Title: BCG infections at high frequency in both AR-CGD and X-CGD patients following BCG vaccination

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Key points: Although AR-CGD patients have more reactive oxygen intermediates than X-CGD patients, we have shown that BCG infections develop at high frequency in both AR-CGD and X-CGD patients, regardless of genotype and mutant forms.

Abstract

Background

Patients with chronic granulomatous disease (CGD) develop severe infections, including *Bacillus Calmette-Guérin* (BCG). Although the autosomal recessive CGD (AR-CGD) patients should hypothetically develop relatively fewer infections compared to the X-linked CGD (X-CGD) patients due to more residual reactive oxygen intermediates, the impacts of BCG vaccination on AR-CGD and X-CGD patients are unclear. Herein, we demonstrated the clinical features of BCG infections, treatments, and genetic factors in CGD patients after BCG vaccination under the Japanese immunization program.

Methods

We collected data retrospectively from 43 patients with CGD and assessed their history of initial infection, age at diagnosis of CGD, BCG vaccination history, clinical course, treatment for BCG infections, and genetic mutations associated with CGD.

Results

Fourteen CGD patients avoided BCG vaccination because of other preceding infections and family history. Of 29 patients with CGD who received BCG vaccination, 20 patients developed BCG infections. Although the age at onset of initial infection in X-CGD patients was significantly younger than that in AR-CGD patients (P<0.01), the onset and frequency of BCG infections were similar in X-CGD and AR-CGD patients. In X-CGD patients, BCG infections equally developed in the patients carrying missense, insertion, deletion, nonsense, and splice mutations of *CYBB*. All CGD patients with BCG infections were successfully treated

with anti-tuberculous drugs.

Conclusions

Although X-CGD patients develop severe infections at a younger age than AR-CGD patients, our data suggested that BCG infections develop at high frequency in both AR-CGD and X-CGD patients, regardless of genotype and mutant forms.

Keywords: Chronic granulomatous disease; *Bacillus Calmette-Guérin*; *Mycobacterium*; vaccine; infection

Introduction

Chronic granulomatosis disease (CGD) is a primary immunodeficiency, characterized by dysfunction of phagocytes to produce reactive oxygen intermediates (ROI). NADPH oxidase is a transmembrane enzyme complex generating ROI; it is principally constructed of gp91^{phox}, p22^{phox}, p47^{phox}, p67^{phox}, and p40^{phox} binding protein subunits [1, 2, 3]. CGD could be caused by defects in any of the components of the NADPH oxidase complex [4]. The defect of *CYBB* encoding gp91^{phox} is associated with X-linked CGD (X-CGD); other defects of the NADPH oxidase complex, namely, *CYBA* encoding p22^{phox}, *NCF1* encoding p47^{phox}, *NCF2* encoding p67^{phox}, and *NCF4* encoding p40^{phox} are associated with autosomal recessive CGD (AR-CGD) [5]. CGD patients experience various bacterial and fungal infections, and are also susceptible to infection by *Mycobacterium*, such as *M. tuberculosis* and *Bacillus-Calmette Guérin* (BCG) [6, 7, 8]. Therefore, BCG vaccination is contraindicated for CGD patients, and BCG infections are an important issue in the countries where BCG vaccination is routinely given. Under the BCG vaccination program in Japan, children are generally recommended to receive the routine BCG vaccination when under one year old, the age group in which the vaccine coverage rate reaches approximately 95%.

Others previously reported that AR-CGD had more residual ROI than that of X-CGD and would develop relatively fewer infections [9-11]. Moreover, in X-CGD patients, the mutations of *CYBB* other than missense mutations had fewer residual ROI than missense mutations of *CYBB*, and would cause more severe infections [12]. It has been known that the differences of residual ROI due to the genotype and mutant forms affect the development of bacterial and fungal infections [9-12], but the impacts of BCG vaccination on CGD patients

and the associations with mutant forms of *CYBB* in X-CGD patients are unclear. Herein, we examine the clinical features of BCG infections in CGD patients under the Japanese immunization program. We investigated the implementation of BCG vaccination, the frequency of BCG infections, the genotype and mutant forms relating to BCG infections, and treatment in AR-CGD and X-CGD cases.

