

# Clinical, Immunological, and Molecular Findings of Patients with p47<sup>phox</sup> Defect Chronic Granulomatous Disease (CGD) in Indian Families

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**Abstract** Chronic granulomatous disease (CGD) is a group of inherited disorder of phagocytes, resulting from mutations in the components of the NADPH oxidase complex. Reduced or absent oxygen radical synthesis seen in these patients leads to impaired killing of intracellular bacteria and fungi. CGD clinically presents with recurrent and life-threatening infections as well as granulomatous inflammatory responses. p47<sup>phox</sup> encoded by the *NCF1* gene is the most common autosomal recessive form of CGD which is often clinically milder. Here, we are presenting the data on clinical and immunological findings in 21 Indian patients with Del GT mutation in the *NCF1* gene. Diagnosis of these patients was based on detailed clinical evaluation, measurement of respiratory burst activity by nitro blue tetrazolium and dihydrorhodamine-1,2,3 assay, expression of p47<sup>phox</sup> by flow cytometry, and molecular confirmation by GeneScan method. Seventeen male and four female patients with median age of onset of 1 year ranging from 1.5 months to 6 years were included

in the study. Sixty-two percent (13 out of 21) of patients belonged to a consanguineous marriage with only one family having a history of a previous sibling death. Significant variability in clinical presentation was observed in spite of identical genetic defect ranging from asymptomatic to very severe presentation leading to early death or requiring transplantation. However, none of these patients showed difference in immunological parameters to account for this variability. Thus, this study highlights the phenotypic heterogeneity seen in these patients with Del GT mutation in the *NCF1* gene and its implication in management of these patients.

**Keywords** AR-CGD · *NCF1* defect · p47<sup>phox</sup> flow cytometry · GeneScan

## Introduction

Chronic granulomatous disease (CGD) is a primary immunodeficiency disorder marked by inability of phagocytes to produce microbicidal reactive oxygen species (ROS) essential for elimination of bacterial and fungal infections. Phagocytes convert molecular oxygen into potent ROS with the help of an enzyme called nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase). NADPH oxidase consists of spatially separated six heterosubunits, namely, gp91<sup>phox</sup>, p22<sup>phox</sup>, p47<sup>phox</sup>, p67<sup>phox</sup>, p40<sup>phox</sup>, and GTPase Rac, that associate in a stimulus-dependent manner to form active enzymes [1]. Two subunits gp91<sup>phox</sup> and p22<sup>phox</sup> are integral membrane proteins that together form heterodimeric flavocytochrome b558 at the catalytic core. Regulatory subunits p47<sup>phox</sup>, p67<sup>phox</sup>, and p40<sup>phox</sup> reside in the cytoplasm under unstimulated conditions. Upon activation, p47<sup>phox</sup> gets phosphorylated and the entire assembly eventually

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translocates to the membrane in order to form an active enzyme complex with b558 [2].

Each component of this enzyme complex is encoded by its own gene: gp91<sup>phox</sup> by the *CYBB* gene (cytochrome b-245 beta subunit) located on chromosome X, p22<sup>phox</sup> by the *CYBA* gene (cytochrome b-245 alpha subunit) located on chromosome 16, p47<sup>phox</sup> by the *NCF1* gene (neutrophil cytosolic factor 1) on chromosome 7, and p67<sup>phox</sup> and p40<sup>phox</sup> by *NCF2* (neutrophil cytosolic factor 2) and *NCF4* (neutrophil cytosolic factor 4) genes located on chromosomes 1 and 22, respectively [3]. Mutation in any one of these genes can lead to inappropriate activation of NADPH oxidase resulting in rare, life-threatening genetic disorders like CGD.

Prevalence of CGD is approximately 1 in 200,000 to 250,000 live births [4]; however, due to the lack of definitive diagnostic facilities, lack of awareness of the doctors to this disease, and the access of the patients to good clinics, the incidence rate is likely to be underestimated especially in India. [5]. Mutation in the *CYBB* gene is reported in approximately 65 to 70 % of all CGD patients, making it largely X-linked disorder (XL-CGD) in the western world [6, 7]. Autosomal recessive (AR-CGD) mutations in the *NCF1* gene affecting the expression of p47<sup>phox</sup> are reported in approximately 30 % of all cases. Defects in genes encoding for p22<sup>phox</sup> and p67<sup>phox</sup> are relatively uncommon, and only one case is reported with p40<sup>phox</sup> defect till now [8–12]. Though the pattern of genetic abnormality is vastly affected by ethnicity, autosomal recessive inheritance of CGD is more common in regions with high rate of consanguinity [13].

