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ORIGINAL ARTICLE

Clinical and Molecular Features of Chronic Granulomatous Disease in Mainland China and a XL-CGD Female Infant Patient After Prenatal Diagnosis

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Abstract

Purpose Chronic granulomatous disease (CGD) is the most common phagocyte defect disease. Here, we describe 114 CGD patients in our center and report a rare female infant with XL-CGD to provide a better understanding of diagnosis, treatment, and prenatal diagnosis of CGD.

Method Patients were diagnosed by DHR-1,2,3 flow cytometry assays and gene analysis. X chromosome inactivation analysis and $gp91^{phox}$ protein test were used for a female infant with XL-CGD.

Results XL-CGD accounts for the majority of cases in China and results in higher susceptibility to some infections than AR-CGD. The DHR assay can help diagnose CGD quickly, and atypical results should be combined with clinical manifestations, genetic analysis, and regular follow-up. For prenatal diagnosis, both gDNA and cDNA genotypes of amniotic fluid cells should be identified, and cord blood DHR assays should be performed to identify female XL-CGD patients.

Keywords Chronic granulomatous disease · dihydrorhodamine-1,2,3 assay · gene analysis · prenatal diagnosis · X inactivation

Introduction

Chronic granulomatous disease (CGD) is a rare inherited immunodeficiency disease caused by defects in the NADPH oxidase complex in neutrophils and macrophages, which cannot effectively produce superoxide anions and their metabolites to kill peroxidase-positive bacteria and fungi. These defects make patients susceptible to recurrent, severe infections and excessive hyper-inflammatory responses at young ages. The NADPH oxidase complex consists of $gp91^{phox}$ (encoded by *CYBB*), $p22^{phox}$ (encoded by *CYBA*), $p47^{phox}$ (encoded by *NCF1*), $p67^{phox}$ (encoded by *NCF2*), and $p40^{phox}$ (encoded by *NCF4*) [1, 2]. After interaction with pathogens, these cells are activated, and NADPH oxidizes to produce reactive oxygen species (ROS) [3]. Mutation of one of these genes will lead a decrease in NADPH oxidase activity to different degrees and CGD. X-linked CGD (XL-CGD) is caused by *CYBB* gene defects and is the most common genotype (70% of CGD cases), while *NCF1* (20%), *NCF2* (5%), *CYBA* (5%), and *NCF4* gene defects result in autosomal recessive CGD (AR-CGD) [4]. However, only a few patients with *NCF4* deficiency have been reported, and they have presented with mild

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CGD [5]. Recently, a new gene, *CYBC1*, related to CGD, has been found in the AR inheritance mode. A defect in the *CYBC1* gene can result in a decrease in the expression of the main subunit of NADPH oxidase ($gp91^{phox}$), which leads to CGD [6].

CGD patients can develop their first clinical manifestation at a young age or even at birth. They often suffer from repeated serious infections caused by bacteria and fungi such as *Staphylococcus aureus*, gram-negative *Enterobacter* sp., *Salmonella enterica*, *Aerobacter* sp., *Klebsiella pneumoniae*, *Serratia marcescens*, *Burkholderia cepacia*, *Aspergillus fumigatus*, and *Candida albicans* in the USA [7]. In large cohort studies, lung infections, including pneumonia and lung abscesses, are the predominant manifestations, and other symptoms include lymphadenitis and skin and gastrointestinal tract infections [8, 9].

To better understand the clinical and laboratory test characteristics, gene features, treatment, and prognosis, as well as the prenatal diagnosis of CGD patients in China, our study collected 114 cases of CGD patients and 15 prenatally diagnosed fetuses from 2003 to 2018 in Children's Hospital of Chongqing Medical University.

Patients and Methods

Patients

From November 2003 to August 2018, we screened 348 children with recurrent infection by dihydrorhodamine (DHR)-1,2,3 tests and enrolled 114 patients diagnosed with CGD based on clinical manifestations and molecular and genetic analysis in Children's Hospital of Chongqing Medical University. The study was approved by the ethics committee of Children's Hospital of Chongqing Medical University, and the consent of children and their parents was obtained.

Neutrophil Oxidative Function Tests

Neutrophil oxidative function was detected by two methods: the nitroblue tetrazolium (NBT) test and the DHR-1,2,3 flow cytometry assay. After stimulation with phorbol 12-myristate 13-acetate (PMA), normal neutrophils reduce the yellow nitroblue tetrazolium dye to formazan, and blue-black deposits of formazan particles are visible upon microscopic inspection, whereas neutrophils from patients with CGD do not reduce the dye [10]. The principle of the DHR-1,2,3 flow cytometry assay is that in the presence of peroxidase or an equivalent catalyst, H_2O_2 oxidizes DHR to rhodamine, which emits fluorescence when stimulated [11]. DHR assays were performed as previously described, with modifications [12]. For each 50-μl blood sample, two reactions were prepared as follows: in one tube, blood was incubated with 20 μl of 1.6 mol/l PMA (Sigma) and 6 μl of 40 mol/l DHR (Sigma); in another tube, blood was incubated with 20 μl of phosphate-buffered saline (PBS) and 6 μl of 40 mol/l DHR. All reactions were incubated for 20 min at 37 °C. After incubation, the samples were analyzed using CellQuest software (BD FACSCalibur™ Flow Cytometer, USA). The results are based on the stimulation index (SI, SI = geometric mean of fluorescence intensity of PMA-incubated neutrophils/geometric mean of fluorescence intensity of PBS-incubated neutrophils).

Genetic Analysis

Genomic DNA (gDNA) and total mRNA from the patients and family members were isolated from whole blood cells according to the manufacturer's recommendations. Genomic DNA was extracted using a Blood Mini Kit (TIANGEN BIOTECH, Beijing, China). Total mRNA was isolated using an RNAprep Pure Blood Kit (BioTeke, Beijing, China) and reverse transcribed into cDNA using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Dalian, China). The *CYBB*, *CYBA*, *NCF1*, and *NCF2* genes were amplified by polymerase chain reaction (PCR) using the Prime Script RT Reagent Kit (TaKaRa, Dalian, China) according to the standard protocol. PCR products were confirmed by gel electrophoresis and then sent to Sangon Bio. (Chengdu, China) for sequencing by the Sanger sequencing method. 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, the possibility of single nucleotide polymorphisms (SNPs) was ruled out.

Prenatal Diagnosis

Female carriers were advised to have prenatal screening performed when they became pregnant again. Amniotic fluid (30 ml) was collected at 20 weeks gestation using ultrasound guidance. DNA and RNA were extracted from amniotic fluid cells. Direct sequencing of gDNA and cDNA regions of CGD proband mutation region in fetal families was 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 whether the fetus was healthy, umbilical cord blood (2 ml) was collected at 26-week gestation to detect the respiratory burst of neutrophils by DHR assay.

