

Differential Expression and Phosphorylation of BTK Protein Domain in X-linked Agammaglobulinemia

Mahsa Sohani^{1‡}, Samaneh Delavari^{1‡}, Amir Hassan Zarnani², Leila Parvaneh¹, Shima Rasouli³, Sepideh shahkarami^{1,4}, Sepideh Babaie², Fatemeh Kiaee^{1,3*}

¹ Research Center for Immunodeficiencies, Pediatrics Center of Excellence, Children's Medical Center, Tehran University of Medical Science, Tehran, Iran

² Department of Immunology, School of Public Health Tehran University of Medical Sciences Tehran, Iran

³ Department of Medical Immunology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

⁴ Medical Genetics Network (MeGeNe), Universal Scientific Education and Research Network (USERN), Tehran, Iran

[‡] These authors are first authors in this work.

Abstract

Background/objectives: X-linked (Bruton's) agammaglobulinemia (XLA) is a rare congenital disorder with defects in early B cell development caused by mutations in the gene encoding BTK (Bruton tyrosine kinase). The aim of this study was to investigate the expression and phosphorylation of BTK protein domain in these patients.

Methods: A total of 19 patients with mutations in BTK gene were analyzed for the expression and phosphorylation of BTK protein through immunoblotting. The correlations between BTK expression and the results of immunoblotting as well as clinical and immunologic phenotypes were evaluated.

Results: Six patients showed normal expression of protein and phosphorylation of BTK and two patients had normal phosphorylation while no expression was observed. There was a significant difference between the groups of patients with normal expression of protein and those without it ($p=0.01$).

Conclusions: Since we identified 6 patients with normal expression and phosphorylation of BTK, and two patients with normal phosphorylation but no expression, thus more studies should be done in order to explore other aspects of the disease. Although there was not any significant correlation between the severity of clinical manifestations and BTK expression, further investigations are necessary to determine the compensatory mechanisms in XLA patients.

Keywords X-linked agammaglobulinemia, Bruton's tyrosine kinase, protein expression and phosphorylation.

*Corresponding Author: Fatemeh Kiaee

1. Research Center for Immunodeficiencies, Pediatrics Center of Excellence, Children's Medical Center, Tehran University of Medical Science, Tehran, Iran

‡ These authors are first authors in this work.

E-mail: fa_kiaei@yahoo.com

Introduction

The most common group of primary immunodeficiency diseases (PIDs) are primary antibody deficiencies (PAD) in which affected patients have an early B cell defects causing agammaglobulinemia (1-3). As a prototype of agammaglobulinemias, XLA is a humoral immunodeficiency disorder manifesting with severe bacterial infections as a result of a significant reduction or absence of B cells and almost lack of all serum immunoglobulin isotypes (4-6). Newborns with XLA are normal in early postnatal months of their life due to the maternal antibodies transferred through the placenta. However, by the end of the half-life of the above-mentioned maternal antibodies, recurrent infections occur especially those of the respiratory and gastrointestinal tracts (7).

Impairments in the expression and function of Bruton's tyrosine kinase (BTK), a cytoplasmic kinase for B-cell receptor (BCR) downstream signaling crucial for B cells development, proliferation, and survival, cause XLA phenotype. (8, 9). X-linked pattern of inheritance makes the diagnosis of XLA likely where the early diagnosis of XLA is confirmed with the presence of mutations in *BTK* gene, resulting in defects in the expression and function of BTK protein (10). This gene contains 19 exons and encodes a multidomain protein composed of five different domains (11, 12). Over 1100 mutations in the genes encoding BTK have been identified in affected patients (13, 14).

The scientific methods applied to the clinical diagnosis are investigations of genomic mutations for *BTK* along with the assessment of the expression and function of BTK protein (15). Indeed, genetics and immunological approaches are robust in the definitive diagnosis of XLA (16). However, in most of the previous reports, the expression and function of the protein had not been analyzed (17). Applying methods such as exon sequencing can identify present mutations, yet detecting these mutations will not confirm the absence of *BTK* gene's expression; thus, studying BTK protein's expression in addition to its function is essential in providing insights into the diagnosis of XLA.

