest symptom (83.3%), followed by otitis media (61%), recurrent diarrhea (44.4%), rheumatic manifestations (44.4%), dermatologic manifestations (33.3%), and neurologic manifestations (33.3%).

Table 1. Demographic and biological characteristics of XLA patients

Variables	ALL patients	Mild mutation	Severe mutation	*P* Value
Number of patients	19	10	9	-
Age at onset, year (IQR)	1.0 (0.7-5)	1.3 (0.6-5.5)	1.0 (0.4-4.5)	0.49
Age at delayed diagnosis, year (IQR)	3.4 (2-6.6)	3.0 (1.3-6.7)	4.0 (2.2-6.8)	0.4
Diagnostic age, year (IQR)	5.0 (4-9)	4.0 (2.6-9.5)	7.6 (5.9-9.5)	0.15
IgG, mg/dl (IQR)	120 (39-221)	78 (2.5-129)	203 (116-385)	0.063
IgA, mg/dl (IQR)	3.0 (0-9.25)	1.0 (0-10)	5.0 (0-7.5)	0.79
IgM, mg/dl (IQR)	18 (4-29.25)	10 (0.5-53)	19 (15.5-27.5)	0.48
B cells (%) (IQR)	0.0 (0-2)	2.0 (0-2)	0.0 (0-0.75)	0.13
BTK expression+	6/19	6/10	0/9	0.011
CD3+ (%) (IQR)	92 (78-94)	92 (64.5-93.5)	92 (80.5-94.5)	0.6
CD4+ (%) (IQR)	44 (32-54)	42 (29.5-54)	44 (28-52.5)	0.78
CD8+ (%) (IQR)	39 (28-47)	36 (20.5-47)	43 (27.5-47)	0.72
Clinical outcome (Severe/Mild)	10/9	6/4	4/5	0.49
Influenced domain (SH2/ SH1-TK/PH)	1/8/10	1/5/4	0/3/6	0.49

Mutation analysis

All patients had confirmed mutations in the *BTK* gene, including 5 missense variations in 9 patients, 3 nonsense mutations in 3 patients, 4 splicing site defects in 5 cases, and 2 small deletions in 2 patients. The mentioned variations were positioned on the SH1/TK domain of the *BTK* protein in 8 out of 19 cases (42.1%), in the PH domain in 10 cases (52.6%), and in the SH2 domain in 1 case (5.2%). Fourteen identified types of mutations were predicted and assigned into two groups of mild (n = 6) or severe (n = 8). The frequency of each type of mutations is shown in **Figure 1**.

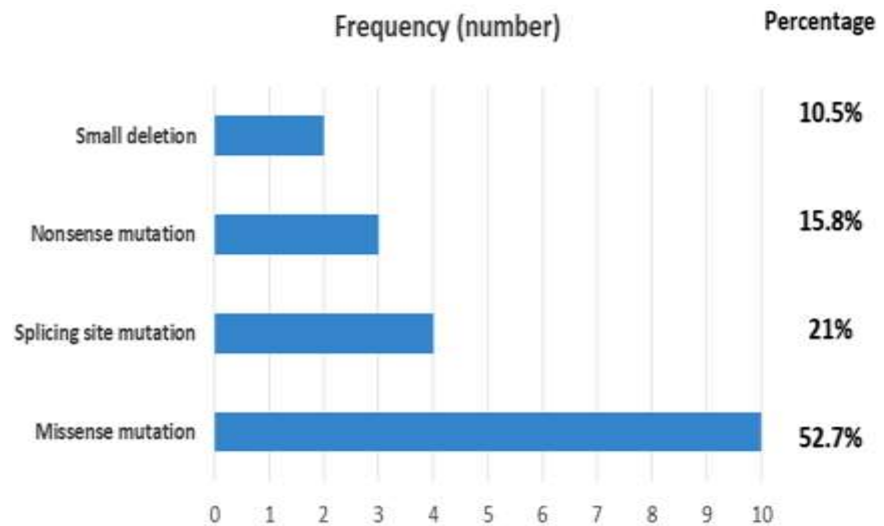

Figure 1. The frequency of different types of mutations (number and percentage) in XLA patients

Correlation of mutational type and clinical/immunological features

A summary of detected mutations and their predicted severity is given in **Table 2**. There was no

significant difference between the groups with mild and severe mutations in terms of demographic data such as age at onset, diagnosis, and delayed diagnosis or immunological data including immunoglobulin levels, B and T cell percentages.