X chromosome Inactivation Analysis

The HUMARA site on the X chromosome contains two methyl-sensitive *Hpa*II restriction sites. X chromosome inactivation can be analyzed by PCR amplification of the

polymorphism of the CAG repeats in HUMARA gene exon 1. When the X chromosome is inactivated, the cleavage site of the methylation-sensitive endonuclease *Hpa*II located near the repeats is methylated and cannot be cut, whereas the site of an active X chromosome exhibits the opposite characteristic [13]. A total of 5 μl of gDNA extracted from the peripheral blood of patients and a healthy female control were digested overnight with *Hpa*II at 37 °C to digest all unmethylated DNA at this locus. PCR amplification of the digested and undigested DNA was performed using FAM-labeled fluorescent primers with the following conditions: 5 s at 95 °C; 30 s at 60 °C; and 15 s at 72 °C for 50 cycles. The products were detected by Sangon Bio. (Shanghai, China). The peak sizes corresponding to the 2 alleles before and after *Hpa*II digestion were compared by the Gene Mapper ID (V3.2, Applied Biosystems) program to determine the X chromosome inactivation ratio in each female. The ratios were determined through the use of a previously described formula [14]; X chromosome inactivation ratios: $X(A/B)\ \text{Inactivation}\% = B_{\text{digested}}/[(B_{\text{undigested}} \times A_{\text{digested}})/A_{\text{undigested}} + B_{\text{digested}}] \times 100\%$.

Gp91^{phox} Detection

Peripheral blood was collected and divided into two tubes, each of which contained 50 ml. The positive tube was incubated at room temperature for 30 min with 1 ml of anti-human cytochrome b558 mAb (MBL Company, USA). The expression of gp91^{phox} in neutrophils was detected using a flow cytometry-based assay.

Statistical Analysis

Statistical analysis was accomplished using SPSS statistical software version 20.0. The clinical manifestations were analyzed, and the differences were determined by *t*-test and X^2 test. p values < 0.05 were considered significant.

Results

Patient Demographics

From 2003 to 2018, we diagnosed 114 patients with CGD, of whom 109 were male, and 5 were female. A total of 76 cases were diagnosed after 2014, and the other 38 cases (P77-P114) were previously reported [15]. Patients were from 20 provinces and cities in Mainland China, and 62 cases (54%) of the patients came from southwestern China, including 29 cases (25.4%) in Sichuan, 20 (17.5%) in Chongqing, and 10 (8.8%) in Guizhou. A total of 114 patients came from 109 families (P44 and P45 are twin brothers, P87 and P110 are brothers, P74 and P109 are brothers, P93 and P94 are twin brothers, and P38 is the younger sister of P93 and P94). Among them, 49 patients (43%) had positive family histories.

The mean age at onset was 4 months (range, 2 days to 72 months; median, 1 month). A total of 50% of patients developed symptoms within 1 month, 85% developed symptoms within 6 months, and the remaining 14% developed symptoms within 2–3 years of age. Only one patient (P67) had a late onset age of 6 years. The mean age at diagnosis was 23.1 months (range, 10 days to 172 months; median, 12 months). A total of 50% of the patients were diagnosed by 1 year of age, and 64% were diagnosed by 2 years of age. By the end of December 2018, of 102 patients who were followed up, 33 had died (including P10 and P94 after HSCT), with a mean age of 40.2 months (range, 6 months to 120 months; median, 31 months). Twelve patients were lost to follow-up.

Infections

Patients presented with recurrent fever (100%), pneumonia (92.1%), abscess (73.7%), lymphadenitis (58.8%), hepatosplenomegaly (58.8%), diarrhea (45.6%), and sepsis (30.7%) as the main manifestation, while other common infections were thrush (16.7%), urinary tract infection (7%), and osteomyelitis (4.4%). A total of 98 cases received the BCG vaccination, of which 54 cases (54/98,55.1%) had definitive BCGosis [suppuration at inoculation site, enlargement of ipsilateral axillary lymph nodes, tuberculosis (TB) manifestations in a short time after BCG inoculation, etc.]. A total of 24 patients (21.1%) had TB infections, and the main infected organs of TB were lungs, followed by bone, brain, and abdominal cavity. However, acid-fast bacilli were detected in only 8 patients (6 were BCGosis and 2 were TB), and the remaining patients were clinically diagnosed (Table 1). The age of onset and diagnosis of patients with XL-CGD was slightly earlier than that of AR-CGD patients, and the incidence of infections was higher for XL-CGD patients than that for AR-CGD patients. The others difference was not significant except for the higher incidence of BCGosis for XL-CGD patients than for AR-CGD patients ($p = 0.009$).

All patients had bacterial infections, and antibiotic treatment was effective; however, only 37 patients exhibited evidence of a bacterial infection. The main bacteria-causing infections were *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Escherichia coli*, *Staphylococcus epidermidis*, and *Moraxella catarrhalis*. A total of 48 patients were diagnosed with or suspected of having deep fungal infections, and 32 patients displayed explicit evidence of fungal infections, mainly due to strains of *Candida albicans* and *Aspergillus fumigatus* (Table 2). There was no significant difference between XL-CGD

Table 1 Comparison of the clinical features of the 114 patients in our study

	XL-CGD ($n = 95$)	AR-CGD ($n = 13$)	Unknown ($n = 6$)	All ($n = 114$)
Age of onset, mean (months)	3.8	4.3	5.3	4.0
Age of diagnosis, mean (months)	22.9	29.9	10.4	23.1
Pneumonia (%)	92.6%	92.3%	83.3%	92.1%
Hepatosplenomegaly (%)	62.1%	38.5%	50.0%	58.8%
Lymphadenitis (%)	61.1%	53.8%	33.3%	58.8%
Probable/disseminated BCG disease (%)	59.8% (49/82)	18.2% (2/11)	50% (2/4)	55.1% (54/98)
Recurrent diarrhea or Enteric infections (%)	47.4%	30.8%	50.0%	45.6%
Perianal abscesses (%)	42.1%	15.4%	50.0%	37.7%
Skin abscesses (%)	41.1%	38.5%	50.0%	41.2%
Sepsis (%)	33.7%	15.4%	16.7%	30.7%
Tuberculosis (%)	22.1%	23.1%	16.7%	21.1%
Deep abscess (%)	20.0%	15.4%	0.0%	18.4%
IBD or Crohn's disease (%)	9.5%	15.4%	33.3%	11.4%
Urinary tract infections (%)	8.4%	0.0%	0.0%	7.0%
Osteomyelitis (%)	5.3%	0.0%	0.0%	4.4%

and AR-CGD patients from an etiological point of view. However, the four main mutation types of XL-CGD were associated with different infection rates for *Klebsiella pneumonia* ($p = 0.01$) and total fungal ($p = 0.04$) infections.

Laboratory Features

Peripheral blood cells count, CRP, and immunoglobulin levels were analyzed in most patients. Refer to the normal values

Table 2 Isolation of microorganisms from the 114 patients in our study

	XL-CGD ($n = 95$)	AR-CGD ($n = 13$)	p value	4 main mutant types of XL-CGD					Unknown ($n = 6$)	All (n = 114)
				Splicing error ($n = 30$)	Deletion ($n = 27$)	Missense ($n = 18$)	Nonsense ($n = 18$)	p value		
Bacteria	32	3	0.44	7	13	5	7	0.22	2	37
Klebsiella pneumoniae	8	1	0.93	1	6	0	1	0.01	0	9
Staphylococcus aureus	7	1	0.97	1	2	2	2	0.66	0	8
Escherichia coli	6	0	0.35	2	3	0	1	0.27	0	6
Staphylococcus epidermidis	5	0	–	2	1	1	1	–	1	4
Burkholderia cepacia	3	0	–	1	2	0	0	–	0	3
Moraxella catarrhalis	2	1	–	0	1	0	1	–	1	4
Haemophilus influenzae	2	0	–	0	1	0	1	–	0	2
Pseudomonas aeruginosa	2	0	–	0	1	1	0	–	0	2
Acinetobacter baumannii	2	0	–	0	0	1	1	–	0	2
Streptococcus pneumoniae	1	0	–	1	0	0	0	–	0	1
Viridans streptococci	1	0	–	0	0	0	1	–	0	1
Clostridium prazmowski	1	0	–	1	0	0	0	–	0	1
Serratia marcescens	0	1	–	0	0	0	0	–	0	1
Fungus	27	3	0.68	4	9	4	9	0.04	2	32
Candida albicans	16	1	0.40	2	5	3	6	0.13	1	18
Aspergillus fumigatus	12	2	0.78	2	4	2	4	0.36	2	16
Acid-fast bacilli	8	0	0.28	1	2	1	2	0.75	2	10

