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Approach to Molecular Diagnosis of Chronic Granulomatous Disease (CGD): an Experience from a Large Cohort of 90 Indian Patients

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Abstract

Background Chronic granulomatous disease (CGD) is characterized by mutation in any one of the five genes coding NADPH oxidase components that leads to functional abnormality preventing the killing of phagocytosed microbes by affecting the progression of a respiratory burst. CGD patients have an increased susceptibility to infections by opportunistic and pathogenic organisms. Though initial diagnosis of CGD using a nitroblue tetrazolium (NBT) test or dihydrorhodamine (DHR) test is relatively easy, molecular diagnosis is challenging due to involvement of multiple genes, presence of pseudogenes, large deletions, and GC-rich regions, among other factors. The strategies for molecular diagnosis vary depending on the affected gene and the mutation pattern prevalent in the target population. There is a paucity of molecular data related to CGD for Indian population.

Method This report includes data for a large cohort of CGD patients ($n = 90$) from India, describing the diagnostic approach, mutation spectrum, and novel mutations identified. We have used mosaicism in mothers and the expression pattern of different NADPH components by flow cytometry as a screening tool to identify the underlying affected gene. The techniques like Sanger sequencing, next-generation sequencing (NGS), and Genescan analysis were used for further molecular analysis.

Result Of the total molecularly characterized patients ($n = 90$), 56% of the patients had a mutation in the *NCF1* gene, 30% had mutation in the *CYBB* gene, and 7% each had mutation in the *CYBA* and *NCF2* genes. Among the patients with *NCF1* gene mutation, 82% of the patients had 2-bp deletion (DelGT) mutations in the *NCF1* gene. In our cohort, 41 different mutations including 9 novel mutations in the *CYBB* gene and 2 novel mutations each in the *NCF2*, *CYBA*, and *NCF1* genes were identified.

Conclusion Substantial number of the patients lack *NCF1* gene on both the alleles. This is often missed by advanced molecular techniques like Sanger sequencing and NGS due to the presence of pseudogenes and requires a simple Genescan method for confirmation. Thus, the diagnostic approach may depend on the prevalence of affected genes in respective population. This study identifies potential gene targets with the help of flow cytometric analysis of NADPH oxidase components to design an algorithm for diagnosis of CGD in India. In Indian population, the Genescan method should be preferred as the primary molecular test to rule out *NCF1* gene mutations prior to Sanger sequencing and NGS.

Keywords Chronic granulomatous disease · molecular characterization · NGS · novel mutations · flow cytometry · Genescan

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Introduction

Chronic granulomatous disease (CGD) is a rare inherited primary immunodeficiency disorder (PID) which results from a defect in any one of the five components of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex. It follows both X-linked (XL) and autosomal recessive (AR) forms of inheritance. Defects in the *CYBB* gene causes XL-CGD, while AR-CGD is caused by defects in either *CYBA, NCF1, NCF2,* or *NCF4* genes [1]. In rare cases, a defect in

Rac1/2 GTP-binding protein causes deficiency in ROS (superoxide) production, and chemotaxis defect also shows similar manifestations as that of CGD [2]. CGD is characterized by the inability of phagocytes to form superoxide upon interaction with bacterial or fungal pathogens [3]. The molecular spectrum of CGD varies with rate of consanguineous marriages in different populations and ethnic diversity. Overall, XL-CGD is the most prevalent type of CGD, accounting for > 65% of CGD cases in China, Japan, Europe, and the USA [4–7]. However, in regions where consanguineous marriage is highly prevalent (or more frequent), such as Turkey, Egypt, Oman, and Iran, a higher rate of AR-CGD is reported [8–11]. According to a previously reported study comprising of 17 patients in India, AR-CGD accounted for 59% of the cases, while XL-CGD accounted for 41% of the cases [12].

Diagnosis of CGD involves the evaluation of neutrophil oxidase activity, in response to neutrophil stimulation. It is measured by the nitroblue tetrazolium test (NBT) or dihydrorhodamine (DHR) test, and further confirmed by molecular analysis. Despite different underlying genetic defects, both XL-CGD and AR-CGD patients are clinically indistinguishable and show similar pattern of abnormalities in the diagnostic tests [13]. Carrier mothers of XL-CGD patients may show a mosaic pattern due to the presence of a heterogeneous population of neutrophils, which demonstrates both defective and normal respiratory burst and thus suggests underlying genetic defect in the *CYBB* gene [14]. A study of specific NADPH oxidase component (gp91^{phox}, p22^{phox}, p47^{phox}, p67^{phox}, and p40^{phox}) expression is required in AR-CGD and XL-CGD patients, whose mothers do not display a carrier pattern. It is performed either by western blot or flow cytometry to get a clue to the underlying genetic defect prior to genetic confirmation [15]. However, a substantial number of XL-CGD patients have been described with normal expression of gp91^{phox} by western blotting but without the activity of this protein. Thus, a normal expression of components does not rule out the mutation in respective gene, as the function may be diminished in spite of normal expression [13]. Flow cytometry using specific monoclonal antibodies (mAbs) is a rapid and sensitive method for expression analysis of specific NADPH oxidase components. Reduction or absence of specific NADPH oxidase component expression guides us for the molecular confirmation [8].

This study describes the molecular spectrum of CGD and novel mutations reported in a large cohort of Indian patients (*n* = 90). It also highlights the utilization of flow cytometry for rapid identification of defective genetic subtype of CGD prior to molecular confirmation. It can also be concluded from this study that there is need to apply population-specific molecular diagnostic strategy for identification of underlying genetic defect, as majority of our population suffer from AR-CGD with the *NCF1* gene being the most commonly affected. The next-generation sequencing (NGS) technology for molecular diagnosis in our cohort will be of limited use and suitable only for the patients with known defective gene expression.

Materials and Methods

Patient Information and Sample Collection

This study consists of 90 well-characterized CGD patients. A minimum of 3-mL peripheral blood sample was collected in EDTA anticoagulated vacutainer from each patient. Additional samples (parents and siblings) were collected in the case of patients with a strong family history after obtaining a proper informed consent. Samples were referred to and processed at the National Institute of Immunohaematology (NIIH), Mumbai, India, which is the center of excellence for PID in India. A clinical protocol has been followed per the guidelines of the Institutional Ethics Committee and Declaration of Helsinki.

Neutrophil Function Tests

NBT and DHR Assays

The assay procedures have been explained in our previously published article [16]. A stimulation index (SI) was calculated for gated neutrophils by estimating the ratio of median fluorescent intensity (MFI) of phorbol myristate acetate (PMA)-stimulated and PMA-unstimulated cells in DHR assay. The SI value of the patient was always paired and simultaneously processed with a healthy control sample at the time of diagnosis. Parent's and sibling's samples were also tested, as required. The carrier status of mother was tested to rule out XL-CGD in case of male patients. The remaining patients were further evaluated for NADPH oxidase component expression by flow cytometry.

Flow Cytometry for the Assessment of NADPH Oxidase Component Expression

For patients with abnormal NBT and DHR assays, NADPH oxidase component expression was analyzed by flow cytometry. Around 100 μL of peripheral whole blood sample was stained with CD20-PerCP and CD3-APC antibody (BD Biosciences, San Diego, USA) for gating of B cells and T cells, respectively. The expression of NADPH oxidase components on neutrophils and B cells was assessed; T cells do not express these components, hence, were considered as an internal negative control to evaluate specificity of antibody binding. The extracellular protein gp91^{phox} was assessed by stain-lyse-wash protocol using surface staining with 7D5 mAb (MBL International Co., Woburn, MA, USA). For assessment of intracellular components like p47^{phox} and

p67[phox], leukocytes were fixed by 10% formaldehyde, permeabilized by 0.1% Triton-X treatment for 30 min at 37 °C, and washed with FACS buffer (PBS with 1% BSA and 0.05% Triton-X). Leukocytes were then incubated for 30 min at 4 °C with mAbs specific for either human p47[phox] (clone 1, BD Biosciences, San Diego, CA, USA) or p67[phox] (clone N-19- PE conjugated, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Leukocytes were then washed with FACS buffer, and anti-p47[phox]-stained tubes were further incubated for 30 min at 4 °C with a 1:100 dilution of goat anti-mouse IgG-FITC antibody (BD Biosciences, San Diego, CA, USA). After extensive washing, the stained leukocytes were analyzed by flow cytometry on BDFACS Aria; gating on neutrophils was based on forward- and side-scatter properties. Analysis of the data was done on BDFACS Diva software. The ratio of median fluorescent intensity (MFI) of neutrophils and B cells (signal/noise = S/N ratio) of stained tube to unstained tube was calculated in patients and healthy control samples.

Molecular Diagnosis Strategy

Molecular diagnosis was done by following a strategy mentioned in Fig. 2. Male patients along with their mother's samples were tested with NBT ($n = 57$ of 90) and DHR ($n = 52$ of 90) assays to rule out XL-CGD carrier status. In case of unavailability of maternal samples ($n = 2$ of 57) or absence of carrier status ($n = 3$ of 57), and female patients ($n = 33$ of 90) were evaluated for NADPH oxidase component expression by flow cytometry. Absent or reduced intracellular component expression (p47[phox] or p67[phox]) is suggestive of a gene defect in NCF1 or NCF2 genes. Abnormal 7D5 mAb expression could be due to a defect in any one of the membrane-bound components (gp91[phox] or p22[phox]), as 7D5 mAb exclusively binds to an extracellular domain of gp91[phox], which requires presence of both p22[phox] and gp91[phox] for its stable expression. Hence, male patients with abnormal 7D5 expression were tested either for CYBB gene first or CYBA gene in case of consanguinity. Evaluation of p40[phox] was considered only when all the other components were found to be normal, as it is a very rare form [17].

DNA Sequencing and Genetic Analysis

Genomic DNA was isolated from peripheral blood collected in EDTA tubes using the whole blood DNA extraction kit (Sigma-Aldrich Co., St. Louis, MO, USA). All the exons, along with the intron-exon boundaries of CYBB, NCF2, and CYBA genes, were amplified by polymerase chain reaction (PCR) and were run on 1.5% agarose gel. The PCR products were sequenced by Sanger sequencing (performed at NIIH, ABI 3130Xl genetic analyzer, Applied Biosystems, USA) and the results were analyzed by the BLAST program.

