Clinical features and mutation analysis of X-linked agammaglobulinemia in 20 Chinese patients

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Background: **X-linked agammagobulinemia (XLA) is a primary immunodeficiency caused by *Bruton's tyrosine kinase (BTK)* gene mutation. XLA patients have an extremely small amount of peripheral B cells and profound deficiency in all immunoglobulin isotypes. We analyzed the clinical, immunologic, and molecular characteristics of children with XLA in an attempt to improve the diagnosis and treatment of XLA in China.**

Methods: **Twenty children with XLA-compatible phenotypes from 18 unrelated families were enrolled in this study. The *BTK* gene was amplified and sequenced, followed by mutation analysis in these children and their female relatives.**

Results: **Eighteen different mutations of the *BTK* gene were identified in the 20 patients. Eleven mutations had been reported previously including eight missense mutations (c.994C>T, c.1987C>A, c.1885G>T, c.502T>C, c.1085C>T, c.1816C>T, c.214C>T, c.1912G>A) and three nonsense mutations (c.1267T>A, c.1793C>G, c.1618C>T). Seven novel mutations of the *BTK* gene were also presented and included five missense mutations (c.134T>A, c.1646T>A, c.1829C>G, c.711G>T, c.1235G>A), one splice-site mutation (c.523+1G>A) and one insertion mutation (c.1024-1025insTTGCTAAAGCAACTGCTAAAGCAAG). Eight out of 18 mutations of the *BTK* gene were located in the TK domain, 4 in the PH domain, 4 in the SH2 domain and 2 in the TH domain. Genetic study for carrier status was carried out in 18 families with definite *BTK* gene mutations. Nine carriers with *BTK* gene mutations were identified. Six families without carriers were detected, and 3 patients were not tested in this study.**

Conclusion: **Our results support that molecular genetic testing represents an important tool for early confirmed diagnosis of congenital agammaglobulinemia and may allow accurate carrier detection and prenatal diagnosis.**

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Key words: **BTK gene;**
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Brief report

Introduction

X-linked agammaglobulinemia (XLA, MIM# 300300) is a prototypical humoral immunodeficiency disease. In 1952, Bruton[1] described the first case of XLA in a male patient with recurrent bacterial infections. XLA is the most common form of inherited antibody deficiency disorder and is characterized by markedly reduced serum levels of all major classes of immunoglobulins and few or no mature circulating B cells.[2] Affected individuals have markedly reduced serum levels of all major classes of immunoglobulins and present with an increased susceptibility to severe and recurrent bacterial infections from early childhood.

In 1993, the cause of XLA was determined as a mutation of *Bruton's tyrosine kinase* (*BTK*) gene which localizes at Xq21.3-Xq22.[3] The *BTK* gene is 37.5 kb in length and includes 19 exons. The BTK protein consists of the following 5 functional domains: pleckstrin homology (PH), Tec homology (TH), Src homology 3 (SH3), SH2, and the kinase (TK) domains.[3] The genetic defect in XLA impairs early development of B cells, resulting in marked reduction of mature B cells in the blood. Currently, more than 930 unique *BTK* gene mutations have been reported in the international mutation database designated BTKbase (*http://bioinf.uta.fi/BTKbase*).

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The Chinese XLA patients have been identified in Hong Kong and the mainland of China.[4-6] Approximately 100 patients have been diagnosed with XLA by molecular genetic analysis. As the estimated prevalence of XLA is 1/200 000, it is thought that XLA may be under-diagnosed in China. This study was undertaken to investigate clinical and molecular characteristics of XLA in 20 patients from 18 unrelated Chinese families.