Methods

Study design and patients

We collected data retrospectively from 51 patients with CGD who visited the National Center for Child Health and Development from 2002 to 2019. Of the 51 patients with CGD, eight patients were excluded from this study because of unidentified BCG vaccination history. We examined the data of 43 patients with CGD who were identified, including their history of initial infection, age at diagnosis of CGD, BCG vaccination history, clinical course of BCG infections, treatment for BCG infections, and genetic mutations associated with CGD. This study was approved by the ethics committee (378) of the hospital, and written informed consent was obtained from all patients or their parents.

Diagnosis of BCG infections

The following diagnostic criteria of BCG infections are (1) or (2) based on previous reports [13, 14]: (1) BCG bacteria were identified by the lesion; (2) lymphadenopathy (10 mm or more), regional ulcer, pneumonia, cutaneous tuberculosis-like lesion, osteomyelitis, or multiple-organ involvement develops after BCG

vaccination. For the identification of BCG, culture with Ogawa medium (Kyokuto Pharm. Ind., Japan) or MGIT medium (Nippon Becton Dickinson, Japan) was used to detect acid-fast bacillus, and polymerase chain reaction (PCR) and sequencing were performed as previously described [15].

Diagnosis of CGD

The diagnosis of CGD was performed by flow cytometry-based ROI production assay using dihydrorhodamine-123 (Sigma, St. Louis, MO) and confirmed by genetic mutation analysis of *CYBB*, *CYBA*, *NCF1*, *NCF2*, and *NCF4* by the Sanger sequencing method.

ROI production assay

The ROI production by neutrophils was assessed with flow cytometry using DHR123. As previously described [16], neutrophils were stimulated with phorbol 12-myristate 13-acetate (PMA), and were analyzed by mean fluorescence intensity (MFI) of DHR123. The data is presented as the median of the DHR stimulation index (DHR-SI) that is the ratio of MFI of stimulated cells divided by that of unstimulated cells.

Statistical analysis

Data are presented as the mean ± standard error. Analysis for vaccination rate and frequency of BCG infections was performed by using Fisher's exact test. Others were performed by using the Mann-Whitney test. *P* values less than 0.05 were considered statistically significant. All statistical tests were calculated using

Prism 7 (GraphPad Software, CA).

Results

AR-CGD patients frequently develop BCG infections as much as X-CGD patients.

Of the 43 patients with CGD, 10 patients had AR-CGD, including five patients, one patient, and four patients who carried gene mutations of *CYBA*, *NCF1*, and *NCF2*, respectively. Of the 10 AR-CGD patients, three patients were male and no patients had a family history of CGD. The other 33 patients had X-CGD, carrying a gene mutation of *CYBB*. All patients with X-CGD were male, and included one group of three brothers (N=3), two pairs of two brothers each (N=4), and one pair of cousins (N=2).

Of the 10 AR-CGD patients, eight patients received BCG vaccination, and six patients developed BCG infections. Of the 33 X-CGD patients, 21 patients received BCG vaccination, and 14 patients developed BCG infections. There was no significant difference in the rate of BCG vaccination between AR-CGD and X-CGD patients (Table 1). The median residual ROI production assessed by DHR-SI was 1.20 (interquartile range, 1.17 to 2.92) and 1.00 (interquartile range, 1.00 to 1.08) in AR-CGD and X-CGD patients, respectively, which was statistically as high as in AR-CGD patients (P=0.015; Fig. 1A, 1B). However, there was no significant difference in the frequency of BCG infections and the age of onset at BCG infection between AR-CGD and X-CGD patients given BCG vaccination (Table 1), indicating that AR-CGD patients showed susceptibility to BCG infections similarly to X-CGD patients.

We also examined CGD patients who avoided BCG vaccination. In AR-CGD patients, two AR-CGD patients

did not receive BCG vaccination because of preceding infections leading to diagnosis of CGD. Of 12 X-CGD patients who did not receive BCG vaccination, six patients had a family history of CGD, and other patients had diagnosed CGD due to development of preceding infections. Therefore, the initial infection due to bacteria and fungi would lead to diagnosis of CGD and contraindication of BCG vaccination even in AR-CGD patients.