Patients with *NCF1* gene defects have absent or non-functional expression of p47<sup>phox</sup> protein. A 2-bp GT deletion at the beginning of exon 2 in the *NCF1* gene is the most common mutation in the *NCF1* gene accounting for more than 95 % cases of p47<sup>phox</sup> defect. This 2-bp deletion is also a signature characteristic of two pseudo-*NCF1* genes ( $\Psi$ *NCF1*) that flank the *NCF1* gene [14]. Due to a random recombination between *NCF1* and  $\Psi$ *NCF1*, this 2-bp GT deletion results in the loss of a functional gene on one allele. An individual who receives a defective gene carrying an allele from both mother and father lacks expression of p47<sup>phox</sup> resulting in autosomal CGD [15]. Due to high degree of sequence homology between *NCF1* and  $\Psi$ *NCF1*, direct sequencing and annotation become difficult, but a rapid GeneScan analysis can not only identify the *NCF1* to  $\Psi$ *NCF1* ratio based on a 2-bp difference but also helps in the identification of patients and carrier status [16]. Generally, the ratio in normal individuals is 2:4, but in some cases 1:1 can also be seen. In *NCF1* defect carriers, the ratio is 1:5 [17, 18].

Clinical manifestations seen in CGD vary significantly depending on the underlying genetic defect [19]. On the whole, XL-CGD is more severe as compared to AR-CGD with defect in the *NCF1* gene [13]. Clinical severity also depends on the

type of mutation seen in these patients resulting in variable residual NADPH activity. p47<sup>phox</sup> defect is the most common form of genetic defect identified in a cohort of Indian CGD patients from our center. Here, we are presenting clinical and immunological characteristics seen in this group with a single common gene defect.

## Materials Methods

### Samples

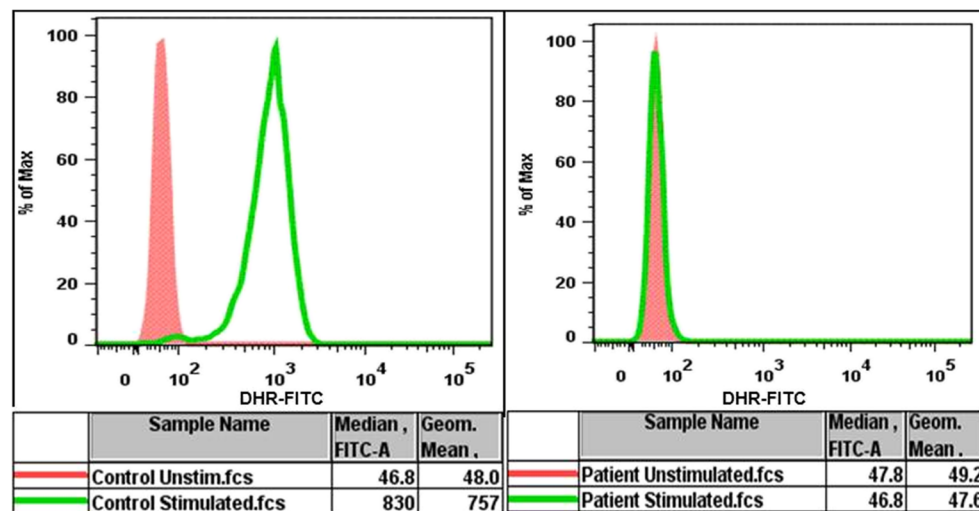
A total of 21 patients with AR-CGD were diagnosed and molecularly confirmed with *NCF1* defect during the study at National Institute of Immunohaematology, ICMR, Mumbai. After obtaining appropriate informed consent as per the guidelines of the institutional ethics committee board under Declaration of Helsinki, 3 ml peripheral blood was collected in EDTA, plain, and heparin vacutainers.

### Laboratory Evaluation and Neutrophil Function Test

Neutrophil functioning analysis was done using nitro blue tetrazolium (NBT), dihydrorhodamine-123 (DHR), and phorbol myristate acetate (PMA) from Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, Missouri, USA). A defect in the NADPH oxidase enzyme complex was detected by NBT dye reduction test where upon activation, blue-black deposits of formazan particles were seen in functional neutrophils after stimulating with PMA; similarly, oxidation of DHR was studied using flow cytometry as a tool [5]. Complete blood counts were evaluated using a Sysmex XS-800i (Sysmex Co., Kobe, Japan) five-part automated hematological analyzer. Lymphocyte subset analysis was done in a few patients using BD Multitest 6-color TBNK reagent, and cells were acquired on FACS Aria-I using FACSDiva software (BD Biosciences, San Jose, CA, USA). Serum immunoglobulin levels were estimated by nephelometry.

