J Clin Immunol (2014) 34:633–641
DOI 10.1007/s10875-014-0061-0

ORIGINAL RESEARCH

Clinical and Molecular Features of 38 Children with Chronic Granulomatous Disease in Mainland China

Huan Xu · Wen Tian · Shu-Juan Li · Lu-Ying Zhang · Wei Liu · Yao Zhao · Zhi-Yong Zhang · Xue-Mei Tang · Mo Wang · Dao-Qi Wu · Ji-Sheng Shi · Yuan Ding · Xiao-Dong Zhao · Xi-Qiang Yang · Li-Ping Jiang

Received: 25 January 2014 / Accepted: 15 May 2014 / Published online: 19 June 2014

Abstract
Purpose Chronic granulomatous disease (CGD) is an inherited disorder, with phagocytes failing to produce antimicrobial superoxide due to deficient NADPH oxidase activity. Mutations in the gene encoding CYBB are responsible for the majority of the CGD cases. To date, there have been no reports on large samples of children with CGD in China. Therefore, in this study, we described the clinical and molecular features of 38 suspected CGD patients from 36 unrelated Chinese families.
Methods Clinical diagnosis was performed using dihydrorhodamine assays detected by flow cytometry. Molecular analysis was used to identify underlying CGD-causative genes.
Results The mean age of onset in our 38 patients was 3.4 months, while the mean age at diagnosis was 31.7 months. Apart from recurrent pneumonia and abscesses, tuberculosis (TB) and Bacille Calmette-Guerin (BCG) infections were notable features in our cohort. Overall, 17 cases died and patient 1 did not participate in the follow-up period . In total, we identified 29 different CYBB gene mutations in 31 patients. We found NCF1 and CYBA mutations in 3 and 2 patients, respectively. In addition, we identified 31 carriers and prenatally diagnosed 4 CGD and 4 healthy fetuses.
Conclusions The results of our study demonstrate that children with BCG infections or recurrent TB infections should have immune function screening tests performed. Moreover, newborns with family histories of primary immunodeficiency diseases should avoid of BCG vaccination. Molecular analysis is an important tool for identifying patients, carriers, and high-risk CGD fetuses.

Keywords Bacille Calmette-Guerin and mycobacterium tuberculosis infections · children · chronic granulomatous disease · clinical features · gene mutation · prenatal diagnosis

H. Xu · W. Tian · S.-J. Li · L.-Y. Zhang · W. Liu · X.-Q. Yang · L.-P. Jiang (✉)
Clinical Immunology Laboratory, Children's Hospital of Chongqing Medical University, Ministry of Education Key Laboratory of Child Development and Disorders, Key Laboratory of Pediatrics in Chongqing, Chongqing International Science and Technology Cooperation Center for Child Development and Disorders, No. 136 Zhongshan 2nd Road, Chongqing 400014, China
e-mail: jianglp2005@126.com

Y. Zhao · X.-D. Zhao
Laboratory Biosafety-2, Children's Hospital of Chongqing Medical University, Ministry of Education Key Laboratory of Child Development and Disorders, Key Laboratory of Pediatrics in Chongqing, Chongqing International Science and Technology Cooperation Center for Child Development and Disorders, No. 136 Zhongshan 2nd Road, Chongqing 400014, China

Z.-Y. Zhang · X.-M. Tang · M. Wang · D.-Q. Wu · J.-S. Shi · Y. Ding
Department of Immunology and Nephrology, Children's Hospital of Chongqing Medical University, Ministry of Education Key Laboratory of Child Development and Disorders, Key Laboratory of Pediatrics in Chongqing, Chongqing International Science and Technology Cooperation Center for Child Development and Disorders, No. 136 Zhongshan 2nd Road, Chongqing 400014, China

Introduction

Chronic granulomatous disease (CGD) is a rare (1:200,000–1:250,000 births in USA) inherited disorder caused by defects in the nicotinamide adenine dinucleotide phosphate oxidase (NADPH) complex [1–3]. The NADPH oxidase complex is composed of two membrane proteins (gp91phox and p22phox), three cytosolic components (p47phox, p67phox, and p40phox), and the Rac GTPase [4].

CGD affected patients suffered frequently from recurrent, life-threatening bacterial and fungal infections, with 2/3 of cases presenting their first manifestations at an early age, sometimes even at birth [5, 6]. Pneumonia is the most common form of infections, although abscesses and lymphnoditis are also frequently observed [4, 6–8]. In North America, infections are mainly arised from five pathogens, specifically, *Staphylococcus aureus*, *Serratia marcescens*, *Burkholderia cepacia*, *Nocardia*, and *Aspergillus* [1, 5]. *Mycobacterium tuberculosis* and Bacille Calmette-Guerin (BCG) infections are frequent in other areas of the world [2, 3, 7, 9], such as Turkey and Hong Kong. Apart from infections, CGD patients are vulnerable to granulomatous formation and autoimmune diseases [1, 10, 11].