Methods

Patients and study design

Chongqing Children's Hospital is a public university-affiliated hospital and the pediatric immunology unit is one of three referral centers for PIDs. Molecular genetic examinations were performed in local patients and those referred from other provinces of China. From 2010 to 2011, 20 children with XLA-compatible phenotypes from 18 unrelated families and 8 provinces of China were enrolled in this study. XLA was diagnosed according to the diagnostic criteria for XLA developed by the Joint European Society for Immunodeficiencies/ Pan American Group for Immunodeficiencies Committee.[7] Male patients were diagnosed with XLA if they had recurrent bacterial infections in the first 5 years of life, serum IgG/A/M of 2SD below normal range for age, $CD19^+$ B cells of <2% (measured by immunofluorescence analysis through a use of anti-CD19 mAb), and absent isohemagglutinins and/or poor response to vaccination. Definitive diagnosis of XLA was made if *BTK* mutation was identified.[7] Informed consent for genetic testing was obtained from patients or their parents. The study was approved by the Ethics Committee of the Chongqing Medical University.

RNA and DNA extraction

Total RNA was extracted from peripheral blood mononuclear cells (MBC) with a Total RNA Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For cDNA synthesis, 1 μg of total RNA was used as a template for reverse transcription-PCR (RT-PCR) with random primers (Takara, Otsu, Japan). Genomic DNA was purified with QIAamp ®DNA MiniKit (Qiagen Inc, Hilden, Germany) according to the manufacturer's instructions.

Amplification of the *BTK* gene by PCR

Amplification of the full length *BTK* gene was carried out using the oligonucleotide primers by RT-PCR as previously reported.[8] In brief, 2 μL of cDNA was added to 23 μL of PCR mixture containing 0.5 μmol/L forward and reverse primers, 250 μmol/L dNTP, 10 μM Tris-HcL and 1.25 U Taq polymerase. The thermocycling protocol was 94°C for 4 minutes, 35 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute, and a final extension at 72°C for 10 minutes. Then, the PCR products were subjected to 1.5% agarose gel electrophoresis and visualized under UV light. PCR amplification of genomic DNA at the mutation region was carried out using primers encompassing each exon/intron of the previously described *BTK* gene.[9]

Sequencing

PCR products of the *BTK* gene were purified by agarose gel with QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and directly sequenced by ABI3100 sequencer (Applied Biosystems, Foster City, CA,USA) using the original PCR primers. Sequencing was performed with BTK reference sequence (Accession: X58957.1) in the National Center for Biotechnology Information Program Basic Local Alignment Search Tool (*http://www.ncbi.nlm.nih.gov/BLAST/*). For novel mutations, sequencing of 100 alleles from normal controls was performed to rule out the possibility of polymorphisms.

Results

Clinical characteristics

Twenty XLA children from 18 families were enrolled in this study, and patients 5 and 6 were brothers as were patients 2 and 3. The mean age of onset of XLA was 1.5 years, and the mean age at diagnosis was 7.7 years. At the time of diagnosis, the clinical infections in these 20 individuals are shown in Table 1. Of the types of infections, respiratory tract infections were the most common (n=18, 90%), followed by otitis media (n=15, 75%), arthritis (n=9, 45%), skin infections (n=3, 15%), diarrhea (n=3, 15%), septicemia (n=2, 10%), and rashes (n=1, 5%). Of note, patient 6 was healthy and had no clinical manifestations of infection at eight months of age when he was diagnosed with XLA. Patient 6 was the younger brother of patient 5 and confirmed with XLA one year later when his sibling was diagnosed with XLA. Osteonecrosis of the left femeral head was found in patient 10 when he was 10 years old. Nine patients had a positive family history, and the mean age at diagnosis among these nine patients was 9.7 years, which was older than that in sporadic cases (5.7 years).

Immunological tests

IgG, IgA, and IgM levels and percentage of B cells are shown in Table 1. Serum IgG was >200 mg/dL in 5 patients at diagnosis. A pair of siblings (patients 5 and 6) had almost normal serum IgG levels (434 mg/

dL and 327 mg/dL), but their IgA and IgM levels were extremely low. The percentage of B cells of the siblings was 0.12% and 0.04%, respectively. Both of them had mild symptoms and were diagnosed at teenagers. The remaining patients had remarkably low serum IgG, IgA, and IgM levels. The percentage of B cells in all patients was lower than 2%.