The present study aimed to provide an informative outlook of clinical and immunological manifestations of XLA along with the evaluation of the expression and phosphorylation of BTK protein in patients with XLA. For studying the expression and function of proteins like BTK, flow cytometry and western blotting are amongst the valid methods for investigation (8, 18, 19). Here, western blotting was preferred for analysis of BTK protein since it has been considered to be gold standard in the detection and characterization of protein mixtures (20).

Materials and methods

Study population and ethical considerations

Twenty male subjects with an established diagnosis of primary agammaglobulinemia were enrolled in the study. Inclusion criteria were designed according to the European Society of Immune Deficiencies (ESID) including very low circulating B cells (<2%) with a normal number of T cells, low level of serum IgG (200 mg/dl and 500 mg/dl in <12 and >12 months of age, respectively) with documented recurrent infections before 5 years of age (21). The procedure of this study was approved by the ethics committee of Tehran University of Medical Science (TUMS) and written informed consents were also obtained from both the adult patients and the children's parents.

Collection of data

The data, obtained from our charts or a survey from reviewing patients' hospital records, included the following: personal information, family history, findings at diagnosis, clinical evolutions, and treatment.

Collection and preparation of samples

Peripheral blood mononuclear cells (PBMCs) were prepared by collecting the lower band of Polymorphprep (Nycomed, Oslo, Norway) separation from the citrated whole blood. A total of 10^7 PMN cells were washed twice in phosphate buffer saline (PBS) and lysed in 1 mL of the cell lysis buffer (150 mM NaCl, 5 mM EDTA and 0.05% NP-40) for 5 min on ice. The obtained lysates were clarified via centrifugation in 18,000g

for 10 min to be used for Western blot analysis.

Western Blot analysis of BTK expression and phosphorylation

The supernatant of prepared lysate was incubated with 7 pL of anti-BTK antiserum (prepared by the same method as described by Tsukada *et al* (22)) on ice for 60 minutes which was followed by overnight incubation with conjugations of protein A sepharose beads at 4°C. The beads were then washed 4 times (2X with 1 mL of the cell lysis buffer). Finally, the samples were loaded onto a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Immobilon P: Millipore Corp, Bedford, MA). The membranes were processed as follows: blocking with 5% bovine serum albumin (BSA) in TBST (20 mmol/L Tris HCl [pH, 7.51], 150 mmol/L NaCl, and 0.05% Tween 20) for 1 hour, incubation with the hybridoma supernatant of 48-28 (diluted 1:s) for 1 hour, washing 3 times with TBST for 10 min each time. BTK was detected using the mAb 8F10 (provided by Dr D. L. Nelson, National Cancer Institute, National Institutes of Health), peroxidase goat anti-mouse as a secondary antibody (Sigma), and the ECL system (Amersham Biosciences, Buckinghamshire, United Kingdom). BTK autokinase capacity was also analyzed through immunoblotting with an anti-phospho-Tyr223-BTK antibody (Cell Signaling Technology, Beverly, Mass). Both protein extracts obtained from total PBMCs were used as controls; membranes were reblotted with anti-beta-actin (Sigma) to demonstrate equal loading.

Statistical analysis

Statistical analyses were performed using the SPSS software package, version 22 (SPSS Inc., Chicago, IL, USA). Kolmogorov-Smirnov test was used to check the normality assumption for variables. Values were expressed as frequency (number and percentage), mean \pm standard deviation and median (interquartile range, IQR) where necessary. Fisher's exact test and chi-square tests were used for 2 \times 2 comparison of categorical variables, while t-tests, one-way ANOVA and their nonparametric equiva-

lent were used to compare numerical variables.

Results

In total, 20 subjects from 13 unrelated families with a median age of 22.0 (16-29) years were enrolled in the present study. One patient was excluded since the lysed sample of their blood was not obtained. Demographic and immunological characteristics of the patients are summarized in **Table 1**. The median age of diagnostic delay was 5 years and the patients were followed up for a median period of 17 years. Also, 11 (57%) of the patients had a family history of XLA. Almost all of the subjects experienced recurrent infections during the follow-up time where otitis media, pneumonia, and recurrent diarrhea were the most common complications. As shown in **Figure 1**, 6 patients showed normal expression of protein and phosphorylation of BTK including P1, P6, P7, P15, P16, and P19 patients. Two patients (P5 and P12) had normal phosphorylation while no expression was observed and there was a significant difference between the two groups of patients with normal expression of protein and those without it ($p=0.01$). Patients who had no BTK phosphorylation have not been shown.