The protein expression level was evaluated in all 19 patients. The results indicated that 6 out of 19 patients had normal levels of *BTK* expression, while no expression was found in 13 patients (data not shown). Statistical analysis showed that there was a significant correlation between the severity of the mutations and *BTK* expression (-p-value <0.05). It was found that all severe predicted mutations had an absence or reduced level of protein production. Six (31.5%) patients (P1, P6, P7, P15, P16, and P19) harboring a mild mutation in the *BTK* gene displayed normal expression of *BTK* as compared with healthy controls. However, the normal expression level of *BTK* was not observed in 4 out of 10 patients with mild mutations.

The data showed that the influenced domain does not have a significant association with mutation severity, nor with the *BTK* expression level. In addition, statistical analysis indicated that there was not a particular domain in which mutations occurred with higher statistical significance. No significant correlation was observed between the patients' clinical outcome and types of *BTK* gene variations in the 19 studied cases (**Table 3**).

Table 2. Summary of detected mutations and their predicted severity in individuals diagnosed with XLA

Patient	Family history	Domain	Mutation	Protein variation	Variation type	Predicted severity
1	-	SH2	c.906_908 delAGG	P.G303del	Small in-frame deletion	Mild
2	-	PH	c.178_180 delAAG	p.K60del	Small in-frame deletion	Mild
3	-	SH1/TK	ivs15-13 delTTG	exon16 skiping	Frameshift nonsense	Severe
4	-	PH	c.110 T<C	p.L37P	Non-frameshift missense	Severe
5	-	SH1/TK	c.1978 C<T	p.L616F	Non-frameshift missense	Mild
6	+	PH	c.214 C<T	p.r28C	Non-frameshift missense	Mild
7	+	PH	c.214 C<T	p.r28C	Non-frameshift missense	Mild
8	+	PH	c.31+5G<C	Splicing defect	Splice-site	Severe
9	-	SH1/TK	ivs14-1G>A	Splicing defect	Splice-site	Severe
10	+	SH1/TK	c.1922G<A	p.R641H	Non-frameshift missense	Mild
11	-	PH	ivs1+5G<C	Splicing defect	Splice-site	Severe
12	+	PH	ivs1+5 G<C	Splicing defect	Splice-site	Severe
13	+	SH1/TK	c.1922G<A	p.R641H	Non-frameshift missense	Mild
14	-	PH	c.349 delA	p.N72fs.120X	Frameshift nonsense	Severe
15	+	SH1/TK	c.1651T<C	p.Y551H	Non-frameshift missense	Mild
16	-	PH	c.214 C<T	p.r28C	Non-frameshift missense	Mild
17	-	SH1/TK	c.1896 G<A	p.W588X	Non-frameshift nonsense	Severe
18	-	PH1	ivs3+2 T>C	Splicing defect	Splice-site	Severe
19	+	SH1/TK	c.1651T<C	p.Y551H	Non-frameshift missense	Mild

Table 3. Correlation of clinical features with mutation severity in 19 XLA patients

Clinical manifestation	Total (n=19)	Severe	Mild	*P* value
Rheumatic manifestation (Number)	8	4	4	1
Otitis (Number)	11	6	5	1
Dermatologic manifestation (Number)	6	3	3	1
Neurologic manifestation (Number)	6	4	2	0.6
Pneumonia (Number)	12	6	6	1
Sinusitis (Number)	13	6	7	1
Diarrhea (Number)	8	6	2	0.15

Discussion

The current study evaluated the correlation of *BTK* gene alterations with the expression level of *BTK* and the clinical/immunological findings of 19 XLA patients. The data showed that all patients with severe mutations represented a severe reduction in *BTK* protein expression, while some patients with mild mutations were associated with normal protein expression. However, heterogeneity was observed in the serum level of immunoglobulins, CD-markers, clinical manifestations, and age at onset in the patients, demonstrating that none of them reflected the consequence of the type of mutation. It was also shown that mutations in all affected domains could cause the disease without any preferences.