To date, *CYBB* (encoding gp91phox), *NCF1* (encoding p47phox), *CYBA* (encoding p22phox), *NCF2* (encoding p67phox), and *NCF4* (encoding p40phox) genetic mutations have been reported in CGD patients [4, 12]. Approximately 65 %–70 % of patients worldwide have X-linked CGD (X-CGD) caused by *CYBB* (Xp21.1) mutations, but in countries with a higher incidence of consanguineous marriage, autosomal recessive CGD (AR-CGD) is the most common form [1, 4]. Neutrophils functional tests (either nitroblue tetrazolium test (NBT) or the dihydrorhodamine (DHR) assay) are rapid tools for the diagnosis of CGD [13, 14], although the DHR assay is more sensitive and preferable [1]. Genetic analysis can identify the disease-causing genes in patients, find carriers and enable development of prenatal tests. Effective prenatal diagnosis (including direct sequencing of DNA from amniotic fluid cells or chorionic villus samples (CVS) and DHR assays on umbilical cord blood) is an important tool for controlling the number of children born with CGD [15, 16].

Conventional CGD treatments include prophylactic antimicrobial agents, surgical drainage or excision [6], and administering interferon-γ (IFN-γ). Hematopoietic stem cell transplantation (HSCT) is the only cure for CGD. Despite improvements in treatment, CGD still has a high mortality rate (2 %–5 % per year in USA) [17].

To date, there have been no reports in a large sample of Chinese children with CGD. Thus, in this study, we described the clinical features, laboratory tests, genetic analysis, prognosis and outcome of 38 CGD patients from mainland China, and perform prenatal diagnosis in eight fetuses.

Methods

Patients

During 2003 to 2013, we enrolled 38 cases (including 3 transplanted patients) diagnosed with CGD in the Children's Hospital of Chongqing Medical University. This study was approved by the ethics committee of Children's Hospital of Chongqing Medical University and informed consent obtained from the children or their parents.

Basic Information and Laboratory Tests

Diagnoses of tuberculosis (TB) included clinically diagnosed and confirmed cases (Diagnostic criteria for pulmonary tuberculosis, China (WS 288–2008)). Clinical diagnosis was made according to clinical manifestations, radiography, laboratory tests (PPD, ESR, TB-Ab, T-SPOT.TB, and lymph node biopsy) and effective anti-tuberculosis treatments. Diagnosis of confirmed cases involved detecting the presence of *Mycobacterium tuberculosis* by either sputum smear microscopy, sputum, pus or gastric liquid cultures, TB-DNA-PCR, or lung biopsy. Diagnosis of BCG disease (regional, extra regional localized, disseminated disease, and other BCG syndromes) was made as described by Hesseling AC et al. [18]. A "probable" diagnosis of disseminated BCG disease was made as described by Lee PP et al. [9].

Methods used to distinguish between TB and BCG vaccinations included spoligotyping and multiplex PCR [19].

DHR Flow Cytometry Assay

DHR flow cytometry assays were performed as previously described [15]. Data were analyzed using CellQuest software (BD FACSCalibur™ Flow Cytometer).

DNA and RNA Extraction

Genomic DNA (gDNA) and total RNA from patients and family members were isolated from whole blood cells according to the manufacturer's recommendations. Total RNA was reverse transcribed into cDNA using the PrimeScriptTM RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Dalian, China).

PCR, RT-PCR and Sequencing

Relevant genes were amplified from gDNA and cDNA using specific oligonucleotide primers (Sangon Biotech, Shanghai, China). PCR products were confirmed by gel electrophoresis and then sent to Sangon Biotech (Shanghai) Co., Ltd for sequencing.

Sequence Analysis

Sequence analyses were performed using Bioedit software and the basic local alignment search tool (BLAST) from the National Center for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi). Before identifying novel

missense mutations, 100 normal alleles were sequenced to rule out the possibility of single nucleotide polymorphisms (SNPs).

Prenatal Diagnosis

Female carriers were advised to have prenatal diagnosis performed when they were pregnant again. Amniotic fluid (30 ml) was collected at 18 weeks gestation using ultrasound guidance. DNA and RNA were extracted from amniotic fluid cells. Direct sequencing of relevant gDNA and cDNA regions were performed. Simultaneously, the sex determining region Y (*SRY*) gene was PCR amplified to determine the gender of the fetus. If direct sequencing failed to determine if the fetus was normal or not, umbilical cord blood (2 ml) was collected at 26 weeks gestation to detect the respiratory burst of neutrophils by DHR assay. After birth, DHR assays were performed to verify the accuracy of the prenatal diagnosis.

Results

Patient Demographics

We enrolled 38 CGD patients (36 male, 2 female) from 36 unrelated families (patients 11 and 34 are brothers and patients 17 and 18 are twin brothers), and 14 provinces and cities of mainland China (Sichuan 29 %, Chongqing 21 %). The type of onset in this cohort included recurrent fever (68.4 %; 26/38), cough (28.9 %; 11/38), and abscess (18.4 %; 7/38). The mean age at onset was 3.4 months (range 2 days–18 months), and the mean age of diagnosis was 31.7 months (range 18 days–172 momths). To date, 17 patients (44.7 %) have died, with the mean age of death being 30.9 months (range 6 months–102 months). Thus, 20 patients are alive (including 2 transplanted patients), with a mean age of 71.1 months (range 3 months–206 months). However, patient 1 did not participate in the follow-up period of the study. Approximately 78.9 % (30/38) of the CGD patients were diagnosed between 2009 and 2013.