None of the immunological findings showed any significant differences with BTK expression in XLA cases as compared to those without expression of BTK (**Table 1**). Age at onset of symptoms was not influenced by the type of *BTK* gene variation ($p=0.4$) and there was no correlation between the immunoglobulin levels of IgG, IgA, as well as IgM serum and *BTK* expression ($p=0.7$, $p=0.1$ and $p=0.3$, respectively), while the occurrence of autoimmune disorders (1/13[7%] vs.3/6[50%], $P=0.07$) was higher in the patients with BTK expression than in those without. Further, the patients without BTK expression showed a tendency towards higher frequency of multiple organ infections (12/13[92%] vs. 3/6[50%], $P=0.07$). However, the variation was not statistically significant.

Molecular genetic analysis was also performed for the patients the results of which are presented in **Table**

3. The most common mutation was a non-frameshift missense in the catalytic kinase (SH1) and pleckstrin homology (PH) domains in both groups. Nevertheless, no significant difference was found between the involved domains and the expression of BTK protein and phospho-BTK protein.

Table 1. Demographic and immunological data of patients with XLA

Parameters	Total (n=19)	BTK expression		p.value*
		NO (n=13)	Yes (n=6)	
Current age (Months), Mean (range)	273.5 (60.0-528.0)	302.8 (60.0-528.0)	210.0 (84.0-348.0)	0.17
Age of onset, Months (IQR)	12.0 (7.0-60.0)	12.0 (9.0-72.0)	7.0 (6.0-40.0)	0.42
Age at diagnosis (Months), Mean (range)	79.73 (10.0-264.0)	89.38 (10.0-264.0)	58.83 (12.0-168.0)	0.33
Diagnosis delay (Months), Mean (range)	41.00 (24.0-79.0)	56.15 (0.0-180.0)	39.83 (0.0-108.0)	0.49
Course of disease, Months (IQR)	244.7 (49.0-479.0)	269.5 (49.0-479.0)	191.0 (69.0-341.0)	0.23
Consanguinity, Number (%)	6 (31%)	3 (23.1%)	3 (50.0%)	0.32
Positive family history, Number (%)	11 (57%)	7 (53.8%)	4 (66.7%)	0.65
IgG, mg/dl (IQR)	120.0 (39.0-221.0)	120.0 (48.5-230.5)	99.0 (19.5-179.5)	0.765
IgA, mg/dl (IQR)	3.0 (0.0-9.25)	5.0 (0.0-8.0)	0.0 (0.0-29.5)	1.00
IgM, mg/dl (IQR)	18.0 (4.0-29.3)	17.0 (5.5-23.50)	30.0 (2.5-78.0)	0.39
CD3+%, Mean (range)	92.0 (81.8-94.0)	92.0 (78.0-94.0)	92.0 (91.0-94.0)	0.63
CD4+%, Mean (range)	44.3 (18.0-73.0)	41.2 (18.0-61.0)	52.4 (42.0-73.0)	0.13
CD8+%, Mean (range)	37.2 (1.0-61.0)	39.2 (1.0-61.0)	32.2 (8.0-47.0)	0.40
CD19+%, (IQR)	0.0 (0.0-2.0)	0.0 (0.0-0.0)	2.0 (0.0-2.0)	0.27
WBC *10 ⁹ /L, Mean (range)	10116 (3870-18000)	9583 (3870-16220)	11181 (6930-18000)	0.44
Lymphocytes (%), Mean (range)	41.1 (14.0-79.0)	42.0 (14.0-79.0)	39.3 (15.0-66.0)	0.79
Neutrophil (%), Mean (range)	48.6 (10.0-85.0)	48.1 (10.0-79.0)	49.5 (22.0-85.0)	0.90
Hb, g/dl (IQR)	12.0 (10.0-13.0)	13.0 (10.9-13.0)	11.0 (10.0-12.5)	0.41
BTK domain				
SH2 domain	1	0	1	0.50
PH domain	10	7	3	
SH1/TK domain	8	6	2	
BTK phosphorylation	8/19 (42%)	2/13 (15%)	6/6 (100%)	0.01*
Type of mutations				
Deletion	2	1	1	0.08
Nonsense	3	3	0	
Missense	9	4	5	
Splicing-site	5	5	0	

IQR: 25th to 75th inter quintile range.

