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ORIGINAL ARTICLE

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Identification of mutations in the Bruton's tyrosine kinase gene, including a novel genomic rearrangements resulting in large deletion, in Korean X-linked agammaglobulinemia patients

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Abstract Mutations in the Bruton's tyrosine kinase (*BTK*) gene are responsible for X-linked agammaglobulinemia (XLA). We identified *BTK* mutations in six patients with presumed XLA from unrelated Korean families. Four out of six mutations were novel: two missense mutations (P565T, C154Y), a point mutation in a splicing donor site (IVS11 + 1G > A), and a large deletion (a 6.1-kb deletion including *BTK* exons 11–18). The large deletion, identified by long-distance PCR, revealed *Alu-Alu* mediated recombination extended from an *Alu* sequence in intron 10 to another *Alu* sequence in intron 18, spanning a distance of 6.1 kb. The two known mutations consisted of one missense (G462D) mutation, and a point mutation in a splicing acceptor site (IVS7−9A > G). This study suggests that large genomic rearrangements involving *Alu* repeats are few but an important component of the spectrum of *BTK* mutations.

Keywords X-linked agammaglobulinemia · Bruton's tyrosine kinase · Mutation · *Alu* repeat recombination · Korea

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Introduction

X-linked agammaglobulinemia (XLA), caused by mutations in Bruton's tyrosine kinase (*BTK*), is the most common form of inherited antibody deficiency (Conley 1992). While pro-B cells in the bone marrow are present in normal or greater numbers, XLA-affected males have no or very low levels of circulating mature B cells (usually less than 1% of total lymphocytes), plasma cells, and immunoglobulins of all isotypes. The XLA patients suffer from recurrent, occasionally life-threatening infections from a very early age. The female carriers remain asymptomatic due to proliferation and maturation of B cells expressing the normal *BTK* gene product, as evidenced by the completely skewed X chromosome inactivation in their B cell lineage (Conley et al. 1986; Fearon et al. 1987).

BTK is located on the long arm of the X chromosome at Xq22 and consists of 19 exons that span 37 kb of genomic DNA (Rohrer et al. 1994). BTK is composed of five distinct structural domains: the pleckstrin homology (PH), Tec homology (TH), Src homology 3 (SH3), SH2, and catalytic kinase (SH1) domains (Ohta et al. 1994; Sideras et al. 1994; Rohrer et al. 1994). A database of *BTK* mutations lists more than 700 mutation entries from 667 unrelated families (http://bioinf.uta.fi/BTKbase).

Mutations in *BTK* are heterogeneous and spread throughout all five domains of BTK, and are associated with reductions in *BTK* mRNA, protein, and kinase activity (Conley et al. 1994; Kornfeld et al. 1996; Tsukada et al. 1993; Saffran et al. 1994; Hashimoto et al. 1996; Bykowsky et al. 1996). Greater than 90% of *BTK* mutations can be identified using single-strand confirmation polymorphism (SSCP) screening of genomic DNA. However, the task of identifying larger genomic alterations may be formidable because of the lack of noncoding sequence data at the *BTK* locus needed to determine deletion breakpoints (Rohrer et al. 1999).

We describe here the *BTK* mutations found in six Korean patients with XLA. Four of them were novel, one of which was a deletion resulting from homologous recombination between *Alu* repeats in introns 10 and 18.

Subjects and methods

Subjects

The six male Korean hypogammaglobulinemic patients from six unrelated families enrolled in this study; none of them have been previously reported. All patients were male, aged between three and six years old, and had low levels of serum immunoglobulins. Clinical and immunological data of six patients are shown in Table 1.

The percentages of peripheral B cells were assessed by an immunofluorescence analysis using anti-CD19 monoclonal antibody (MoAb; Pharmingen, USA). All six patients showed markedly reduced B-cell numbers ($<1\%$), which implied that they had XLA.

Analysis of BTK expression

Expression of BTK protein in monocytes from the four patients (P2, P3, P4, and P5) was assessed by flow cytometric assay using anti-BTK monoclonal antibody as previously described. (Futatani et al. 1998).