BCG infections were the most common initial infection in AR-CGD patients.

For CGD patients with and without BCG vaccination, we examined the initial clinical presentation in AR-CGD and X-CGD patients. The age at onset of initial infection including bacterial, fungal, and BCG infections was 18.0±19.4 months and 4.9±5.8 months in AR-CGD and X-CGD patients, respectively, which was significantly older in AR-CGD patients (P<0.01; Fig. 2A). Accordingly, the age at diagnosis of CGD was 65.1±106.0 months and 15.9±22.2 months in AR-CGD and X-CGD patients, respectively, which was significantly older in AR-CGD patients (P<0.01; Fig. 2B).

As compared with the frequency of BCG infections and other infections, the common initial diseases were bacterial and fungal infections, including pneumonia, perianal abscess, and lymphadenitis rather than BCG lymphadenitis in X-CGD patients; however, BCG lymphadenitis was the most common disease as the initial infection in AR-CGD patients (Table 2). The frequency of BCG lymphadenitis as the initial infection in AR-CGD patients was significantly higher than that of X-CGD patients (60.0% and 18.2% for AR-CGD and X-CGD, respectively; P=0.017; Table 2). As compared with the age of BCG infections and other infections as

initial infection, X-CGD patients developed bacterial and fungal infections significantly earlier than that of BCG lymphadenitis (P<0.01; Table 2), which would lead to the earlier onset of the initial infection compared to AR-CGD patients. These data indicated that AR-CGD patients hardly developed bacterial and fungal infection at early infancy with the exception of BCG infections.

X-CGD patients can develop BCG infections, regardless of the specific gene affected.

For X-CGD patients, we examined BCG infections based on the type of the mutations in *CYBB* because it is known that the mutations of *CYBB* other than missense mutations have fewer residual ROI than missense mutations of *CYBB* [12]. We genetically identified 21 mutations of *CYBB*, including four missense mutations and 17 other types of mutations in X-CGD patients, and eight mutations in AR-CGD patients, all of whom received BCG vaccination (Table 3). In our patients, the median residual ROI production assessed by DHR-SI was 1.08 (interquartile range, 1.06 to 2.89) and 1.00 (interquartile range, 1.00 to 1.02) in X-CGD patients carrying missense mutations and other mutations of *CYBB*, respectively, which was not a significant difference (P=0.12). BCG infections developed in all patients carrying missense mutations of *CYBB*, and in 10 of 17 patients carrying other types of mutations, which showed no significant difference in development of BCG infections (P=0.24). It is also known that X-CGD patients with missense mutations affecting $gp91^{phox}$ amino acids greater than 310 have lower residual ROI production than 1 to 309 [12]. However, in this study, all of the missense mutations of *CYBB* were located in the amino acid substitution positions 1 to 309; therefore, we could not compare the impact of missense mutations of *CYBB* located in the amino acids

substitution positions greater than 310 on the development of BCG infections.

Treatments for BCG infections were successful in all CGD patients, regardless of the genotype.

In the infections associated with BCG vaccination, BCG lymphadenitis was the most common disease in both X-CGD and AR-CGD patients. It should be noted that BCG pneumonia developed in both AR-CGD and X-CGD cases, and that systemic BCG infection and BCG sepsis developed in X-CGD cases (Table 4).

As the treatments for BCG infections in 20 patients with CGD, combination therapy of isoniazid and rifampicin was administered in seven patients (35%), and monotherapy of isoniazid in three patients (15%) (Table 5). For the patients who developed BCG infections in multiple organs, clarithromycin and streptomycin were added to the combination therapy of isoniazid and rifampicin. Pyrazinamide was concomitantly used in cases of suspected *M. tuberculosis* infections. In seven of 20 patients who developed BCG infections, prophylactic treatment with interferon-gamma (IFN-γ) was maintained during treatments for BCG infections, and no patients developed severe adverse events associated with IFN-γ during treatment for BCG infections. There were six patients (30%) affected with localized and low-activity BCG infections who did not require treatment for BCG infections. Regardless of the genotype of CGD, all patients had a good prognosis for BCG infections, with or without treatment. Although some affected tissue was detected by computed tomography at the end of treatment for BCG infections in 11 patients, no patients appeared to show exacerbation of BCG infections during treatment with hematopoietic stem cell transplantation.