*p-value is statistically significant <0.05

WBC: White Blood Cell; HB: Hemoglobin; CD: Cluster of Differentiation

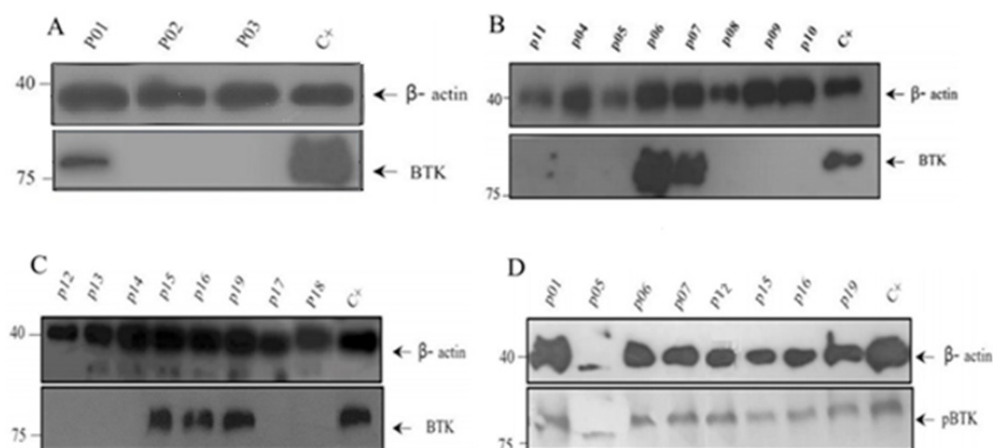


Figure 1. BTK expression (A), (B) and (C); BTK phosphorylation (D)

Table 2. Clinical features of the 19 XLA patients

Manifestations	Total (n=19)	BTK expression		p. value*
		NO (n=13)	Yes (n=6)	
Respiratory tract infection, Number (%)	15/19 (78%)	10/13 (76%)	5/6 (83%)	1.00
Pneumonia, Number (%)	11/19 (57%)	9/13 (69%)	2/6 (33%)	0.32
Otitis media, Number (%)	12/19 (63%)	8/13 (61%)	4/6 (66%)	1.00
Bronchiectasis, Number (%)	7/19 (36%)	5/13 (38%)	2/6 (33%)	1.00
Clubbing of nails, Number (%)	4/19 (21%)	2/13 (15%)	2/6 (33%)	0.55
Sinusitis, Number (%)	13/19 (68%)	9/13 (69%)	4/6 (66%)	1.00
Enteropathy, Number (%)	5/19 (26%)	4/13 (30%)	1/6 (16%)	0.63
Lymph proliferation, Number (%)	2/19 (10.5%)	1/13 (7%)	1/6 (16%)	1.0
Recurrent diarrhea, Number (%)	7/19 (36%)	6/13 (46%)	1/6 (0.16%)	0.33
Autoimmune, Number (%)	4/19 (21%)	1/13 (7%)	3/6 (50%)	0.07
Gastrointestinal, Number (%)	7/19 (36%)	6/13 (46%)	1/6 (16%)	0.33
Rheumatologic, Number (%)	9/19 (47%)	7/13 (53%)	2/6 (33%)	0.62
Neurologic, Number (%)	6/19 (31%)	6/13 (46%)	0/6 (0.0%)	0.10
Dermatologic, Number (%)	6/19 (31%)	4/13 (30%)	2/6 (33%)	1.00
Multiple organ infections, Number (%)	15/19 (78%)	12/13 (92%)	3/6 (50%)	0.07