"–" indicates too few to analysis

Table 3 DHR-1,2,3 assay and NBT test results in different types of CGD

	XL-CGD		AR-CGD		Unknown		All	
SI, mean (IQR)	1.58 (0.98, 1.72)	$n = 95$	1.81 (1.14, 2)	$n = 13$	1.58 (1.07,1.57)	$n = 6$	1.62 (1.01, 1.76)	$n = 113$
NBT, mean (IQR)	0.60 (0, 0)	$n = 84$	1.25 (0, 2)	$n = 12$	0.60 (0, 0)	$n = 5$	0.68 (0, 1)	$n = 101$

IQR interquartile range

range of healthy children of different ages in China to interpret the results [16, 17]. Routine blood examination was performed on all patients. The white blood cell count (WBC) and C-reactive protein (CRP) of most patients were higher than normal. An immunoglobulin test (performed in 109/114 patients) showed that 31 patients had hyper-IgM, 26 had hyper-IgA, 21 had hyper-IgE, and 20 had hyper-IgG. Lymphocyte classification (94/114) showed that the proportion of T cells, B cells, and (or) natural killer (NK) cells was altered in 60 cases.

NBT test (101/114) results ranged from 0 to 6% with a mean of 1%. The patients' SI of DHR assay (113/114) ranged from 0.1 to 15 with a mean of 1.67; 95 patients (84%) had an SI less than 2. Only 11 patients had SI more than 3, while 3 patients had SI more than 5, and one patient had SI of 15 (Table 3). DHR assay results were obtained for a healthy control, XL-CGD patients, a carrier, and a BCGosis patient without CGD. The stimulated peak of a healthy control (who was not related to patient) had a complete shift (Fig. 1a). The PMA-stimulated peak of most of CGD patients (P7) should almost overlap with the unstimulated peak (Fig. 1b). A *CYBB* mutation gene carrier (mother of P7) showed two different peaks after PMA stimulation: one peak overlapped with the unstimulated peak, while the other peak was completely shifted (Fig. 1c). There were some patients showed variations: the SI of P105 was 15; the result showed that the PMA-stimulated peak was obviously shifted compared with that of the unstimulated peak, but the displacement was substantially smaller than that of the healthy control (Fig. 1d). Another noteworthy result was in a BCGosis patient, who came to the hospital at the age of 1 year because of repeated fevers and convulsions. She had pneumonia, purulent meningitis, sepsis, suppuration in the inoculation site of BCG, and ipsilateral axillary lymph node swelling and abscess. At that time, her SI was only 0.94, and no suspicious gene mutation was found. After 4 months of treatment, she underwent a second

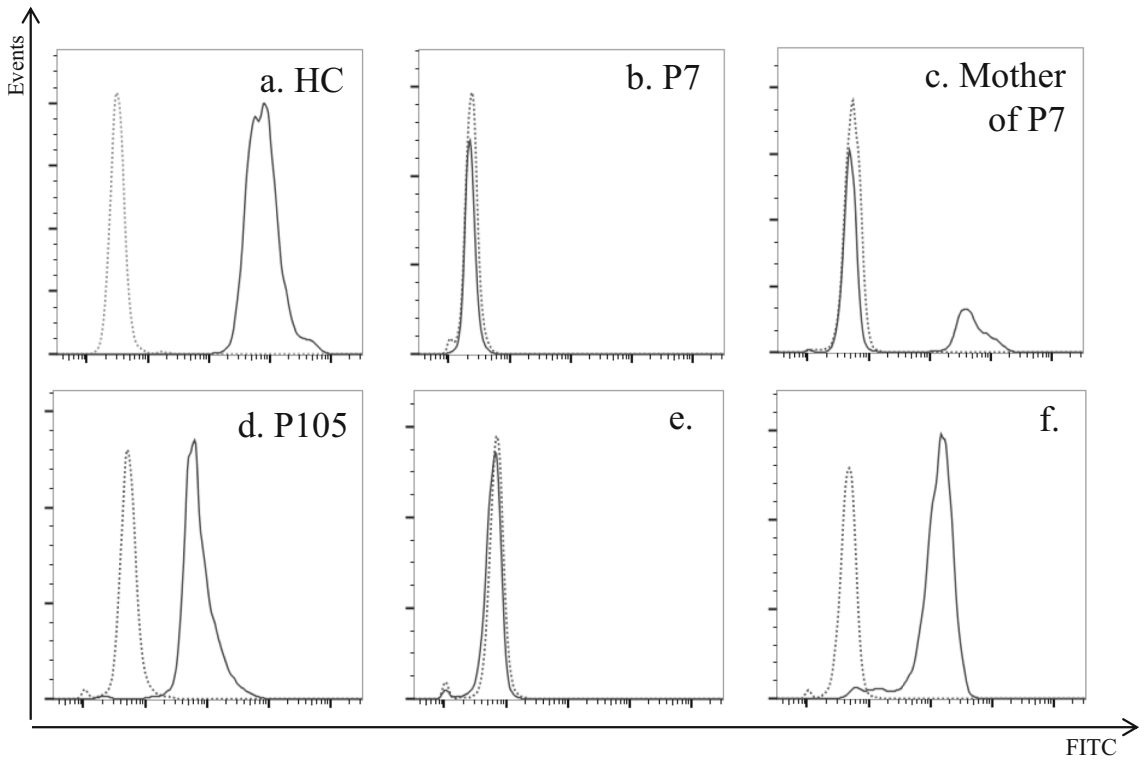

Fig. 1 DHR-1,2,3 flow cytometry results and variations. Unstimulated (gray dotted line), PMA-stimulated (black line). **a** A healthy control. **b** A XL-CGD patient. **c** A carrier. **d** A XL-CGD patient with variation. **e**, **f** Two different results for the BCGosis patient in January and May 2018.

DHR assay after obvious improvement with an SI reaching 28.76; therefore, she was not diagnosed with CGD at the time and needed further follow-up (Fig. 1e and f).

