Variant Type X91⁺ Chronic Granulomatous Disease: Clinical and Molecular Characterization in a Chinese Cohort

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Abstract

Purpose: We aimed to report the clinical and immunological characteristics of variant type X91⁺ CGD in a Chinese cohort.

Methods: The clinical manifestations and immunological phenotypes of X91⁺ CGD patients were collected. Dihydrorhodamine (DHR) analysis was performed to evaluate neutrophil function. Gp91^{phox} protein expression was determined by flow cytometry-based extracellular staining with the monoclonal antibody (mAb) 7D5.

Results: X91+CGD patients accounted for 8% (7/85) of all patients with GCD. The median onset age in the 7 X91⁺ CGD patients was 4 months. Six patients received the same BCG vaccine strain, and three had probable BCG infections. Moreover, 4 patients were highly suspected of having *Mycobacterium tuberculosis* infection. Recurrent infections of the lungs and soft tissues (3/7) were the most common symptoms. Two patients had noninfectious recurrent oral ulcers and received interferon gamma (IFN- γ) treatment afterward. In our cohort, the stimulation index (SI) of the 7 X91⁺ CGD patients ranged widely from 1.9 to 67.5, while the SI ranged from 1.2 to 35.7 in patents with X91⁰ CGD. The level of SI between these two groups was statistically significant ($P < 0.05$). CYBB mutations associated with X91+CGD were usually located in or near the FAD and NADPH binding domains. Three new X91+ CGD related mutations (c.1462-2 A>T, c.1243C>T and c.925G>A) were identified.

Conclusions: Variant type X91⁺ CGD may have varied severities of clinical manifestations. Moreover, the laboratory findings of X91⁺ CGD could present with a moderate neutrophil stimulation index. We should deepen our understanding of the X91⁺ variant CGD to prevent missed diagnosis.

1. Introduction

Chronic granulomatous disease (CGD) is a rare inherited primary immunodeficiency disease with an incidence of 1 case per 200,000 to 250,000 live births worldwide [1]. The pathogenesis of CGD is characterized by dysfunctional nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and decreased or no superoxide anion (O_2^-), resulting in significantly decreased bactericidal activities of neutrophils and other phagocytes. The clinical patterns of CGD patients generally include early onset recurrent bacterial, tuberculous or fungal infection, especially catalase-positive microbes, involving the lungs, lymph nodes, livers and bones [2]. The respiratory burst of neutrophils has been previously detected by the nitroblue tetrazolium (NBT) test and is currently detected by the dihydrorhodamine-1,2,3 (DHR) test in clinical practice. The respiratory burst of neutrophils is defective in CGD patients.

NADPH oxidase is a complex composed of membrane-bound gp91phox and p22phox and cytosolic p47phox, p67phox and p40phox. Gp91phox and p22phox together form the heterodimer cytochrome b559. Gp91phox is the enzymatic core of the NADPH oxidase complex, while other components play a regulatory role in the NADPH oxidase complex. The gp91phox is encoded by the cytochrome b-245 β

chain (*CYBB*) gene mapped at Xp21.1-p11.4, and its mutation results in X-linked CGD, accounting for 70% of CGD cases overseas and approximately 85% of cases in China [1, 3]. Most X-linked CGD patients have neither H₂O₂ production ability nor gp91phox expression, and this kind of CGD is named classic type X-linked CGD (X91⁰ CGD). However, some patients with *CYBB* mutations have low expression levels of functional or partially functional gp91phox (X91⁻ CGD) or normal expression levels of nonfunctional or partially functional gp91phox (X91⁺ CGD) [4]. X91⁻ CGD and X91⁺ CGD with *CYBB* mutations are named variant type X-linked CGDs. The characteristics of variant type X-linked CGD, especially X91⁺ CGD, differ from classical CGD, which may interfere with the rapid diagnosis of X-linked CGD using the stimulation index (SI) of DHR and the expression of gp91 protein in clinical practice.

However, few X91⁺ CGD patients have been reported to date. In this study, we reported the clinical and immunological characteristics of 7 Chinese patients with X91⁺ CGD, paid attention to the relationship with SI, and then reviewed the literature to further document the rare *CYBB* gene mutations resulting in variant type X-linked CGD.

2. Methods

This study was approved by the Ethics Committee of Children's Hospital of Fudan University. Written informed consent was obtained from the parents of all patients.

2.1 Patients and Clinical Data

Children with CGD admitted to our hospital between January 2018 and March 2021 were enrolled in this study. CGD was diagnosed according to the clinical manifestations, laboratory findings and genetic data. The relevant data were summarized in detail. Previous variant type X-linked CGD patients and mutations reported in PubMed Medline (<https://www.ncbi.nlm.nih.gov/pubmed/>) from 1987 to November 2021 were reviewed. The diagnostic criteria of BCG disease were described in a previous publication [2].