Genescan Analysis

The NCF1 gene and two flanking ΨNCF1 (pseudogene) genes were amplified using 6-FAM tag-labeled primers as per the sequences provided earlier [18]. The primers anneal with regions both in ΨNCF1 and NCF1, around the GTGT sequence at the start of exon 2. The PCR conditions were as mentioned in article [19]. The mixture of NCF1 product and ΨNCF1 product, differing two nucleotides in length (ΨNCF1 contains GT and NCF1 contains GTGT), was analyzed using GeneMapper software (Life Technologies, Carlsbad, CA, USA) on an Applied Biosystems 3130xl analyzer. Peak height corresponding to the copy number of genes was calculated for both NCF1 gene and ΨNCF1. The ratio of NCF1 to ΨNCF1 was determined in order to establish the patient, normal, and carrier status [19]. The replacement of NCF1 gene with (part of) its pseudogenes is mentioned as DelGT mutation in the rest of the manuscript.

In patients with NCF1 gene defect, other than DelGT mutations were identified using NGS for 9 patients at the Dept. of Blood Cell Research, Sanquin Blood Supply Organization, Amsterdam, The Netherlands. The analysis was done using Ion Torrent sequencing by using an Ampliseq™ custom panel (Thermo Fisher Scientific, Waltham, MA, USA). The panel includes the coding regions of the following genes: CYBB, CYBA, NCF1, NCF2, NCF4, G6PD, and RAC2. Library preparation and sequencing was performed according to manufacturer protocols on an Ion S5 system (Thermo Fisher Scientific).

Statistical Method

Data were statistically described in terms of median and percentages, whenever required as appropriate. Unpaired T test was used to compare SI values for control and patient, and one-way analysis of variance (ANOVA) test was used for comparison of > 2 groups. Mann-Whitney U test was used for comparing groups with non-parametric data. The p values less than 0.05 were considered statistically significant. All statistical calculations were done using SPSS software (Chicago, IL, USA) version 15 and GraphPad Prism 7 for Microsoft Windows.

In Silico Analysis

On sequencing, the sequences obtained were compared with the reported gene database (CYBB, CYBA, NCF1, NCF2) at NCBI using the BLASTN program [http://www.ncbi.nlm.nih.gov/BLAST]. Any novel sequence variant was analyzed for its effect on the respective protein by using different prediction software, i.e., PolyPhen-2 (www.http://genetics.bwh.harvard.edu/pph2/), SIFT (www.http://sift.jcvi.org/), and MutationTaster (http://www.mutationtaster.org/). SIFT scores of ≤ 0.05 are usually taken as indicative of deleterious

substitutions [20]. PolyPhen-2 scores of 0 to 1 show low to high confidence for probability of protein damage [21]. MutationTaster is used for evaluating disease-causing potential of sequence alterations [22]. For splice region variants, Human Splicing Finder tool is used [23].
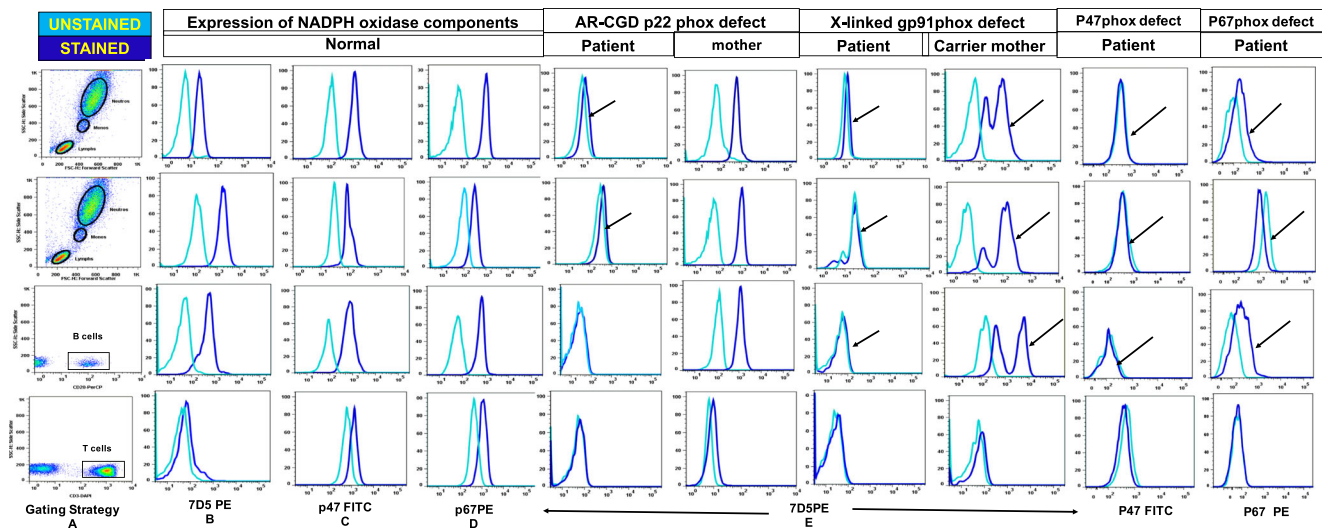
Results

Neutrophil Function Test

A total of 90 patients (male, 57; female, 33) from 84 families were diagnosed with CGD and were included in this study. Of these, 4 families included ≥ 2 affected siblings. Overall, 64% were male and 36% were females with a male to female ratio of 1.77:1. Consanguinity was noted in 32% of the patients. Seventy-five percent were Hindu, 21% were Muslim, and 4% were Christian by religion, suggestive of ethnic diversity. The diagnosis of CGD was done using both NBT ($n = 90$) and DHR ($n = 85$) assays. Among the male patients ($n = 57$), mothers of 23 patients were identified as carriers based on NBT and DHR assays. One female patient had XL-CGD as a result of extreme skewed X chromosome inactivation. The median (range) percentage positivity of NBT and DHR assays in XL-CGD carrier mothers was 54% (range, 10–65%) and 55% (range, 10–65%), respectively. Following the next step delineated in the diagnostic strategy, the remaining patients were evaluated for NADPH oxidase component expression by flow cytometry based on our molecular diagnosis strategy.

Consistent reduction in the SI value of the CGD patients was noted in comparison with the healthy control sample (Table 2). The SI value of 60 was taken as a cutoff based on median value of healthy controls evaluated in our laboratory. In alignment with the previous report, we found no significant correlation of SI values based on its genotype and severity for XL-CGD (median SI, 1.1 (range = 0.7–9)) vs AR-CGD patients (median SI, 1.3 (range = 0.2–13.3), P value 0.35 [24]. There was no statistically significant difference observed when the SI values in different subgroups (by one-way ANOVA test) were compared. However, statistically significant reductions were observed in SI values of patients with null mutations (nonsense, frameshift deletion, or insertion mutations leading to introduction of premature stop codon) ($n = 52$, mean $\pm$ SD = 1.7 $\pm$ 1.8, median = 1.1, range = 0.2–9.8) vs. SI values of patients with missense mutations ($n = 21$, mean $\pm$ SD = 3.8 $\pm$ 3.5, median = 2.3, range = 1.1–13.3). Though there is a trend of high SI value in patients with $NCF1$ defect [21], significantly lower SI values in patients with DelGT mutation (causing premature stop codon) ($n = 37$, mean $\pm$ SD = 1.7 $\pm$ 1.8, median = 1.1, range = 0.2–9.8) compared to those in patients with other than DelGT mutations ($n = 9$, mean $\pm$ SD = 6.5 $\pm$ 4.3, median = 7.3, range = 1.1–13.3, P value < 0.0013) were observed.

NADPH Component Expression and Classification by Flow Cytometry

Of the total patients ($n = 90$), 23 males had carrier mothers and of them, 16 patients were evaluated for 7D5 mAb expression.

Fig. 1 NADPH oxidase component expression by flow cytometry. Gating of neutrophils, monocytes, B cells, and T cells, gated using FSC vs SSC and using specific marker for B and T cells. (b–d) the results of expression of gp91phox, p47phox, and p67phox in blood leukocyte populations from healthy individual, surface expression of gp91phox analyzed by 7D5mAb (b), intracellular expression of p47phox (c), p67phox (d) in neutrophils, monocytes, CD3+ T, and CD20+ B from a healthy control. Sky blue lines indicate unstained tube and dark blue lines represent staining of mAbs specific for the NADPH oxidase subunits. Arrow showing abnormal expression of p22phox and gp91phox expression in AR-CGD (e), XL-CGD (e) and carrier mother. Similarly, absent or reduced expression of p47phox (f) and p67phox (g) in AR-CGD patients

Samples of carrier mothers of XL-CGD patients also showed the same mosaic pattern. Of the remaining 67 patients, 52 were evaluated for NADPH oxidase by flow cytometry. Of the 52 patients, 10 (male = 5, female = 5) patients showed reduction or absence of 7D5 mAb expression on both neutrophils and B cells compared to healthy control sample. They were suspected for either *CYBB* or *CYBA* gene defect (AR-CGD). Similarly, anti-p47^{phox} and anti-p67^{phox} antibody expression was found to be reduced or absent in 38 and 4 patients, respectively (Figs. 1 and 2). Data for 21 patients with anti-p47^{phox} antibody expression were published in our previous article [16]. The S/N ratio of affected component for patient and control samples is given in Table 1 and Table 2. Based on their suspected genotype, no statistically significant difference was observed in the expression pattern of 7D5, anti-p47^{phox}, and anti-p67^{phox} antibodies on neutrophils or B cells (Table 2 and Fig. 3).