### Dihydrorhodamine Assay

DHR was dissolved in DMSO to prepare 29 mM working stock stored at –20 °C. Out of this stock, 0.75  $\mu$ l of DHR is added to 100  $\mu$ l of EDTA anticoagulated whole blood sample in each unstimulated and stimulated tubes. PMA was dissolved in DMSO to give a 1- $\mu$ g/ $\mu$ l stock solution, and aliquots were stored at –20 °C. Stimulation was carried out by adding 2  $\mu$ l of PMA working solution, which is a 1/10 dilution of the stock solution. It is then incubated at 37 °C for 15 min. BD FACS lysing solution (1 $\times$ ) was used for RBC lysis. For analysis, at least 10,000 neutrophils are acquired (Fig. 1).



**Fig. 1** Oxidation of dihydrorhodamine-123 (DHR) assay in control and *NCF1* defect patient after stimulation with PMA

### Flowcytometric Evaluation of NADPH Oxidase Component Expression

Expression of the NADPH component was evaluated using monoclonal antibodies including 7D5 (MBL International Co., Woburn, MA, USA) against gp91<sup>phox</sup> and p22<sup>phox</sup>, and against p47<sup>phox</sup> (clone 1, BD Biosciences, San Diego, CA, USA) and p67<sup>phox</sup> (clone D-6, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) in a flow cytometry-based approach. Stain-lyse-wash protocol was used to study the transmembrane components gp91<sup>phox</sup> and p22<sup>phox</sup>. The expression was evaluated on neutrophils and B and T lymphocytes, which were gated on phycoerythrin-Cy5-conjugated anti-CD20 and allophycocyanin-conjugated anti-CD3, respectively (Becton Dickinson, San Diego, CA, USA). Cytoplasmic components were evaluated using the previously described permeabilization protocol [20]. A minimum of 10,000 neutrophils per tube were acquired on BD FACSAria-I using FACSDiva software, and data was further analyzed using FlowJo software (Tree Star Inc., Ashland, USA).

### GeneScan Analysis

Genomic DNA was isolated using whole blood DNA extraction kit (Sigma-Aldrich Co., St. Louis, Missouri, USA). The *NCF1* gene and two flanking  $\Psi$ *NCF1* genes were amplified using 6-FAM tag-labeled primers as per the sequences provided earlier [21]. GeneScan analysis was done 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. Ratio of *NCF1* to  $\Psi$ *NCF1* was determined in order to establish normal, patient, and carrier status [19].

### Results

A total of 21 CGD patients belonging to 19 families were found to harbor the *NCF1* gene defect. Sixty-two percent of these patients (13 out of 21) belonged to families with a consanguineous setting. Only one family had a history of death of patient's twin sibling at the age of 2.5 years. Male predominance was seen in *NCF1*-defective patients as 17 out of 21 patients were male. Though the median age of onset of symptoms for these patients was found to be 1 year, the median age at diagnosis was about 2.6 years showing a considerable delay in diagnosis. Basic clinical parameters of CGD patients with *NCF1* defect are given in Table 1.

### Laboratory Investigation and Molecular Analysis

Superoxide burst activity of neutrophils was measured by NBT test ranging from 0 to 1 % ( $n=21$ ) and DHR assay ranging from 0 to 5 % ( $n=17$ ) positive, respectively. Lymphocyte subsets as evaluated in 17 patients showed normal count of B cells and with mild elevation of T and NK cells in almost half of the patients as given in Table 2. Leukocytosis was present in 52 % of the patients; majority of them showed neutrophilia. Similarly, serum immunoglobulin levels were elevated in 50 % of the patients. Anemia was also observed in 90 % of the patients due to chronic infections.