Mutation analysis

Mutation analysis of the *BTK* gene was performed in these 20 children and 18 different mutations were identified, including missense mutation in 14 patients, nonsense mutations in 3, splice-site mutations in 2, and insertion mutations in 1 (Table 2). Of the 18 *BTK* gene mutations, 11 had been reported previously including

Table 1. Clinical characteristics of children with suspected X-linked agammaglobulinemia (XLA)

Patient	Age of onset (y)	Age at diagnosis (y)	IgG (g/L)	IgA (g/L)	IgM (g/L)	B cells (%)	Clinical presentations
1	0.5	9.1	2.975	0.027	0.061	0	Recurrent pneumonia, arthritis, otitis media
2	3.2	10.6	4.340	0.006	0.052	0.12	Recurrent pneumonia, diarrhea, skin infection
3	1.5	17.0	3.270	0.015	0.115	0.04	Recurrent upper respiratory tract infection
4	1.5	5.1	1.360	0.243	0.229	0	Recurrent pneumonia, otitis media, rash, arthritis
5	1.2	3.6	0.022	0.003	0.005	0	Recurrent pneumonia, otitis media, nasosinusitis
6	Unknown	0.7	0.822	0.067	0.049	0	No clinical presentation at the diagnosis of 8 months
7	0.7	4.5	0.333	0.067	0.057	0.02	Recurrent pneumonia, otitis media
8	2.0	11.2	0.130	0	0.010	0	Recurrent pneumonia, arthritis, otitis media
9	1.5	17.0	2.360	0.305	0.092	0	Recurrent pneumonia, otitis media, nasosinusitis
10	0.9	8.9	0.613	0.067	0.036	0	Recurrent pneumonia, osteonecrosis of the femeral head, arthritis
11	1.2	3.2	2.170	0.067	0.103	0.14	Recurrent pneumonia, otitis media, nasosinusitis
12	1.3	11.2	1.610	0.039	0.229	0	Recurrent pneumonia, otitis media, arthritis
13	1.1	4.1	1.560	0.220	0.030	0	Recurrent pneumonia, arthritis
14	1.4	7.2	1.530	0.220	0.177	0	Recurrent pneumonia, otitis media
15	2.1	5.7	0.530	0.070	0.100	0	Recurrent pneumonia, otitis media
16	3.0	12.2	1.560	0.250	0.180	0	Recurrent pneumonia, otitis media, nasosinusitis, arthritis
17	1.2	2.1	1.767	0.389	0.101	0	Recurrent pneumonia, arthritis, otitis media, rash, septicemia, diarrhea
18	0.9	7.2	1.910	0.059	0.250	1.74	Recurrent pneumonia, otitis media, nasosinusitis, skin infection
19	3.7	6.2	0.980	0.100	0.010	0.03	Recurrent pneumonia, otitis media, skin infection
20	0.6	8.6	0.440	0.070	0.260	0	Recurrent pneumonia, arthritis, diarrhea, septicemia, otitis media, nasosinuitis

Unknown: patient 6 had no clinical presentations of infection when he was diagnosed with XLA.