Discussion

Under the BCG vaccination program in Japan, children are generally recommended to receive the routine BCG vaccination when under one year old. We examined the clinical features of BCG infections in CGD patients under the Japanese immunization program to identify the impacts of BCG vaccination on AR-CGD and X-CGD patients. Although AR-CGD patients are less likely to develop severe bacterial and fungal infections due to more residual ROI than X-CGD patients [9-12], there was no difference in the frequency of BCG infections and the age of onset at BCG infection between AR-CGD and X-CGD patients vaccinated with BCG in this study. It is thought that ROI can exert their bactericidal effect mainly by direct oxidation of mycobacterial elements and thereby damage *Mycobacterium* spp. including BCG [17, 18]. In an animal study using CGD mice, there was no difference in the frequency of BCG infections between *CYBB* and *NCF1* defect mice [19]. In this study, residual ROI production by neutrophils in AR-CGD patients was higher than that of X-CGD, whereas AR-CGD patients developed BCG infections after BCG vaccination as often as those with X-CGD. These suggest that AR-CGD and X-CGD patients would produce ROI insufficiently to prevent BCG infections, and therefore, BCG infections would develop at high frequency in all genotypes of CGD patients.

We also assessed the genetic background and clinical features of BCG infections in X-CGD patients, based on a report that missense mutations of *CYBB* tended to retain residual ROI production more than other mutant forms of *CYBB* [12]. In this study, there was no difference in the frequency of BCG infections between missense mutations and other mutant forms of *CYBB* in X-CGD. Although X-CGD patients with missense mutations affecting $gp91^{phox}$ amino acids 1 to 309 had higher residual ROI production than those of amino

acids greater than 310 [12], all X-CGD patients carrying missense mutations affected in gp91^{phox} amino acids 1 to 309 developed BCG infections after BCG vaccination. Together with these data, it is possible to speculate that BCG vaccination causes development of BCG infections at high frequency in CGD patients, regardless of mutant forms of *CYBB*.

In this study, almost 80% of X-CGD patients developed a severe infection before the recommended age of BCG vaccination under the Japanese program, and had CGD diagnosed at a younger age compared to those of the patients with AR-CGD. In general, CGD patients are suspected of having primary immunodeficiency due to refractory infections, opportunistic infections, inflammatory bowel disease, and family history [20]; therefore, the age at diagnosis of CGD would depend on the time of appearance of these clinical symptoms. In fact, AR-CGD patients rarely developed severe infection before BCG vaccination, resulting in 80% of BCG vaccine coverage in AR-CGD patients. This may be associated with the more residual ROI in neutrophils of AR-CGD compared to that of X-CGD as in previous reports [9-12]. Our results suggest that the high rate of BCG vaccination coverage would lead to BCG infections as the initial clinical presentation in AR-CGD patients. It is of note that the variant X-CGD patients, who carry *CYBB* missense mutations leading to a less severe deficiency of ROI, appear to have a less severe phenotype. In such a variant X-CGD, the nosocomial pathogen *Burkholderia cepacia* can cause severe infection, which would be one of the essential factors to make the diagnosis of CGD [21].

In Japan, almost a million infants receive BCG vaccination every year, and the adverse events associated with BCG vaccination occur at 0.02% in all subjects, including healthy subjects and patients without