Gene Analysis

A total of 97 different mutation sites were identified, 34 of which were novel mutation sites (Table 4, not reported) [15, 18–21]. There were 95 (83.3%) patients with *CYBB* gene mutations (94 male and 1 female) consisting of 30 (31.6%) splicing errors, 27 (28.4%) deletion mutations (including 3 large gene deletions), 18 (18.9%) missense mutations, 18 (18.9%) nonsense mutations, 1 promoter mutation, and 1 insertion. A total of 8 (7%) patients had *CYBA* gene mutations, 3 (2.6%) had *NCF1* gene mutations, and 2 (1.8%) had *NCF2* gene mutations. In 6 cases, no mutation gene was found in the *CYBB*, *CYBA*, *NCF1*, *NCF2*, and *NCF4* genes in either gDNA or cDNA. The *CYBC1* gene was also investigated in two patients (P4 and P9), but no mutation site was found. Among the patients with a *CYBB* mutation, c.252G>A was found in 4 unrelated patients, c.676C>T in 3 unrelated patients, and c.868C>T in 4 unrelated patients. For the *CYBA* gene, c.152T>A was found in 3 unrelated patients (Fig. 2).

Prenatal Diagnosis

Through gDNA and cDNA sequencing of amniotic fluid exfoliated cells and sex detection, we screened 15 cases consisting of 7 patients (6 XL-CGD and 1 AR-CGD), 5 healthy fetuses (3 male and 2 female), and 3 carriers.

P38 is one of 3 carriers who had a prenatal diagnosis as a female carrier with a heterozygous mutation at the gDNA level at the same site as her two twin brothers (P93 and P94) with XL-CGD. At the age of 2 months and 21 days after birth, she came to the hospital with a perianal abscess as the first symptom. She was born with neonatal ABO hemolysis and mild eczema, vaccinated with BCG at 1 month, while the inoculation began to form ulcers at 2 months, ulcers formed a scar after about 20 days. Some laboratory test results were as follows: WBC = 12.75 (10^9/l) with 49% neutrophils and 41% lymphocytes; CRP< 8 (mg/l); and NBT = 0%. For further diagnosis, we performed DHR assay, gene sequencing, X chromosome inactivation test, and $gp91^{phox}$ test. Her SI was 0.65 (Fig. 3a); $Gp91^{phox}$ was not expressed at all on the neutrophils of patients and was on 22.4% of her mother's neutrophils (Fig. 3b); the gDNA showed a heterozygous deletion mutation in *CYBB* exon 9 (c.1123delG), while a homozygous deletion mutation in the cDNA resulted in a change in p.Glu375SerfsX11 (Fig. 3d). X chromosome inactivation results showed that the maternal chromosome of P38 produced a 292-bp product, and the paternal chromosome produced a 287-bp product; only one 292-bp product was found after *Hpa*II digestion (Fig. 3c). The paternal X chromosome of P38 was inactivated, and a skewed ratio of 99.5% was observed. The X chromosome of the mother was partially inactivated, and the ratio was 76.5%. The patient did not take drug to prevent infection and has had 4 bouts of pneumonia, 1 axillary lymph node ulceration, 1 cervical lymph node suppuration, and repeated diarrhea episodes before the age of one and a half. After she was hospitalized for pneumonia and hemoptysis, she started taking sulfamethoxazole and itraconazole regularly. Currently, the patient is more than 2 years old and is waiting for transplantation.

Treatment and Outcome

Attention should be paid to the care of patients in daily life, including avoiding excessive exposure to a complex public environment and contact with items containing more *Aspergillus fumigatus*. We suggest that all CGD patients take lifelong prophylactic sulfamethoxazole (30 mg/kg/d, divided twice daily) and itraconazole (5 mg/kg/d) to prevent infection. Antibiotics were selected according to the etiological sensitivity results of blood culture and sputum culture. By December 2018, 12 patients were lost to follow-up, and 29 underwent transplantation (2 of whom died of rejection). Of the remaining 73 nontransplant patients, 48 (66%) failed to take long-term prophylactic treatment. The mean age of follow-up patients was 36.9 months, the median was 30.5 months, and 25 (52%, 25/48) patients died. Meanwhile, other 25 (34%) patients who had long-term prophylactic treatment had occasional fever, diarrhea, pneumonia, lymphadenitis, and other infections. Among these patients, the mean age was 58.6 months, the median was 42 months, and 6 patients (24%, 6/25) died.

To date, 29 of the patients have undergone HSCT, 2 of them died of rejection and severe infection after transplantation, and one of them failed. Twenty-six patients were successfully transplanted, and the mean age at transplantation was 34.4 months (range, 7 to 112 months). Seventeen patients received stem cells from unrelated donors (including 3 umbilical cord blood sources), 8 patients received HLA-matched sibling donors, and 4 patients received HLA-mismatched paternal stem cells (Table 5). Patients were required to take cyclosporine and receive gamma globulin monthly for at least half a year after transplantation and to take sulfamethoxazole and itraconazole for several months. Most patients showed good conditions or only slight rejection after transplantation, and both the SI and NBT results returned to normal levels within 3 months. P53, who has been transplanted for 15 months, received his carrier sister's stem cells and is currently in good condition. His DHR assay results show two peaks similar to carriers, with an SI = 35.8. The gene mutation has not yet been found for P4 and P96; their SIs were 3.26 and 1.22, respectively, and both developed pneumonia, BCGosis, TB, and hepatosplenomegaly at 2–3 months of age. P96 also had multiple skin and perianal abscesses and inflammatory

Table 4 Genetic mutations and carriers of the 114 CGD patients and their family members

Patient no	Affected gene	Exon/ intron	Nucleotide change	Mutation	Predicted codon change	Reported or not [ref]	Carriers
1	CYBB	Promoter	c.-66T>C	Promoter	NA	N	Mother
2	CYBB	Exon7	c.676C>T	Nonsense	p.Arg226X	[18]	Mother
3	CYBB	Exon9	c.958delG	Deletion	p.Glu320LysfsX23	[18]	Mother
4	–	–	–	–	–	–	–
5	CYBB	Exon3	c.252G>A	Splice site	del exon3 p.Ser48_Ala84del	[18]	Mother
6	CYBB	Intron1	c.45+1G>A	Splice site	del exon1? p.Met1_ Ile15del? no start protein prod	[18]	Mother
7	CYBB	Exon7	c.746C>A	Nonsense	p.Ser249X	N	Mother
8	CYBB	Intron2	c.141+4A>G	Splice site	del exon2? p.Leu16_ Gly47del	N	Mother
9	–	–	–	–	–	–	–
10	CYBB	Exon2	c.46_141del96	Deletion	del exon2? p.Lue16_ Gly47del	N	Mother
11	CYBB	Exon9	c.1149_1150delTA	Splice site	p.Lys384ArgfsX2, p.Ile385X	N	Mother
12	CYBB	Exon7	c.801delT	Nonsense	p.Met268X	N	Mother
13	CYBB	Exon8	c.868C>T	Nonsense	p.Arg290X	[18]	Mother
14	CYBB	Exon5	c.338_483del146bp	Deletion	del exon5? p.Ile114_ Lys161del	N	Mother
15	CYBB	Exon9–13	Large deletion	Deletion	del exon9–13?	N	–
16	CYBB	Exon5	c.469C>T	Nonsense	p.Arg157X	[18]	Mother
17	CYBB	Exon1–7	Large deletion	Deletion	del exon1–7?	N	Mother
18	CYBB	Exon7	c.769T>C	Missense	p.Cys257Arg	[18]	Unidentified
19	CYBB	Exon11	c.1359_1361GTT>TG	Deletion	p.Trp453fsX501	N	Mother
20	CYBB	Exon9	c.916_919delAAAAinsAAG*	Deletion	p.Lys306fsX6	N	Mother
21	CYBB	Exon10	c.1244C>A	Missense	p.Pro415His	[18]	Mother
22	CYBB	Exon12	c.1468_1474delCACTTTG	Deletion	p.His490fsX499	N	Mother
23	CYBB	Exon4	c.254_312del59	Deletion	p.Cys86MetfsX17	[19]	Unidentified
24	CYBB	Intron7	c.804+1G>A	Splice site	del exon7 p.Arg226fsX5	[18]	Mother
25	CYBB	Intron3	c.252+5G>C	Splice site	del exon3? p.Ser48_ Ala84del?	[18]	Mother
26	CYBB	Intron2	c.141+5G>A	Splice site	del exon2 p.Lue16_ Gly47del	[18]	Unidentified
27	CYBB	Exon9	c.1085C>T	Missense	p.Thr362Ile	[18]	Mother
28	CYBB	Intron4	c.337+1G>T	Splice site	del exon4 p.Cys85ArgfsX15	[18]	Mother
29	CYBB	Intron9	c.1152-1G>A	Splice site	alt splice site>1 nt del p.Ile385X	[18]	Mother
30	CYBB	Exon9	c.925G>A	Missense	p.Glu309Lys	[18]	Mother
31	CYBB	Exon5	c.394delA	Deletion	p.Asn132IlefsX8	N	Mother
32	CYBB	Exon12	c.1531_1534delTATG*	Deletion	p.Tyr511AspfsX21	N	Mother
33	CYBB	Exon9	c.1104delT	Deletion	p.Cys369ValfsX17	N	Mother
34	CYBB	Exon2	c.99T>G	Nonsense	p.Tyr33X	N	Mother
35	CYBB	Exon7	c.737dupA	Insertion	p.Ile248AsnfsX36	N	Mother
36	CYBB	Exon3,4	c.203_313del111bp	Nonsense	p.Arg226X	N	Unidentified
37	CYBB	Exon2	c.65A>T	Missense	p.Asn22Ile	N	Mother
38	CYBB	Exon9	c.1123delG	Deletion	p.Glu375SerfsX11	[15]	Mother
39	CYBB	Exon9	c.931C>T	Nonsense	p.Gln311X	N	Mother
40	CYBB	Intron6	c.674+4_+7delAGTG	Splice site	Multiple splice products	[18]	Mother
41	CYBB	Intron1	c.45+2delT	Splice site	del exon1? p.Met1_ Ile15del?	N	–