Table 2. Analysis of *BTK* gene mutation in 18 X-linked agammaglobulinemia children

Patients	Exon/intron	Domain	Mutation	Predicted change in codon	Mother status
1*	exon 2	PH	c.134T>A	p.M1K	Carrier
2	exon 2	PH	c.214C>T	p.R28C	Carrier
3	exon 2	PH	c.214C>T	p.R28C	Carrier
4	exon 5	PH	c.502T>C	p.W124R	NE
5	intron 5	PH	c.523+1G>A	Aberrant splicing	Carrier
6*	intron 5	PH	c.523+1G>A	Aberrant splicing	Carrier
7*	exon 7	TH	c.711G>T	p.E193D	NC
8	exon 10	SH2	c.994C>T	p.R288W	Carrier
9*	exon 10	SH2	1024-1025ins TTGCTAAA GCAACTGCTAAAGCAAG	Frameshift	Carrier
10	exon 11	SH2	c.1085C>T	p.S318F	NE
11*	exon 13	SH2	c.1235G>A	p.G368E	NC
12	exon 15	TK	c.1618C>T	p.Q496X	NC
13*	exon 15	TK	c.1646T>A	p.V505D	NC
14	exon 17	TK	c.1793C>G	p.Q554X	NE
15	exon 17	TK	c.1816C>T	p.R562W	Carrier
16*	exon 17	TK	c.1829C>G	p.P566R	NC
17	exon 18	TK	c.1267T.>A	p.Q379X	Carrier
18	exon 18	TK	c.1885G>T	p.V585F	Carrier
19	exon 18	TK	c.1912G>A	p.G594R	NC
20	exon 18	TK	c.1987C>A	p.P619T	Carrier

The cDNA nucleotide position corresponds to the sequence reported by Vetrie et al.[3] GenBank access number X58957. NE: not examined; NC: non carrier. *: novel mutation.

8 missense mutations (c.994C>T, c.1987C>A, c.1885 >T, c.502T>C, c.1085C>T, c.1816C>T, c.214C>T, c.1912G>A) and 3 nonsense mutations (c.1267T>A, c.1793C>G, c.1618C>T). Seven novel mutations of the *BTK* gene included 5 missense mutations (c.134T>A, c.1646T>A, c.1829C>G, c.711G>T, c.1235G>A), 1 splice-site mutation (c.523+1G>A) and 1 insertion mutation (1024-1025insTTGCTAAAGCAACTGC TAAAGCAAG). In addition, 8 of the 18 mutations of the *BTK* gene were located in the TK domain, 4 in the PH domain, 4 in the SH2 domain and 2 in the TH domains. Genetic study for carrier status was carried out in 18 families with definite *BTK* gene mutations and 11 carriers with *BTK* gene mutations were identified. Mutation at the splice site of intron 5 (c.523+1G>A) was identified in patients 5 and 6, which led to deletion of 81 nucleotides and 27 amino acids. Patient 5 was diagnosed with XLA at age 4 and had clinical features that included recurrent pneumonia, otitis media and nasosinusitis. The age of onset of XLA was 1 year and 2 months in patient 5. Patient 6 was diagnosed with XLA at 8 months but had no clinical manifestations up to the time of diagnosis. Patients 2 and 3 were brothers. A missense mutation (c.214C>T) was observed in the brothers and led to an arginine to cysteine substitution at the codon 28. Patient 2 was diagnosed with XLA when he was 10 years and 8 months old, while confirmed diagnosis was achieved in patient 3 when he was 17 years old. Furthermore, insertion of 25 nucleotides was observed in patient 9, resulting in a frame-shift mutation. In the 25 nucleotides, 18 had the duplication of sequence from 1007 to 1024 (Accession: X58957.1) in the exon 10.

Discussion

In the present study, we reviewed the clinical data of 20 children with compatible-XLA from 18 unrelated Chinese families in 8 provinces and cities, including Beijing, Shandong, Henan, Chongqing, Sichuan, Hunan, Guzhou, and Anhui. Our results showed that all children had typical clinical presentations including recurrent bacterial infections and severe hypogammaglobulinemia with few or no circulating B cells. Increased susceptibility to infection was the major clinical manifestation, and of these upper respiratory tract infections (URTIs) and lower respiratory tract infections (LRTIs) were the most frequent as previously reported.[5,6,10-13] Besides URTI and LRTI prior to diagnosis, a relatively high incidence of arthritis, diarrhea and skin infection was also observed. Respiratory tract infections and otitis media were the main clinical manifestations of the patients in this study. The high incidence of arthritis in the current study was consistent with the findings in other clinical reports in China.[5,6] Thus, it is important for clinicians to recognize XLA as a cause of arthritis in Chinese children.