BTK mutation detection

The *BTK* mutations were detected by the direct sequencing of *BTK* cDNA and by using polymerase chain reaction (PCR)-based single-strand conformation polymorphism (SSCP) analysis and direct sequencing of the corresponding exons, as described (Hashimoto et al. 1996; Jo et al. 2001; Kanegane et al. 2001). Total RNA was extracted from PBMC with TRIzol Reagent (Life Technologies, Grand Island, N.Y.) and used for first-strand cDNA synthesis. PCR amplification of the cDNA involved seven overlapping primers (Hashimoto et al. 1996). The resulting PCR products were used as templates for direct sequencing by an automated ABI PRISM 310 DNA Genetic Analyzer (Applied Biosystems, Foster City, Calif.). Genomic DNA was purified from PBMC with a QIAamp Blood Kit (Qiagen, Hilden, Germany) and amplified by using primers encompassing each exon-intron boundary of the *BTK* gene (Vorechovsky et al. 1995). For SSCP analysis, the amplified products were electrophoresed on a Gene Excel 12.5/24 polyacrylamide gel (Amersham Pharmacia Biotech, Buckinghamshire, UK) for 90 min and were visualized by silver staining. When abnormal mobility was detected in the SSCP gel, the corresponding exons were sequenced as described above. Long-distance PCR of genomic DNA was performed with pairs of oligonucleotide primers specific to introns 9 and 19 of the *BTK* gene (5′-ATCACTGACATGGACAAGCC-3′ and 5′-TTGCTCAGAAGCCACTATCC-3′). The reaction was carried out using Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen, Carlsbad, Calif.).

Results

The clinical features of the six patients with XLA are shown in Table 1. Before intravenous immunoglobulin replacement, all patients except P5 had serum IgG levels <300 mg/dl, and the serum IgA and IgM levels were undetectable in some of these patients. A transient neutropenia (absolute neutrophil count, $462/\mu l$) was observed in P6. All patients had recurrent bacterial infections, and all patients except P3 had pneumonia histories. However, they had no specific familial histories. Three patients (P1, P3, and P5) had recurrent otitis media. The P5 suffered from various recurrent bacterial infections, including orbital cellulites, erysipelas, abscess, otitis media, sinusitis, and pneumonia. In addition, he had sepsis induced by *Streptococcus pneumoniae* infection. Currently, the six patients receive intravenous immunoglobulin replacement therapy, and are in apparently healthy states without any clinical symptoms.

Flow cytometric evaluation of BTK expression disclosed that BTK deficiency (1.7–14.1%) was found in the four patients, suggesting that they were XLA patients (Table 2). Thereafter, we examined the *BTK* gene in patients with presumed XLA and the sequencing analysis detected *BTK* mutations in all six patients (Table 2). Among six XLA patients we studied, three

Table 1 Patient laboratory data and family history (*y* years, *URTI* upper respiratory tract infections)

Patient no.	Present age	Age of onset	Age at diagnosis	Ig level (mg/dl) at onset			Peripheral B cells (%)	Clinical presentation
				IgG	IgM	IgA		
P1	6 y	4 y	4.5 y	70	35	15	0	Pneumonisa, otitis media, sepsis
P2	3 y	2 y	3 y	227	33	7	1.0	Frequent pneumonia
P3	5 y	3 y	3 y	192	5	7	0.1	Sinusitis, otitis media, frequent URTI
P4	4 y	1 y	2 y	33	17	7	0	Frequent URTI, pneumonia
P5	6 y	2 y	6 y	499	21	3	0.6	Cellulitis, erysipelas, otitis media, peumonia, sepsis
P6	3 y	2 y	2 y	255	70	20	0	Pneumonia

Table 2 *BTK* mutations and BTK protein expression identified in the present study (*NT* not tested)

Patient no.	BTK expression	Intron/exon	Domain	Nucleotide aberration	Amino acid aberration
P1[a]	NT	Intron 11	SH2	IVS11 + 1G > A	G325ins + 14 (in frame)
P2[a]	14.1%	Exon 17	Kinase	1825C > A	P565T
P3	12.4%	Intron 7	TH	IVS7–9A > G	I197ins201X
P4[a]	2.8%	From intron 10 to intron 18	SH2 and kinase	6,074 bp deletion between introns 10 and 18	Exon 11–18 skipping
P5[a]	1.7%	Exon 18	Kinase	593G > A	C154Y
P6	NT	Exon 15	Kinase	1517G > A	G462D

[a]Novel mutations

(P2, P5, and P6) had missense mutations. P2 showed a C to A change at position 1825, which resulted in a proline to threonine change at residue 565. The P565T mutation was a novel one, although a C to T point mutation at the same position was reported to cause a P565L substitution (Stewart et al. 2001). Another novel missense mutation, a G to A nucleotide change at position 593, was detected in P5, which resulted in a cysteine to tyrosine substitution at residue 154 (C154Y). In the previous report, the mutation 593G > C caused a cysteine to serine substitution (Vihinen et al. 1997). In P6, the 1517G > A aberration caused the amino-acid change G462D, as previously submitted to the BTKbase (http://bioinf.uta.fi/BTKbase).