Nearly 40% of the patient's samples were shipped and had to travel a long distance, of which 14% were for XL-CGD patients and the remaining were for AR-CGD patients (86%). The paired controls had a range of 40 to 85% of neutrophils positive in DHR assay after PMA stimulation as compared to fresh control sample, but > 75% neutrophils showed expression of intact NADPH oxidase component (> 95% in fresh sample) or reduction in MFI as compared to fresh sample (Fig. 4). The details of neutrophil function test and NADPH oxidase component expression by flow cytometry are given in Tables 1 and 2. The sensitivity and specificity are 98%. Based on the assessment of affected protein component of NADPH oxidase complex by flow cytometry, CGD patients were further classified as per their respective gene defect as a starting point for their molecular diagnosis (Fig. 2).

Genetic Analysis

Our molecular strategy was based on the results of mother's carrier status in NBT and DHR assays and NADPH oxidase component expression by flow cytometry. Normal DHR or NBT test does not completely rule out the carrier status in females and in cases of extreme lyonization. The abnormal protein expression guided the selection of candidate gene for Sanger sequencing. Of the total patients characterized by NBT, DHR, and NADPH component studies, 38 patients had p47^{phox} deficiency suggesting *NCF1* defect, 23 patient's mothers were carrier suggesting *CYBB* defect, and 4 patients had reduced p67^{phox} suggesting *NCF2* defect. Of the 10
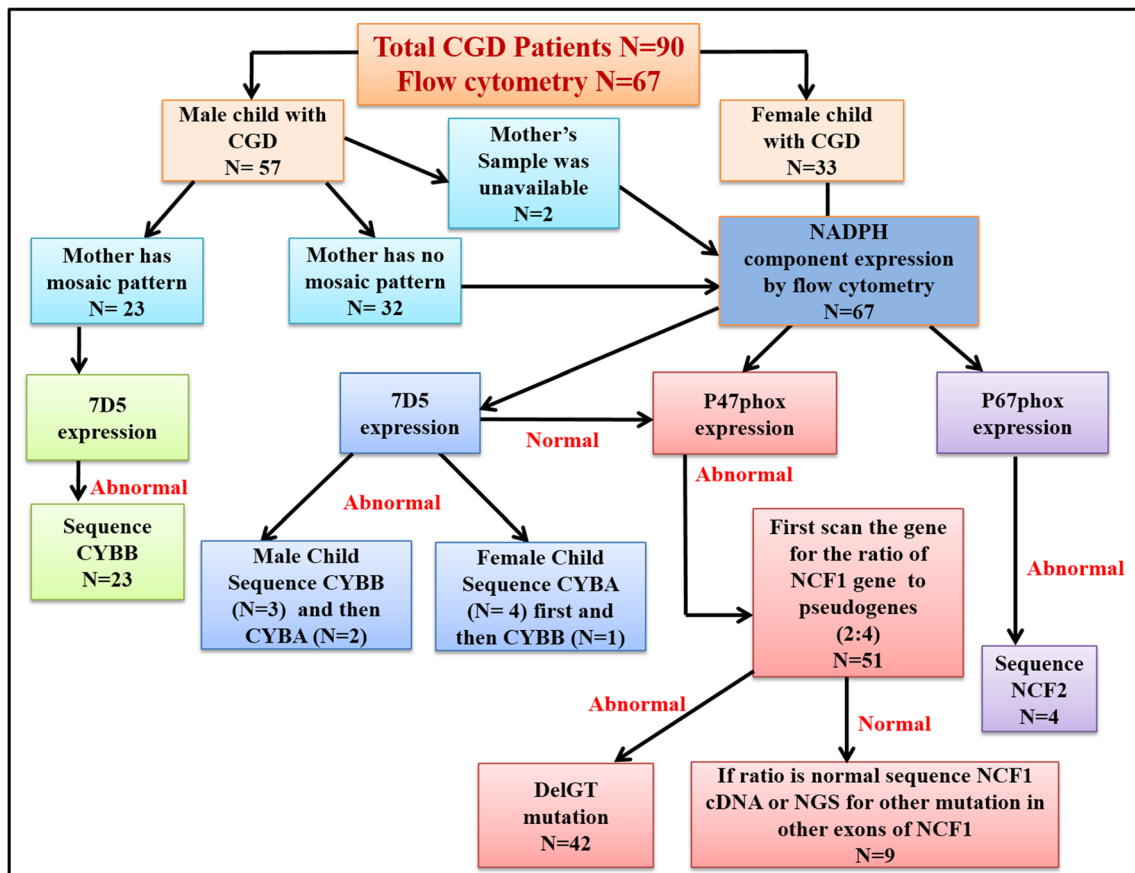

Fig. 2 Diagnostic algorithm for molecular diagnosis of CGD patients at a starting point

Table 1 Laboratory Investigation of CGD Patients

Pt. no.	Age In months	Sex	DHR (SI)	DHR (%) (n = 85)	NBT (%) (n = 90)	NBT (%) In mother	DHR (%)	Defective NADPH component	Defective gene	Expression on neutrophils (S/N ratio)		Expression on B cells (S/N ratio)	
										Patient	Control	Patient	Control
Patient with abnormal p47phox expression with AR-CGD (n = 51)													
1	15	M	ND	ND	0%	98%	ND	p47phox	NCF1	ND	ND	ND	ND
3	120	M	ND	ND	0%	98%	ND	p47phox	NCF1	ND	ND	ND	ND
7	17	M	ND	ND	0%	98%	ND	p47phox	NCF1	ND	ND	ND	ND
9	264	M	ND	ND	0%	98%	ND	p47phox	NCF1	ND	ND	ND	ND
12	29	M	ND	ND	0%	98%	ND	p47phox	NCF1	ND	ND	ND	ND
18	138	F	1.1	0%	0%	98%	98%	p47phox	NCF1	2.1	13.1	1.5	1.2
19	156	F	1.2	0%	0%	98%	98%	p47phox	NCF1	ND	ND	ND	ND
24	24	F	1.7	0%	0%	98%	98%	p47phox	NCF1	2.4	25.2	1.1	2.2
25	72	M	1.4	0%	0%	98%	98%	p47phox	NCF1	ND	ND	ND	ND
26	132	F	1.2	0%	0%	98%	98%	p47phox	NCF1	ND	ND	ND	ND
30	6	M	1.2	0%	0%	98%	98%	p47phox	NCF1	ND	ND	ND	ND
37	96	M	1.2	0%	0%	98%	98%	p47phox	NCF1	ND	ND	ND	ND
39	7	M	1.1	0%	0%	98%	98%	p47phox	NCF1	ND	ND	ND	ND
40	6	M	0.9	0%	0%	98%	98%	p47phox	NCF1	ND	ND	ND	ND
50	69	F	1.0	0%	0%	ND	ND	p47phox	NCF1	ND	ND	ND	ND
57	168	M	0.9	0%	0%	98%	98%	p47phox	NCF1	2.0	10.7	1.0	1.9
62	36	F	0.8	0%	0%	98%	98%	p47phox	NCF1	0.3	5.7	1.0	1.8
63	252	M	0.4	0%	0%	ND	ND	p47phox	NCF1	0.5	14.1	0.9	1.5
64	84	M	0.2	0%	0%	98%	98%	p47phox	NCF1	1.0	10.7	1.2	1.9
65	108	F	0.2	0%	0%	98%	98%	p47phox	NCF1	0.9	10.7	1.5	1.9
71	29	M	0.9	0%	0%	98%	98%	p47phox	NCF1	0.7	5.2	1.0	2.1
73	24	M	0.6	0%	0%	98%	98%	p47phox	NCF1	0.4	3.7	1.8	2.8
74	60	M	7.3	0%	0%	ND	ND	p47phox	NCF1	1.8	15.5	1.3	3.5
75	24	M	1.2	0%	0%	98%	98%	p47phox	NCF1	0.8	8.3	1.6	2.8
78	30	M	0.6	0%	0%	98%	98%	p47phox	NCF1	0.6	15.5	1.7	2.4
79	32	M	0.3	0%	0%	98%	98%	p47phox	NCF1	0.7	15.5	1.4	3.4
82	1	F	8.6	5%	0%	ND	ND	p47phox	NCF1	1.0	73.8	1.3	4.7
84	6	F	4.0	0%	0%	98%	98%	p47phox	NCF1	2.0	24.1	6.4	5.8
85	3	F	1.0	0%	0%	ND	ND	p47phox	NCF1	1.7	9.4	1.9	5.7
89	48	F	3.2	0%	0%	98%	98%	p47phox	NCF1	2.8	65.9	1.7	4.9
91	27	F	2.8	0%	0%	98%	98%	p47phox	NCF1	6.5	49.8	1.0	3.5
95	84	M	13.3	0%	0%	ND	ND	p47phox	NCF1	1.5	3.5	1.3	3.3
96	36	F	7.6	0%	0%	ND	ND	p47phox	NCF1	1.6	3.5	1.2	3.3
97	60	F	3.4	0%	0%	ND	ND	p47phox	NCF1	2.2	3.5	4.4	3.1
98	60	M	10.6	0%	0%	ND	ND	p47phox	NCF1	1.6	3.5	1.4	3.3
99	108	M	4.1	0%	0%	ND	ND	p47phox	NCF1	3.8	9.8	4.4	2.4
104	120	F	9.8	0%	0%	98%	98%	p47phox	NCF1	3.5	12.1	4.8	15
110	120	F	3.4	2%	0%	98%	98%	p47phox	NCF1	1.0	11.9	0.9	33.8
111	31	F	1.4	2%	0%	ND	ND	p47phox	NCF1	1.2	11.0	ND	ND
112	192	M	0.8	0%	0%	98%	98%	p47phox	NCF1	2.7	27.5	2.7	13.6
113	53	F	1.1	0%	0%	98%	98%	p47phox	NCF1	2.0	23.1	2.0	10.8
121	53	F	1.0	0%	0%	98%	98%	p47phox	NCF1	2.5	8.1	2.1	3.1
124	120	F	1.2	0%	0%	ND	ND	p47phox	NCF1	5.1	18.8	6.0	10
126	120	F	1.4	0%	0%	ND	ND	p47phox	NCF1	2.2	107.0	1.8	7.5
128	108	F	1.1	0%	0%	98%	98%	p47phox	NCF1	1.9	8.5	2.8	5.1

Table 1 (continued)