GeneScan analysis of 21 patients showed an *NCF1*/ $\Psi$ *NCF1* ratio of 0:6 indicating homozygous Del GT, while their parents had a ratio ranging from 1:5 to 1:7 showing a carrier status, except in two carrier parents with a ratio which is seen to be 2:4 (Table 3; Fig. 2). Normal controls showed a ratio of 2:4, except in two controls showing a ratio of >0.8. Expression of the p47<sup>phox</sup> antibody using flow cytometry was performed on 10 patients and found to be abnormal. Similarly,

**Table 1** Basic and Clinical parameters of *NCF1* defect patients

Basic parameters	Median	Range
°Age of onset	1 year	1.5 month–6 years
°Age of diagnosis	2.5 years	3 months–22 years
°WBC	16025	7350–44810
°ANC	7175	3455–25542
°ALC	5024	1872–17476
°Hb	9.8	5.2–13
°NBT	0 %	0–1 %
°DHR	0 %	0–5 %
° <i>NCF1</i> /pseudo- <i>NCF1</i>	0	0:06
Clinical findings	Number	%
°Pneumonia	16	76
°Tuberculosis	10	48
°Lymphadenopathy	9	43
°Diarrhea	7	34
°Abscess	5	24
°Skin infection	4	19
Organism isolated	Number	%
° <i>Aspergillus</i> sp.	4	19
° <i>Staphylococcus aureus</i>	4	19
° <i>Chromobacterium violaceum</i>	3	14
° <i>Candida</i> sp.	3	14
° <i>Klebsiella</i> sp.	2	10
° <i>E. coli</i>	2	10
° <i>Aspergillus fumigatus</i>	1	5
° <i>Burkholderia cepacia</i>	1	5
°Acinetobacter	1	5

analysis in eight carrier parents showed reduced intensity of expression of cytoplasmic p47<sup>phox</sup> as compared to healthy controls (Fig. 3; Table 4).

### Clinical Features

Pneumonia and tuberculosis were the most frequent clinical manifestation present in 76 and 48 % patients, respectively. Lymphadenitis, skin infections, and chronic diarrhea were among the other common presenting features as listed in Table 1. Interestingly, sternum osteomyelitis due to *Aspergillus* infection and lupus vulgaris was also seen in two cases. Apart from skin abscesses, no other deep abscesses were reported in these patients. Only one patient was documented to have chronic granulomatous infiltrations in lung requiring repeated immunosuppressive therapy.

Since most of the patients received empirical antimicrobial therapy, culture status could not be established. Of the confirmed culture reports, *Aspergillus* species and *Staphylococcus aureus* were isolated in four patients. *Candida* species and *Chromobacterium violaceum* were isolated in three patients as shown in Table 1.

All the patients were started with antibacterial and antifungal prophylaxis after the diagnosis. None of these patients were treated with IFN $\gamma$  therapy. Out of 21 patients, 5 died due to severe infections and 2 underwent matched sibling donor transplantation for severe disease.

### Discussion

CGD accounts for 12.5 % of primary immunodeficiency diseases in the ESID registry [22] and 8.5 % [23] in the US registry of primary immunodeficiency disorder. However, the incidence is reported to be higher where consanguinity is practiced; for example, in an Arab population, it is twice that in western parts of the world [8, 24]. Incidence in India is not known; however, CGD constitutes approximately 14 % among the diagnosed population of the primary immunodeficiency disorder cohort [25, 26].

A report from an Indian group suggests that the autosomal recessive (59 %) pattern of inheritance is more common than X-linked (41 %) CGD [5] and *NCF1* gene defect accounts for 41 % (7 out of 17) of all the cases. Thus, identifying cases with *NCF1* gene mutations from a cohort of CGD patients is important for management as well as for genetic counseling. If a mother's neutrophils show a mosaic pattern on NBT and DHR assay, it clearly suggests XL-CGD, but if it shows normal NBT or DHR results, still the male child could have XL-CGD due to spontaneous mutation that requires molecular confirmation. The expression of p22<sup>phox</sup>, p47<sup>phox</sup>, and p67<sup>phox</sup> needs to be studied further for identifying the autosomal recessive genetic defect [27]. Western blot analysis is routinely practiced to infer the specific genotype of the patient, but it is very time consuming, laborious, and requires a large amount of blood sample. Most of the patients belong to the pediatric group where availability of a large amount of sample is often difficult. Direct molecular analysis is also time consuming and costly, and very few centers provide this facility in India. Flow cytometry offers a rapid and sensitive tool for studying the expression of NADPH oxidase component proteins p22<sup>phox</sup>, p47<sup>phox</sup>, and p67<sup>phox</sup> on neutrophils and B lymphocytes [20]. All the 10 patients tested by flow cytometry showed a consistent reduction in expression of p47<sup>phox</sup> compared to XL-CGD patients and healthy normal controls, thereby suggesting utility of this assay as a rapid screening tool at the time of diagnosis.