Overall, 39.5 % (15/38) had positive family histories including known family histories of CGD and early death due to recurrent infections. The mean diagnosis age of patients with positive family histories was 32.3 months (range 0.7 months–172 months), similar to those with negative family histories (31.3 months, range 1.9 months–116 months).

Infection

In this cohort, 32 cases (84.2 %) developed their first clinical manifestations before 6 months. The most common infections were pneumonia (84.2 %; 32/38) and abscesses (84.2 %; 32/38) including skin, perianal, liver, and spleen abscesses. Additionally, 36.8 % (14/38) suffered form recurrent diarrhea, but only 3 children presented with colitis confirmed by colonoscopy, 44.7 % (17/38) with hepatosplenomegaly, and 42.1 % (16/38) with lymphadenitis. The main pathogens were *Staphylococcus aureus* and *Candida albicans*.

The most common types of *Candida albicans* infections were thrush (7 cases) and mycotic pneumonia (5 cases). We identified *Candida albicans* in six patients (15.8 %; 6/38). Results of fungal cultures with sputum, bronchoalveolar lavage fluid, or feces showed *Candida albicans* in 4 cases, 1 case, or 1 case, respectively.

BCG Complications

In total, 32 children received BCG vaccination (BCG strain D2 PB302) at birth or later as part of the National Vaccination Program. Fifteen cases did not suffer from BCG disease, while seventeen cases had BCG disease including regional (10 cases), extra regional localized (17 cases), disseminated (4 cases), and probable disseminated (10 cases). 21 patients presented with pulmonary tuberculosis including 19 clinically diagnosed and 2 confirmed cases. The common site of occurrence for disseminated BCG disease was the lungs.

Laboratory Features

Routine blood tests, immunoglobulin levels, NBT, and DHR flow cytometry assays were shown in Table 1. Overall, 36 patients had elevated white blood cells (WBC), mainly neutrophils. All the children except three cases had elevated C-reactive protein (CRP). Blood sedimentation tests were performed on 26 patients, and 21 had high erythrocyte sedimentation rates (ESR). Considering their age, 32 patients had remarkably high serum IgG levels, and some had high serum IgE levels. The NBT (0 %–3 %) and neutrophil oxidative function were all significantly lower than health controls.

Gene Analysis

In total, we tested 36 male children for CYBB gene mutations, and identified 29 different mutations in 31 patients, including 14 reported mutations and 15 novel mutations (51.72 %). There were 12 splice errors (including 4 suspected splicing errors) (patient 31, Fig. 1), 7 deletion mutations, 5 nonsense mutations, 4 missense mutations and 1 large gene deletion (*XK*, *CYBB* and *DYNLT3* genes) . Two splicing products were identified in cDNA from patient 8 (*CYBB* c.483-484ins115bp, p.K161fsX13; and c. 338-483del146bp, 483-484ins115bp,

Table 1 Laboratory features of 38 CGD patients in mainland China

Patient No	WBC (10^9/L)	N%	Immunoglobulin ($g \cdot L^{-1}$)				NBT	NOI
			IgG	IgA	IgM	IgE		
1	14.1	70	11.51	1.66	2.53	5.5	0	1.01
2	11.3	37	18.74	2.42	3.09	52.4	0	–
3	8.68	77	22.17	2.64	1.68	497.4	0	1.63
4	18.28	85	15.9	1.92	2.68	677.5	0	1.64
5	14.6	70	17.46	1.19	2.88	949.8	0	1.14
6	19.83	56	8.65	0.53	1.56	60	0	1.15
7	25.65	51	12.9	0.45	1.59	8.5	0	1.65
8	9.71	51	15.16	2.04	2.37	50.4	0	1.07
9	13.16	47	11.38	2.63	1.92	41.1	0	1.2
10	13.93	51.5	7.82	0.52	1.00	2090	0	1.88
11	23.7	48	8.64	0.73	0.62	32.6	0	1.85
12	32.57	60	16.65	0.89	1.04	172.4	0	1.09
13	20.49	63	7.32	0.37	0.45	32.1	0	1.17
14	11.37	70	16.33	1.24	1.24	351.6	0	2.16
15	14.3	70	26.17	3.59	2.49	200.7	0	1.52
16	11.52	67	20.5	7.8	1.12	–	0	1.46
17	21.14	46	8.02	0.33	0.53	12.4	0	1.66
18	24.49	60 53	12.24	0.31	1.53	12.2	0 24,45	1.72 131
After transplantation	8.18	53	4.83	0.24	1.42	20.5	24,45	131
19	17.07	57	15.23	4	1.12	250.3	0	1.53
20	26.31	22	13.8	1.68	1.78	769	0	1.22
After transplantation	6.94	40	8.52	1.06	1.23	74.8	0 45,62	124
21	25.26	67	11.3	1.16	1.53	81.2	0	1.45
22	30.34	70	19.7	3.52	2.52	31.8	0	1.98
23	10.42	64	23.8	4.62	2.81	280	0	1.92
24	22.59	76	11.3	1.74	1.71	12.5	0	1.45
25	19.82	72	15.6	1.75	1.8	41.6	0	1.23
26	22.77	62	37.55	1.93	1.45	348.2	0	1.61
27	10.11	75	16.14	4.51	1.14	265.5	0	1.71
28	12.2	73	11.2	3.46	1.52	4.2	0	2.61
After transplantation	6.22	72	6.11	1.5	0.571	0.8	22,33	16.3
29	12.2	65	10.9	1.18	2.74	47	3	15
30	21.01	58	6.59	0.186	1.1	61.7	0	3.46
31	19.54	48	16	0.684	1.74	4.9	0	1.99
32	16.56	65	16.2	3.74	1.66	334.7	0	1.86
33	27.85	47	7.7	0.359	0.646	5.8	2	1.14
34	23.73	61	6.78	0.609	0.876	6.3	0	1.29
35	14.55	56	4.48	0.417	1.2	4.9	3	1.16
36	18.51	80	–	–	–	–	3	1.11
37	23.68	39	15.7	1.83	2.4	11.27	0	1.17
38	21.42	53	13	0.684	1.2	22.6	0	1.2