Pt. no.	Age In months	Sex	DHR (SI)	DHR (%) (n = 85)	NBT (%) (n = 90)	NBT (%) In mother	DHR (%) In mother	Defective NADPH component	Defective gene	Expression on neutrophils (S/N ratio) Patient	Expression on neutrophils (S/N ratio) Control	Expression on B cells (S/N ratio) Patient	Expression on B cells (S/N ratio) Control
129	156	M	1.5	0%	0%	98%	98%	p47^{phox}	NCF1	2.0	7.9	2.7	4.1
131	24	M	2.0	0%	0%	98%	98%	p47^{phox}	NCF1	2.0	9.0	1.8	5.3
132	72	F	1.0	0%	0%	98%	98%	p47^{phox}	NCF1	2.8	10.1	4.9	8.0
137	60	M	2.2	0%	0%	98%	98%	p47^{phox}	NCF1	2.3	5.0	1.7	3.8
139	138	F	1.3	0%	0%	ND	ND	p47^{phox}	NCF1	4.6	14.4	2.0	2.4
140	84	F	1.0	0%	0%	ND	ND	p47^{phox}	NCF1	5.9	14.6	1.5	2.4
Patient with abnormal 7D5 expression with XL-CGD (n = 27)													
6	4	M	ND	0%	0%	50%	55%	Gp91^{phox}	CYBB	ND	ND	ND	ND
15	20	F	1.8	10%	8%	98%	98%	Gp91^{phox}	CYBB	1.6	3.3	1.2	20.9
16	8	M	ND	0%	0%	54%	56%	Gp91^{phox}	CYBB	ND	ND	ND	ND
20	5	M	ND	0%	0%	60%	63%	Gp91^{phox}	CYBB	ND	ND	ND	ND
42	24	M	ND	0%	0%	50%	54%	Gp91^{phox}	CYBB	ND	ND	ND	ND
46	24	M	1.3	0%	0%	50%	31%	Gp91^{phox}	CYBB	4.0	34.8	ND	ND
53	60	M	0.8	0%	0%	ND	ND	Gp91^{phox}	CYBB	1.7	22.1	2.8	93.4
54	41	M	4.2	13%	5%	45%	50%	Gp91^{phox}	CYBB	6.5	10.6	ND	ND
61	24	M	0.9	0%	0%	40%	45%	Gp91^{phox}	CYBB	1.0	22.3	1.0	17.9
66	8	M	0.9	0%	0%	60%	59%	Gp91^{phox}	CYBB	1.5	32.2	ND	ND
67	5	M	1.1	0%	0%	50%	52%	Gp91^{phox}	CYBB	5.8	7.4	0.7	48
70	3	M	1.6	0%	0%	98%	98%	Gp91^{phox}	CYBB	8.1	22.3	40.8	79.4
72	7	M	1.0	0%	0%	60%	63%	Gp91^{phox}	CYBB	ND	ND	ND	ND
76	48	M	3.5	0%	0%	54%	50%	Gp91^{phox}	CYBB	1.2	3.0	18.8	18.3
83	6	M	0.9	0%	0%	62%	65%	Gp91^{phox}	CYBB	0.6	3.8	3.8	20.9
90	3	M	2.3	0%	0%	ND	ND	Gp91^{phox}	CYBB	1.2	11.5	3.2	20.4
93	24	M	0.7	0%	0%	50%	52%	Gp91^{phox}	CYBB	ND	ND	ND	ND
103	5	M	2.0	0%	0%	65%	63%	Gp91^{phox}	CYBB	0.7	8.3	3.7	30.9
106	0.8	M	1.6	0%	0%	55%	65%	Gp91^{phox}	CYBB	1.0	5.2	4.1	32.3
107	24	M	2.3	0%	0%	15%	10%	Gp91^{phox}	CYBB	1.0	5.9	5.5	41.2
109	13	M	1.1	0%	2%	50%	36%	Gp91^{phox}	CYBB	1.4	3.2	0.8	8.8
115	24	M	1.3	0%	0%	50%	39%	Gp91^{phox}	CYBB	1.9	12.5	1.2	29.3
119	3	M	1.1	0%	0%	60%	62%	Gp91^{phox}	CYBB	1.03	5.9	1.2	1.6
120	2	M	1.0	0%	0%	30%	28%	Gp91^{phox}	CYBB	1.3	4.9	ND	ND
122	84	M	1.1	0%	0%	70%	65%	Gp91^{phox}	CYBB	ND	ND	ND	ND
127	5	M	1.5	0%	0%	70%	65%	Gp91^{phox}	CYBB	2.7	27.5	3.6	6.9
133	3	M	1.1	0%	0%	35%	13%	Gp91^{phox}	CYBB	2.7	83.2	7.3	9.2
Patient with abnormal 7D5 expression with AR-CGD (n = 6)													
47	39	F	1.9	0%	0%	98%	98%	p22^{phox}	CYBA	0.7	3.4	7.7	14.3
49	10	M	1.1	0%	0%	98%	98%	p22^{phox}	CYBA	1.3	8.0	8.0	15.0
52	3	F	1.2	0%	0%	98%	98%	p22^{phox}	CYBA	1.7	4.1	1.1	9.9
55	72	F	1.1	0%	0%	98%	98%	p22^{phox}	CYBA	1.1	13.3	3.4	4.5
80	1	F	3.6	0%	0%	98%	98%	p22^{phox}	CYBA	0.9	9.4	8.2	23.2
105	41	M	1.8	6%	0%	98%	98%	p22^{phox}	CYBA	1.6	7.5	ND	ND
Patient with abnormal p67^{phox} expression with AR-CGD (n = 6)													
43	29	M	2.3	0%	0%	98%	98%	p67^{phox}	NCF2	4.5	11.3	5.4	5.6
51	6	F	1.3	0%	0%	98%	98%	p67^{phox}	NCF2	7.2	13.0	16.3	33.3
60	144	F	1.4	0%	0%	ND	ND	p67^{phox}	NCF2	ND	ND	ND	ND
77	96	M	2.7	0%	0%	98%	98%	p67^{phox}	NCF2	13.0	50.8	15.6	46.0

Table 1 (continued)

Pt. no.	Age In months	Sex	DHR (SI)	DHR (%) (n = 85)	NBT (%) (n = 90)	NBT (%) In mother	DHR (%)	Defective NADPH component	Defective gene	Expression on neutrophils (S/N ratio)		Expression on B cells (S/N ratio)	
										Patient	Control	Patient	Control
92	10	F	1.5	0%	0%	98%	98%	p67^{phox}	NCF2	3.5	16.4	3.0	20.5
117	1.5	M	1.1	0%	0%	98%	98%	p67^{phox}	NCF2	ND	ND	ND	ND

%, positive neutrophils after stimulation with PMA; *ND*, not determined

patients having abnormal or reduced 7D5, 5 females were first screened for defect in the *CYBA* gene, 2 males that belonged to consanguineous marriage were first screened for defect in the *CYBA* gene, and the remaining 3 males were screened for *CYBB* gene defect first.

A total of 79 patients were analyzed by Sanger sequencing or Genescan, and NGS was performed in 11 patients. Parents' samples were analyzed to screen their carrier status for most of the identified mutations. More than half of the patients, 56% (*n* = 51), had *NCF1* gene defect suggesting it as the most common subtype in the Indian cohort, followed by *CYBB* gene defect (in 30% of the patients). Gene defects in *CYBA* and *NCF2* genes were the least common subtype (7% of patients each). A total of 41 different mutations were identified, including 25 mutations in the *CYBB* gene, 5 mutations each in *CYBA* and *NCF2* genes, and 6 mutations in the *NCF1*

gene. Fifteen (37%) novel mutations were identified in this cohort: 9 in the *CYBB* gene, 2 in the *CYBA* gene, 2 in the *NCF2* gene, and 2 in the *NCF1* gene. A list of mutations identified in CGD patients in the Indian cohort is given in Table 3. The spectrum of mutations in our cohort reveals 58% patients had small deletions, 21% missense, 16% nonsense, 3% splice site, and 1% each insertion and duplication mutations (Table 4).

XL-CGD Mutations

For the *CYBB* gene, 13 nonsense, 7 small deletion, 4 missense, 1 insertion, 1 duplication, and 1 splicing error mutations were identified. Amongst 9 novel mutations: 5 were deletions, 1 insertion, 1 duplication, 1 missense mutation, and 1 nonsense mutation. A female carrier of XL-CGD (P15) was found to have a heterozygous mutation in