More than 95 % of the patients with *NCF1* gene defect show presence of a single Del GT mutation at the beginning of exon 2 [28]. Molecular analysis by direct PCR sequencing is very difficult in the *NCF1* gene due to presence of highly homologous pseudogenes. A rapid GeneScan method calculating the peak height of *NCF1* and  $\Psi$ *NCF1* genes provides a reliable tool for identification of this particular mutation. Altered *NCF1* to  $\Psi$ *NCF1* gene ratio is seen in carriers of this mutation [29]. A

**Table 2** Laboratory investigations of *NCF1* defect patients

Patient no.	Age at onset (in months)	Age at diagnosis (in months)	Sex	Hb	Total WBC count Cell/ $\mu$ l	ANC	ALC	Lymphocyte subset analysis						Serum immunoglobulin levels				DHR (%)	SI (stimulation index)	NBT (%)
								CD19+	CD3+	CD4/CD3+	CD8/CD3+	CD56+ 16/CD3-	IgG (g/l)	IgA (g/l)	IgM (g/l)	% Abs. count	% Abs. count			
1	1	15	M	9.20* 10.5–	18900 <sup>^</sup> 6000–	10584 <sup>^</sup> 1000–	7084 3600–	63 2100–	44 3177	15 1063	10 708	12.1 3.5–16.2	6.14 <sup>^</sup> 0.01–	2.25 0.30–	ND	ND	0			
3	72	120	M	10.0* 11.5–	15300 <sup>^</sup> 4500–	8500 1800–	5202 <sup>^</sup> 1900–	ND 270–	ND 650–	ND 370–	ND 100–	22.9 <sup>^</sup> 3.5–16.2	4.96 <sup>^</sup> 0.17–	2.36 0.30–	ND	ND	0			
7	12	17	M	9.40* 10.5–	14400 6000–	5472 1000–	8784 3600–	78 720–	59 5183 <sup>^</sup>	14 1230	14 1230 <sup>^</sup>	ND 3.5–16.2	ND 0.17–	ND 0.30–	ND	ND	0			
9	NA	264	M	12.6* 13.5–	7800 4500–	5070 1800–	1872 1400–	6.4 110–	29 543	52 973 <sup>^</sup>	3.6 67	ND 6.5–16.2	ND 0.57–	2.65 0.30–	ND	ND	1			
25	12	72	M	8.2* 11.5–	21210 <sup>^</sup> 4500–	15695 <sup>^</sup> 1500–	3818 2300–	31 390–	17 700–	40 1527 <sup>^</sup>	8 305	ND 3.5–16.2	ND 0.17–	2.65 0.30–	0	1.41	0			
26	29	132	F	11.8* 12.0–	9990 4500–	5495 1800–	3197 1900–	12 270–	32 1023	49 1566 <sup>^</sup>	5 160	ND 6.4–17.0	ND 0.52–	2.65 0.34–	2	1.19	0			
30	4	6	M	10.2 9.5–	31110 <sup>^</sup> 6000–	18666 <sup>^</sup> 1000–	9333 <sup>^</sup> 3900–	ND 430–	ND 1800–	ND 590–	ND 170–	ND 3.5–16.2	ND 0.01–	0.30–	0	1.23	0			
37	24	96	M	9.6* 11.5–	26500 <sup>^</sup> 4500–	22260 <sup>^</sup> 1500–	2385 1900–	26 270–	24 572* 650–	38 906	7 167	13.61 3.42 <sup>^</sup>	0.91 3.18	2.65 0.30–	0	1.23	0			
39	6	7	M	6.6* 10.5–	20150 <sup>^</sup> 6000–	15113 <sup>^</sup> 1500–	4232 3400–	N 110–	N 530–	N 330–	N 70–	N 3.5–16.2	N 0.01–	0.30–	0	1.10	0			
40	5	6	M	8.80* 9.5–	36890 <sup>^</sup> 6000–	22503 <sup>^</sup> 1000–	8485 3900–	14 430–	63 5346 <sup>^</sup> 1800–	18 1527	4 339	16.5 <sup>^</sup> 3.5–16.2	2.73 <sup>^</sup> 0.01–	2.64 0.30–	0	0.99	0			
50	10	69	F	5.2* 11.5–	44810 <sup>^</sup> 5000–	25542 <sup>^</sup> 1500–	17476 2300–	ND 390–	ND 700–	ND 490–	ND 130–	12.89 4.0–15.9	3.49 <sup>^</sup> 0.17–	2.61 0.34–	0	1.00	0			
57	60	168	M	9.9* 11.5–	10400 4500–	5096 1800–	4472 <sup>^</sup> 1400–	9 110–	26 1163	43 1923 <sup>^</sup>	12 537 <sup>^</sup>	16.1 6.5–16.2	3.86 0.57–	1.31 0.30–	3	0.98	0			
62	1.5	3	F	10.1 9.5–	16750 6000–	6700 1000–	8040 <sup>^</sup> 3400–	11 110–	26 3377 <sup>^</sup> 530–	26 2090 <sup>^</sup>	10 804 <sup>^</sup>	10.3 4.0–15.9	1.27 0.17–	1.27 0.34–	0	1.25	0			
63	6	264	M	13* 13.5–	7350 4500–	3455 1800–	3014 1400–	ND 110–	ND 530–	ND 330–	ND 70–	ND 6.5–16.2	ND 0.57–	0.30–	5	2.16	0			
64	60	108	M	10.1* 11.5–	13210 4500–	6209 1800–	5284 1900–	18 270–	35 1587 <sup>^</sup> 650–	26 1179	14 635	32.2 <sup>^</sup> 3.5–16.2	5.81 <sup>^</sup> 0.17–	1.34 0.30–	1	3.5	0			
65	NA	84	F	8.5* 13.5–	9890 13500	3560 8000	4846 <sup>^</sup> 3700	16 719	36 1618 <sup>^</sup> 2600	21 944	15 674 <sup>^</sup>	25.3 <sup>^</sup> 5.9 <sup>^</sup>	2 3.18	2.65	1	1.3	0			

