

Clinical and mutational characteristics of X-linked agammaglobulinemia and its carrier identified by flow cytometric assessment combined with genetic analysis

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Background: X-linked agammaglobulinemia (XLA), caused by mutations in Bruton's tyrosine kinase (*BTK*), is the most common form of inherited antibody deficiency. We previously reported that a flow cytometric evaluation of *BTK* expression in monocytes could easily detect XLA as well as its carrier.

Objective: Our purpose was to perform further flow cytometric analysis in additional XLA families in Japan.

Methods: In all, 106 hypogammaglobulinemic males (from 91 families) of various ages with a lack of mature B cells (<1%) were investigated.

Results: Flow cytometric assessment revealed the deficient *BTK* expression status in 78 families (93 patients), and mutations in *BTK* were identified in 76 of 78 families with presumed XLA. Of the patients with normal *BTK* expression, 2 showed missense mutations in which the normal amount of altered *BTK* transcript would cause the XLA phenotype. As many as 30% of these patients with XLA were clinically or genetically recognized beyond 5 years of age. Higher concentrations (>300 mg/dL) of serum IgG were evident in the cases diagnosed among adults, seemingly preventing severe infections. Fifty-seven of 70 mothers of patients with *BTK* deficiency were diagnosed as obligate carriers on the basis of a bimodal *BTK* expression pattern. Nine of the remaining 13 mothers showing nonmosaic *BTK* expression had no mutations in 2 alleles; surprisingly, the other 4 mothers had the mutated alleles.

Conclusions: A diagnostic approach based on flow cytometric assessment for XLA should be initially considered in genetic investigation of antibody deficiencies, regardless of the patient's age. (*J Allergy Clin Immunol* 2001;108:1012-20.)

Key words: X-linked agammaglobulinemia, Bruton's tyrosine kinase, hypogammaglobulinemia, B cells, flow cytometry, genetic analysis, obligate carrier

Antibody deficiency syndromes, clinically recognizable as agammaglobulinemia or hypogammaglobulinemia, are the most frequently reported primary immunodeficiency diseases in the world.¹ X-linked agammaglobulinemia (XLA) is the most common form of inherited antibody deficiency disorder.² XLA is characterized by markedly reduced levels of all major classes of immunoglobulins in the serum and few or no mature B cells in the blood.^{3,4} Typically, affected males begin to exhibit susceptibility to recurrent and severe bacterial infections in the first few years of life.

In 1993, two groups identified the gene responsible for XLA, which has been named *Bruton's tyrosine kinase* (*BTK*).^{5,6} The *BTK* protein consists of 5 functional domains: the pleckstrin homology (PH) domain, the Tec homology (TH) domain, the Src homology 3 (SH3) domain, the SH2 domain, and the kinase (SH1) domain.^{7,8} Mutations in all domains of the *BTK* gene have been shown to cause XLA.^{9,10}

Although antibody deficiencies are easy to diagnose and treat, defining the genetic etiology of antibody deficiencies is important for subsequent genetic counseling, carrier detection, and prenatal diagnosis. Diagnosis of XLA is readily achieved as a result of the presence of maternal male relatives showing similar antibody deficiencies. Otherwise, physicians might consider other genetic immunodeficiencies in hypogammaglobulinemic patients without family histories. We have reported that a number of patients with XLA are misregistered as having common variable immunodeficiency in the Japanese Immunodeficiency Registry.¹¹ More than 50% of patients with mutations in *BTK* have been shown to have no family history.¹² In addition, there exist cases of detected atypical XLA showing milder clinical symptoms, higher serum Ig concentrations, or later onset than expected.¹³⁻¹⁶

The majority of *BTK* mutations result in deficient *BTK* expression, reflecting a reduction in *BTK* mRNA or an instability of the produced protein.^{5,17-21} On the basis of the observation that *BTK* is expressed in monocytes as

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Abbreviations used

BTK: Bruton's tyrosine kinase
XLA: X-linked agammaglobulinemia

well as B cells, we previously developed a flow cytometric assay with the anti-BTK mAb for detection of XLA by demonstration of deficient BTK expression in monocytes.²¹ 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. It has recently been reported that BTK is expressed in platelets, and flow cytometric analysis of BTK in platelets can make detection of XLA and its carrier possible.²² Since our description of a simple method for XLA detection, we have examined hypogammaglobulinemic Japanese males with a lack of circulating mature B cells. The goal of our efforts has been identification of *BTK* mutations in XLA candidates screened through use of flow cytometric assessment. For the past 3 years, we have newly identified 95 patients with XLA (from 80 families) among 106 hypogammaglobulinemic males (from 91 families) with a lack of circulating B cells. We here 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 carrier.