Table 2 Details of Investigations

Sr. no.	Variable	No. of patients tested	Patient median (range)	Healthy control (n = 100) median (range)
1	NBT	90	0%	98% (85%–99%)
2	DHR	85	0%	98% (85%–99%)
Stimulation index (SI)				
3	CYBB	22	1.1 (0.8–9.0)	60 (40–100)
4	CYBA	06	1.5 (1.1–3.6)	60 (40–100)
5	NCF1	51	1.3 (0.2–13.3)	60 (40–100)
6	NCF2	06	1.9 (1.3–2.7)	60 (40–100)
NADPH components expression on neutrophils (S/N ratio)				
7	7D5 (XL-CGD)	20	1.6 (0.7–8.1)	9.6 (3.0–83.2)
8	7D5 (AR-CGD)	06	1.2 (0.7–1.7)	9.6 (3.0–83.2)
9	P47^{phox} (AR-CGD)	38	1.9 (0.3–6.5)	10.7 (3.1–107)
10	P67^{phox} (AR-CGD)	04	7.2 (3.5–13)	13 (11.3–53.8)
NADPH components expression on B cells (S/N ratio)				
11	7D5 (XL-CGD)	16	3.4 (0.7–40.8)	20.9 (1.6–93.4)
12	7D5 (AR-CGD)	06	1.2 (1.1–8.2)	20.9 (1.6–93.4)
13	P47^{phox} (AR-CGD)	37	1.7 (0.9–6.4)	3.4 (1.2–33.8)
14	P67^{phox} (AR-CGD)	04	12.3 (5.4–16.3)	20.8 (5.6–46)
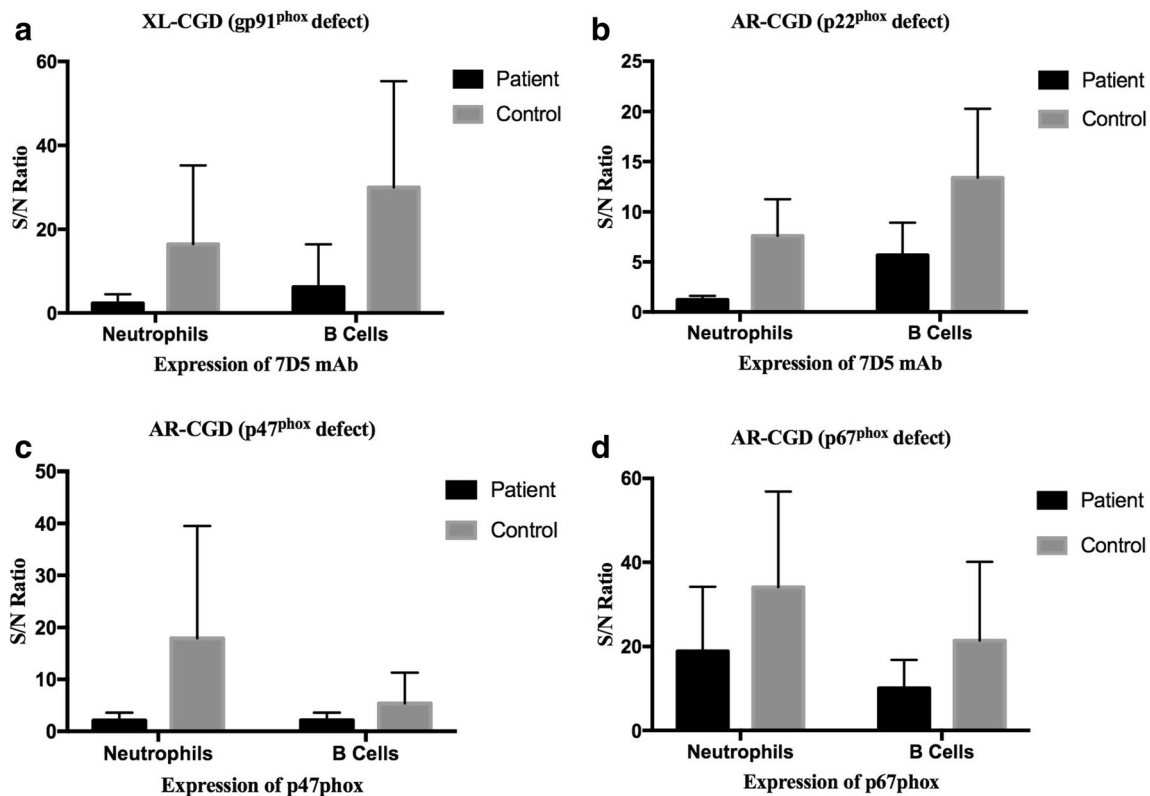
Fig. 3 Comparison of NADPH oxidase component expression on neutrophils and B cells. **a** 7D5 mAb flow cytometric expression analysis on neutrophils and B cells of XL-CGD patients and healthy control peripheral blood. **b** 7D5 mAb flow cytometric expression analysis on neutrophils and B cells of AR-CGD CYBA patients and healthy control peripheral blood. **c** p47phox flow cytometric expression analysis on neutrophils and B cells in AR-CGD NCF1 patients and healthy control peripheral blood. **d** p67phox flow cytometric expression analysis on neutrophils and B cells in AR-CGD NCF2 patients and healthy control peripheral blood

the *CYBB* gene. This patient had a defective NADPH oxidase activity, with 8% positive neutrophils in the NBT assay, 10% positive neutrophils in the DHR assay, and clinical symptoms consistent with CGD. Further exon sequencing in this patient did not reveal any other CGD-related gene mutation. This finding suggests that a heterozygous deletion in the *CYBB* gene and an extremely skewed X-inactivation may have resulted in CGD phenotype in this female patient. The effect of the novel mutations was determined to be disease causing by in silico analysis tools like SIFT, PolyPhen, Human Splicing Finder, and MutationTaster (Table 3, Fig. 5).

AR-CGD Mutations

For the *NCF2* gene, 5 mutations were identified in our cohort. Among them, 2 were novel mutations: 1 missense mutation and a 2-bp deletion mutation. One patient (P92) had deletion of 2 exons (exon 1 and exon 2) which is previously published in article [25].

For the *CYBA* gene, 5 mutations including 2 novel mutations were identified: 1 missense mutation and 1 deletion mutation.

NCF1 Gene Analysis

For the *NCF1* gene, 6 mutations including 2 novel splice region variants were found, c.153+5G>C in P82 probably causing skipping of exon 2. The Human Splice Finder shows the wild-type site with c.153+5G>C is broken with a score of − 13.65. The MaxEnt score is 7.83 for the wildtype and 3.03 for the mutant. In P113, c.574+1G>A probably causing skipping of exon 6 were identified. For c.574+1G>C, the Human Splice Finder shows the wild-type site is broken with a score of − 31.15. The MaxEnt score is 3.34 for the wildtype and − 4.83 for the mutant [23].

Genescan Analysis

Genescan analysis of 51 patients showed an *NCF1/ΨNCF1* ratio of 0:6 in 42 patients indicating homozygous DelGT mutation, while their parents had a carrier ratio (range, 1:5 to 1:7) showing a carrier status, and some carrier parents and patients with other than DelGT mutation showed a ratio of 2:4 [16]. The flow cytometric and molecular analysis revealed that 63 patients (70%) had AR-CGD and 27 patients (30%) had XL-CGD in our cohort. A varied spectrum of 15 novel mutations was identified: 7 deletion, 3 missense, 2 splice site mutation, 1
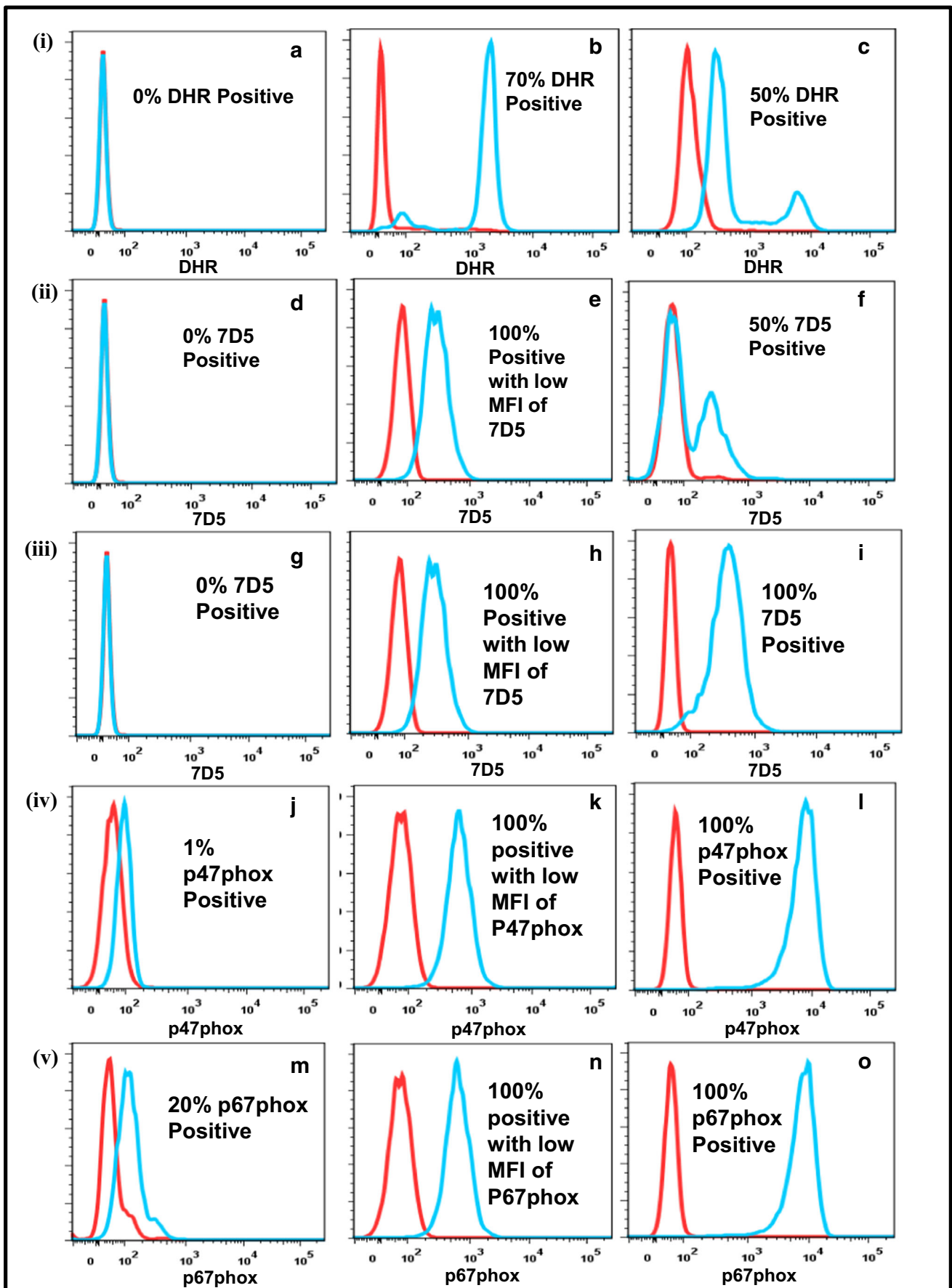
◀ **Fig. 4** Flow cytometric analysis in traveled sample. Unstained tube (red), stained tube (blue), (i) DHR analysis in patient (A), traveled control (B), and mother (C); (ii) 7D5 mAb expression analysis in XL-CGD patient (D), traveled control (E), and carrier mother (F); (iii) 7D5 mAb expression analysis in AR-CGD patient (G), traveled control (H), and mother (I); (iv) p47^{phox} expression analysis in AR-CGD patient (J), traveled control (K), and fresh control (L); (v) p67^{phox} expression analysis in AR-CGD patient (M), traveled control (N), and fresh control (O)

nonsense, 1 duplication, and 1 insertion mutations. (Tables 3 and 4, Fig. 5). The effect of the novel mutations was determined to be disease causing by in silico analysis tools like SIFT, PolyPhen, Human Splicing Finder, and MutationTaster.