–not examined

p.A113fsX3), while his cousin was diagnosed with CGD in the fetal period by prenatal diagnosis but had different splicing products (*CYBB* c.483-484ins115bp, p.K161fsX13; and c.483-484ins115bp, 484-674del191bp, p.K161fsX13). A GT homozygous deletion in *NCF1* (c.75-76 del GT) was found in Patient 27 and 32, and a heterozygous *NCF1* mutations

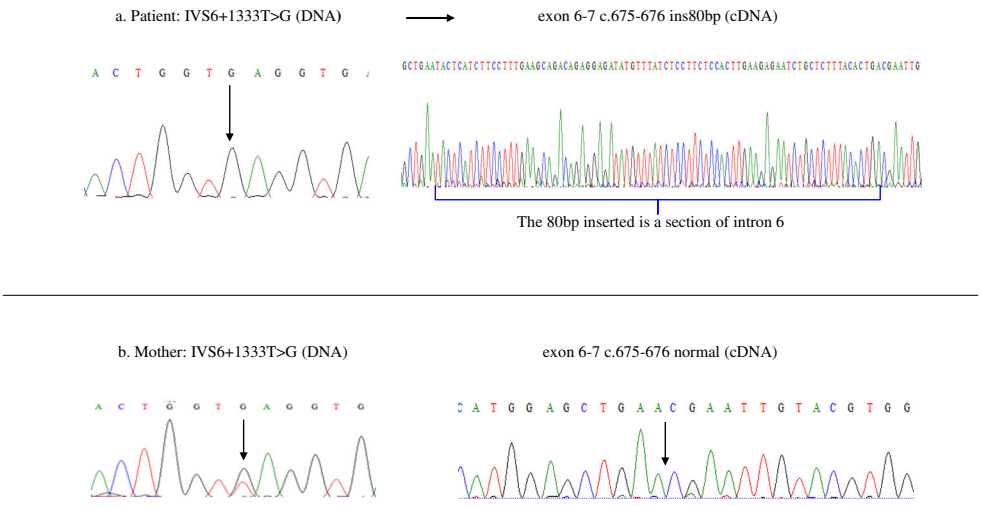

Fig. 1 CYBB gene mutation analysis of patient 31 and his mother. (**a**). A nucleotide substitution (IVS6+1333 T>G) located far from any splice sites, leading to insertion of a section of intron 6 (80 bp) between exons 6 and 7 (**b**). Mother was a heterozygote at this point (IVS6+1333 T>G) and cDNA sequence was normal

(c.764-801del 38 bp and 923C>T) in patient 30. In the *CYBA* gene, 1 homozygous missense mutation and 1 homozygous deletion mutation were found in patients 26 and 33, respectively. No *CYBB* gene mutations were identified in gDNA, and because patient 1 did not participate in the follow-up period, the cDNA of CYBB gene and other genes were not detected. Although *CYBB*, *CYBA*, *NCF1*, *NCF2* and *NCF4* were all analyzed, no mutations were identified in patient 20, and additional novel genes may be required for further analysis of this patient. Overall, we identified 31 carriers in 23 unrelated families (Table 2).

Prenatal Diagnosis

Combined with direct sequencing analysis, gender identification and DHR assays, we identified 4 fetuses with CGD (from families 8, 11, 19 and 26) and 4 healthy fetuses (from families 6, 8 and 19).

Treatment and Outcome

Upon diagnosis, all CGD patients were advised to receive prophylactic treatment with cotrimoxazole (24 mg/kg/d, divided twice daily) and itraconazole (patients age<13 years or <50 kg, 100 mg/d; > 13 years or>50 kg, 200 mg/d) to prevent bacterial and fungal infections.