METHODS

Patients

The patients were referred to our laboratory by physicians all over Japan. 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 a combined use of anti-CD19 and anti-CD20 mAbs. In all, 106 male patients (from 91 unrelated families) ranging in age from 2 months to 41 years were recruited during the period 1998-2000. Of these, 7 patients (P15, P21, P29, P46, P50, P54, and P79) were reported to be patients with XLA misregistered as common variable immunodeficiency in the Japanese Immunodeficiency Registry.¹¹ Two patients (P2 and P65) were described as having atypical XLA.¹⁶ Ten patients (P5, P23-1, P45, P52-1, P52-2, P57-1, P57-2, P73-1, P75, and P78) were enrolled in a study on bone marrow B-lineage cells.²³ Two patients (P48-1 and P48-2) were reported to be siblings with XLA as a result of maternal germinal mosaicism.²⁴

After informed consent was received, 5 to 10 mL of venous blood was collected into heparin-containing syringes and delivered to our laboratory within 48 hours. When available, the 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.²¹ 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 anti-mouse IgG1 antibody (Southern Biotechnology Associates, Inc, Birmingham, Ala) for 20 minutes. The stained cells were analyzed with by means of a flow cytometer (EPICS XL-MCL; Beckman Coulter KK, Tokyo, Japan).

BTK mutation detection

The *BTK* mutation detection was performed as described previously.^{11,17} Total RNA was extracted from PBMCs through use of Trizol Reagent (Life Technologies, Grand Island, NY) and subjected to RT-PCR. The *BTK* gene was amplified from cDNA through use of 7 overlapping PCR primers. Through use of a QIAamp Blood Kit (QIAGEN GmbH, Hilden, Germany), genomic DNA was also extracted from PBMCs. Sets of the appropriate primers containing the flanking intron sequences were used for the DNA-based PCR. The PCR products were purified by means of Microcon 100 microcentrators (Amicon, Inc, Beverly, Mass). The sequencing reaction was carried out through use of a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif) with an automated ABI PRISM 310 DNA sequencer (Applied Biosystems). Detected mutations were confirmed by sequencing from the opposite direction and sequencing genomic DNA encompassing exon-intron boundaries.

RESULTS

Flow cytometric analysis of BTK expression in monocytes

We previously demonstrated by flow cytometric analysis that defective BTK expression in monocytes was seen in most patients with XLA.²¹ Fig 1 shows representative profiles in a patient with XLA and in his mother as an obligate carrier. Although the patient exhibits negligible BTK expression, the bimodal or mosaic pattern of BTK expression is demonstrable in the heterozygous mother, reflecting the random X-chromosome inactivation in monocytes.

We first examined for monocyte BTK expression in patients through use of a flow cytometric assay. Among 106 males (from 91 families) of various ages with a lack of circulating mature B cells, we found that 93 patients (88%) in 78 families (86%) exhibited less BTK expression than normal individuals (>95%; Table I). Although the degree of BTK deficiency varied from person to person, patients showing deficient BTK expression were assessed as presumably having XLA.²¹ The same family members with presumed XLA were to the same (or a similar) extent deficient in BTK expression. The mosaic pattern of BTK expression in monocytes, indicating obligate XLA carriers, was obtained in most mothers.