**Table 2** (continued)

Patient no.	Age at onset (in months)	Age at diagnosis (in months)	Sex	Hb	Total WBC count Cell/ $\mu$ l	ANC	ALC	Lymphocyte subset analysis						Serum immunoglobulin levels			DHR (%)	SI (stimulation index)	NBT (%)				
								CD19+		CD3+		CD4/CD3+		CD8/CD3+		CD56+ 16/CD3-				IgG (g/l)	IgA (g/l)	IgM (g/l)	
								%	Abs. count	%	Abs. count	%	Abs. count	%	Abs. count	%							Abs. count
71	12	29	M	11.5–13.5*	4500–13500	1500–8000	1900–3700	270–860	1200–2600	650–1500	370–1100	34–3153	30–490	30–278	4.0–15.9	0.17–2.90	0.34–3.48	0	0.90	0			
73	24	24	M	8.8*–10.5–	8500–6000–	3655–1500–	4420–2300–	1400–390–	1400–3700	700–2200	30–1326	30–490	30–221	18.1^ <0.239	3.18–2.65	0.30–2.62	0	1.5	0				
75	6	24	M	10*–10.5–	17000–22800^	8500–10716^	9804^–2300–	1400–390–	3700–5400	2200–700–	23–2255^	23–490	23–588	24.1^ 2.81	3.18–2.22	0.30–2.65	0	1.15	0				
78	24	30	M	9.7*–11.5–	11800–5500–	10384^–1500–	1180–2300–	1400–390–	3700–5400	2200–700–	24–283*	15–177*	15–83*	11.3 3.33^	3.18–3.46^	0.30–2.65	0	1.17	0				
79	24	32	M	12.5–9.1*–	15500–19300^	8500–16019^	2895–5400	1400–390–	3700–5400	2200–700–	31–897	20–579	3–87*	14.1 1.73	1.89–0.30–	2.65	0	1.33	0				
				11.5–12.5	5500–15500	1500–8500	2300–5400	1400–3700	2200–3700	2200–1300	20–1300	3–1300	3–1300	3.5–16.2	0.17–3.18	0.30–2.65							

Pt no: 57, 64, 65 siblings belonged to one family, Pt no: 3, 7, 25, 50, 78 expired due to severe disease, Pt no: 1 and 37 underwent HSCT

SI Stimulation index, ND Not Done, N Normal, NA Not available, Normal Ranges for HB, WBC count, ALC, ANC, Lymphocyte subset populations and Serum Immunoglobulins are mentioned below.