Table 4 (continued)

Patient no	Affected gene	Exon/ intron	Nucleotide change	Mutation	Predicted codon change	Reported or not [ref]	Carriers
42	CYBB	Exon3	c.252G>A	Splice site	no start protein prod del exon3 p.Ser48_Ala84del	[18]	Mother
43	CYBB	Exon9	c.1064T>A	Missense	p.Ile355Asn	N	Mother
44	CYBB	Exon9	c.1064T>A	Missense	p.Ile355Asn	N	Mother
45	CYBB	Intron7	c.804+2T>C	Splice site	del exon7 p.Arg226LeufsX5	[18]	Unidentified
46	CYBB	Exon11	c.1459C>T	Nonsense	p.Gln487X	N	Mother
47	CYBB	Exon1	c.25G>A	Missense	p.Gly9Arg	N	Mother
48	CYBB	Intron1	c.46-2A>G	Splice site	del exon2 p.Leu16_ Gly47del	[18]	Unidentified
49	–	–	–	–	–	–	–
50	CYBB	Exon7	c.781C>T	Nonsense	p.Gln261X	[18]	Mother
51	CYBB	Exon8	c.897G>A	Splice site	del exon8 p.Thr269_ Lys299del	[18]	Mother
52	CYBB	Exon2	c.211_214delGTCT	Deletion	p.Cys72GlufsX4	[20]	Mother
53	CYBB	Exon12	c.1546T>C	Missense	p.Trp516Arg	[18]	Mother
54	CYBB	Exon1	c.1A>G	Missense	p.Met1Val, startcodon lost	[18]	Mother
55	CYBB	Exon4	c.301C>G	Missense	p.His101Asp	N	Mother
56	CYBB	Intron2	c.141+1G>A	Splice site	del exon2 p.Leu16_ Gly47del	[18]	Mother, his sister
57	CYBB	Exon6	c.560C>G	Missense	p.Ile187Arg	N	Mother, his sister
58	CYBB	Exon8	c.868C>T	Nonsense	p.Arg290X	[18]	Mother
59	–	–	–	–	–	–	–
60	CYBB	Exon12	c.1598_1600delGAG	Deletion	p.Gly533del	[18]	Mother
61	CYBB	Exon6	c.613delT	Deletion	p.Trp206GlyfsX8	N	Unidentified
62	CYBB	Exon3	c.252G>A	Splice site	del exon3 p.Ser48_Ala84del	[18]	–
63	CYBB	Exon8	c.824delG	Deletion	p.Gly275ValfsX38	N	Mother
64	CYBB	Exon3	c.252G>A	Splice site	del exon3 p.Ser48_Ala84del	[18]	Mother
65	CYBB	Exon8	c.868C>T	Nonsense	p.Arg290X	[18]	Mother
66	CYBB	Exon6	c.665A>G	Missense	p.His222Arg	[18]	Mother
67	CYBB	Exon1	c.4C>T	Missense	p.Gly2Trp	N	Unidentified
68	CYBB	Exon11	c.1328G>A	Nonsense	p.Trp443X	N	Mother, maternal grandmother
69	CYBA	Exon1,2	c.7C>T, c.70G>A	Missense, non-sense	p.Gly24Arg, p.Gln3X	[21]	Mother, father
70	CYBA	Exon4	c.205G>T	Nonsense	P.Gly69X	N	Mother
71	CYBA	Exon1,5	c.1A>G, c.353G>A	Missense	p.Met1Val, p.Ser118Asn	N	Mother, his sister
72	CYBA	Exon3	c.152T>G	Missense	p.Leu51Arg	[15]	Unidentified
73	CYBA	Exon3,4	c.152T>G, c.205T>G	Missense, nonsense	p.Leu51Arg, p.Gly69X	N	Mother, father
74	CYBA	Exon4	c.246_273del28bp	Deletion	p.Phe83SerfsX99	[15]	Unidentified
75	NCF2	Exon3	c.304C>T	Nonsense	p.Arg102X	[21]	Mother, father
76	NCF2	Exon2	c.233G>A	Missense	p.Gly78Glu	[21]	Mother, father
77	–	–	–	–	–	–	–
78	CYBB	Exon11	c.1327delT	Deletion	p.Trp443GlyfsX58	[18]	Mother, his 2 sisters, maternal grandmother
79	CYBB	Exon6	c.577T>C	Missense	p.Ser193Pro	[18]	Mother
80	CYBB	Exon8	c.868C>T	Nonsense	p.Arg290X	[18]	Unidentified
81	CYBB	Intron10	c.1315-2A>C	Splice site	p.Ile439_Gln487del?	[18]	Mother
82	CYBB	Exon6	c.565_568delATTA	Deletion	p.Ile189SerfsX23	[18]	

Table 4 (continued)