By the end of December 2013, three patients had undergone HSCT. The first patient (patient 18) was given an HLA-B and HLA-C_W mismatched unrelated cord blood (CB) graft after receiving busulfan (BU)+cyclophosphamide (CY)+anti-thymocyte albumin (ATG) as a pretreatment regimen. Unfortunately patient 18 had acute graft-versus-host disease (GVHD, grade two) affecting the skin and gut, followed by four bouts of pneumonia at 3 months (*Respiratory Syncytial Virus* and *Cytomegalovirus*), 6 months (fungi and *Respiratory Syncytial Virus*), 10 (*Moraxella catarrhalis*), and 22 months after transplantation. He suffered from infectious diarrhea twice at 3 months (*Enterobacter aerogenes*) and 15 months (*Pseudomonas aeruginosa*) after transplantation. 13 months after transplantation, the child developed septic arthritis (*Pseudomonas aeruginosa*), and 22 months after transplantation died of severe pneumonia, acute respiratory distress syndrome, and sepsis (*Acinetobacter baumannii*, fungi, *Cytomegalovirus*, and *Epstein Barr virus*). After a conditioning regimen with BU+CY, the second patient (patient 20) was transplanted using a HLA-identical sibling donor, and is now healthy and doing well. The last patient (patient 28) was also transplanted using a HLA-matched sibling donor. He had grade-1 GVHD and presented with sepsis after transplantation. DHR assay were recovered normal in the first two children, but presented with a mosaic pattern in the third one after transplantation.

Discussion

Here we described detailed clinical features, laboratory tests, gene analysis and outcome of 38 CGD patients from mainland China. In our cohort, children suffered from first infections at an early age (between 0 and 6 months), but diagnosis was delayed significantly (between 13 and 40 months). Lack of awareness and screening tests for CGD may be important reasons for this. With significant progress in medicine in China, the diagnostic rate is higher than before, with approximately 78.9 % of CGD patients were diagnosed in the past 5 years.

In accordance with previous studies, pneumonia was the most common form of infections [3, 20, 21]. We also frequently detected abscesses, mainly skin abscesses (42.1 %).

Table 2 Genetic mutations and carriers of 38 CGD patients and their families in mainland China

Patient no	Mutant gene	Exon/Intron	Nucleotide change	Mutation	Predicted codon change	Carriers
1	–	–	–	–	–	–
2	CYBB	exon 11	c.1327delT	deletion	W443fsX452	mother, her 2 sisters, maternal grandmother
3	CYBB	exon 6	c.577 T>C	missense	S193P	mother
4	CYBB	exon 8	c.868C>T	nonsense	R290X	unidentified
5	CYBB	intron 10	IVS10-2A>C★	splicing error	I439fsX484	mother
6	CYBB	exon 6	c.565-568delATTA	deletion	I189fsX212	mother, maternal grandmother
7	CYBB	intron 10	IVS10+2dup T★	splicing error	skip exon10	mother
8	CYBB	exon 5	c. 483-484ins115bp c. 338-483del146bp 483-484ins115bp★	splicing error▲	K161fsX173 A113fsX3	unidentified
9	CYBB	exon 7	c.576G>T	nonsense	R226X	mother
10	CYBB	intron 5	IVS5+1G>A	splicing error	skip exon 5	mother
11	CYBB	exon 9	c.1077del G★	deletion	D360fsX385	mother
12	CYBB	exon 10	c.1168delC★	deletion	F391fsX404	mother
13	CYBB	exon7	c.725-726delCA★	deletion	T242fsX244	mother
14	CYBB	exon5	c. 469C>T	nonsense	R157X	mother
15	CYBB	exon7	c.676C>T	nonsense	R226X	mother
16	CYBB	exon2	c.46-92del 46 bp★	splicing error▲	L16fsX18	unidentified
17						
18	CYBB	exon9	c.1122delG★	deletion	E375fsX385	mother
19	CYBB	exon9	c.1082G>T★	missense	W361L	mother
20	–	–	–	–	–	–
21	CYBB	intron 2	IVS1-2A>G★	splicing error	L16-G47del	mother
22	CYBB	exon 5, 6	c.338_674del, c.484_674del★	splicing error▲	A113fsX17, N162fsX17	unidentified
23	XK、CYBB、DYNLT3	the whole three genes★	deletion	skip XK, CYBB, DYNLT3	unidentified	
24	CYBB	exon 6, 7	c.484-804del★	splicing error▲	N162-M268del	unidentified
25	CYBB	intron 6	IVS6+1delG★	splicing error	A113fsX17	mother
26	CYBA	exon 3	c.152 T>G★	missense	L51A	unidentified
27	NCF1	exon 2	c.75-76delGT	deletion	Y26HfsX27	unidentified
28	CYBB	exon12	c.1548G>C	missense	W516C	mother, his sister
29	CYBB	exon3	c.162G>C	missense	R54S	mother
30	NCF1	exon8, 10	c.764-801del 38 bp, 923C>T ★	deletion missense	E254-R267del A308V	mother
31	CYBB	intron 6	IVS6+1333 T>G★	splicing error	c.675-676ins80bp	mother
32	NCF1	exon2	c.75-76delGT	deletion	Y26HfsX27	mother, father
33	CYBA	exon4	c.246-273del 28 bp★	deletion	L79fsX190	mother, father
34	CYBB	exon 9	c.1077del G★	deletion	D360fsX385	mother
35	CYBB	exon 9	c.963delG	deletion	G322DfsX21	unidentified
36	CYBB	exon 9	c.1120C>T	nonsense	Q374X	unidentified
37	CYBB	intron5	IVS5+1G>C	splicing error	skip exon 5	mother
38	CYBB	intron 7	IVS7-1G>T	splicing error	skip exon 8	mother

– unidentified ★ novel mutation ▲ suspected splicing error, but we did not find mutation of splice site

This differs from the USA [21] and Israel [22], where deep abscesses, including liver, spleen, brain or pulmonary abscesses, are the major type (46 %–76 %).