Discussion

Diagnosis of CGD is complex due to involvement of multiple genes and overlapping clinical manifestations with different genotypes. Currently, NBT and DHR assays are the most widely used for the diagnosis of CGD. Upon stimulation with PMA, around 85% to 98% neutrophils show reduction of NBT dye and oxidation of DHR dye. In our cohort, the median of percent positive neutrophils is 0% in both NBT (range, 0–13%) and DHR (range, 0–8%) assays. Flow cytometry-based DHR assay has largely replaced NBT assay for its sensitivity and specificity. It is useful to distinguish between the 2 genotypes based on SI and coefficient of variation (CV) of the peak after stimulation for, e.g., p47^{phox} deficiency and gp91^{phox} deficiency [26]. A previous report has conclusively shown an association of the residual NADPH activity value with the clinical severity of the disease [27]. Although XL-CGD is more severe than AR-CGD p47^{phox} deficiency [6, 27, 28], there is no difference between AR-p22^{phox}, AR- p67^{phox}, and AR-p47^{phox} gene defects [1, 8]. A similar observation was noted by previous reports suggesting the effect of other genes, and epigenetic and environmental factor also plays an important role in having such diversity compared to other published populations from the Western world [12, 16]. Recent reports suggest that the amount of residual NADPH oxidase activity is influenced by the type of mutation rather than the gene affected, irrespective of the mode of inheritance [27]. However, a significant correlation of the SI value with the nature of mutation (P value < 0.0001) was observed in our study.

The mothers of all male patients were tested for carrier status using NBT/DHR assays, considering the possibility of the absence of a mosaic pattern in mothers of some male patients with newly generated mutation in the *CYBB* gene and female carriers with extreme lyonization. This factor should be considered when giving genetic counseling, as well as gonadal mosaicism. However, in cases of traveled samples (analyzed after > 24 h), a population of neutrophils with varying intensities mimicking a mosaic pattern was observed in healthy controls which is considered to be due to loss of

viability on storage. The risks of misinterpretation of DHR results in shipped blood had been already described in report by Roesler [29]. In such cases, the use of flow cytometry in investigation of the NADPH oxidase components helped us to determine the genotype, as component expression remained relatively stable in these patients even after storage up to 48 h (Fig. 4).

Analysis of component expression by western blot is both time consuming and labor-intensive and requires large amount of sample, which is difficult in case of pediatric population. Western blot analysis of various components like gp91^{phox}, p22^{phox}, p47^{phox}, and p67^{phox} can now be reliably assessed by using flow cytometry with fluorescently tagged specific mAbs [30]. Expression of gp91^{phox} and p22^{phox} is interdependent for stable and mature expression. If there is a defect in gp91^{phox}; expression of p22^{phox} will also be abnormal. The 7D5 mAb stains the extracellular epitope of gp91^{phox} protein; hence, abnormal expression is observed in both XL-CGD and AR-CGD with p22^{phox} defect [31]. Abnormal 7D5 mAb expression in patients with both gp91^{phox} and p22^{phox} defects and a mosaic pattern in gp91^{phox}-deficient carrier mother gives a clue to the underlying genetic pattern. The expression of p40^{phox} can also be studied by flow cytometry; however, our cohort did not include any patient with p40^{phox} defect.

Molecular diagnosis of CGD is challenging as 7 candidate genes with > 55 target exons have been identified to be associated with CGD [32]. Currently, 2 molecular diagnostic strategies are used to identify genetic defects: Sanger sequencing and NGS. Sanger sequencing is considered as the gold standard for identification of the pathogenic mutation and is preferred when the gene of interest is known. Unless the gene of interest is known, sequencing of all the candidate genes becomes expensive and time consuming. The advent of NGS technology has helped to overcome these limitations by designing small gene panels for screening multiple causative genes for CGD [33, 34]. However, NGS also has some challenges such as need of technical expertise for processing, sorting, and validation of the huge data set generated by this high-throughput technique. The NGS data analysis is also complicated in genes that have deep intronic variants, splice site mutations, gene duplications, loss of entire gene or exons, and presence of pseudogenes. It also requires a diverse approach, based on the type of variant to be analyzed. It is essential to note that the targeted NGS panels are designed to screen for specific regions of the gene. For instance, some commercial panels screen only for DelGT mutation [33]; hence, patients with other than DelGT mutation are not diagnosed or may be missed [35].

Thus, a comprehensive approach with detection of a mosaic pattern in mother's sample along with component expression analysis is recommended before planning the molecular diagnostic strategy. Using this strategy, mutations could be identified in 88% of the patients using either

Table 3 List of mutations identified in Indian CGD patients

Pt. no.	Defective protein	Defective gene	Location	Nucleotide change	Protein change	Mutation type	Allele	Carrier status		Reference
								Mother	Father	
List of mutations in the *NCF1* gene ($n = 51$)										
1	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	Carrier	Carrier	Roos et al., 2010, 2nd [37]
3	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	Carrier	Carrier	Roos et al., 2010, 2nd [37]
7	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	Carrier	Carrier	Roos et al., 2010, 2nd [37]
9	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	NA	NA	Roos et al., 2010, 2nd [37]
12	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	NA	NA	Roos et al., 2010, 2nd [37]
18	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	NA	NA	Roos et al., 2010, 2nd [37]
19	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	NA	NA	Roos et al., 2010, 2nd [37]
24	p47phox	NCF1	Exon 6	c.574G>A	Skip Exon 6	Splicing error	Homozygous	ND	ND	Roos et al., 2010, 2nd [37]
25	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	NA	NA	Roos et al., 2010, 2nd [37]
26	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	Carrier	Carrier	Roos et al., 2010, 2nd [37]
30	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	Carrier	Carrier	Roos et al., 2010, 2nd [37]
37	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	Carrier	Carrier	Roos et al., 2010, 2nd [37]
39	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	NA	NA	Roos et al., 2010, 2nd [37]
40	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	Carrier	Carrier	Roos et al., 2010, 2nd [37]
50	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	NA	NA	Roos et al., 2010, 2nd [37]
57	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	Carrier	Carrier	Roos et al., 2010, 2nd [37]
62	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	Carrier	Carrier	Roos et al., 2010, 2nd [37]
63	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	NA	NA	Roos et al., 2010, 2nd [37]
64	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	Carrier	Carrier	Roos et al., 2010, 2nd [37]
65	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	Carrier	Carrier	Roos et al., 2010, 2nd [37]
71	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	Carrier	Carrier	Roos et al., 2010, 2nd [37]
73	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	Carrier	Carrier	Roos et al., 2010, 2nd [37]
74	p47phox	NCF1	Exon 2	c.124C>T	p. R42W	Missense	Homozygous	NA	NA	Wolach et al., 2016 [1]
75	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	Carrier	Carrier	Roos et al., 2010, 2nd [37]

Table 3 (continued)

Pt. no.	Defective protein	Defective gene	Location	Nucleotide change	Protein change	Mutation type	Allele	Carrier status		Reference
								Mother	Father	
78	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	Carrier	Carrier	Roos et al., 2010, 2nd [37]
79	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	Carrier	NA	Roos et al., 2010, 2nd [37]
82	p47phox	NCF1	Exon 2	c.153+5G>C	Skip Exon 2	Probably splicing error	Homozygous	ND	NA	This study
84	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	NA	NA	Roos et al., 2010, 2nd [37]
85	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	Carrier	Carrier	Roos et al., 2010, 2nd [37]
89	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	NA	NA	Roos et al., 2010, 2nd [37]
91	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	Carrier	Carrier	Roos et al., 2010, 2nd [37]
95	p47phox	NCF1	Exon 8	c.784G>A	p. G262S	Missense	Homozygous	NA	NA	Roos et al., 2010, 2nd [37]
96	p47phox	NCF1	Sibling of Pt. 95							
97	p47phox	NCF1	Sibling of Pt. 95							
98	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	NA	NA	Roos et al., 2010, 2nd [37]
99	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	NA	NA	Roos et al., 2010, 2nd [37]
104	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	Carrier	Carrier	Roos et al., 2010, 2nd [37]
110	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	Carrier	NA	Roos et al., 2010, 2nd [37]
111	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	NA	NA	Roos et al., 2010, 2nd [37]
112	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	Carrier	Carrier	Roos et al., 2010, 2nd [37]
113	p47phox	NCF1	Exon 6	c.574+1G>A	Skip Exon 6	Probably splicing error	Homozygous	ND	ND	This study
121	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	NA	NA	Roos et al., 2010, 2nd [37]
124	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	NA	NA	Roos et al., 2010, 2nd [37]
126	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	NA	NA	Roos et al., 2010, 2nd [37]
128	p47phox	NCF1	Exon 2	c.124C>T	p. R42W	Missense	Homozygous	Carrier	Carrier	Wolach et al., 2016 [1]
129	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	Carrier	Carrier	Roos et al., 2010, 2nd [37]
131	p47phox	NCF1	Sibling of Pt. 128							
132	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	NA	NA	Roos et al., 2010, 2nd [37]
137	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	Carrier	Carrier	Roos et al., 2010, 2nd [37]
139	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	Carrier	Carrier	Roos et al., 2010, 2nd [37]

Table 3 (continued)

