Deficient Expression of Bruton's Tyrosine Kinase in Monocytes from X-Linked Agammaglobulinemia as Evaluated by a Flow Cytometric Analysis and its Clinical Application to Carrier Detection

Asghar Aghamohammadi[1*], Ali Akbar Amirzargar[2], Nima Parvaneh[1], Paul Marjousef[2], Mostafa Moin[1], Abolhassan Farhoudi[1], Mehdi Yeganeh[1], Toshio Miyawaki[3]

[1]Department of Clinical Pediatric Immunology, Children's Medical Center Hospital, Tehran University of Medical Sciences, Tehran, Iran, [2]Department of Immunogenetics, Tehran University of Medical Sciences, Tehran, Iran, [3]Department of Pediatrics, Faculty of Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan

ABSTRACT

Background: The B-cell defect in X-linked agammaglobulinemia (XLA) is caused by mutations in the gene for Bruton's tyrosine kinase (BTK). BTK mutations result in deficient expression of BTK protein in peripheral blood monocytes. **Methods:** Using the anti-BTK monoclonal antibody (48-2H), a flow cytometric analysis of intra cytoplasmic BTK protein expression in monocytes was performed to identify Iranian patients with XLA phenotype. To examine the possible identification of XLA patients and female carriers by this assay, we studied 13 XLA families. **Results:** The flow cytometric assay showed deficient expression of the BTK protein in 12 (92%) families. One patient exhibited a normal level of BTK expression. The cellular mosaicism of BTK expression in monocytes from obligate carriers was clearly shown in 9 of 12 (75%) families. **Conclusion:** The results suggested that most XLA patients have deficient expression of the BTK protein; therefore we conclude that deficient expression of BTK protein can be evaluated by a flow cytometric assay.

Keywords: Bruton's Tyrosine Kinase(BTK), X-linked Agammaglobulinemia (XLA), BTK Expression

*Corresponding author: Dr. Asghar Aghamohammadi, MD, Department of Clinical Pediatric Immunology, Children's Hospital Medical Center, Tehran University of Medical Sciences, Tehran, Iran, Tel: (+) 98 21 66428998, Fax: (+) 98 21 66923054, e-mail: aghamohammadi@sina.tums.ac.ir

INTRODUCTION

X-linked agammaglobulinemia (XLA) is the prototypical humoral immunodeficiency first described by Bruton in 1952(1). It is characterized by a paucity of circulating B cells and a marked reduction in serum levels of all immunoglobulin isotypes, which causes susceptibility to recurrent and severe bacterial infections in affected males (2-4). The defect in XLA is considered to be due to inefficient expansion of pre-B cells into later B-cell stages or incomplete differentiation of B-cell precursors to pre-B cells (4,5). In 1993, the gene responsible for XLA was identified to reside on Xq21 as a cytoplasmic tyrosine kinase, named Bruton's tyrosine kinase (BTK)(6,7). BTK belongs to a group of related cytoplasmic tyrosine kinases, known as the Tec family, and consists of five distinct structural domains, which encompass the N-terminus, Pleckstrin homology (PH) domain, Tec homology (TH) domain, Src homology 3 (SH3) domain, SH2 domain, and the catalytic kinase (SH1) domain (8-13). The clinical spectrum of XLA varies, not only from family to family (4), but also within families with more than one affected member (14). Because of this variability in the clinical phenotype, the diagnosis of XLA may be difficult in some cases (4). In addition, more than 50% of patients with mutations in BTK have no family history (15). The most decisive approach for confirming the diagnosis of XLA is mutation analysis of BTK. However, sequence analysis of the BTK gene requires a specialized laboratory, is time consuming and may be difficult. The majority of BTK mutations result in deficient BTK expression, reflecting a reduction in BTK mRNA or instability of the produced protein (7, 16-20).On the basis of the observation that BTK is expressed in monocytes as well as B cells, a flow cytometric assay with the anti-BTK mAb for detection of XLA has been developed (20). In this method BTK expression is evaluated in peripheral blood monocytes of suspected cases. This assay is also useful in the detection of female XLA carriers, because the random X-chromosome inactivation occurs in their monocytes, leading to the bimodal or mosaic BTK expression pattern (21). Here we have examined hypogammaglobulinemic Iranian males with a lack of circulating mature B cells. The goal of our efforts is to identiy BTK mutations in XLA candidates screened through use of flow cytometric assessment. We will also discuss the clinical usefulness and limitations of flow cytometric assessment and the necessity of final genetic analysis of BTK in definitive diagnosis of XLA and its carriers.