^Above the normal range

\*Below the normal range

**Table 3** *NCF1* GeneScan analysis

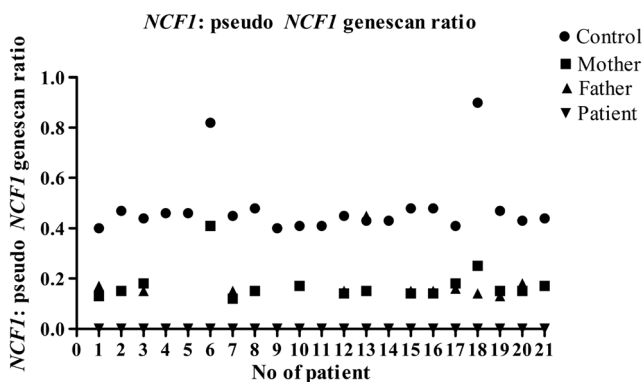
No.	Pt. no.	<i>NCF1</i> GeneScan analysis											
		Control			Patient			Mother			Father		
		<i>NCF1</i>	$\Psi$ <i>NCF1</i>	<i>NCF1</i> / $\Psi$ <i>NCF1</i>	<i>NCF1</i>	$\Psi$ <i>NCF1</i>	<i>NCF1</i> / $\Psi$ <i>NCF1</i>	<i>NCF1</i>	$\Psi$ <i>NCF1</i>	<i>NCF1</i> / $\Psi$ <i>NCF1</i>	<i>NCF1</i>	$\Psi$ <i>NCF1</i>	<i>NCF1</i> / $\Psi$ <i>NCF1</i>
1	1	191	403	0.40	0	2881	0	212	1595	0.13	214	1455	0.17
2	3	1842	4292	0.47	0	8372	0	864	5578	0.15	NA	NA	NA
3	7	718	1639	0.44	0	945	0	577	3250	0.18	436	2848	0.15
4	9	254	543	0.46	0	3502	0	NA	NA	NA	NA	NA	NA
5	25	1149	2502	0.46	0	3431	0	NA	NA	NA	NA	NA	NA
6	26	2060	2502	0.82	0	1184	0	1422	3419	0.41	NA	NA	NA
7	30	554	1244	0.45	0	3419	0	242	1958	0.12	490	3381	0.15
8	37	593	1274	0.48	0	4403	0	864	5578	0.15	NA	NA	NA
9	39	560	1384	0.40	0	1055	0	NA	NA	NA	NA	NA	NA
10	40	402	987	0.41	0	1454	0	902	5203	0.17	333	1945	0.17
11	50	1712	4130	0.41	0	1743	0	NA	NA	NA	NA	NA	NA
12	57	1267	2827	0.45	0	7963	0	787	5500	0.14	766	5018	0.15
13	62	462	1085	0.43	0	3895	0	963	6355	0.15	1270	2850	0.45
14	63	1389	3267	0.43	0	4859	0	NA	NA	NA	NA	NA	NA
15	64	3248	6676	0.48	0	4150	0	787	5500	0.14	766	5018	0.15
16	65	3248	6676	0.48	0	2629	0	787	5500	0.14	766	5018	0.15
17	71	910	2205	0.41	0	1880	0	1248	6798	0.18	886	5607	0.16
18	73	856	955	0.9	0	2969	0	66	255	0.25	536	3829	0.14
19	75	1455	3068	0.47	0	5692	0	541	3592	0.15	365	2772	0.13
20	78	519	1215	0.43	0	8041	0	379	2526	0.15	643	3580	0.18
21	79	617	1418	0.44	0	2086	0	1611	9476	0.17	NA	NA	NA

GeneScan ratio of 1:1 occurs in approximately 10 % of individuals from western countries, suggesting that they carry one copy of each gene. As per the study reported by Heyworth et al., there are three different haplotypes of *NCF1*/ $\Psi$ *NCF1* gene ratios observed in the general population due to reciprocal crossing over events during meiosis. These are 2:4, 1:1, and 4:2; this also explains one of the two carrier parents (pt 26, pt 62) showing a ratio of 2:4. In this case, testing their