*p-value is statistically significant <0.05

Table 3. Genetic and analysis in subjects with XLA

Patient	Domain	Mutation	Variation type	BTK exp (WB)	PBTK exp	BTK exp (flow)
1	SH2	c.906_908 del AGG	Small in-frame deletion	N	N	UN
2	PH	c.178_180 del AAG	Small in-frame deletion	NU	NU	ND
3	SH1/TK	ivs15-13 del TTG	Frameshift nonsense	NU	NU	UN
4	PH	c.110 T>C	Non-frameshift missense	NU	NU	ND
5	SH1/TK	c.1978 C>T	Non-frameshift missense	NU	N	N
6	PH	c.214 C>T	Non-frameshift missense	N	N	ND
7	PH	c.214 C>T	Non-frameshift missense	N	N	ND
8	PH	c.31+5G>C	Splice-site	NU	NU	ND
9	SH1/TK	ivs14-1G>A*	Splice-site	NU	NU	UN
10	SH1/TK	c.1922G>A	Non-frameshift missense	NU	NU	ND
11	PH	ivs1+5G>C*	Splice-site	NU	NU	UN
12	PH	ivs1+5 G>C*	Splice-site	NU	N	UN
13	SH1/TK	c.1922G>A	Non-frameshift missense	NU	NU	ND
14	PH	c.349 del A	Frameshift nonsense	NU	NU	N
15	SH1/TK	c.1651T>C	Non-frameshift missense	N	N	UN
16	PH	c.214 C>T	Non-frameshift missense	N	N	UN
17	SH1/TK	c.1896 G>A	Non-frameshift nonsense	NU	NU	N
18	PH	ivs3+2 T>C	Splice-site	NU	NU	N
19	SH1/TK	c.1651T>C	Non-frameshift missense	N	N	UN

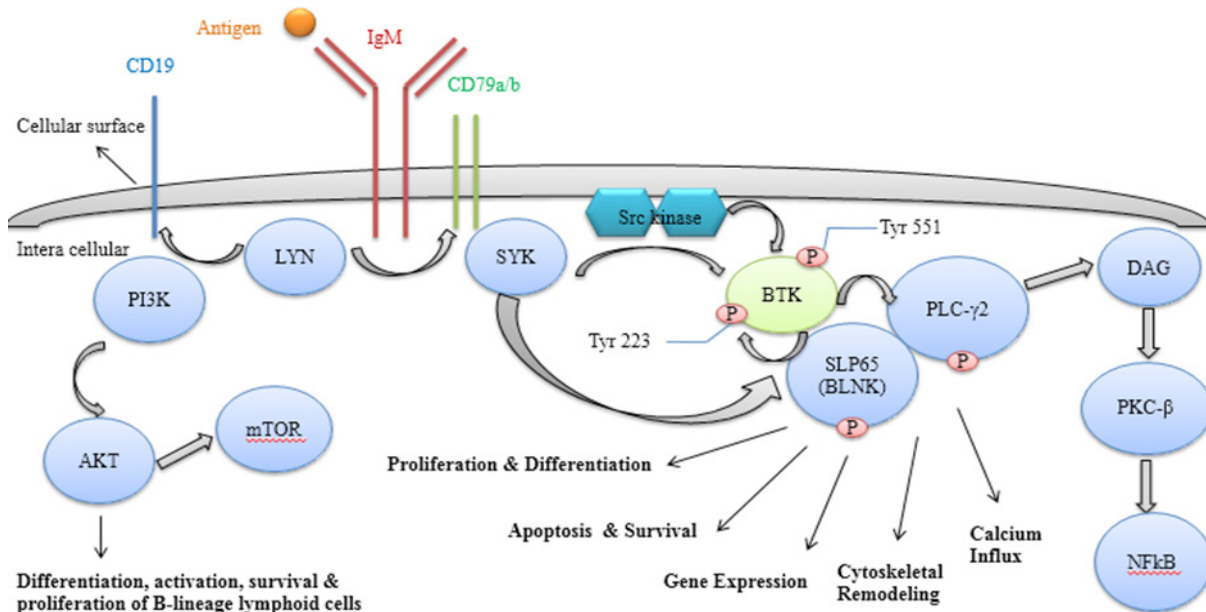


Figure 2. BTK is involved in multiple signal-transduction pathways including survival regulation, activation, proliferation, and differentiation of B-lineage lymphoid cells. Initiation of BCR signaling involves LYN and SYK, part of the Src family. LYN phosphorylates the intracellular domain of the BCR, leading to the recruitment and phosphorylation of SYK. Activated SYK then phosphorylates the adaptor protein SLP65 (BLNK), which results in the recruitment of BTK and its phosphorylation by SYK at Tyr551. BTK, in turn, becomes autophosphorylated at Tyr223 for its full activation, and then phosphorylates PLC-γ2. LYN also activates the BCR co-receptor CD19, leading to the activation of the PI3K/AKT pathway, which is responsible for B-cell differentiation, survival, and proliferation

Discussion

Bruton's disease, as the X-linked form of agammaglobulinemia, originates from the primary defect in the development of B lymphocytes causing hypo-gammaglobulinemia, a sharp decline in the number of peripheral B lymphocytes, reduced size of lymph nodes and tonsils along with recurrent bacterial infections (23).