Two splicing errors were found in the genetic material of patients P1 and P3. A point mutation was found in intron 11 (+1G > A) by sequencing in the genomic DNA of P1. The IVS11 + 1G > A usually causes exon 11 skipping; however, P1 showed an insertion of 42 bp at the 5′ end of intron 11 [G325ins + 14 (in frame)]. In this case, intron 11 + 43 + 44 gt might be a new splice acceptor site. Although a previous report demonstrated the first G nucleotide deletion at the splice donor site for intron 11 (Conley et al. 1994), this is a novel splicing mutation at the exon 11/intron 11 border. The other splicing change in P3 was found at the −9 position of intron 7 (−9A > G), which created an aberrant splicing receptor site. This change resulted in the addition of a novel exon-like sequence of 8 bp between exons 7 and 8. The resulting frameshift introduced a protein termination signal at codon 201 (I197ins201X). The same mutation was already submitted to the BTKbase (http://bioinf.uta.fi/BTKbase); however, the current study confirms the resulting amino acid change of this genetic alteration.

The final mutation was found to be a 6.1-kb deletion including *BTK* exons 11–18. To better understand the mechanism by which this mutation had occurred, we characterized this alteration in detail. Analysis of P4 genomic DNA by a PCR method showed that there is no product from exons 11 to 18. Since PCR amplification for exons 10 and 19 were normal, the sense primer for the exon 10 boundary and the anti-sense primer for the exon 19 boundary were used to PCR-amplify the patient's genomic DNA. These primers, normally separated by 9.9 kb of genomic DNA, specifically amplified a 3.8-kb fragment when using patient DNA (Fig. 1). Sequence analysis of the PCR product revealed that an inverted *Alu* sequence repeat (position 62318–62619; U78027) within intron 10 had homologously recombined with an inverted *Alu* sequence repeat (position 68391–68559; U78027), resulting in the formation of a new full-length *Alu* and the deletion of 6.1 kb (Fig. 2).

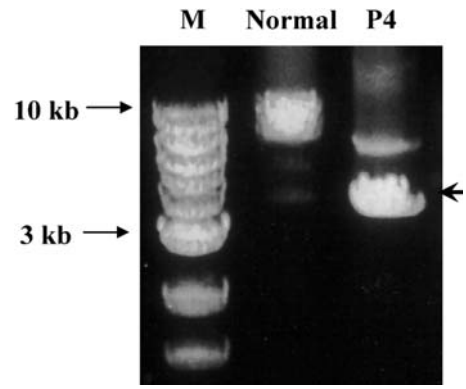

Fig. 1 Long-distance PCR of genomic DNA from P4. PBMCs were isolated from P4, and normal control and genomic DNAs were purified as described in the text. Long-distance PCR of genomic DNA from P4 was performed with pairs of oligonucleotide primers specific to introns 9 and 19 of the *BTK* gene. An *arrowhead* shows the deletion of 6.1 kb; *M* standard size markers

In conclusion, we described here the molecular identification of six XLA patients in Korea. Our data emphasize that initial characterization of *BTK* mutations – not readily detectable by PCR-SSCP analysis – should include a search for *Alu* and other repetitive elements to facilitate subsequent PCR-based mutation analysis. Further identifications of *BTK* mutations should be compiled and analyzed to provide data for clarifying correlation between the severity of the disease and the type and location of the mutations found in XLA patients.

Discussion

Although more than 700 *BTK* mutations have been reported in the patients with XLA (http://bioinf.uta.fi/BTKbase), most alterations in *BTK* are single base-pair

Fig. 2 Homologous sequences from patient DNA and *Alu* repeat of introns 10 and 18. Sequence data of the PCR product from P4 show homologies with *Alu* sequence repeats in introns 10 and 18 of the *BTK* gene. The nucleotide numbers were designated by GenBank accession U78027

substitutions or small insertions or deletions that are readily detectable by PCR-SSCP analysis. However, some mutations are not readily detectable by conventional SSCP analysis. Particular genetic alterations, such as retroposon insertion, inversion, duplication, and large deletions could be detected by large-scale sequencing. The repetitive DNA elements, including *Alu* repeats, played a significant role in the underlying molecular mechanisms in these alterations (Rohrer et al. 1999). Human *Alu* elements play a major role in genome remodeling, both by de novo insertion and by promoting deletions and re-duplications as a result of unequal recombination between dispersed repeat elements (Deininger and Batzer 1999). *Alu* repeats are composed of two internal tandem repeats – a left arm, 132 bp in length, and a right arm, 159 bp long (Deininger et al. 1981) – and have reached a copy number in excess of 500,000 copies per human genome. These elements have been proposed to have a number of functions in the human genome, and have certainly had a major impact on genomic architecture (Deininger and Batzer 1999). The frequency of mutagenesis caused by unequal homologous recombination between *Alu* repeats is estimated as 0.3% of human genetic diseases, and *Alu* elements continue to contribute to a significant portion of human genetic diseases (Deininger and Batzer 1999). To our knowledge, this is the fifth mutation involving *Alu* repeat recombination in *BTK* gene.

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