In general, genotype-phenotype correlation studies seem to be controversial as the clinical manifestations in XLA disease are mostly variable. As previously mentioned, *BTK* belongs to the Tec family of non-receptor protein tyrosine kinases. The Tec family includes Tec, Itk/Emt/Tsk, Bmx, and Txk/Rlk. We have previously demonstrated that severe mutations do not always lead to severe phenotypes. We have shown that Tec can influence the clinical phenotype of XLA patients by substituting for *BTK*. In fact, the presence of single nucleotide polymorphisms in Tec was in part attributed to a tendency toward mild XLA phenotype despite the severe mutations (20). Consistent with our previous data, we demonstrated that the severe mutations were accompanied by mild clinical phenotypes in some cases. This may necessitate further consideration of gene modifiers affecting XLA severity (16). In line with the our previous report, the present study also indicated that severe genotypes such as splicing and frameshift mutations attributed to splice site defects and premature stop codons, respectively (20). This notion further implicates a direct relevance between the mutational defect and the resultant protein.

To confirm XLA disease, direct and indirect investigation of *BTK* mutations is essential (8, 21-23); however, *BTK* gene mutations are not always reflective of the level of expression or functional activity of the protein. Three patients in the present study represented the same non-frame missense mutation (R28C substitution) and almost normal *BTK* expression. This data was in agreement with the study by Kanegane *et al.* which showed that the mentioned alteration may result in normal *BTK* expression (24). In a previous report, we could identify one patient with an almost 40% reduction

in protein expression of this mutant by flowcytometry and western immunoblotting methods. This discrepancy is probably due to the unknown individual and environmental factors which could affect protein expression (20). However, the precise underlying reason still remains elusive. Interestingly, there were two cases with the same Y551H mutation. Lyn mediated trans-phosphorylation of *BTK* at tyrosine 551 induces auto-phosphorylation of the protein at Y-223 with the ultimate increment in *BTK* enzymatic activity (25). We have previously demonstrated a patient with Y551H mutation and increased *BTK* protein expression, while the protein expression was almost normal in the two patients in the present study; this result reinforces the individual variations in the protein's integrity.

An interesting point in the present study is related to patients who had the same mutations but different clinical manifestations. P10 and P13 were relatives with the same mutation (c.1922G>A) in their SH1/TK domain of *BTK*. However, clinical presentations were more severe in P10 than in his nephew. P10 represented Hodgkin's lymphoma along with other XLA manifestations, including pneumonia, sinusitis, and bronchiectasis as well as rheumatic, dermatologic, and neurologic manifestations. Similarly, while P15 was suffering from Kawasaki disease, his brother (P19) did not manifest the same complication. These divergences may suggest other ambient factors and genetic modifiers that alter the course of the disease.

The current study has also identified a previously described mutation in a patient with a mild phenotype of an in-frame deletion of arginine (P.G303del) affecting two consecutive glycines [20]. An interesting observation regarding this mutation is the deletion of the first glycine and the presence of the second one with a modified codon at the context of the protein.

No significant correlation was found between age at onset of symptoms, the percentage of peripheral B-cells or plasma IgM levels, and *BTK* gene variations, which could be probably explained by the small sample size.

Conclusion

Finding a genotype-phenotype correlation among XLA patients may not be well established at the level of clinical outcome of the disease. It seems that environmental and individual factors as well as other unidentified modifiers may profoundly affect disease severity, especially in cases with identical gene mutations and variable clinical representations.

Conflict of interest

The authors declare no conflicts of interest.

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