Genetic analysis of the *BTK* gene

We next examined whether the BTK deficiency revealed by flow cytometry assessment might be due to mutations in the *BTK* gene. By means of genetic analysis, we identified various mutations in *BTK* in 76 (91 patients) of 78 families (93 patients; Table I). The mutations were as follows: 28 missense and 11 nonsense mutations, 29 deletions, 6 insertions, and 2 multiple

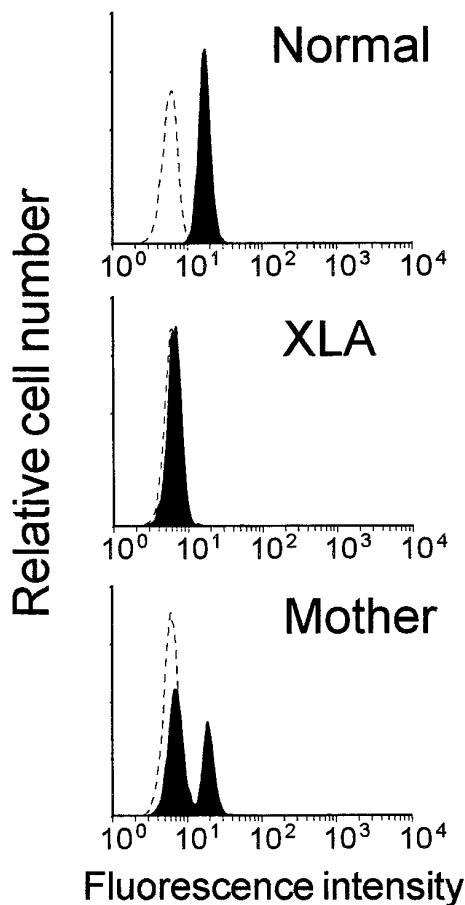


FIG 1. Flow cytometric analysis of monocyte BTK expression in a normal donor and in a patient with XLA (P54) and his mother. The closed areas and dashed lines indicate the staining with anti-BTK (48-2H) and control IgG1 mAbs in monocytes, respectively.

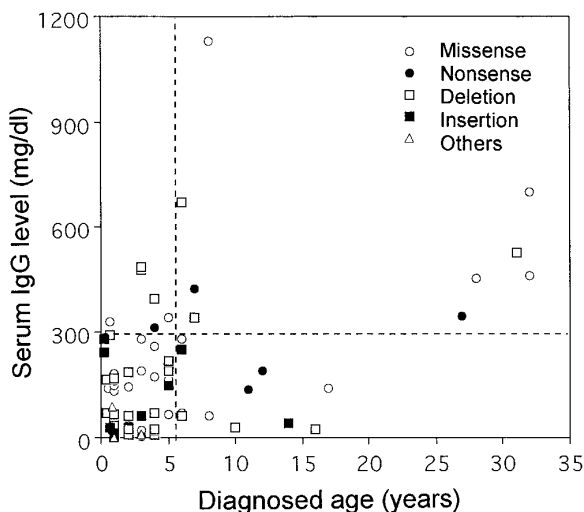


FIG 2. The serum levels of IgG at diagnosed age in patients with XLA. The illustration is based on clinical data of patients with XLA for whom laboratory records were available.

alterations. In 2 remaining families (P79 and P80), no mutations in the coding or promoter regions were detected. The mosaic BTK expression in monocytes was demonstrated in the mother of P79, indicating that the patient had XLA. P74 seemed to be in the same situation.

We have observed that some missense mutations in *BTK* lead to normal levels of BTK expression in monocytes.²¹ Thirteen patients showing normal BTK expression were analyzed for the *BTK* gene. We found that 2 patients (P1 and P12) had missense mutations in *BTK* (R28C and R307S). The former R28C substitution in the PH domain was the same one seen in murine X-linked immunodeficiency.²⁴ The latter R307S substitution was located in the functionally specialized amino acid sequence in the BTK-SH2 domain, which binds to a B-cell specific adapter protein, BLNK.²⁵ Thus, both missense mutations appeared to cause the B-cell defect in affected males.

Relation between BTK expressions and mutations in *BTK*

We wished to learn how mutations in *BTK* might lead to deficient production of the BTK protein. It was likely that missense mutations in *BTK* might produce the measurable amounts of BTK protein. As Table I shows, the relatively intense BTK expression (tentatively, >10%) was seen in a proportion of the patients with missense mutations (P1, P8, P11-13, P18, P20, P25-P27, and P30), which were distributed broadly from the PH to the SH1 domains. The anti-BTK mAb used here was generated against the SH3 domain.¹⁷ It was predicted that the negligible BTK expression pattern would be obtained in patients with nonsense mutations (P31-P35), deletions (P42-P54), or insertions (P71-P74), causing the premature termination at the 3' terminal regions from the SH3 domain. Certainly, all patients with these mutations exhibited negligible BTK expression. On the other hand, flow cytometric assessment might detect the truncated protein likely produced in mutations not affecting the SH3 domain. Relatively intense expression of BTK was demonstrated in the patients with the following mutations: nonsense mutations (P36 and P38), deletions (P56, P59, P61-P65, P67, and P70), and insertion (P75).