A significant feature of our cohort was a high incidence of mycobacteria infections. Based on our findings and those in previous studies [3, 9], it appears that CGD patients are prone

to TB and BCG complications. The types of BCG disease include regional, extra regional localized, disseminated disease, and other BCG syndromes [18]. Disseminated BCG disease is the most serious complication, and associated with a high mortality rates (60 %–83 % in immunocompromised patients) [23]. We found 55.3 % and 10.5 % of cases suffered from pulmonary TB and disseminated BCG disease, respectively. In addition, we did not identify BCG in 10 cases (26.3 %), therefore these patients likely have probable disseminated BCG disease. Similarly, 55.9 % of CGD patients in Iran presented with BCGitis [20]. Moreover, in a study from Hong Kong, 41.2 % (7/17) and 47.1 % (8/17) patients presented with TB and BCG infections [9]. The high incidence of BCG infections demonstrates that the respiratory burst of neutrophils plays an important role in limiting BCG replication. Apart from CGD, BCG infections are reported in other primary immunodeficiency diseases (PID), including severe combined immunodeficiency syndrome (SCID), Mendelian susceptibility to mycobacterial diseases (MSMD), and X-linked hyper-IgM syndrome (XHIM) [24]. The pathway of IL-12/23-IFN-γ plays a crucial role in the host response to infections with mycobacteria. SCID and MSMD caused by deficiency in these pathways, so the patient with SCID and MSMD often suffered from the most critical complications of BCG. In CGD, BCG lymphadenitis is the most common form of BCG complications. CGD patients are usually respond well to anti-TB regimens (excluding pyrazinamide as BCG is uniformly resistant to it) [25] compared with SCID and MSMD. In our study, the children with BCG disease were recommended to receive anti-TB treatments (isoniazid, rifampicin and ethambutol), but because of side effects (hepatorenal damage) and for financial reasons, most patients did not persist with regular treatment, and seven cases (50 %; 7/14) died of probable/disseminated BCG disease. Thus, we suggest that newborns with a family history of PID should avoid BCG vaccination. Patients with BCG infections are suggested to take immune function screening tests, including the respiratory burst assay. In addition to mycobacterial infections, we also found *Candida albicans* infections were more common compared with other reports.

Hypergammaglobulinemia is a common finding in CGD patients [26, 27]. In our cohort, five cases had normal IgG levels, while the others had elevated IgG levels. Some cases had high serum IgE levels as described by Patiroglu T *et a l* [27].

There were 31 patients (31/38; 81.6 %) with X-CGD, but because of the higher incidence of consanguineous marriage in other parts of the world (e.g. Middle Eastern countries, North Africa, and much of western, central, and southern Asia), AR-CGD is the most common form. In a study of 38 CGD patients form Israel [22], Baruch Wolach et al. demonstrated autosomal recessive inheritance in 63 % of cases. Similarly, in Iran, 87.1 % of cases inherited a form of AR-CGD [20].

In a previous study, 20 %–30 % of all cases resulted from defects in *NCF1* (A47°CGD), the most common form of AR-CGD [28]. In contrast to other CGD types, there is a "hot mutation" (a GT homozygous deletion, ▵GT) at the beginning of exon 2 in *NCF1*. We detected this mutation in patients 27 and 32. Recombination between *ψNCF-1*(*NCF-1* pseudogenes) and *NCF1* functional gene causes high incidence of the ▵GT mutation. To date, *NCF1* has at least two pseudogenes, both of which show highly homology to *NCF1* and coexist on chromosome 7q11.23 [28, 29]. A remarkable feature of *ψNCF-1* is the GT deletion at the beginning of exon 2, as if this deletion occurs in *NCF1* it can cause CGD. In addition to this, there are other difference between *NCF1* and *ψNCF-1* as Paul G. Heyworth et al. described [28].

We identified four suspected splicing errors in our cohort, but no mutations in coding regions or splice sites of the *CYBB* gene, in gDNA for patients 8, 16, 22, and 24. However we identified a large fragment deletion in *CYBB* cDNA, indicating that mutations may occur in intronic regions outside the splice sites. In patient 31 (Fig. 1), we found a nucleotide substitution (IVS6+1333 T>G) located far from any splice sites, leading to insertion of a section of intron 6 (80 bp) between exon 6 and 7. This is an unexpected splicing pattern, similar as described by Noack D et al. [30]. Thus in genetic analysis, detection of mutations in cDNA is better than that of gDNA for the discovery of splicing errors.

Patient 8 and his cousin had X-CGD caused by different mutations in the *CYBB* gene. We speculate that a *de novo* mutation occurred during embryogenesis in the maternal grandmother, with unsuccessful DNA repair resulting in two aberrant alleles. Therefore, the maternal grandmother of patient 8 had triple somatic mosaicism (a normal allele, the mother's allele, and the aunt's allele). One of the two aberrant alleles has been inherited by patient 8 and his mother, and the other by his cousin and aunt. Unfortunately at present, we have not identified the carriers (including the mother, aunt, and maternal grandmother), but we will perform further studies in this family with this intention [30, 31].