Patient no	Affected gene	Exon/ intron	Nucleotide change	Mutation	Predicted codon change	Reported or not [ref]	Carriers
							Mother, maternal grandmother
83	CYBB	Intron10	c.1151+2dupT	Splice site	del exon1 p.Ile385SerfsX62	[15]	Mother
84	CYBB	Intron5, exon5, exon6	c.483-484ins115bp, c.338_483del146bp, c.484_674del191bp	Splice site	p.Lys161ValfsX12, p.Ala113GlnfsX2, p.Asn162ThrfsX14	[15]	Unidentified
85	CYBB	Exon7	c.676C>T	Nonsense	p.Arg226X	[18]	Mother
86	CYBB	Intron5	c.483+1G>A	Splice site	del exon5, p.Ala113GlnfsX2	[18]	Mother
87	CYBB	Exon9	c.1078delG	Deletion	p.Asp360ThrfsX25	[15]	Mother
88	CYBB	Exon10	c.1170delC	Deletion	p.Phe391LeufsX13	[15]	Mother
89	CYBB	Exon7	c.725_726delCA	Deletion	p.Thr242SerfsX2	[15]	Mother
90	CYBB	Exon5	c.469C>T	Nonsense	p.Arg157X	[18]	Mother
91	CYBB	Exon7	c.676C>T	Nonsense	p.Arg226X	[18]	Mother
92	CYBB	Exon2	c.46_92del47	Splice site	p.Leu16ArgfsX2	[15]	Unidentified
93	CYBB	Exon9	c.1123delG	Deletion	p.Glu375SerfsX10	[15]	Mother
94	CYBB	Exon9	c.1123delG	Deletion	p.Glu375SerfsX10	[15]	Unidentified
95	CYBB	Exon9	c.1082G>T	Missense	p.Trp361Leu	[15]	Mother
96	–	–	–	–	–	–	–
97	CYBB	Intron2	c.46-2A>G	Splice site	del exon2, p.Leu16_Gly47del	[18]	Mother
98	CYBB	Exon5	c.338_674del337	Splice site	p.Ala113AspfsX16	[15]	Unidentified
99	CYBB,XK, DYNLT3		All three genes deleted	Deletion	del XK,CYBB,DYNLT3	[21]	Unidentified
100	CYBB	Exon6,7	c.484_804del321	Splice site	p.Asn162_Met268del	[15]	Unidentified
101	CYBB	Intron6	c.483+1delG	Splice site	p.Asn113AspfsX16	[15]	Mother
102	CYBA	Exon3	c.152T>G	Missense	p.Leu51Arg	[15]	Unidentified
103	NCF1	Exon2	c.75_76delGT	Deletion	p.Tyr26HisfsX25	[15]	Unidentified
104	CYBB	Exon12	c.1548G>C	Missense	p.Trp516Cys	[15]	Mother, his sister
105	CYBB	Exon3	c.162G>C	Missense	p.Arg54Ser	[18]	Mother
106	NCF1	Exon8,9	c.763_800del38bp, c.923 T>C	Deletion, missense	p.Val255GlufsX92, p.Val308Ala	[15]	Mother
107	CYBB	Intron6	c.483+1336T>G	Splice site	c.675-676ins81bp, p.Arg226TyrfsX18	[15]	Mother
108	NCF1	Exon2	c.75-76delGT	Deletion	p.Tyr26HisfsX25	[21]	Mother, father
109	CYBA	Exon4	c.246_273del28bp	Deletion	p.Phe83SerfsX98	[15]	Mother, father
110	CYBB	Exon9	c.1078delG	Deletion	p.Asp360ThrfsX25	[15]	Mother
111	CYBB	Exon9	c.965delG	Deletion	p.Gly322AspfsX20	[18]	Unidentified
112	CYBB	Exon9	c.1120C>T	Nonsense	p.Gln374X	[15]	Unidentified
113	CYBB	Intron5	c.483+1G>C	Splice site	del exon5, p. A113QfsX2	[15]	Mother
114	CYBB	Intron7	c.805-1G>T	Splice site	del exon8?, p.Thr269_Lys299del?	[15]	Mother

"–" indicates unidentified. *N*, not reported

bowel disease (IBD). Although no gene mutations were found, HSCT was performed for both of them. One year after transplantation of P4 and 6 years after transplantation of P96, they are in good condition with no obvious symptoms of recurrent infection, and their SIs increased to 106.85 and 124, respectively.

Discussion

Here, we described in detail the clinical features, laboratory tests, gene analysis, and outcomes of 114 CGD patients from Mainland China and a female infant XL-CGD patient after prenatal diagnosis. The median age of onset of the 114

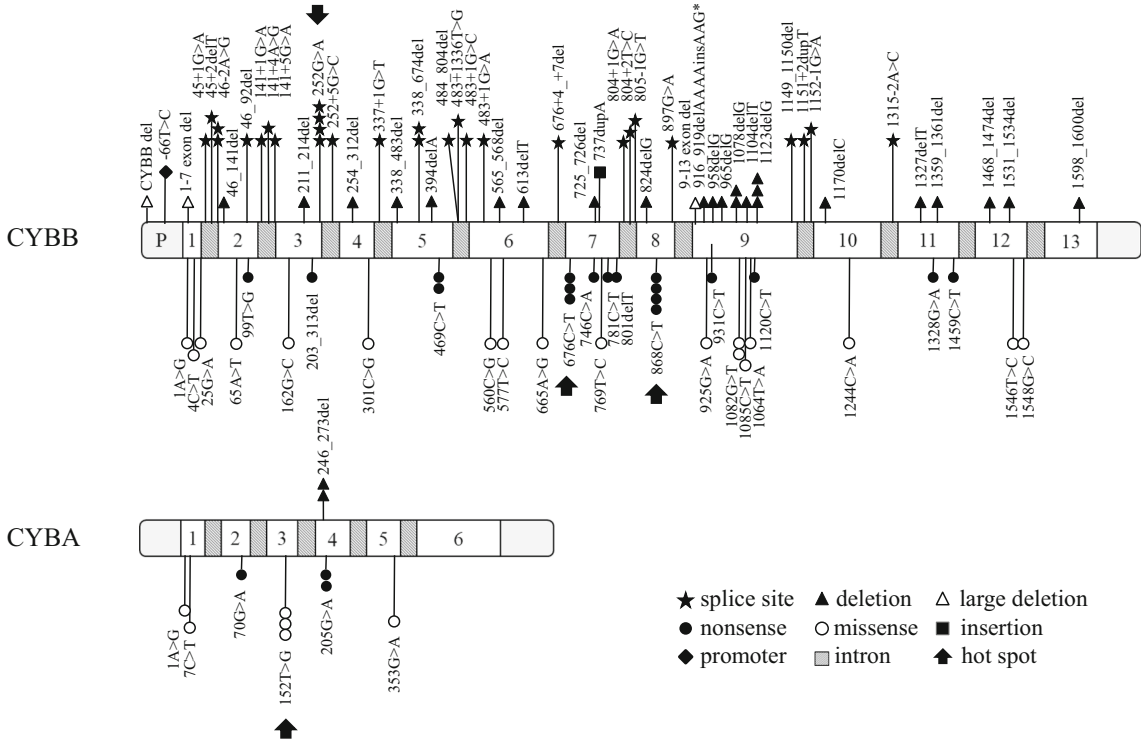

Fig. 2 Schematic overview of the distribution of CYBB and CYBA gene mutation sites

patients was 1 month, and the median age at diagnosis was 12 months. These findings were similar to those of a Shanghai center in China [22, 23] but less than other those of centers outside of China [4, 8, 9, 22–24]. China requires that newborns be vaccinated with BCG within 24 h of birth, which may explain the younger age of onset and diagnosis of the patients in this study. XL-CGD patients had earlier onset and diagnosis ages than AR-CGD patients, but the differences were not significant (Table 1).