2.2 Routine Immune Function Evaluation

Routine blood counts and immunological functional analyses were performed. We used the nephelometry method to detect immunoglobulins, including IgG, IgA, and IgM, as previously reported, and lymphocyte subsets were measured by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

2.3 Neutrophil Function Evaluation

Dihydrorhodamine (DHR) analysis was performed to measure the respiratory burst of neutrophils by testing superoxide production. Briefly, neutrophils were stimulated with 10 µg/ml phorbol-12-myristate-14-acetate (PMA) after incubation with dihydrorhodamine-1,2,3. Flow cytometry analysis was performed as previously described using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) [5]. The stimulation index (SI), which was defined as the geometric fluorescence intensity of PMA-stimulated

neutrophils relative to the geometric fluorescence intensity of unstimulated neutrophils, was used to evaluate neutrophil function [6].

2.4 Expression of gp91^{phox} protein

The expression of gp91^{phox} protein was determined by flow cytometry-based extracellular staining with the monoclonal antibody (mAb) 7D5, which detects flavocytochrome b558 in the NADPH oxidase complex at the cell surface. Neutrophils were stained with mAb 7D5 or isotype-matched control antibody followed by fluorescent isothiocyanate-conjugated or Alexa Fluor 488 anti-mouse antibody. The stained neutrophils were analyzed on a FACSCalibur flow cytometer.

2.5 Gene Analysis

Genomic DNA was extracted from the EDTA-anticoagulated blood of patients and their parents using the QIAmp® DNA Blood Mini Kit (Qiagen, Hilden, Germany). The concentration and quantity of the DNA samples were measured using a NanoDrop ultraviolet spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and then prepared for next-generation sequencing by panel, which included all previously reported immunodeficiency genes.

In accordance with the instructions of the SureSelect Human All Exon Kit, the genomic DNA underwent ultrasonic fragmentation, end repair, adapter connection, and hybridization. The captured DNA library was sequenced on the Illumina HiSeq 2000 platform (Illumina, San Diego, CA). The raw data were converted to a VCF file containing the basic information for the mutation sites through splicing and comparison. We completed the variation annotation using the ANNOVAR and VEP software. The annotation of the mutation frequency referred to databases, including the 1000 Genomes Project, the ExAC Browser and internal databases. Mutation predictions were performed using the SIFT, PolyPhen-2 and MutationTaster software. Deletions were identified by the NGS coverage depth analysis [7], and point mutations were confirmed by Sanger sequencing.

3. Results

3.1 CGD Gene distribution

Between January 2018 and March 2021, a total of 85 CGD patients were admitted to the Children's Hospital of Fudan University. Patients were diagnosed with CGD according to their clinical manifestations, immunophenotype, and finally genetic results. The most common mutation of the *CYBB* gene inherited from the X chromosome was detected in 68 patients (80%). Autosomal recessive CGD (AR-CGD) was found in the minority of the patients, with *CYBA* mutations (7%), *NCF1* mutations (7%) and *NCF2* mutations (6%) (Fig. 1).

Based on the expression level of the gp91 protein, we further classified X-CGD patients with *CYBB* mutations into X91⁰ CGD, X91⁻ and X91⁺ CGD. Compared to other PIDs, NCF1/NCF2 mutant CGD and

healthy adults, we defined the gp91 expression level of X91⁺ as >70%, X91⁻ as 5%-70%, and X91⁰ as <5%. X91⁺ CGD accounted for 8% (7/85) of the total CGDs, which was higher than expected (Fig. 2a).

3.2 Clinical Manifestations of X91⁺ CGD

Here, we report seven X91⁺ CGD patients with different neutrophil stimulation indices and varying clinical presentations (Table 1). None of the patients was consanguineous marriages. The median age of onset was 4 months (range, 0.5 months- 2 years), and 2 patients presented their first clinical manifestations during the neonatal period. However, the diagnostic age of the 7 patients was later, even 9 years old. Patient 7 (P7) had a positive family history. His two elderly brothers died at the age of 5 and 13 years old due to pulmonary infection and mycobacterium tuberculosis infection; however, he was not diagnosed with CGD until 5 years of age. In addition, the uncle of P5 died of unknown causes at the age of 10 years of age.