Clinical and laboratory features of patients with XLA

The clinical and laboratory features of our 95 patients with XLA (from 80 families) are summarized in Table I. More than one half of the XLA families (69%; 55 of 80 families) had no family histories. Most of these patients were suspected by their physicians of having XLA because of markedly reduced numbers of circulating mature B cells. Some patients (P22-2, P22-3, P23-2, P34-2, P67-2, and P73-2) were probands' male relatives who were by chance discovered to have XLA during this study. P34-2 and P73-2 were healthy infants less than 6 months of age. Though the probands were younger, 3 patients (P22-2, P22-3, and P23-2) had never experienced recurrent or severe bacterial infections.

It was noted that 29 (31%) of 95 patients with XLA were clinically or genetically diagnosed beyond the age of 5 years. Thirteen patients were recognized beyond the age of 10 years. Of importance, 6 patients (P2, P14, P23-2, P40, P65, and P67-2) had already entered into adult life. Fig 2 depicts the relationship between serum IgG level and age at diagnosis. It was noted that a substantial number of patients (20%; 16 of 80 cases) had serum IgG levels higher than 300 mg/dL before intravenous replacement of immunoglobulin. This tendency was evident in the patients diagnosed beyond 5 years of age, especially the adults. Surprisingly, serum IgG levels were as high as 1000 mg/dL in P18, who had been misdiagnosed with selective IgA deficiency. Although serum IgA and IgM were undetectable in most patients, some patients had serum IgA and IgM levels higher than 20 mg/dL.

Carrier detection in XLA families

The blood samples obtained from 70 mothers of patients with XLA were checked for monocyte BTK expression (Table I). In 57 mothers (81%), the bimodal or mosaic pattern of BTK expression was obtainable, indicating that they were obligate XLA carriers. In the other 13 mothers, a normal single pattern of BTK expression was demonstrated. It was most likely that the latter cases might be derived from the de novo mutation in oocytes or, rarely, from gonadal mosaicism. Another possibility was that the extremely skewed inactivation of the mutated X-chromosome might occur in monocytes of heterozygous mothers. We examined the *BTK* gene in 13 mothers showing the normal BTK expression pattern. Reasonably, a genetic analysis revealed that 9 (P11, P17, P21, P26, P41, P48, P75, and P78) of these 13 mothers had no mutations in both alleles of the *BTK* gene. Interestingly, one of them had borne 2 children (P48-1 and 48-2) with the same *BTK* mutation. We have described this family as the maternal germinal mosaicism of XLA elsewhere.²⁴ Unexpectedly, the remaining 4 mothers (P4, P24, P46, and P74) had the mutated and normal alleles. Nonmosaic BTK expression in these mothers possibly resulted from the extremely skewed inactivation of the mutated X-chromosome, as seen in a small number of normal women.

DISCUSSION

Definitive diagnosis of XLA is basically accomplished by means of mutation studies of the *BTK* gene.²⁷ However, genetic analysis fails to identify *BTK* mutations even if they fulfill the criteria for XLA.^{11,17} In a previous study, we developed a rapid and simple method of detecting XLA and its carrier by flow cytometric assessment of BTK expression in monocytes.²¹ This assay was designed to demonstrate deficient or mosaic BTK expression in monocytes for patients with XLA or female carriers, respectively. The validity of the assay was assessed in 41 unrelated families with XLA, in most of whom mutations in *BTK* had been identified. We thereafter attempted to identify additional Japanese patients with XLA by a combination of flow cytometric assess-

ment and genetic analysis. To elicit the wide clinical spectrum of *BTK* mutations, we examined all hypogammaglobulinemic patients regardless of family history, age, serum Ig level, infection, or B-cell number. Through such an investigation, we learned that the candidates for XLA were found among males with markedly decreased numbers of circulating mature B cells (<1%).