Mutations in *BTK* gene and consequently lack of the corresponding BTK protein or impairment in its function as the underlying causes, lead to the failure in the maturation of pre-B cells causing XLA (8, 24). Evaluating the expression and function of BTK protein was the subject of current study using western blot analysis along with the data from the hospital records of subjects including immunological, genetics, and clinical information in patients with the confirmed clinical diagnosis of XLA. Mutational analysis results of the current study (Table 1) indicated that almost all of

the nonsense and splice-site mutations which have been predicted to be severe were associated with null expression and function of BTK protein ($p=0.08$). This is exactly consistent with the necessity of BTK's presence for activation of its downstream mechanisms in various pathways such as IKK and NFκB pathways as important signals for survival regulation, activation, growth, and proliferation of B cells (Figure 2) (25-28). Additionally, in the study conducted by Kanegane *et al.*, all of the patients with normal expression and function of BTK protein were reported to be diagnosed with mild predicted mutations (29). The observed BTK protein expression in 6 patients revealed that some mutations in domains had no effects on protein stability in these patients, while they may have led to the instability of the protein in those patients without BTK expression. Weakly positive results for the functionality of BTK in these patients may suggest a

variation in their domains that did not affect the kinase activity or the stability of altered proteins. Further, this may be indicative of a compensatory role of the Tec kinase for the detected protein (17), rather than its essential role as a regulatory factor in apoptosis and JNK/SAPK kinase activity (30). These are also in agreement with the results of Holinski-Feder *et al.* from immunoblotting analyses of cases with confirmed mutations in BTK (31). They reported lack of BTK expression in subjects with splice-site and nonsense mutations, while some of those with missense mutations showed BTK expression and in some cases positive BTK kinase activity, using western blot analyses.

Investigations for genotype-phenotype correlation (**Table 2**) revealed almost a positive correlation between null BTK expression and autoimmunity manifestations along with multiple organ infections (both $p=0.07$) in corresponding patients. These results are in concordance with the fact that less severe mutations with less detrimental effects on the expression and function of the BTK protein result in a higher percentage of B-cells and higher levels of immunoglobulins (32, 33) thus less infection and a lower probability to be affected by an autoimmune disorder (34, 35).

In a study by Abolhassani *et al.*, the level of protein expression was evaluated in 27 patients with XLA including 12 patients of our study using flow-cytometry (14). As reported in **Table 3**, our results are similar to their findings except for 2 patients (P1 and P5). This discrepancy can be explained due to use of different antibodies which recognize distinct epitopes of BTK. Indeed, the monoclonal anti-BTK antibody used in the current study have been declared to be produced by immunizing rabbits with a synthetic peptide corresponding to residues surrounding Asp195 of human Btk protein while the anti-BTK antibody used in the above-mentioned study may be bounded to other residues. Further, the reported normal kinetic function of the protein can be attributed to the presence of mono-

cytes in the prepared lysate in our study. Teimourian *et al.* investigated the expression of BTK using flow cytometry in 22 subjects including P5 of the present study and reported normal BTK expression in contrast to null BTK expression in our study. This can be explained regarding possible false positive results of flow cytometry (36). Also, normal phosphorylation with no BTK expression in P5 and P12 may be due to Phospho-Btk (Tyr223) mAb which recognizes Btk protein only when it is phosphorylated at Tyr223 within the SH3 domain, while Btk mAb recognizes total Btk protein.

However, the data of our study are almost in concordance with the results of another study by Lopez-Herrera *et al.* They found BTK expression in 21.4% of subjects with XLA. Specifically, they investigated the mutations in BTK gene and expression of BTK protein in 14 patients with clinical and immunological criteria of ESID for XLA using both western blotting and flow cytometry (37). Western blot analysis demonstrated very low expression of BTK only in three subjects with XLA, while flow-cytometry of monocytes showed a low mean fluorescent intensity (MFI) for most of the subjects with XLA. In this regard, Kanegane *et al.* investigated hypogammaglobulinemic males through flow-cytometric analysis of BTK protein expression in monocytes and genetic analyses of related BTK gene (29).