SUBJECTS AND METHODS

Subjects. Male patients with markedly reduced circulating B-cell numbers (<1%) were subjected to investigation regardless of age, serum Ig level, infection, and family history. The percentages of circulating B cells were evaluated by immunofluorescence analysis through use of anti-CD19 mAbs. In all, 16 male patients (from 13 unrelated families) were recruited during the period 2003-2005. After informed consent was received, 5 to 10 mL of venous blood was collected into heparin-containing syringes and delivered to our laboratory within 24 hours. When available, blood of the patient's mother was also obtained.

Flow Cytometric Analysis of BTK Expression in Monocytes. Intracellular BTK staining of monocytes was performed as described previously (20). Briefly, PBMCs

were separated by Ficoll-Hypaque gradient centrifugation. PBMCs were first stained with phycoerythrin-labeled anti-CD14 (IgG2a; Dako Japan, Kyoto, Japan) mAb for 20 minutes. The cells were fixed in 4% paraformaldehyde in phosphate-buffered saline solution for 15 minutes and then permeabilized in 0.1% Triton X-100 for 5 minutes. Subsequently, the cells were reacted with 2 µg/mL anti-BTK (48-2H) or control IgG1 (Dako Japan) mAb for 20 minutes and further incubated with a 1:2000 dilution of FITC-conjugated goat antimouse IgG1 antibody (Dako Japan) for 20 minutes. The stained cells were analyzed by means of a flow cytometer (Partec PAS, Munster, Germany).

RESULTS

The clinical and laboratory features of 16 patients with XLA (from 13 families) are summarized in Table1. Age at onset of symptoms ranged from 5 to 85 (mean 22) months with a mean serum IgG level of 141 mg/dl. Of these, seven patients from 4 families had a family history consistent with an immunodeficiency disorder. About 70 percent of the XLA families (9 of 13 families) had no family history. Most of these patients were suspected by their physicians of having XLA because of markedly reduced numbers of circulating mature B cells. P11 was healthy brother of P10 who was by chance discovered to have XLA during this study. It was noted that 5 (31%) out of 16 patients with XLA were clinically diagnosed before they were 5 years old. Two patients were recognized before age 10. We examined for monocyte BTK expression in patients through use of a flow cytometric assay. Among 13 families who had patients with XLA phenotype, we found that patients in 12 families (92%) exhibited less BTK expression than normal individuals (Table 1). P14 showed a normal pattern of BTK expression. Although the degree of BTK deficiency varied from person to person, patients showing deficient BTK expression were assessed as presumably having XLA. The members of a same family with presumed XLA were deficient in BTK expression to a similar extent. We found no correlation between extent of BTK expression, serum IgG levels, and age at onset of disease.The mosaic pattern of BTK expression in monocytes, indicating obligate XLA carrier mothers, was obtained in 9 out of 12 (75%) studied families. Figure1 shows representative profiles in a patient with XLA (P13) and in his mother as an obligate carrier in comparison to a normal person. Although the patient exhibits diminished BTK expression, the bimodal or mosaic pattern of BTK expression is demonstrable in the heterozygous mother, reflecting the random X-chromosome inactivation in monocytes.