diagnosed immunodeficiency disease [3], whereas the adverse events associated with BCG vaccination occurred at high frequency in AR-CGD and X-CGD patients. As for BCG infections, lymphadenitis is the most common in healthy subjects and CGD patients, which persist for a long period during treatment with antitubercular antibiotics in CGD. Regarding the treatment for BCG infections, combination therapy of isoniazid, rifampicin, and clarithromycin is recommended by guidelines for tuberculosis infections [22], and the combination therapy of isoniazid and rifampicin successfully treated BCG lymphadenitis in CGD [23-25]. In this study, the combination therapy of isoniazid and rifampicin was administered for BCG infections in most of the CGD patients, and other drugs were added for disseminated BCG infections, which successfully treated all of the patients. Although the therapeutic effect of IFN-γ was not assessed in this study because of the small number of patients, IFN-γ would be effective in BCG infections and could be considered for severe BCG infections [14, 26]. Some patients were not treated for BCG lymphadenitis, and no patients appeared to have exacerbations of BCG infections in this study. This may be explained by the fact that BCG is an attenuated strain of *Mycobacterium bovis* [27]. Although BCG infections occasionally improved without treatment in CGD patients [28], others reported that the overall 10-year survival rate of disseminated BCG infections was 34% [13], and mortality of BCG infections was 50% in CGD patients [26, 29, 30]. Together with our data, the disseminated BCG infections are at high risk and refractory in CGD patients; therefore, the appropriate treatment would be recommended in all of the genetic forms of CGD patients.

Conclusions

We examined BCG infections in CGD patients. Although the patients who already had diagnosed CGD and had a family history could have a contraindication for receiving the BCG vaccination, 67% of CGD patients received BCG vaccination under the Japanese immunization program. There was no difference in the frequency and the age of onset at BCG infection after BCG vaccination in AR-CGD and X-CGD patients. The AR-CGD patients develop relatively fewer infections compared to the X-CGD patients; however, BCG vaccination causes BCG infections at high frequency in all CGD patients, regardless of genotype and mutant forms.

NOTES

Author Contributions

T.I., M.O., T.U., M.O., and T.K. managed the patients, and designed research. T.I. and E.M. performed research and analyzed data. E.M., T.U., and M.O. performed DHR-123 analysis and genetic analysis. T.I. and T.K. contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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Disclosure of Conflicts of Interest

The authors declare no conflicts of interest.

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Figure legends

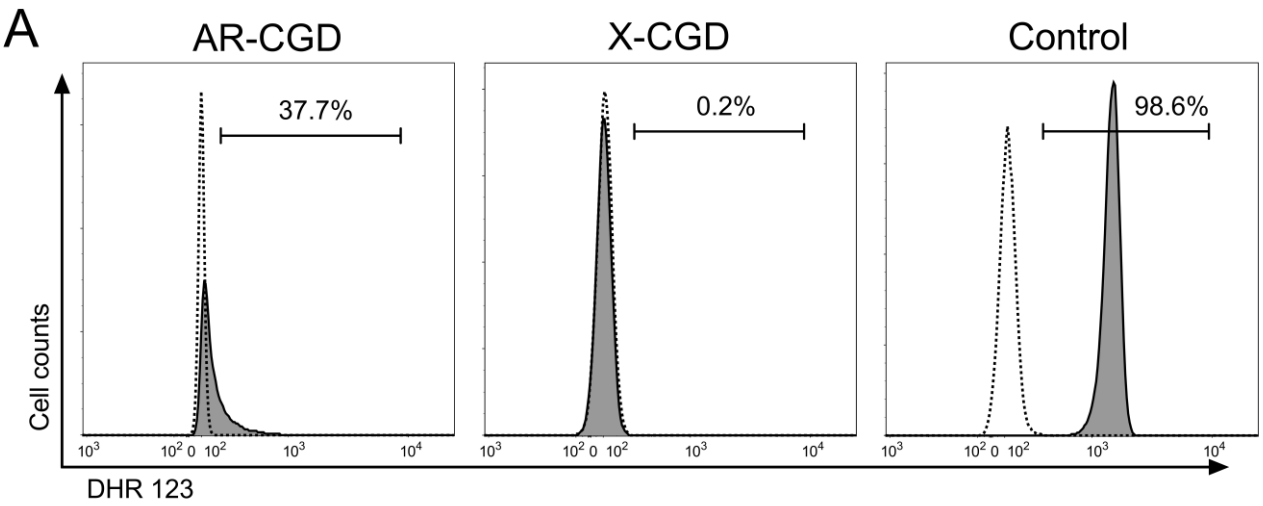
Figure 1. Residual reactive oxygen intermediates (ROI) production by the neutrophils in AR-CGD and X-CGD patients

(A) The shown is representative data of flow cytometry analysis of DHR for assessment of ROI production by the neutrophils in the patient with AR-CGD, X-CGD, and healthy control. (B) The data is DHR stimulation index (DHR-SI) in AR-CGD and X-CGD patients. The horizontal lines show the median value of DHR-SI.