Regarding treatment for CGD, prophylaxes with antibacterial and antifungal agent are used worldwide, although the efficacy of IFN-γ therapy is still in dispute [11, 32]. Research by the International Chronic Granulomatous Disease Cooperative Study Group found that IFN-γ treatment was effective regardless of the inheritance mode, and if taking antibiotics or not [33]. However, a long term follow-up of 60 CGD patients by Martire et al. [11], demonstrated that IFN-γ treatment did not reduce the incidence of infections (severe and mild infections), and that there are adverse events (e.g. fever, myalgia, and headache) to adherence to IFN-γ

prophylaxis. In our cohort, cotrimoxazole and itraconazole were recommended and IFN-γ was not routinely used. Seventeen cases (44.7 %) died of various infections at an early age due to diagnosis delay, unclear pathogens, and substandard treatment. Until recently, *Aspergillus* species infections were the main cause of mortality in CGD patients [17]. We found only one patient had an *Aspergillus* infection, although it is perhaps due to limitations in the detection methods that we did not detect more evidence for *Aspergillus* infection in the other cases. There is controversy about optimum timing and indications in HSCT. As previously discussed, transplantation with an HLA-matched sibling donor at an earlier age is the best choice [17, 32]. However, in a multicenter study by Tayfun Güngör et al. [34], 56 CGD patients (75 % patients with high-risk features) were enrolled. With reduced-intensity conditioning regimens, all 21 HLA-matched related-donor and 35 HLA-matched unrelated-donor (HLA-9/10 or HLA-10/10 match with the recipient) transplants had a better outcome (overall survival rate 93 %, event-free survival rate 89 %), suggesting a reduced-intensity conditioning regimen is safe and effective for high-risk CGD patients with HLA-matched related- or unrelated-donors.

Prenatal diagnosis is an effective way to control the birth of children with CGD. Direct sequencing of gDNA/RNA from amniotic fluid cells or CVS can provide an earlier diagnosis, but careful attention must be paid to avoid contamination with maternal DNA. Although DHR-assays are another reliable and accurate method for prenatal diagnosis, umbilical cord blood cannot be collected until 18 weeks gestation [16]. Whereas a prior study described a lower than normal respiratory burst of neutrophils at 22 weeks gestation, in our study, DHR-assays were performed at 26 weeks gestation (using a male fetus from family 8 and a female fetus from family 26). A combination of direct sequencing and DHR assays is an effective and accurate way for prenatal screening for CGD.

There are some shortcomings in our study, specifically, this is a single center study with the patients mainly from Sichuan and Chongqing (50 %), and therefore may not completely and accurately describe CGD in China. In this regards, we are setting up a system of "CARE CENTER FOR PID IN CHINA". In our cohort, most patients had no etiological evidence of CGD; this may be due to limitations in detection methods.

Conclusion

We have described the clinical and molecular features of 38 CGD patients from mainland China. A remarkable feature of our cohort is a high incidence of TB and BCG infections. Molecular analysis is an important tool for identifying patients, carriers and high-risk CGD fetus.

Acknowledgment This study was supported by grants from Key Project of Chongqing Health Bureau (2012-1-048), the Scientific and technology Committee of Yuzhong District of Chongqing (20110313) and Public Welfare Scientific Research Project of China (201402012). We thank the patients and their family members for their cooperation.

Conflict of interest statement There was no conflict of interest to declare.

References

1. Holland SM. Chronic granulomatous disease. Hematology/Oncology Clinics of North America. 2013;27:89–99.
2. Holland SM. Chronic granulomatous disease. Clinical Reviews in Allergy and Immunology. 2010;38:3–10.
3. Mahdaviani SA, Mohajerani SA, Rezaei N, et al. Pulmonary manifestations of chronic granulomatous disease. Expert Review of Clinical Immunology. 2013;9:153–60.
4. Gardiner GJ, Deffit SN, McLetchie S, et al. A role for NADPH oxidase in antigen presentation. Frontiers in Immunology. 2013;4:295.
5. Segal BH, Leto TL, Gallin JI, et al. Genetic, biochemical, and clinical features of chronic granulomatous disease. Medicine (Baltimore). 2000;79:170–200.
6. Roos D, de Boer M. Molecular diagnosis of chronic granulomatous disease. Clinical and Experimental Immunology. 2014;175(2):139–49.
7. Köker MY, Camcıoğlu Y, van Leeuwen K, et al. Clinical, functional, and genetic characterization of chronic granulomatous disease in 89 Turkish patients. The Journal of Allergy and Clinical Immunology. 2013;132:1156–63.
8. van den Berg JM, van Koppen E, et al. Chronic granulomatous disease: the European experience. PLoS One. 2009;4:e5234.
9. Lee PP, Chan KW, Jiang L, et al. Susceptibility to mycobacterial infections in children with X-linked chronic granulomatous disease: a review of 17 patients living in a region endemic for tuberculosis. Pediatric Infectious Disease Journal. 2008;27:224–30.
10. Liese J, Kloos S, Jendrossek V, et al. Long-term follow-up and outcome of 39 patients with chronic granulomatous disease. Journal of Pediatrics. 2000;137:687–93.
11. Martire B, Rondelli R, Soresina A, et al. Clinical features, long-term follow-up and outcome of a large cohort of patients with Chronic Granulomatous Disease: an Italian multicenter study. Clinical Immunology. 2008;126:155–64.
12. Williams DA, Tao W, Yang F, et al. Dominant negative mutation of the hematopoietic- specific Rho GTPase, Rac2, is associated with a human phagocyte immunodeficiency. Blood. 2000;96:1646–54.
13. Kim HY, Kim HJ, Ki CS, et al. Rapid determination of chimerism status using dihydrorhodamine assay in a patient with X-linked chronic granulomatous disease following hematopoietic stem cell transplantation. Annals of Laboratory Medicine. 2013;33:288–92.
14. Vowells SJ, Sekhsaria S, Malech HL, et al. Flow cytometric analysis of the granulocyte respiratory burst: a comparison study of fluorescent probes. Journal of Immunological Methods. 1995;178:89–97.
15. Sun J, Wang Y, Liu D, et al. Prenatal diagnosis of X-linked chronic granulomatous disease by percutaneous umbilical blood sampling. Scandinavian Journal of Immunology. 2012;76:512–8.
16. Yavuz Köker M, Metin A, Ozgür TT, et al. Prenatal diagnosis of chronic granulomatous disease in a male fetus. Iranian Journal of Allergy, Asthma, and Immunology. 2009;8:57–61.
17. Åhlin A, Fugeläng J, de Boer M, et al. Chronic granulomatous disease - haematopoietic stem cell transplantation versus conventional treatment. Acta Paediatrica. 2013;102:1087–94.