Their observed less BTK expression in 88% of subjects with confirmed mutations for BTK as compared to normal healthy subjects. In another study, Tani *et al.* employed flow-cytometric analyses of BTK expression on 16 Brazilian males with confirmed mutations of BTK causing XLA; 13 subjects (81.2%) showed a complete form of BTK deficiency (38). It can be concluded that these findings suggest more consistency for results of genetic studies with the findings of Western blot analysis of proteins and this technique is superior to flow cytometry for tracking BTK protein.

On the other hand, P10 and P13, P15, and P19, as three distinct groups of patients from the same fami-

lies showed different clinical manifestations despite the same mutations. P10 and P15 had Hodgkin's lymphoma and Kawasaki disease, respectively, while another member of their family, P13 and P19, did not manifest the same complication suggesting that patients from the same family with the same mutations are not similar concerning the disease severity, and other different mechanisms are involved in the course of the disease which are yet to be identified.

Conclusion

Although some patients have normal BTK protein expression without BTK protein phosphorylation, there are some patients who have both normal BTK protein expression and phosphorylation. So, it is necessary to determine which protein domain is involved in these patients. Further, western blotting using different anti-BTK antibodies would be more beneficial for the detection of different defective binding sites, though confirmation of relative gene defects using mutational analyses is required.

Conflict of interest The authors declare no conflict of interest.

Acknowledgments

We are grateful to the entire staff in the Immunology Department

References

- Gatti RA, Berkel I, Boder E, Braedt G, Charmley P, Concannon P, et al. Localization of an ataxia-telangiectasia gene to chromosome 11q22-23. *Nature*. 1988;336(6199):577-80.
- Sanal O, Ersoy F, Tezcan I, Metin A, Turul T, Gariboglu S, et al. Antibody response to a seven-valent pneumococcal conjugated vaccine in patients with ataxia-telangiectasia. *J Clin Immunol*. 2004;24(4):411-7.
- Ersoy F, Berkel AI, Sanal O, Oktay H. Twenty-year follow-up of 160 patients with ataxia-telangiectasia. *Turk J Pediatr*. 1991;33(4):205-15.
- Lefton-Greif MA, Crawford TO, Winkelstein JA, Loughlin GM, Koerner CB, Zahurak M, et al. Oropharyngeal dysphagia and aspiration in patients with ataxia-telangiectasia. *J Pediatr*. 2000;136(2):225-31.
- Chopra C, Davies G, Taylor M, Anderson M, Bainbridge S, Tighe P, et al. Immune deficiency in Ataxia-Telangiectasia: a longitudinal study of 44 patients. *Clin Exp Immunol*. 2014;176(2):275-82.
- Davies EG. Update on the management of the immunodeficiency in ataxia-telangiectasia. *Expert Rev Clin Immunol*. 2009;5(5):565-75.
- Schubert R, Reichenbach J, Zielen S. Deficiencies in CD4+ and CD8+ T cell subsets in ataxia telangiectasia. *Clin Exp Immunol*. 2002;129(1):125-32.
- Khanna KK, Yan J, Watters D, Hobson K, Beamish H, Spring K, et al. Defective signaling through the B cell antigen receptor in Epstein-Barr virus-transformed ataxia-telangiectasia cells. *J Biol Chem*. 1997;272(14):9489-95.
- Driessen GJ, Ijspeert H, Weemaes CM, Haraldsson A, Trip M, Warris A, et al. Antibody deficiency in patients with ataxia telangiectasia is caused by disturbed B- and T-cell homeostasis and reduced immune repertoire diversity. *J Allergy Clin Immunol*. 2013;131(5):1367-75.
- Sanal O, Ersoy F, Yel L, Tezcan I, Metin A, Ozyurek H, et al. Impaired IgG antibody production to pneumococcal polysaccharides in patients with ataxia-telangiectasia. *J Clin Immunol*. 1999;19(5):326-34.
- Stray-Pedersen A, Aaberge IS, Fruh A, Abrahamsen TG. Pneumococcal conjugate vaccine followed by pneumococcal polysaccharide vaccine; immunogenicity in patients with ataxia-telangiectasia. *Clin Exp Immunol*. 2005;140(3):507-16.
- Conley ME, Notarangelo LD, Etzioni A. Diagnostic criteria for primary immunodeficiencies. Representing PAGID (Pan-American Group for Immunodeficiency) and ESID (Eu-