Figure 2. Age at onset of initial infection and at diagnosis of CGD in AR-CGD and X-CGD patients

(A) Age at onset of initial infection in AR-CGD and X-CGD patients. (B) Age at diagnosis of CGD in AR-CGD and X-CGD patients. The horizontal lines show the average value of age in AR-CGD and X-CGD patients.

Figure 1



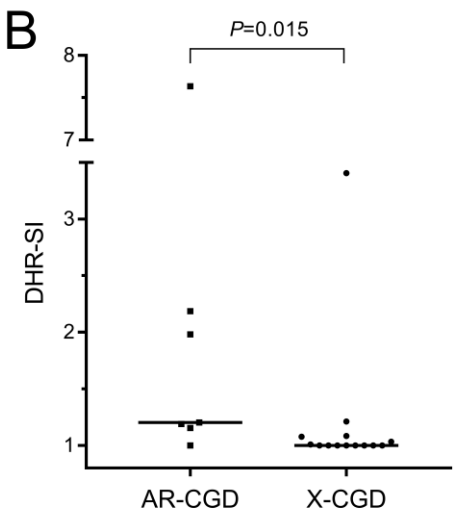
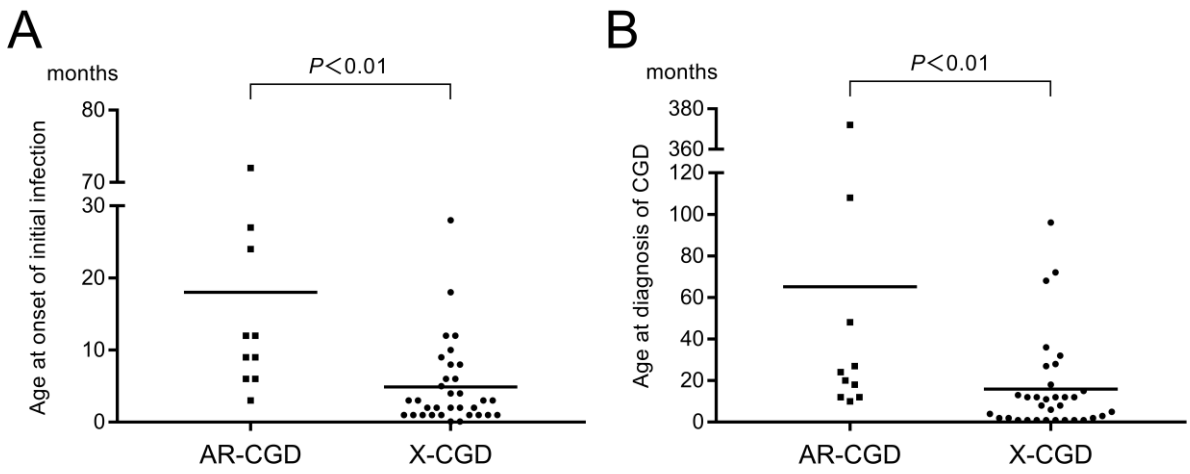


Figure 2



Tables

Table 1. BCG infections following BCG vaccination in AR-CGD and X-CGD patients

	AR-CGD n=10	**X-CGD** n=33	***P* value**	**All patients** n=43
Rate of BCG vaccination	8 (80%)	21 (64%)	0.46	29 (67%)
Developed BCG infections	6 (75%)	14 (67%)	>0.9	20 (69%)
Age at BCG infections (months)	22.3±21.7	14.5±8.7	0.43	18.1±16.6

P value is calculated by comparing AR-CGD and X-CGD.