In this study, we identified 95 patients with XLA (from 80 unrelated families) among 106 hypogammaglobulinemic males (from 91 unrelated families) with a lack of mature B cells who were recruited during the period 1998-2000. Premature stop codons, frameshift mutations, and splice defects in the *BTK* gene result in the reduction of *BTK* mRNA.^{5,17-19} In addition, a high proportion of missense mutations in *BTK* renders the produced protein unstable.^{20,21} This observation verifies the clinical usefulness of flow cytometric evaluation of monocyte BTK expression for the detection of XLA. Using flow cytometric assessment, we demonstrated that 78 (98%) of 80 families with XLA were significantly deficient in production of the BTK protein. As expected, mutation studies demonstrated that 76 of 78 families showing BTK deficiency had various mutations in *BTK*. Two patients with XLA (P78 and P79) with BTK deficiency did not have any mutations in the coding or promoter regions of the *BTK* gene. Of note, 2 patients with XLA (P1 and P12), both of whom expressed the BTK protein to the normal extent, had missense mutations (R28C and R307S, respectively).

It has been recognized that the genotype-phenotype correlations are not seen in patients with XLA.²⁹ It was found that patients with XLA sometimes have higher levels of serum immunoglobulin than expected or have relatively mild infections.^{11,15,16,28} One family with XLA, in which affected members with the same mutation in *BTK* had different clinical phenotypes, has been reported.²⁸ We encountered such XLA families (P22, P23, and P67) as well. Although typical patients with XLA exhibit the clinical symptoms within the first few years, when the maternal-derived IgG disappears in the body,²⁹ approximately 30% of patients with XLA identified here were diagnosed beyond 5 years of age. Relatively high concentrations (>300 mg/dL) of serum IgG were found in patients diagnosed beyond 5 years of age. Our search detected 6 patients with atypical XLA (P2, P14, P23-2, P40, P65, and P67-2); they exhibited mild or even clinical symptoms until adult life. Of note, levels of serum IgG in 5 adult cases were as high as 300 mg/dL, seemingly preventing these patients from severe infections. A leaky generation of mature B cells has been found in patients with XLA.^{30,31} It is plausible that immunoglobulins produced by a small number of leaked mature B cells, possibly distributed in local lymphoid tissues, such as bone marrow or lymph nodes, might delay the onset of recurrent or severe infections.

Flow cytometric assessment appears to provide a powerful tool for detection of XLA carriers, especially in atypical or sporadic cases. When patients with XLA have BTK deficiency, this assay will demonstrate the bimodal and mosaic pattern of BTK expression in monocytes from carriers. Although more than one half of patients with XLA identi-

TABLE I. Clinical data, BTK protein expression, and *BTK* mutations in 95 patients with XLA from 80 families