- ropean Society for Immunodeficiencies). *Clin Immunol.* 1999;93(3):190-7.
13. Al-Herz W, Bousfiha A, Casanova JL, Chapel H, Conley ME, Cunningham-Rundles C, et al. Primary immunodeficiency diseases: an update on the classification from the international union of immunological societies expert committee for primary immunodeficiency. *Front Immunol.* 2011;2:54.
 14. Lumsden JM, McCarty T, Petiniot LK, Shen R, Barlow C, Wynn TA, et al. Immunoglobulin class switch recombination is impaired in *Atm*-deficient mice. *J Exp Med.* 2004;200(9):1111-21.
 15. Reina-San-Martin B, Chen HT, Nussenzweig A, Nussenzweig MC. ATM is required for efficient recombination between immunoglobulin switch regions. *J Exp Med.* 2004;200(9):1103-10.
 16. Callen E, Jankovic M, Difilippantonio S, Daniel JA, Chen HT, Celeste A, et al. ATM prevents the persistence and propagation of chromosome breaks in lymphocytes. *Cell.* 2007;130(1):63-75.
 17. Nowak-Wegrzyn A, Crawford TO, Winkelstein JA, Carson KA, Lederman HM. Immunodeficiency and infections in ataxia-telangiectasia. *J Pediatr.* 2004;144(4):505-11.
 18. Mohammadinejad P, Abolhassani H, Aghamohammadi A, Pourhamdi S, Ghosh S, Sadeghi B, et al. Class switch recombination process in ataxia telangiectasia patients with elevated serum levels of IgM. *J Immunoassay Immunochem.* 2015;36(1):16-26.
 19. Zaki-Dizaji M, Akrami SM, Abolhassani H, Rezaei N, Aghamohammadi A. Ataxia telangiectasia syndrome: moonlighting ATM. *Expert Rev Clin Immunol.* 2017;13(12):1155-72.
 20. Ghiasy S, Parvaneh L, Azizi G, Sadri G, Zaki Dizaji M, Abolhassani H, et al. The clinical significance of complete class switching defect in Ataxia telangiectasia patients. *Expert Rev Clin Immunol.* 2017;13(5):499-505.
 21. Roifman CM, Gelfand EW. Heterogeneity of the immunological deficiency in ataxia-telangiectasia: absence of a clinical-pathological correlation. *Kroc Found Ser.* 1985;19:273-85.
 22. Weemaes CM, The TH, van Munster PJ, Bakkeren JA. Antibody responses in vivo in chromosome instability syndromes with immunodeficiency. *Clin Exp Immunol.* 1984;57(3):529-34.
 23. Eisen AH, Karpati G, Laszlo T, Andermann F, Robb JP, Bacal HL. IMMUNOLOGIC DEFICIENCY IN ATAXIA TELANGIECTASIA. *N Engl J Med.* 1965;272:18-22.
 24. Porro M, Saletti M, Nencioni L, Tagliaferri L, Marsili I. Immunogenic correlation between cross-reacting material (CRM197) produced by a mutant of *Corynebacterium diphtheriae* and diphtheria toxoid. *J Infect Dis.* 1980;142(5):716-24.
 25. Gupta RK, Collier RJ, Rappuoli R, Siber GR. Differences in the immunogenicity of native and formalinized cross reacting material (CRM197) of diphtheria toxin in mice and guinea pigs and their implications on the development and control of diphtheria vaccine based on CRMs. *Vaccine.* 1997;15(12-13):1341-3.
 26. Stray-Pedersen A, Jonsson T, Heiberg A, Lindman CR, Widing E, Aaberge IS, et al. The impact of an early truncating founder ATM mutation on immunoglobulins, specific antibodies and lymphocyte populations in ataxia-telangiectasia patients and their parents. *Clin Exp Immunol.* 2004;137(1):179-86.
 27. Shelly MA, Pichichero ME, Treanor JJ. Low baseline antibody level to diphtheria is associated with poor response to conjugated pneumococcal vaccine in adults. *Scand J Infect Dis.* 2001;33(7):542-4.