Table 2. Initial diseases of CGD patients

Initial diseases	AR-CGD		X-CGD	
	Events n=10	**Age at onset** (months)	**Events** n=33	**Age at onset** (months)
BCG infections				
Lymphadenitis	6 *	24.0±23.0	6 *	12.7±8.1
Skin lesions	0	–	0	–
Systemic/Sepsis	0	–	1	28.0
Pneumonia	0	–	1	12.0
Other infections				
Pneumonia	2	7.0, 12.0	7	1.8±1.5
Perianal abscess	0	–	7	3.0±2.8
Lymphadenitis	0	–	6	2.2±1.9
Skin lesions	1	12.0	3	2.0±0.8
Systemic/Sepsis	0	–	2	0.1, 1.0
Urinary tract infection	1	12.0	0	–

* P=0.017, P value is calculated by comparing the number of patients with BCG lymphadenitis between AR-CGD and X-CGD. The infections with only two patients indicate their respective ages.

Table 3. Genetic mutations and BCG infections in CGD patients who received BCG vaccination

Patient no.	Mutation gene	Nucleotide change	Amino acid change	BCG infections
1	*CYBB*	c.332A>G	p.His111Arg	+
2	*CYBB*	c.625C>T	p.His209Tyr	+
3	*CYBB*	c.665A>G	p.His222Arg	+
4	*CYBB*	c.769T>C	p.Cys257Arg	+
5	*CYBB*	c.6insG	p.Gly2fs	+
6	*CYBB*	c.121insT	p.Tyr41fs	+
7	*CYBB*	c.252G＞A	p.Ser48_Ala84del	+
8	*CYBB*	c.483+5G>C	p.Ala113fs	+
9	*CYBB*	c.484-8T>G	p.Val27fs	+
10	*CYBB*	c.490G>T	p.Glu164Ter	+
11	*CYBB*	c.674+5G＞A	exon6 deletion	+
12	*CYBB*	c.1038delT	p.Pro346fs	+
13	*CYBB*	exon 1_13 deletion	complete deletion	+
14	*CYBB*	exon 11_13 deletion	exon 11_13 deletion	+
15	*CYBB*	c.80delTCTG	p.Val27fs	−
16	*CYBB*	c.142-12C＞A	p.Ser48fs	−
17	*CYBB*	c.733G＞T	p.Glu245Ter	−
18	*CYBB*	c.1120C>T	p.Gln374Ter	−

19	*CYBB*	c.1314+1delG	p.lli439fs	−
20	*CYBB*	c.1314+1delG	p.lli439fs	−
21	*CYBB*	c.1354G>T	p.Glu452Ter	−
22	*CYBA*	c.70G＞A	p.Gly24Arg	+
23	*NCF2*	c.66G＞A, c.304C＞T	p.Trp22Ter, p.Arg102Ter	+
24	*NCF2*	c.304C＞T	p.Arg102Ter	+
25	*NCF2*	c.304C＞T, c.365A＞G	p.Arg102Ter, p.Glu122Gly	+
26	*NCF2*	c.1231A＞T	p.Lys411Ter	+
27	*NCF1*	Unknown	Unknown	+
28	*CYBA*	c.70G＞A, c.371C＞T	p.Gly24Arg, p.Ala124Val	−
29	*CYBA*	c.214T＞C, c.521T＞C	p.Try72His, p.Val174Ala	−

Table 4. Diseases associated with BCG infections in AR-CGD and X-CGD patients

BCG infections	AR-CGD	X-CGD	All patients
All diseases	8	19	27
Lymphadenitis	6 (75%)	12 (63%)	18 (67%)
Skin lesions	0	4 (21%)	4 (15%)
Pneumonia	2 (25%)	1 (5%)	3 (11%)
Systemic/Sepsis	0	2 (11%)	2 (7%)

Data is shown by the number of diseases. In AR-CGD, two patients developed lymphadenitis and pneumonia. In X-CGD, five patients developed BCG infections in multiple organs, which were lymphadenitis and skin lesions in three patients, and lymphadenitis and systemic/sepsis in two patients.

Table 5. Treatments for BCG infections

Treatments	Patients n=20
Isoniazid + Rifampicin (with IFN-γ)	7 (5)
Isoniazid (with IFN-γ)	3
Isoniazid + Rifampicin + Pyrazinamide (with IFN-γ)	2 (1)
Isoniazid + Rifampicin + Clarithromycin	1
Isoniazid + Rifampicin + Streptomycin	1
No treatment (with IFN-γ)	6 (1)

The number of the patients treated with IFN-γ is shown in parentheses. IFN-γ, interferon-gamma