Patient no.	Present age	Age at diagnosis	Serum Ig level (mg/dL)*			Family history†	
			IgG	IgA	IgM		
Missense mutation							
P1	4 y	4 y	174	<29	<18	(-)	
P2§	41 y	32 y	462	<8	<7	(-)	
P3-1	21 y	17 y	142	1	7	P3-1 and -2 are brothers	
P3-2	9 y	4 y	258	2	20		
P4	20 y	3 y	NA	NA	NA		(-)
P5¶	11 y	5 y	167	63	95		(-)
P6	6 y	6 y	279	2	5	(-)	
P7	26 y	5 y	65	<8	<6	(-)	
P8	6 y	1 y	134	<8	<8	(-)	
P9	10 y	4 y	7	3	2	(-)	
P10	33 y	6 y	70	12	16	(-)	
P11	30 y	15 y	NA	NA	NA	(-)	
P12	36 y	6 y	NA	8	7 (34 y)	(-)	
P13	3 y	1 y	148	<5	13	(-)	
P14	34 y	32 y	702	185	<25	(-)	
P15	15 y	3 y	282	7	7	(+)	
P16-1	14 y	2 y	34	0	13	P16-1 and -2 are cousins	
P16-2	1 y	6 mo	141	<2	9		
P17	10 y	3 y	191	<10	52	(-)	
P18	12 y	8 y	1130	24	145	(-)	
P19	2 y	1 y	<180	<8	22	(-)	
P20	12 y	4 y	<20	<8	<7	Brother is XLA	
P21	20 y	5 y	341	<7	<6	(-)	
P22-1	2 y	2 y	143	18	25	P22-1, -2, and -3 are brothers	
P22-2	6 y	6 y	NA	NA	NA		
P22-3	8 y	8 y	NA	NA	NA	P23-1 and -2 are brothers	
P23-1¶	29 y	16 y	NA	<1	<1 (29 y)		
P23-2	28 y	28 y	454	95	38	(P23-1 and -2 are brothers)	
P24	17 y	5 y	341	14	82		(-)
P25	19 y	8 y	60	<5	18		(-)
P26	3 y	1 y	160	5	45		(-)
P27	2 y	2 y	54	2	30		(-)
P28	7 y	3 y	22	<8	16		(-)
P29	10 y	8 mo	330	82	40		(-)
P30	9 mo	9 mo	21	1	27		(-)
Nonsense mutation							
P31	1 y	1 y	23	<2	13		(-)
P32	6 y	4 y	315	9	27		(-)
P33	3 y	3 y	3	0	4		(+)
P34-1	1 y	1 y	44	1	14	P34-1 and -2 are brothers	
P34-2	4 mo	4 mo	NA	NA	NA		
P35	21 y	8 y	NA	1	1 (21 y)	(-)	
P36	34 y	12 y	190	<5	9	(-)	
P37	8 y	7 y	423	63	36	(-)	
P38	27 y	5 y	NA	5	15 (27 y)	(-)	
P39	25 y	11 y	137	1	2	(+)	
P40	27 y	27 y	346	16	8	(-)	
P41	2 y	1 y	NA	NA	NA	(-)	
Deletion							
P42	14 y	10 y	28	8	11	(-)	
P43	5 y	5 y	220	<10	28	(-)	
P44	4 y	4 y	9	1	21	(-)	
P45¶	3 y	1 y	<6	<7	5	(+)	
P46	16 y	4 y	24	70	38	(-)	

BTK expression		BTK mutation			
Patient	Mother	Nucleotide change	Consequence	Domain	BTK base
95.0%±	NT	214C >T	R28C	PH	R
1.2%	Mosaic	227T >C	L32S	PH	R
0.6%	Mosaic	227T >C	L32S	PH	R
1.2%					
0.9%	Normal	248A >G	Y39C	PH	N
2.2%	Mosaic	322G >T	V64F	PH	R
3.5%	Mosaic	424T >G	F98V	PH	N
4.8%	Mosaic	464T >G	L111R	PH	N
14.9%	Mosaic	481A >C	T117P	PH	R
7.8%	Mosaic	994C >T	R288W	SH2	R
9.9%	Mosaic	994C >T	R288W	SH2	R
42.2%	Normal	1051A >G	R307G	SH2	R
98.5%	NT	1053A >G	R307S	SH2	N
57.3%	Mosaic	1659G >T	M509I	SH1	R
7.7%	NT	1706G >A	R525Q	SH1	R
9.2%	Mosaic	1742T >A	V537E	SH1	R
6.2%	Mosaic	1816C >T	R562W	SH1	R
7.7%	Mosaic				
2.7%	Normal	1816C >T	R562W	SH1	R
59.1%	Mosaic	1833A >C	E567D	SH1	N
4.1%	Mosaic	1877C >T	A582V	SH1	R
26.6%	NT	1885G >T	V585F	SH1	N
0.9%	Normal	1907C >A	S592Y	SH1	R
7.2%	Mosaic	1912G >C	G594R	SH1	R
5.1%					
8.9%					
9.3%	Mosaic	1924T >G	Y598D	SH1	R
4.7%					
1.2%	Normal	1925A >C	Y598S	SH1	N
51.0%	Mosaic	1976G >C	R615P	SH1	N
13.7%	Normal	1978C >A	L616I	SH1	N
40.6%	Mosaic	1988C >T	P619L	SH1	N
1.3%	Mosaic	2053C >T	R641C	SH1	R
4.3%	Normal	2054G >A	R641H	SH1	R
66.1%	Mosaic	2060C >T	T643I	SH1	N
3.2%	Mosaic	169C >T	R13X	PH	R
3.4%	Mosaic	850G >T	E240X	SH3	R
5.8%	Mosaic	888G >A	W252X	SH3	R
0.5%	Mosaic	895C >T	R255X	SH3	R
0.5%					
4.7%	Mosaic	895C >T	R255X	SH3	R
36.4%	NT	1524C >A	C464X	SH1	N
2.5%	Mosaic	1621C >T	Q497X	SH1	R
53.9%	NT	1630G >T	E500X	SH1	N
5.8%	NT	1690C >T	R520X	SH1	R
8.1%	Mosaic	1705C >T	R525X	SH1	R
7.8%	Normal	1705C >T	R525X	SH1	R
2.7%	Mosaic	251-273del (23nt)	Frameshift	PH	N
8.0%	Mosaic	274-275del AG	Frameshift	PH	N
6.8%	Mosaic	288del G	Frameshift	PH	N
1.9%	Mosaic	524-652del (129nt)	Inframe	PH	R
1.8%	Normal	524-652del (129nt)	Inframe	PH	R