Infections were the most common clinical symptoms, varying in severity. Patients except P3 received the same strain of BCG vaccine (Danish strain BCG823) at birth according to the vaccination records, and three (P1, P4, and P5) had probable BCG infections. Patients presented left axillary (P1 and P4), clavicle and scapula (P5) lymph node enlargement at 3~5 months with delayed healing at BCG vaccination sites. Mycobacterium tuberculosis infection was considered a prominent manifestation of X91⁺ CGD patients. P4 had persistent lymph node enlargement with intermittent fever, their CT showed bilateral lung infection, and the PPD test showed a strong positive result (+++) at the age of 15 months. At 7 years old, P5 developed recurrent fever with cervical lymph node enlargement again, and the pathology of lymph node puncture suggested tuberculous changes, although no mycobacterium was detected by metagenomic sequencing (mNGS). In addition, P7 suffered from recurrent fever and lymph node enlargement at 4 years of age, and his pulmonary CT showed miliary tuberculosis and lymph node calcification. The chest CT scan showed bilateral lung diffuse lesions, and the PPD test was positive (++) for P6; however, mycobacterium was not detected by PCR.

Moreover, patients developed conditions such as pneumonia, diarrhea, perianal abscess, osteomyelitis, and skin and soft tissue infection. Pneumonia was common, most with recurrent infections. *Ureaplasma urealyticum* was detected in sputum by PCR, and *Corynebacterium striatum* (reads, 200) was also found in broncho-alveolar lavage fluid (BALF) by mNGS in P4. P1 was considered to have a fungal infection due to a positive GM test in BALF. Three patients (P1, P2, and P6) had soft tissue infections, and two (P4 and P6) had perianal abscesses. *Burkholderia gladioli* (reads, 69) from P1 and *Klebsiella pneumoniae* from P6 were detected from pus via mNGS and bacterial culture separately. Magnetic resonance imaging (MRI) and positive emission tomography/computed tomography (PET/CT) bone scanning of P1 confirmed multifocal bone destruction and osteomyelitis involving his left finger, right lower leg and foot. *Mycobacterium neoaurum* (reads, 78) was further identified in blood by mNGS. Two (P2 and P7) had recurrent noninfectious oral ulcers.

Most patients except P5 and P7 received prophylactic sulfamethoxazole (SMZ). Six patients underwent antifungal treatment, including voriconazole (P1 and P2), fluconazole (P3 and P6) and itraconazole (P3, P4, P6, and P7). Symptomatic patients received antituberculosis treatment with double, triple or quadruple medications consisting of isoniazide and rifampicin without (P6) or with ethambutol (P4, P5, and P7) or pyrazinamide (P5 and P7), whereas two patients (P2 and P7) who received interferon gamma (IFN- γ) therapy were in good condition during the follow-up period. Three patients received umbilical cord blood stem cell transplantation (UCBSCT), and all survived.

3.3 Comparison of SI between X91⁺ and other different X-CGD types

X-CGD patients with CYBB mutations were divided into classic type X-linked CGD (X91⁰ CGD) and variant type X-linked CGD, including X91⁻ CGD and X91⁺ CGD (Fig. 2b). In our cohort, the SI of 7 X91⁺ CGD patients widely varied from 1.9 to 67.5, including five with SI $\geq$ 20 (Fig. 3). SI ranged from 1.2 to 35.7 in X91⁰ CGD patients, including 74.5% (38/51) of patients with an SI $\leq$ 5, while the SI in X91⁻ CGD patients was less than 5, with one exception. The difference in SI between the X91⁺ CGD and X91⁰ or X91⁻ CGD groups was statistically significant ($P<0.05$).

3.4 X91⁺ CGD Gene Mutations and Review of the literature

Six missense mutations and one splice site of the CYBB gene were detected in the 7 X91⁺ CGD patients. Four X91⁺ CGD-related CYBB mutations were reported previously, including c.162G>C, c.170C>A, c.1223G>A, and c.1244C>A, while c.1462-2 A>T and c.1243C>T resulted in amino acid (AA) changes similar to those previously reported at the same location. Moreover, the c.925G>A mutation has been reported to be X91⁻ CGD-related previously.

Then, we searched PubMed and found 89 cases of variant type X-linked CGD (Table 2) with record data, including 35 X91⁺ CGD cases [1, 2, 4, 8–31] with 26 mutation sites and 54 X91⁻ CGD cases [1, 4, 10, 17, 18, 20, 22, 25, 32–57] with 33 mutation sites. The distribution of loci at the DNA and protein levels is summarized in Figure 4.