Pt. no.	Defective protein	Defective gene	Location	Nucleotide change	Protein change	Mutation type	Allele	Carrier status Mother	Carrier status Father	Reference
140	p47phox	NCF1	Exon 2	c.75_76delGT	p. Y26HfsX26	Deletion	Homozygous	Carrier	Carrier	Roos et al., 2010, 2nd [37]
List of mutations in the CYBB gene (n = 27)										
6	gp91phox	CYBB	Exon 7	c.676C>T	p. R226X	Nonsense	Homozygous	Carrier	ND	Roos et al., 2010, 3rd [38]
15	gp91phox	CYBB	Exon 7	c.771_777delCCCAATC	p. P258LfsX8	Deletion	Heterozygous	Normal	Normal	This study
16	gp91phox	CYBB	Exon 4	c.217C>T	p. R73X	Nonsense	Homozygous	Carrier	ND	Roos et al., 2010, 3rd [38]
20	gp91phox	CYBB	Exon 11	c.1418del T	p. F473SfsX28	Deletion	Homozygous	Carrier	ND	This study
42	gp91phox	CYBB	Exon 4	c. 271C>T	p. R91X	Nonsense	Homozygous	Carrier	ND	Roos et al., 2010, 3rd [38]
46	gp91phox	CYBB	Exon 7	c.714_715insTA	p. H239fsX3	Insertion	Homozygous	Carrier	ND	This study
53	gp91phox	CYBB	Exon 7	c.676C>T	p. R226X	Nonsense	Homozygous	ND	ND	Roos et al., 2010, 3rd [38]
54	gp91phox	CYBB	Exon 9	c.925G>A	p. E309K	Missense	Homozygous	Carrier	ND	Roos et al., 2010, 3rd [38]
61	gp91phox	CYBB	Exon 5	c.400del T	p. S134LfsX5	Deletion	Homozygous	Carrier	ND	This study
66	gp91phox	CYBB	Exon 11	c.1415del G	p. G472AfsX30	Deletion	Homozygous	Carrier	ND	Roos et al., 2010, 3rd [38]
67	gp91phox	CYBB	Exon 7	c.752G>A	p. W251X	Nonsense	Homozygous	Carrier	ND	Roos et al., 2010, 3rd [38]
70	gp91phox	CYBB	Exon 10	c.1234G>A	p. G412R	Missense	Homozygous	Normal	ND	Roos et al., 2010, 3rd [38]
72	gp91phox	CYBB	Exon 5	c.388C>T	p. R130X	Nonsense	Homozygous	Carrier	ND	Roos et al., 2010, 3rd [38]
76	gp91phox	CYBB	Exon 3	c.170C>A	p. A57E	Missense	Homozygous	Carrier	ND	Roos et al., 2010, 3rd [38]
83	gp91phox	CYBB	Exon 9	c.1038Del T	p. E347RfsX39	Deletion	Homozygous	Carrier	ND	Roos et al., 2010, 3rd [38]
90	gp91phox	CYBB	Exon 3	c.217C>T	p. R73X	Nonsense	Homozygous	ND	ND	Roos et al., 2010, 3rd [38]
93	gp91phox	CYBB	Exon 5	c.380_381dupGT	p. N128X	Duplication	Homozygous	Carrier	ND	This study
103	gp91phox	CYBB	Exon 10	c.815 G>A	p. W272X	Nonsense	Homozygous	Carrier	ND	Roos et al., 2010, 3rd [38]
106	gp91phox	CYBB	Exon 11	c.1426G>A	p. W453X	Nonsense	Homozygous	Carrier	ND	Roos et al., 2010, 3rd [38]
107	gp91phox	CYBB	Exon 9	c.1085 C>A	p. T362K	Missense	Homozygous	Carrier	ND	This study
109	gp91phox	CYBB	Exon 5	c.475A>T	p. W159X	Nonsense	Homozygous	Carrier	ND	This study
115	gp91phox	CYBB	Exon 6	c.644_646 del TCT	p. F215X	Deletion	Homozygous	Carrier	ND	This study
119	gp91phox	CYBB	Exon 5	c.388C>T	p. R130X	Nonsense	Homozygous	Carrier	ND	Roos et al., 2010, 3rd [38]
120	gp91phox	CYBB	Exon 8	c.834_841del TCTGTAT-C	p. W280X	Deletion	Homozygous	Carrier	ND	This study
122	gp91phox	CYBB	Exon 3	c.252G>A	Skip Exon 3	Splicing error	Homozygous	Carrier	ND	Roos et al., 2010, 3rd [38]
127	gp91phox	CYBB	Exon 3	c.217C>T	p. R73X	Nonsense	Homozygous	Carrier	ND	Roos et al., 2010, 3rd [38]

Table 3 (continued)

| Pt. no.	Defective protein	Defective gene	Location	Nucleotide change	Protein change	Mutation type	Allele	Carrier status		Reference
								Mother	Father	
133	gp91phox	CYBB	Exon 9	c. 1120C>T	p. Q374X	Nonsense	Homozygous	Carrier	ND	Roos et al., 2010, 3rd [38]
List of mutations in the CYBA gene (n = 6)										
47	p22phox	CYBA	Exon 3	c.157G>C	p. E53Q	Missense	Homozygous	Carrier	Carrier	This study
49	p22phox	CYBA	Exon 4, Exon 5, Exon 6	Large deletion	Exon 4, Exon 5, Exon 6	Deletion	Homozygous	Carrier	NA	This study
52	p22phox	CYBA	Exon 4	c.269G>A	p. R90Q	Missense	Homozygous	Carrier	Carrier	Roos et al., 2010, 2nd [37]
55	p22phox	CYBA	Exon 4	c.269G>A	p. R90Q	Missense	Homozygous	Carrier	Carrier	Roos et al., 2010, 2nd [37]
80	p22phox	CYBA	Exon 4	c.268C>T	p. R90W	Missense	Homozygous	NA	NA	Roos et al., 2010, 2nd [37]
105	p22phox	CYBA	Exon 6	c.371C>T	p. A124V	Missense	Homozygous	Carrier	Carrier	Roos et al., 2010, 2nd [37]
List of mutations in the NCF2 gene (n = 6)										
43	p67phox	NCF2	Exon 2, Exon 5	c.196C>T, c.551G>C	p. R66X p. R184P	Missense	Compound Heterozygous	Carrier	Carrier	Roos et al., 2010, 2nd [37]
51	p67phox	NCF2	Exon 12	c. 1148_1149 TG del	p. L382RfsX7	Deletion	Homozygous	Carrier	Carrier	This study
60	p67phox	NCF2	Exon 5	c.550C>T	p. R184X	Nonsense	Homozygous	NA	NA	Roos et al., 2010, 2nd [37]
77	p67phox	NCF2	Exon 2	c. 73G>A	p. A25T	Missense	Homozygous	NA	NA	This study
92	p67phox	NCF2	Exon 1, Exon 2	Large deletion	Exon 1 Exon 2	Deletion	Homozygous	NA	NA	Roos et al., 2010, 2nd [37]
117	p67phox	NCF2	Exon 5	c.550C>T	p. R184X	Nonsense	Homozygous	NA	NA	Roos et al., 2010, 2nd [37]

NA, not available; *ND*, not done

specific Genescan/Sanger sequencing method. Only 12% (11 out of 90) of the patients required NGS analysis. Most of our patients had an *NCF1* gene defect, which has 2 pseudogenes, and we preferred the traditional approach of

Table 4   Molecular spectrum of Indian CGD patients

Variable	CYBB No. of patient (%)	CYBA No. of patient (%)	NCF1 No. of patient (%)	NCF2 No. of patient (%)	Total No. of patient (%)
Patients	27	6	51	6	90
Families	27	6	45	6	84
Male	26 (96%)	2 (33%)	17 (33%)	3 (50%)	48 (53%)
Female	1 (4%)	4 (67%)	34 (67%)	3 (50%)	42 (47%)
Mutations	25	5	6	5	41
New mutations	9	2	2	2	15 (37%)
Nonsense	13 (48%)	–	–	1 (17%)	14 (16%)
Missense	4 (15%)	5 (83%)	6 (12%)	3 (50%)	18 (21%)
Deletions	7 (26%)	1 (17%)	42 (82%)	2 (33%)	52 (58%)
Insertions	1 (4%)	–	–	–	1 (1%)
Splice site	1 (4%)	–	3 (6%)	–	4 (3%)
Duplication	1 (4%)	–	–	–	1 (1%)
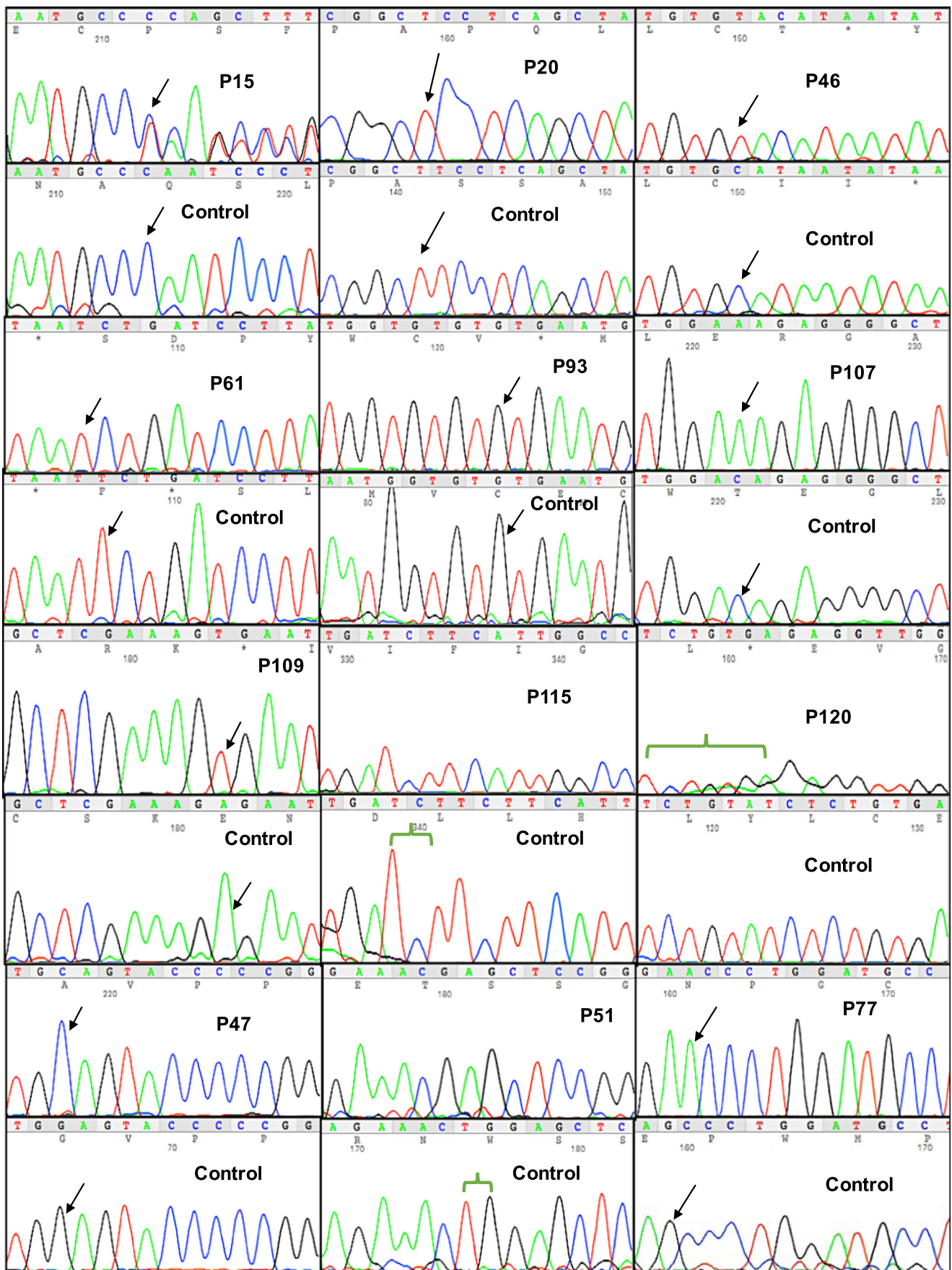
◀ **Fig. 5** Novel mutations identified in Indian CGD cohort. (i) Sequence analysis of novel mutation in CYBB, CYBA, and NCF2 genes with healthy control sample. (ii) Identification of NCF1 gene mutation. (A) Screenshot from the Integrative Genome Viewer (Broad Institute, Cambridge, MA) shows the exon 2 sequence reads from the NCF1 gene of the P82 obtained with the Ion Torrent S5 system. All reads show the c.153+5G>C. (B) Screenshot from the Integrative Genome Viewer (Broad Institute, Cambridge, MA) shows the exon 6 sequence reads from the NCF1 gene of the P113 obtained with the Ion Torrent S5 system. All reads show the c.574+1G>A