The main clinical manifestations of our patients were pneumonia, lymphadenitis, hepatosplenomegaly, diarrhea, sepsis, TB, and abscesses at different locations. The degree of pneumonia in CGD patients was often more severe than that in children without PID and was not easy to cure and required long-term hospitalization. In addition, these patients readily develop pneumonia complications, such as a lung abscess and other serious lung diseases. Some patients had lymph node ulcers (28.1%) and it was often difficult for these wounds to heal without surgery. CGD patients had high frequency of hospitalization. The average length of hospitalization due to pneumonia or diarrhea for children without CGD is 3–7 days, while CGD patients require at least half a month or even several months. At least 54 (47.4%) patients in our study had recurrent and refractory diarrhea, but only 13 (24.1%) of them were diagnosed with IBD by enteroscopy, and 2 (3.7%) of them had Crohn's disease. Usually, the incidence of gastrointestinal complications in CGD patients is more than one-third [25, 26]. In a study by Marciano et al., IBD in XL-CGD patients was at least 43% [27]. IBD has a high incidence in CGD patients, which may be related to genetic susceptibility to IBD as a risk factor for bowel inflammation in CGD patients [26]. Our patients had a lower incidence of IBD than that has been observed at other centers, and the main reason may be that only a few patients were willing to undergo enteroscopy. Another major symptom was infection after BCG vaccination, which was the first clinical manifestation of some of our patients. The proportion of BCGosis was high (55.1%, 54/98). XL-CGD patients had a higher rate of BCGosis than AR-CGD patients in our study ($p = 0.01$). In addition, 18 patients were vaccinated shortly before study conclusion, and it was not possible to determine whether there was BCGosis. Many studies found a high frequency of BCGosis in CGD patients [22, 23, 24]. Deficiency of oxidative burst may explain why CGD patients are susceptible to BCGosis [28]. Therefore, for infants with a suspected family history, BCG vaccination should be delayed, and for confirmed CGD infants, vaccination should be prohibited. Furthermore, infants with an abnormal BCG vaccination should be screened for CGD.

Through sputum, pus, and blood culture, the main bacterial species-causing infection was *Klebsiella pneumoniae* in our study, which is different from *Staphylococcus aureus* that had been reported in other centers [8, 9, 23]. The infection rate of *Candida albicans* was slightly higher than that of *Aspergillus fumigatus*, which was contrary to reports in the USA but consistent with those in India [7, 8]. Although it was difficult to

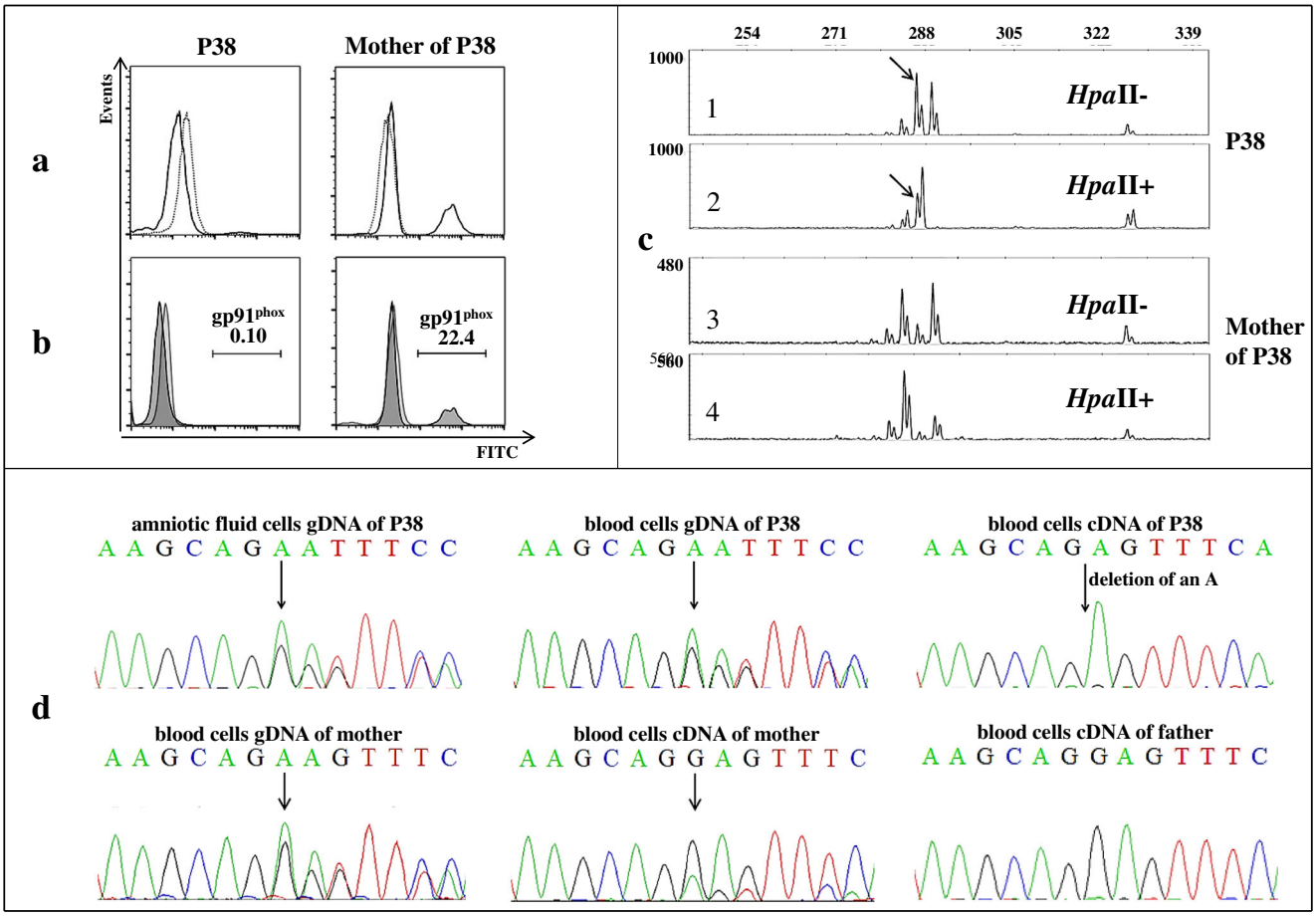

Fig. 3 (a–d) DHR-1,2,3 assay, gp91^{phox} protein test, gene analysis, and X chromosome inactivation detection results of P38

find direct evidence of fungal infections, more patients can be diagnosed with fungal infections by imaging examination and antifungal drugs than by direct observation of the pathogen. The detection rates of fungi and bacteria in this study were lower than those reported abroad, which may be due to the early diagnosis and application of antibiotics in our patients. There was no significant difference between XL-CGD and AR-CGD from an etiological point of view. However, infection rates of total *Klebsiella pneumonia* ($p = 0.01$) and fungal ($p = 0.04$) infections in XL-CGD patients with the four main mutation types (splicing error, deletion, missense, and nonsense) were different (Table 2).