Twenty Chinese male children from 18 unrelated families with XLA phenotype were examined for *BTK* gene mutation, and in 18 different mutations of the *BTK* gene 7 were novel mutations. There were 4 mutations in the PH domain, 2 in the TH domain, 4 in the SH2 domain, and 8 in the TK domain. The mutations were the most common (40%) at the TK domain followed by the PH domain and SH2 domain (20%). These findings were in accordance with the distribution of mutations in different domains in BTKbase (http://bioinf.uta.fi/BTKbase), in which 49.9% (464/930) were seen in the TK domain and 21.5% (200/930) in the PH domain. Among the 7 novel mutations, 5 missense mutations, 1 spice site mutation and 1 insertion mutation were identified.

Patients 2 and 3 had a missense mutation (c.214C>T), which resulted in an arginine to cysteine substitution at codon 28 (R28C) in the PH domain. R28C (codon GCG) is a common mutation and accounts for 34.8% of missense mutations. The clinical presentations of patients 2 and 3 were mild. Conley et al[14] reported that a patient with an R28C mutation died of chronic pulmonary disease at 45 years of age in 1998. He survived until middle age whereas most XLA patients do not survive to adulthood.

In this study, patients 2 and 3 had mild clinical presentations and changes in laboratory parameters were slight. The serum immunoglobulin concentrations of patients 2 and 3 were moderate (Table 1). The IgG concentrations in patients 2 and 3 were 4.34 g/L and 3.27 g/L, respectively, although the percentage of their peripheral blood B cells was 0.12% and 0.04%, respectively. Phthisis was observed in patient 3 at age of 14 years. Besides, he had no clinical manifestations of infection. Interestingly, the same mutation has been found in X-linked immunodeficiency (XID) mice, which have a relatively mild defect in B cell development.[15] The milder phenotype in the XID mice as compared to XLA patients might be attributed to the R28C mutation having less severe consequences than other mutations of the *BTK* gene.[16,17] Kanegane et al[18] reported a patient with the mutation (R28C) that lead to a normal amount of altered BTK protein.

The most common site of mutation in *BTK* is the arginine-coding CpG dinucleotides (e.g., R28 and R562). This site contains the sequence purine-CpG-pyrimidine, which is a single commonly mutated tetranucleotide.[19] Three missense mutations were detected in 4 patients from 2 unrelated families in this study. One mutation (c.214C>T) was detected

in two siblings, resulting in amino acid substitution of R28C at the PH domain. The R28 residue (codon CGC) is a common site of missense mutation in the PH domain. In patient 15, another missense mutation led to the substitution of R562W (codon CGG) in the TK domain. The substitution of R288W in the SH2 domain was detected in patient 3 in this study. R28C, R562W and R288W mutations could cause structural defects affecting BTK function. However, not all mutations affecting CpGs are likely to cause XLA. Only 8 of 18 CpGs containing arginine codons were found to be mutated.[20] According to BTKbase, missense mutation at the TK domain is the most common mutation of the *BTK* gene and accounts for 59.6% among all missense mutations in unrelated families.

Molecular analysis of the *BTK* gene is a favorable tool for the diagnosis of XLA and for accurate carrier determination, which is essential for subsequent genetic consulting. Attention to the "warning signs" of primary immunodeficiency, especially in the symptom-free individuals with a family history of the disease, is very important. Provision of genetic consulting and pre-symptomatic mutational screening of members of affected families is essential to ensure early diagnosis and timely treatment. As the development of intravenous immunoglobulin, more patients survive into adulthood, and long-term complications including bronchiectasis and malignancies should be followed up.

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