18. Hesseling AC, Rabie H, Marais BJ, et al. Bacille Calmette-Guérin vaccine-induced disease in HIV-infected and HIV-uninfected children. Clinical Infectious Diseases. 2006;42:548–58.
19. Huard RC, Lazzarini LC, Butler WR. PCR-based method to differentiate the subspecies of the mycobacterium tuberculosis complex on the basis of genomic deletions. Journal of Clinical Microbiology. 2003;41:1637–50.
20. Fattahi F, Badalzadeh M, Sedighipour L, et al. Inheritance pattern and clinical aspects of 93 Iranian patients with chronic granulomatous disease. Journal of Clinical Immunology. 2011;31:792–801.
21. Winkelstein JA, Marino MC, Johnston Jr RB, et al. Chronic granulomatous disease. Report on a national registry of 368 patients. Medicine (Baltimore). 2000;79:155–69.
22. Wolach B, Gavrieli R, de Boer M, et al. Chronic granulomatous disease in Israel: clinical, functional and molecular studies of 38 patients. Clinical Immunology. 2008;129:103–14.
23. Shojaei H, Rahimi-Hajiabadi H, Heidarieh P, et al. Molecular microbiological investigation of post-vaccination bacille Calmette-Guérin infection in Iranian patients. The International Journal of Tuberculosis and Lung Disease. 2011;15:1497–503.
24. Norouzi S, Aghamohammadi A, Mamishi S, et al. Bacillus calmette-guérin (BCG) complications associated with primary immunodeficiency diseases. Journal of Infection. 2012;64:543–54.
25. Dankner WM, Waecker NJ, Essey MA, et al. Mycobacterium bovis infections in San Diego: a clinicoepidemiologic study of 73 patients and a historical review of a forgotten pathogen. Medicine (Baltimore). 1993;72:11–37.
26. Hanoglu D, Ozgür TT, Ayvaz D, et al. Chronic granulomatous disease presenting with hypogammaglobulinemia. Journal of Investigational Allergology and Clinical Immunology. 2011;21:310–2.
27. Patiroglu T, Gungor HE, Lazaroski S, et al. Chronic granulomatous disease with markedly elevated IgE levels mimicking hyperimmunoglobulin E syndrome. Acta Microbiologica et Immunologica Hungarica. 2013;60:155–62.
28. Heyworth PG, Noack D, Cross AR. Identification of a novel NCF-1 (p47-phox) pseudogene not containing the signature GT deletion: significance for A47° chronic granulomatous disease carrier detection. Blood. 2002;100:1845–51.
29. El Kares R, Barbouche MR, Elloumi-Zghal H, et al. Genetic and mutational heterogeneity of autosomal recessive chronic granulomatous disease in Tunisia. Journal of Human Genetics. 2006;51:887–95.
30. Noack D, Heyworth PG, Kyono W, et al. A second case of somatic triple mosaicism in the CYBB gene causing chronic granulomatous disease. Human Genetics. 2001;109(2):234–8.
31. de Boer M, Bakker E, Van Lierde S, et al. Somatic triple mosaicism in a carrier of X-linked chronic granulomatous disease. Blood. 1998;91(1):252–7.
32. Kang EM, Malech HL. Advances in treatment for chronic granulomatous disease. Immunologic Research. 2009;43:77–84.
33. The International Chronic Granulomatous Disease Cooperative Study Group. A controlled trial of interferon-gamma to prevent infection in chronic granulomatous disease. New England Journal of Medicine. 1991;324:509–16.
34. Güngör T, Teira P, Slatter M, et al. Reduced-intensity conditioning and HLA-matched haemopoietic stem-cell transplantation in patients with chronic granulomatous disease: a prospective multicentre study. Lancet. 2014;383(9915):436–48.