DISCUSSION

In this study, we used flow cytometric analysis to evaluate monocytes from XLA patients for the presence of BTK and to test their mothers for possible carrier status. We studied 13 Iranian XLA families and found that 92% of our studied families had patients with deficient BTK expression. Previous studies have shown deficient BTK expression in 78 to 98% of studied families (20-22). Seventy percent of our cases had no family history, thus an X-linked pattern of inheritance is a specific but not a sensitive

method for definitive diagnosis of XLA in a patient with diminished B cells. In addition 75% of studied mothers in our study had a mosaic pattern of BTK expression

Table1. Clinical data and BTK expression in 16 patients with XLA phenotype from 13 families

No	Age at onset (mo)	Age at Dx	IgG(mg/dl)	IgA(mg/dl)	IgM(mg/dl)	CD19+ B cells	BTK expression	Mother	Family history
P1	72	7 y	152	12	13	<1%	↓	Carrier	-
P2	6	11 y	40	0	0	<1%	↓	Carrier	+ (P2 is P8's uncle)
P3	7	3 y	14	0	0	<1%	↓	Carrier	+
P4	12	6 y	10	0	0	<1%	↓	Carrier	+ (P4 and P5 are brothers)
P5	12	7 y	120	0	0	<1%	NA	Carrier	+
P6	84	14 y	50	0	0	<1%	↓	NA	-
P7	36	5 y	100	10	20	<1%	↓	Carrier	-
P8	8	10 mo	50	0	0	1%	↓	Carrier	+ (P8 is P2's nephew)
P9	9	4 y	150	0	0	<1%	↓	Carrier	+
P10	24	3 y	680	0	63	<1%	↓	Carrier	+ (P10 and P11 are brothers)
P11	-	6mo	110	10	40	<1%	NA	Carrier	+
P12	5	1.5y	100	20	10	<1%	↓	NA	+
P13	24	3.5	100	10	56	<1%	↓	Carrier	-
P14	16	3	380	5	48	<1%	NL	non-carrier	-
P15	9	13mo	24	3	10	<1%	↓	non-carrier	-
P16	6	8 mo	170	390	52	<1%	↓	non-carrier	-

which is consistent with other studies (20-22). The results of our study are depicted in table 2 in comparison to previous studies. A pattern of X-linked inheritance, severely decreased numbers of peripheral B lymphocytes, and decreased immunoglobulin production makes a clinical diagnosis of XLA possible (23). Because approximately one third to one half of XLA cases are sporadic, and there are also several autosomal gene mutations (BLNK, μ heavy chain, λ5, Igα) in which their clinical and immunologic features are indistinguishable from XLA, alternative mechanisms of diagnosis are necessary. Definitive diagnosis of XLA is basically accomplished by means of mutation studies of the BTK gene (24). Mutation analysis for XLA normally involves screening the exons of the BTK gene. Single-stranded conformational polymorphism (SSCP) analysis is the most commonly used technique before direct sequencing of the affected exon (23). SSCP analysis can be a time consuming

Table2: Summary of studies performed on BTK expression in XLA patients

Reference	Method	Target cells	No of Families	Families with decreased BTK expression (%)	Families with carrier Mothers(%)
Futatani et al, *2001*	FACS	Platelet	45	78 %	59%
Futatani et al, *1998*	FACS	Monocyte	41	98%	85%
Kanegane et al, *2001*	FACS	Monocyte	80	98%	81%
Gaspar et al, *1998*	Western blot	Monocyte	13	92%	-----
Current study	FACS	Monocyte	14	92%	75%