(continued)

TABLE I. Continued

Patient no.	Present age	Age at diagnosis	Serum Ig level (mg/dL)*			Family history†
			IgG	IgA	IgM	
P47	2 y	1 y	66	5	9	(-)
P48-1#	8 y	7 y	343	114	31	P48-1 and -2 are brothers
P48-2#	5 y	5 y	190	69	4	
P49	13 y	8 mo	292	1	1	Brother is XLA
P50	6 y	2 y	<9	<7	17	(-)
P51	16 y	3 y	480	<30	<28	(-)
P52-1¶	18 y	2 y	29	0	0	P52-2's uncle
P52-2¶	4 mo	4 mo	167	2	17	
P53	7 y	6 y	671	37	10	(-)
P54	13 y	1 y	35	<13	26	(-)
P55-1	4 y	2 y	63	2	4	P55-2's nephew
P55-2	23 y	1 y	170	<5	42	
P56	26 y	5 y	NA	7	8 (25 y)	Brother is XLA
P57-1¶	6 y	3 y	485	<5	67	P57-1 and -2 are brothers
P57-2¶	1 y	5 mo	165	<5	30	
P58	2 y	2 y	24	24	26	(-)
P59	10 mo	5 mo	71	<24	17	(-)
P60	25 y	4 y	20	5	<3	(-)
P61	9 y	1 y	4	0	36	(-)
P62	11 y	4 y	69	10	13	(-)
P63	26 y	3 y	NA	<10	<10 (24 y)	(-)
P64	20 y	6 y	60	5	43	(-)
P65§	26 y	24 y	NA	0	1 (26 y)	(+)
P66-1	18 y	2 y	184	7	8	P66-1 and -2 are brothers
P66-2	12 y	1 y	40	7	52	
P67-1	4 y	4 y	397	<5	40	P67-2's nephew
P67-2	32 y	31 y	527	8	30	
P68	4 y	1 y	12	2	18	(-)
P69	25 y	16 y	24	0	20	Cousin is XLA
P70	34 y	10 y	NA	16	13 (34 y)	(-)
Insertion						
P71-1	14 y	8 mo	30	0	0	P71-1 and -2 are brothers
P71-2	12 y	3 mo	280	0	0	
P71-3	4 y	3 y	60	<6	7	P72-1 and -3 are cousins
P72	14 y	14 y	<40	<2	44	(+)
P73-1¶	1 y	11 mo	11	<6	7	P73-1 and -2 are brothers
P73-2	2 mo	2 mo	242	<6	8	
P74	24 y	6 y	<250	0	0	Cousin is XLA
P75¶	18 y	5 y	<150	<3	<20	(-)
P76	13 y	1 y	10	<5	11	(-)
Multiple alterations						
P77	1 y	1 y	<1	3	18	(+)
P78¶	4 y	3 y	8	<26	66	(-)
No mutation in the coding region						
P79	6 y	10 mo	88	<8	17	(-)
P80	14 y	9 y	NA	NA	NA	(-)

NA, Not applicable; NT, not tested; nt, nucleotide; R, reported; N, novel.

*Serum levels of immunoglobulins at age of diagnosis are shown. In some cases, data were obtained after clinical diagnosis, at the later age shown in parentheses.

†The plus sign (+) indicates that male members of the same pedigree died at a young age because of severe infections, implying immunodeficiencies.

‡Data indicate the percentages of anti-BTK-stained cells exceeding the upper limit of the control staining with the isotype-matched irrelevant mAb.