Mutations related to X91⁺ CGD mainly included 24 missense mutations, one splicing mutation, and one deletion mutation located at exons 3, 9, 10, 12 and 13. On the structure of the protein encoded by the CYBB gene, there were 2 mutations (p. Arg54Ser and p. Ala57Glu) located in regions encoding the second transmembrane region, 2 mutations (p. Pro338Arg and p. Thr341Lys) located at the potential FAD binding domain, 4 mutations (p. Thr362Arg, p. Cys369Arg, p. Gly408Arg, and p. Gly408Glu) located at regions effecting the tail of the FAD binding site and NAD pyrophosphate binding region, 10 mutations (p. Pro415His, p. Pro415Ser, Δ 488-497, p. Ala488Asp, p. Asp500Tyr, p. Asp500Gly, p. Asp500Glu, p. Thr503Lys, p. Leu505Arg, and p. Cys537Arg) located at a potential NADPH binding domain, and 2 mutations (p. Cys546Pro and p. Glu568Lys) located at regions encoding the C-terminal tail, which is essential for NADPH oxidase complex assembly. Therefore, these loci were mostly located in the intracellular segment.

Among 21 X91⁺ CGD patients with definite clinical records of recurrent infection, pulmonary infection including pneumonia and pulmonary abscess was the most common presentation (11/21). Other clinical manifestations included lymphadenitis and skin involvement (10/21), hepatic abscess (4/21), diarrhea (4/21) and sepsis (4/21). One patient experienced DIC due to *Pseudomonas cepacia* infection. Most patients recovered after antibacterial or antifungal treatment, although recurrent infection was common. Only 1 patient died at the age of 5 due to severe pneumonia.

X91⁻ CGD-related mutations included splicing mutations, deletion mutations and missense mutations. Except for exon 1, X91⁻ CGD-related deletion or missense mutations were found in all other exon regions. However, mutation sites of X91⁻ CGD have no apparent association with different domains.

4. Discussion

CGD is a rare primary immunodeficiency resulting from the inability of neutrophils to generate respiratory bursts to produce O₂⁻. *CYBB* mutations encoding gp91phox can result in X-linked CGD. Typical X-linked CGD accounts for more than 90% of CGD cases [10], with a pattern of early onset disease with no gp91phox expression and low neutrophil SI. Therefore, the combination of low SI and unexpressed gp91phox is often used in the rapid clinical diagnosis of X-linked CGD.

Gp91phox is composed of 570 amino acids. Its N-terminal half has 6 transmembrane regions, in which two hemes are coordinated by four histidines. The C-terminus of gp91phox is a cytosolic tail containing NADPH and FAD-binding sites. The cytosolic tail of gp91phox at its C-terminus is responsible for transferring electrons from intracellular NADPH to FAD, and this reaction activity is called iodonitrazolium (INT) diaphorase activity. Two hemes are lined in series and are responsible for transferring electrons from FADH₂ to extracellular or intravesicle O₂. Resting state gp91phox binds with p22phox and embeds on the cellular membrane. After receiving activation signals, p47phox, p40phox and p67phox translocate to the gp91phox-p22phox heterodimer to form the NADPH oxidase complex. Western blotting was used to analyze gp91phox in the past, while flow cytometry-based extracellular staining with monoclonal antibody (mAb) 7D5 is currently used to analyze gp91phox.

Different mutation sites lead to different mechanisms of protein function. Mutations p. Arg54Ser and p. Ala57Glu are located at the second transmembrane region of gp91phox. Positively charged Arg54 facilitates electron exit from heme. Mutated gp91phox with uncharged Ser54 has a redox potential of -300 mV, thus blocking electrons exiting from heme to form O₂⁻ [58]. In vitro experiments confirmed normal FADH₂ [59] but no H₂O₂ production ability of the Arg54Ser mutation [17]. A similar mechanism is observed for p. Ala57Glu. Ala57 is an uncharged amino acid; however, glutamic acid is negatively charged and much larger than Ala, thus inhibiting electron exit from the transmembrane passage. E309 is located between the transmembrane region and dehydrogenase domain surface and is involved in p65phox/p22phox binding [60]. P415 is located at the intracellular NADPH binding region of gp91phox. The p. P415H and p. P415S mutations disrupt the consensus sequence for an NADPH-binding site (GXGXGXXPF) and cause NADPH binding defects [8, 61]. Therefore, p. P415H-mutated gp91phox could

not produce O_2^- , although it had normal gp91phox expression levels, FAD incorporation and NADPH oxidase assembly. p. P415S has similar patterns to p. P415H presumably. Studies proved that the p. Gly408Glu mutation strongly inhibited the translocation of p47phox and p67phox to the phagosomal membranes of the mutants. Defect FAD incorporation results in absent NADPH oxidase activity. This mutation presumably causes defective complex assembly, direct interference of FAD/NADPH binding and electron transfer disturbance [59]. According to the previously reported mutations c.1462-2 A>G and c.1462-2 A>C, we hypothesize that the c.1462-2A>T mutation removes the 3' splice acceptor site of intron 11 and results in a partial p. Ala488_Glu497del in the gp91phox protein [1, 9]. In wild-type gp91phox, residues 484 to 503 form an α -helical loop lying over the NADPH binding cleft. For c.1462-2 mutated patients, loss of this structure subsequently disturbs NADPH binding and electron transfer to FAD. Therefore, the normally expressed gp91 is nonfunctional and results in defective neutrophils in these patients.