Genescan analysis over NGS for establishing a diagnosis. This highlights the importance of identifying patients with p47phox defect especially in an Indian cohort, as the molecular strategy differs in these patients. Of the 56% patients with abnormal p47phox expression ($n = 51$), 82% ($n = 42$) carried DelGT mutation. An abnormal p47phox expression suggests a defect in the *NCF1* gene. Patients with abnormal p47phox expression, as well as patients for whom the
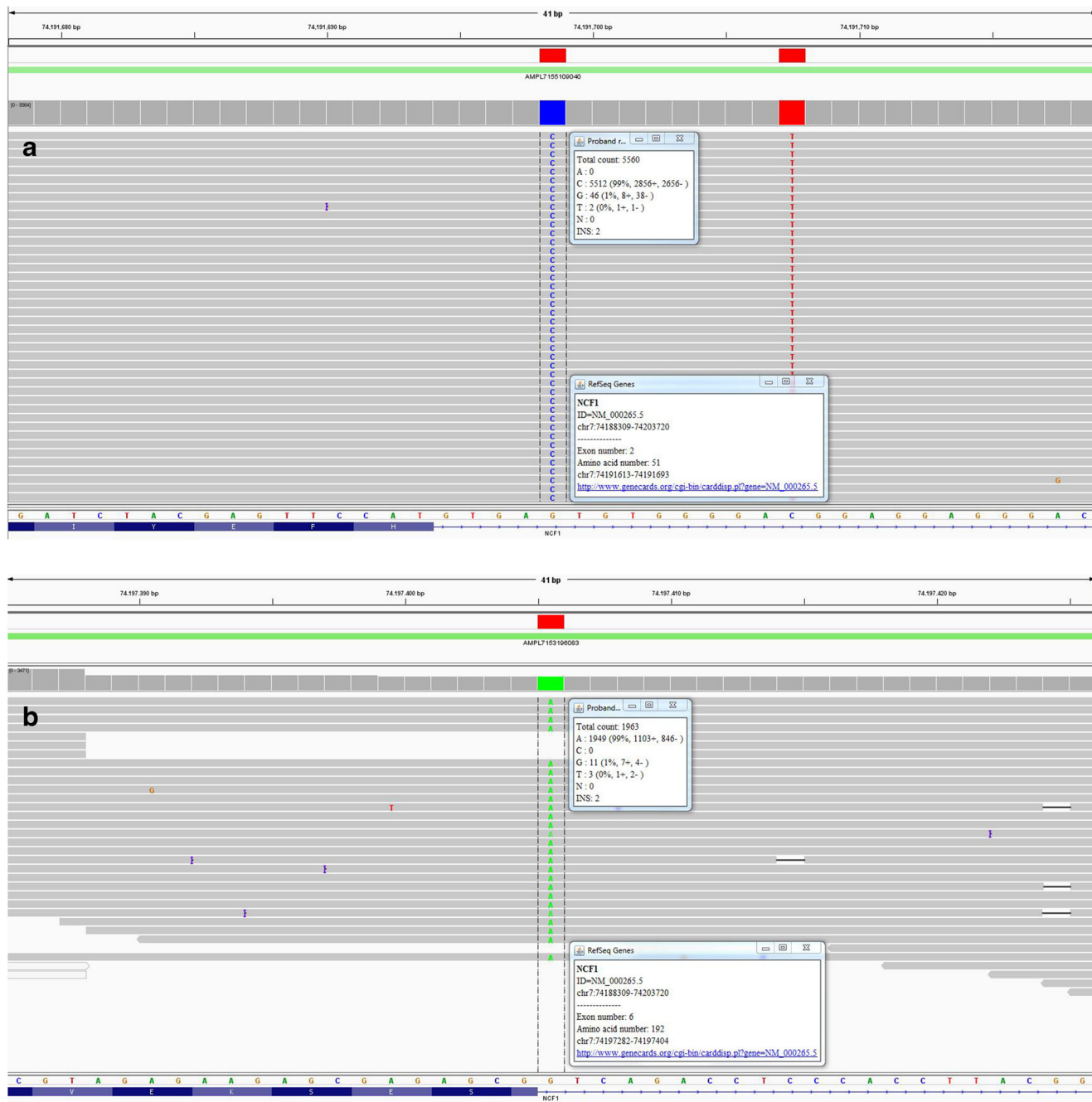
Fig. 5 (continued)

mother does not show a carrier pattern, should be first screened by Genescan analysis for DelGT mutation in the *NCF1* gene. In our study, this approach helped in the diagnosis of 13 patients [16].

Analysis of *NCF1* is more difficult as it is accompanied on each side by one Ψ*NCF1* gene, which is > 99% homologous to *NCF1* but lacks a GT sequence at the start of exon 2. Therefore, sequencing by *NCF1*-specific PCR is difficult, and hence a Genescan is recommended to determine the presence of either one/two *NCF1* genes or only GT deletion-containing pseudogenes in the patient's DNA [32]. A rapid Genescan analysis calculating the peak height of *NCF1* and Ψ*NCF1* genes provides a reliable tool for identification of this particular mutation. Altered ratio of *NCF1* to Ψ*NCF1* gene may be seen in carriers of this mutations as well as in patients with other than DelGT mutation [36]. In the case of other than DelGT mutation, sequencing of *NCF1*-cDNA usually reveals another mutation than the GT deletion which is further confirmed with genomic DNA [18]. In our cohort, other than DelGT mutations were identified by NGS analysis as the cDNA analysis could not be performed due to unavailability of patient's mRNA from fresh blood sample. However, cDNA is preferred for the analysis of other than DelGT mutations. We identified 15 novel mutations in our cohort: 9 mutations are in the *CYBB* gene and 2 mutations in each *NCF2, NCF1,* and *CYBA* genes were identified, suggesting a diverse genetic pattern in the Indian population. XL-CGD comprised of 30% (27/90) of the patients in our cohort. All presently known mutations in the *CYBB* gene were previously listed [37, 38], where nonsense mutations were the most common type (29.8%). According to recently published large cohort data from China [4], single-nucleotide substitution (nonsense—32.7%, missense—20.7%, splicing error—22.7%) mutations were most commonly observed in Chinese population. In our cohort, nonsense mutations (13 of 27 [48%]) were more commonly followed by deletion (6 of 27 [22%]), missense (4 of 27 [15%]), insertion, duplication, and splicing (1 of 27 [4%]) mutations. Among the novel mutations identified in the *CYBB* gene, small deletion mutations (1 to 8 bp) were the most commonly observed (5 of 9 [56%]) in our cohort.

Autosomal recessive CGD (AR-CGD) accounted for the majority of the Indian patients ($n = 63$). Among them, the *NCF1* gene is the most affected gene in our cohort with 51 cases molecularly characterized. DelGT ($n = 42$) is the most common mutation observed in our cohort followed by 3 splice site and 2 missense mutations (c.124 C>T and c.784 G>A, both with a SIFT score of 0.0 and PolyPhen score of 1.0). Overall, defects in *CYBA* and *NCF2* genes are relatively rare (comprised of 5% of total CGD patients) [33]. We have identified 6 such patients in each group with 2 novel mutations in both *CYBA* and *NCF2* genes. This suggests wide heterogeneity in terms of spectrum of mutations in Indian patients.

In summary, this large study on molecular characterization of CGD from India emphasizes the influence of ethnic and cultural practices on the spectrum of CGD and also highlights the utilization of flow cytometry. Flow cytometry-based further classification of subtypes of CGD facilitates rapid identification of affected genes for molecular confirmation. This is essential in a developing country like India considering all constraints with current diagnostic strategies.

Compliance with Ethical Standards

Pateint's sample and additional samples (parents and siblings) were collected in the case of a strong family history after obtaining a proper informed consent. A clinical protocol has been followed per the guidelines of the Institutional Ethics Committee and Declaration of Helsinki.

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

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