Laboratory tests showed that almost all patients had a high inflammatory state, with high WBC and CRP levels, but the proportion of neutrophils was within the normal range overall. Fifty-six percent of patients had hyperimmunoglobulinemia, a phenomenon that has been reported in many studies [29] [30]. However, the high proportion of patients with hyper IgE (> 150 IU/ml) is not common, and the cause of the increase needs further study. In terms of lymphocyte classification, 64% of

Table 5 Transplant-related data in 29 patients with HSCT

Donor type	*N*	Age of transplant (median [month])	Age[§] (median [month])	Mean of SI (before/after HSCT)	Survival rate (%)
UD	14	18	30	1.89/108.28	100%
UCB	3	17	29	2.69/60.6	33.3%
RD (siblings)	7	38	40	1.62/77.86	100%
RD (father)	4	39	56	1.41/102.82	100%
RD (carrier sister)	1	8	21	0.39/38.62	100%

UD, unrelated donor stem cells; *UCB*, unrelated cord blood; *RD*, related donor stem cells; [§] until October 2018

patients had changes in the proportion of lymphocytes [31–34]. Whether there is a definite change in the number and proportion of lymphocytes in patients with CGD requires further observation and testing.

The main method of diagnosing CGD was the NBT test in the past; however, in recent years, DHR assay has gradually replaced NBT because of its convenience, reliability, and sensitivity [7, 35]. We found that the DHR assay of some patients had variations: the peak of PMA-stimulated cells had some displacement compared with that of most patients' cells, but the displacement degree was substantially smaller than that of healthy people, and the SI was also higher (≥ 3) than that of most patients but less than the limit (85.2–264.4) [12]. Wolach B et al. found that CGD patients with null ROS (SI< 3) had significantly earlier disease onset and diagnosis than those with residual ROS (SI≥ 3) [4]. Approximately 7.2% (6/83) of XL-CGD patients had residual ROS (SI≥ 3). However, although the SI of these patients was relatively as high as some carriers, they also have been many infections. P105 had an SI= 15 and developed pneumonia, BCGosis, TB, hepatosplenomegaly, and lymphadenitis at the age of 1 year. Ang EY et al. reported that five patients had the same condition as the BCGosis female patient [36]. DHR assay results may be transiently abnormal during acute illness and may be positive when assessed during an acute illness. When the symptoms of infection improved, although the SI of the BCGosis female patient had recovered, it had still not reached a normal level; thus, CGD could not be completely ruled out, and further follow-up was needed. Moreover, patients with myeloperoxidase (MPO) mutations may have false-positive results [11, 37]. Therefore, when patients exhibit DHR assay results with variations, we should make diagnose based on clinical manifestations and genetic analyses combined with the DHR assay, and these patients should be followed up strictly.

In our study, XL-CGD and AR-CGD accounted for 83.3% and 11.4% of CGD patients, respectively, which were proportions similar to those observed at a Shanghai center [22] but differed from those of other countries [4, 8, 9, 23, 24]. The proportion of AR-CGD cases of this study was significantly lower than that observed in other studies, possibly due to fewer consanguineous marriages in China. Of the four main mutation types of the *CYBB* gene, a splicing error was the most common, followed by deletion, nonsense, and missense mutations. A study in Latin America differed from ours, the authors of the study found that the most common type was nonsense mutations, followed by missense and splicing error, and deletion was the least common [23]. Moreover, we found four sites (c.252G>A, c.676C>T, and c.868C>T in *CYBB*; c.152T>A in *CYBA*) with a relatively high mutation frequency in our cohort, excluding the same mutation in one family (Fig. 2). In the *CYBB* gene, c.252G>A was found in 8 cases in Shanghai and in 4 cases in our center, while c.676C>T was found in 9 patients in Shanghai and in 3 patients in our center. Those sites may be hot spot mutations in Mainland China.

With regard to a prenatal diagnosis, we can determine whether the fetus has CGD to determine whether BCG vaccination should be administered after birth and whether umbilical cord blood cells can be reserved for transplantation as a donor for a sibling with CGD. In addition, if the fetus has CGD, we can perform HSCT during the prenatal period in future. Two methods can be used for prenatal diagnosis. One method is the direct sequencing of gDNA and cDNA from amniotic fluid cells or placental villi sampling (CVS); the other is DHR assay with cord blood. The best approach is to combine the two methods for the diagnosis of female XL-CGD infants. As fetus P38 had too few amniotic fluid cells to successfully extract RNA, therefore, cDNA sequencing could not be performed. Because of poor health of the mother during pregnancy, no cord blood cell DHR assay was performed. After disease onset, we found that the female carrier infant with CGD symptoms was born with an extremely skewed X inactivation ratio. A carrier with a *CYBB* gene defect secondary to an extremely skewed X inactivation event is rare (< 2% of all reported CGD cases) but has been observed [14, 38–43]. In contrast to other female XL-CGD cases, which begin at the age of 16–80 years, our patient developed infections from birth. P38 reminds us of the necessity of simultaneous analysis of gDNA and cDNA combined with cord blood DHR assays for the prenatal diagnosis of CGD.

At present, the main treatment of CGD is the prophylactic use of antibacterial (sulfamethoxazole) and antifungal (itraconazole) drugs and the controversial application of IFN-γ [22, 44–47]. Sulfamethoxazole is effective against most gram-negative bacteria and *Staphylococcus aureus* [48]. The main antifungal prophylactic drug itraconazole, which is mainly effective for *Aspergillus fumigatus*, showed a clear effect in the double-blind controlled experiment by Gallin et al. [49, 50]. According to reports from the USA, the mortality rates of XL-CGD and AR-CGD are approximately 21% and 8.6%, respectively [8]. Among our patients, the mortality rate was 28.9%, and it was higher in early stages (52.6% in 2003–2013). Only 9 (11.8%) patients who were diagnosed in the past 5 years have died. Patients taking long-term prophylaxis had a lower mortality rate (24%) than patients without long-term prophylaxis (52%). However, due to our patients coming from the entire country and a lack of follow-up, more detailed information could not be obtained. At present, HSCT is the main way to cure CGD, and the current graft survival rate is 70–100% [51, 52]. In our study, 25.4% (29/114) of patients underwent HSCT, and the success rate was 93.1% (27/29). The mean age of transplantation was younger than that of other centers in recent years [53, 54]. Four of the HSCT patients received hematopoietic stem cells provided by half HLA-matched

fathers, 1 received carrier sister's stem cells, and 2 of them had not had the mutation sites discovered yet; the CGD symptoms of these patients improved significantly, and the individuals are now in a healthy state. Therefore, CGD can be clinically diagnosed in patients with consistent clinical manifestations and defective neutrophil oxidative function for whom mutant genes cannot be found, and timely treatment should be applied to improve their quality of life.

There are still some deficiencies in this study. We were not able to analyze patients in China as a whole, as most patients were from southwestern China. Moreover, the number of AR-CGD patients was small, and patient management and follow-up were inadequate.

Conclusion

Here, we describe the clinical data, laboratory tests, gene mutations, and outcomes of 114 cases of CGD and a special case of XL-CGD in a female infant after prenatal diagnosis in Mainland China. DHR assay can diagnose CGD quickly. XL-CGD accounts for the majority of cases than AR-CGD. Lifelong prophylactic sulfamethoxazole and itraconazole can reduce infections. In prenatal diagnosis, both gDNA and cDNA should be analyzed. It is necessary to do cord blood DHR assays for female carriers and with uncertain genetic analysis.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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