§Described in a previous report.¹⁶¶Reported in a previous study.¹¹¶Enrolled in a study of bone marrow B-cell analysis of XLA.²³#Reported as an XLA family of maternal germinal mosaicism.²⁴

BTK expression		BTK mutation			
Patient	Mother	Nucleotide change	Consequence	Domain	BTK base
0.5%	Mosaic	524-1698del (1475nt)	Frameshift	PH	N
6.4%	Normal	563del C	Frameshift	TH	N
6.1%					
2.4%	Mosaic	604-607del ACAG	Frameshift	TH	N
1.8%	Mosaic	774-777del TGAG	Frameshift	TH	N
2.4%	Mosaic	785del A	Frameshift	SH3	R
9.5%	Mosaic	787del G	Frameshift	SH3	R
4.6%	Mosaic				
4.3%	NT	803del A	Frameshift	SH3	N
1.4%	Mosaic	909-971del (63nt)	Inframe	SH3	R
0.5%	Mosaic	1027-1106del (80nt)	Frameshift	SH2	N
1.2%	Mosaic				
54.5%	NT	1033-1035del GAA	Inframe	SH2	N
6.2%	Mosaic	1089-1090del GT	Frameshift	SH2	R
8.3%					
8.2%	Mosaic	1107-1234del (128 nt)	Frameshift	SH2	N
23.5%	Mosaic	1213-1215del TAC	Inframe	SH2	N
5.7%	NT	1235-1247del (13nt)	Frameshift	SH2	N
28.6%	Mosaic	1238-1239del TC	Frameshift	SH2	N
27.8%	Mosaic	1482-1763del (282nt)	Inframe	SH1	N
62.0%	Mosaic	1643del A	Frameshift	SH1	N
16.9%	NT	1764-1920del (157nt)	Frameshift	SH1	N
36.1%	Mosaic	1942-1943del AG	Frameshift	SH1	N
3.6%	Mosaic	2008-2040del (33nt)	Inframe	SH1	N
5.1%					
14.5%	Mosaic	2008-2040del (33nt)	Inframe	SH1	N
18.2%	NT				
2.4%	Mosaic	2043del A	Frameshift	SH1	N
6.3%	NT	2044-2046del GCA	Inframe	SH1	N
47.0%	Mosaic	2061del T	Frameshift	SH1	N
3.3%	Mosaic	144-145ins G	Frameshift	PH	N
0.5%					
5.2%	Mosaic				
2.1%	NT	441-442ins (21nt)	Inframe	PH	N
7.1%	Mosaic	612-613ins A	Frameshift	TH	R
5.4%					
0.3%	Normal	720-721ins (7nt)	Frameshift	TH	N
46.7%	Normal	1176-1177ins G	Frameshift	SH2	R
0.1%	Mosaic	1359-1360ins A	Frameshift	SH1	N
5.8%	Mosaic	909-1106del (198nt)	Frameshift	SH3	R
0.5%	Normal	1310-1763del (454nt)	Frameshift	SH1	N
		1482-2040del (559nt)/	Inframe	SH1	
		1699-2040del (342nt)			
1.0%	Mosaic				
6.8%	NT				

fied here had no family history, approximately 80% of mothers of patients were determined to be obligate carriers by flow cytometric assessment. The normal single pattern of monocyte BTK expression in the mother would predict the de novo mutation in the maternal germ line. This pattern was obtained in 13 mothers. Genetic analysis disclosed that 9 of these mothers were normal in both alleles of the *BTK* gene. Surprisingly, the 4 remaining mothers (P4, P24, P46, and P74) were genetically heterozygous. Contrary to the ordinary rule of X-chromosome inactivation in women, the nonrandom or skewed inactivation of the X-chromosomes is known to occur in a small population of women.^{32,33} The extremely skewed inactivation of the mutated X-chromosome might occur in the mothers. Therefore, a genetic analysis is ultimately indispensable in carrier detection.

In conclusion, this report has described our trial to identify XLA in Japan by flow cytometric assessment combined with genetic analysis. The clinical usefulness of the flow cytometric assay for detection of XLA and its carrier was proved in a large group of newly identified 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, we propose that a hypogammaglobulinemic male with a lack of circulating B cells should be initially examined through use of a flow cytometric assay. Needless to say, a mutation analysis is finally necessary for a definitive diagnosis of XLA or subsequent genetic counseling.

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