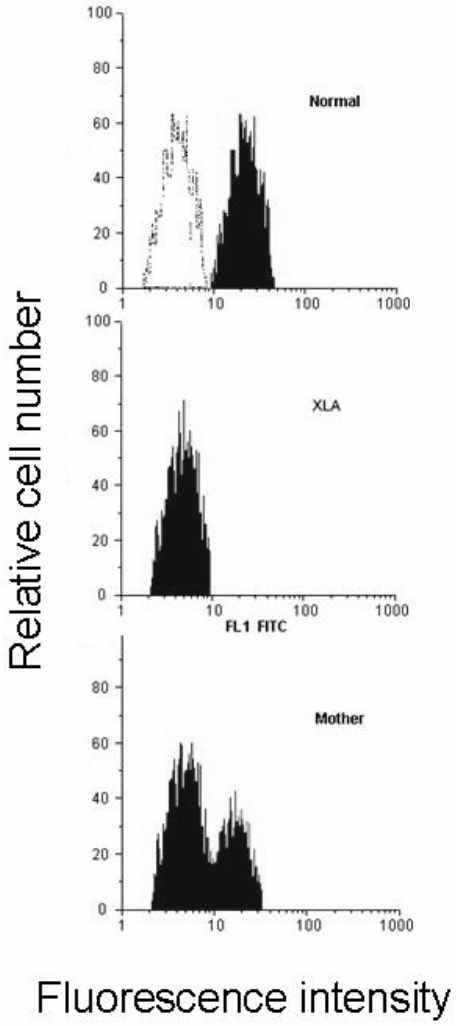

Figure1. Flow cytometric analysis of cellular BTK expression in a normal person, a representative patient (P13) and his mother. BTK expression in monocytes was evaluated by gating on $CD14^+$ population. The dashed line indicates the control antibody. Three thousand cells were evaluated in each gated population.

and expensive procedure, and a definitive diagnosis may take weeks to achieve. Furthermore, some mutations may not be detected by SSCP because the sensitivity of the technique is only 85% to 90 % (23). In some circumstances, genetic analysis fails to identify BTK mutations in the coding region even if they fulfill the criteria for XLA (16, 25). Alternative methods have been used to establish a molecular diagnosis

of XLA in suspected cases (19, 26). A novel strategy to establish a diagnosis has been introduced by Futatani et al (20), who demonstrated that peripheral blood monocytes of XLA patients lack BTK protein and those carrier females have monocytes that show random X-chromosome inactivation with mosaic pattern of BTK expression. This method has been successfully used to diagnose XLA patients who have erroneously been registered as common variable immunodeficiency (CVID) from Japanese immunodeficiency registry (25). Consistent with several documented studies, we did not observe correlation between the level of BTK expression, serum levels of IgG and age at onset of clinical symptoms (20-22, 27). One of our patients (P14) showed normal BTK expression. Some missense and splice site mutations may produce normal levels of nonfunctional BTK protein (21, 28). As his mother also was not a carrier, he most likely is affected by an autosomal form of agammaglobulinemia with a phenotype like XLA. Using a flowcytometric assay, we can detect XLA patients and carriers, especially in cases which are sporadic or have atypical presentation. When patients with XLA have BTK deficiency, this assay will demonstrate the bimodal and mosaic pattern of BTK expression in monocytes from carriers. Although seventy percent of patients with identified XLA here had no family history, approximately 75% of patients' mothers were determined to be obligate carriers by flow cytometric assessment. Three mothers did not show a mosaic pattern of BTK expression. The normal single pattern of monocyte BTK expression in these mothers has two possible explanations: De novo mutation in the maternal germ line, or extreme skewed X chromosome inactivation resulting predominantly in the use of the normal X chromosome. Such skewing of X-chromosome inactivation in normal females has been well recognized (29, 30). In conclusion, this report has described our trial to identify XLA patients in Iran by flow cytometric assessment of BTK expression. The clinical usefulness of the flow cytometric assay for detection of XLA and its carrier was proved in patients with XLA. Its few limitations were also demonstrated, especially in detection of BTK mutations causing normal BTK expression and in carrier detection. Because of the rapidity and simplicity of flow cytometric assessment, it is currently advised that a hypogammaglobulinemic male with a lack of circulating B cells should be initially examined through use of a flow cytometric assay (19, 28). Because a normal pattern of BTK expression does not rule out XLA and rare problems encountered in carrier detection, mutation analysis is finally necessary for definitive diagnosis of XLA or subsequent genetic counseling.

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