In our cohort, X91⁺ variant-type CGD patients had a higher SI, though some patients had milder symptoms. It is worth noting that a high neutrophil SI by the DHR123 test cannot indicate mild symptoms or good prognosis of variant type X-linked CGD patients. Although DHR has the advantages of small blood volume, short time and convenience compared with direct H_2O_2 detection [9], it has limitations in terms of molecular mechanism [62]. Both DHR123 and NBT can react with FADH₂, despite being a milder degree and lower priority than reactive oxygen species (ROS). For neutrophils with normal FADH₂ production but no H_2O_2 production, neutrophil oxidative burst tests review the production of FADH₂ instead of ROS so the SI cannot fully reflect H_2O_2 levels. AR-CGD patients were considered to have a higher SI and better prognosis than X-linked CGD patients [63]. In contrast, according to a single center study of 48 CGD patients in China [64], 3 of 7 (43%) patients with an SI>5 died, and 2 of them (29%) died before their second birthday, while 7 of 26 (27%) patients with an SI<5 died, and 5 of them (19%) died before their second birthday. The neutrophil SI was not very low in Patient A (44.1) and Patient C (24.5), though both had early onset disseminated infections. We suspect that SI has more value in the diagnosis of CGD, but SI should be interpreted carefully.

In clinical practice, experimental data of X91⁺ and X91⁻ variant type CGD patients vary widely, though the pathogenicity of some gene mutations is unavailable in the database. In addition, the same mutations in different cases sometimes have different phenotypes. For example, the mildest case of *CYBB* c.1462-2 A>G mutation stayed healthy until the age of 69, while one of his grandsons died at the age of 5. In this study, the infection symptoms of Patient B were not serious, and their SI fluctuated between 5.8 and 28.5. Similar conditions were reported in other mutations as well [10, 55], indicating other factors associated with the O_2^- production ability of NADPH oxidase [1, 9, 65]. Bacterial killing tests and H_2O_2 quantification tests should be performed if necessary. Clinical symptoms, SI, protein expression and gene analysis should be considered comprehensively in the diagnosis of variant type X-linked CGD patients, especially X91⁺ CGD.

Declarations

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Conflicts of Interest

All the authors declare that they have no conflicts of interest.

Author Contributions

Bijun Sun and Zeyu Zhu contributed to the study conception, design and drafted the manuscript. Xiaoying Hui completed the experimental data section. Jinqiao Sun, Wenjie Wang, Wenjing Ying, Qinhua Zhou, Haili Yao contributed to study design and data collection. Jia Hou and Xiaochuan Wang gave academic feedback and revised the manuscript. All authors have reviewed the final manuscript and agreed to be accountable for the work. All authors read and approved the final manuscript.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Compliance with Ethical Standards

The study was approved by the ethics committee of Children's Hospital of Fudan University.

Consent to participate

Written informed consent was obtained from the parents.

Consent for publication

Not applicable.

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Tables

Due to technical limitations, table 1 and 2 xlsx are only available as a download in the Supplemental Files section.

Figures

Figure 1

Distribution of genes and GP91 protein in CGD patients.

Figure 2

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Comparison of GP91 protein expression and stimulation index in X-linked chronic granulomatosis disease. A: GP91 protein expression levels in different groups, including $X91^+$, $X91^-$, $X91^0$, CGD with NCF1/NCF2, other PIDs and healthy adults. B: Stimulation index comparison between different groups of X-linked CGDs.

Figure 3

GP91 protein expression levels and stimulation index in $X91^+$ CGD.

A: GP91 protein expression levels in 7 $X91^+$ CGD patients. B: Stimulation index in 7 $X91^+$ CGD patients.

Figure 4

CYBB gene mutations of X91⁺ CGD in our cohort and previous reports. The bar above shows the distribution of the CYBB gene mutations in X91⁻ (top) and X91⁺ (low) CGD in cDNA. The bar below shows the distribution of the CYBB gene mutations in X91⁺ CGD on protein structure. The mutation sites in our cohort are marked in red.

Supplementary Files

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