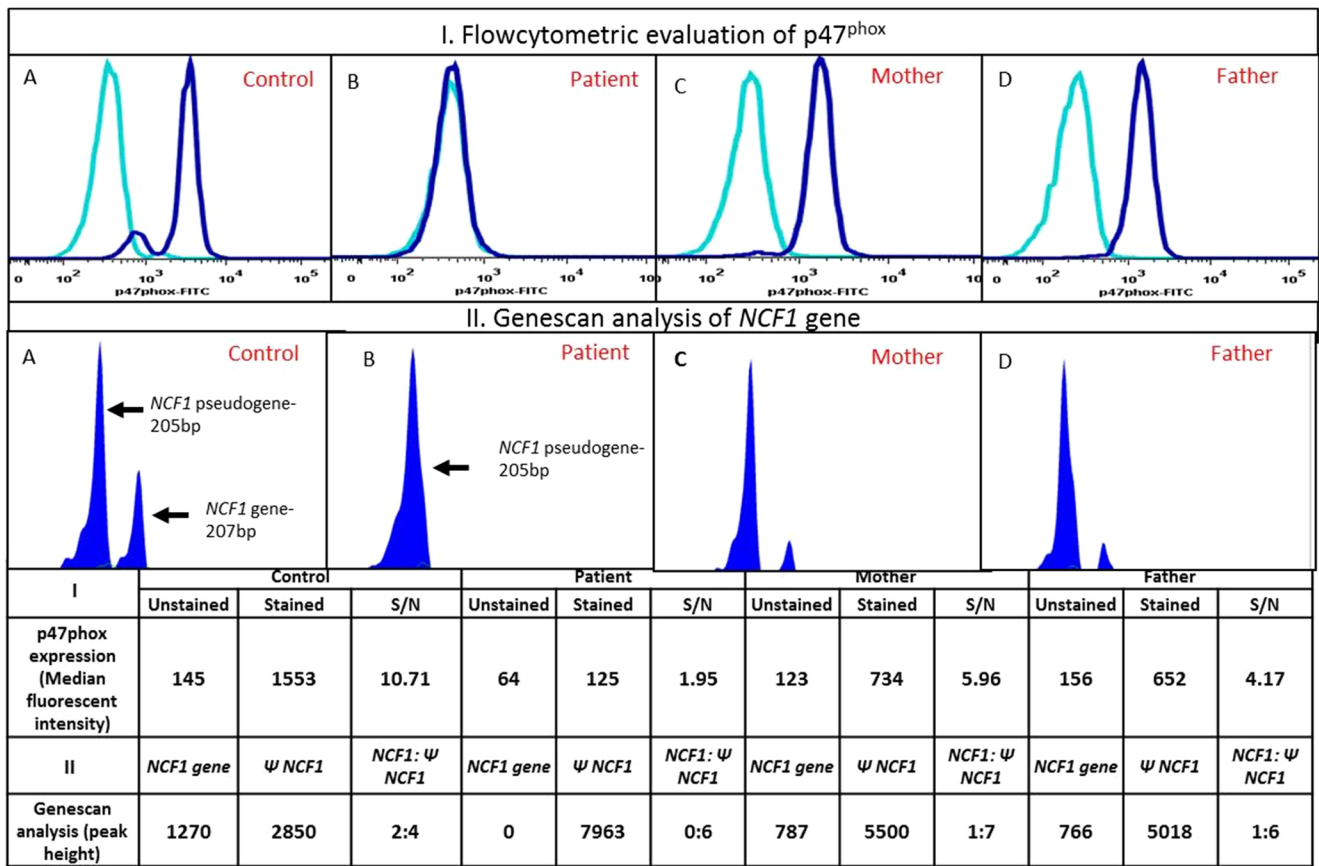
grandparents and other family members should then give a clue for the carrier haplotype in their family [29].

Defect in  $p47^{\text{phox}}$  is the most common form of AR-CGD. Although clinical manifestations in different subtypes of CGD are found to be similar, differences in the pattern of severity and frequency of incidence are observed [30]. Male predominance is seen in this cohort of *NCF1* patients with a male to female ratio of 4.25:1, perhaps due to gender bias in referring patients to hospitals. Ten patients are Hindu, nine Muslim, and two Christian, suggesting diversity in their ethnic background. Two patients were (pt. nos. 64, 65) diagnosed with CGD on family screening of the index case. They were asymptomatic till the age of 9 and 7 years of diagnosis, respectively. This conveys the significance of screening family members of affected patients. A similar observation reported in a series of an Egyptian cohort where siblings were diagnosed at 6 and 3.5 years of age after index case was diagnosed at 6 years of age [31].

Though lung is the most commonly affected organ among these patients, few patients also presented with infections in unusual sites such as brain, skin, bone, gastrointestinal tract, and urinary tract. There was a significant respiratory morbidity seen in these patients, with recurrent pneumonia and



**Fig. 2** Scatter plot of ratio of *NCF1* to pseudo-*NCF1* by GeneScan analysis in control, father, mother, and patient sample



**Fig. 3** (I) Flow cytometric evaluation of expression of the anti-p47<sup>phox</sup> antibody on neutrophils of **a** control, **b** patient, **c** mother, and **d** father, where the patient shows an absent expression of the p47<sup>phox</sup> antibody; parents show reduced expression as compared to healthy control. Unstained tube (sky blue), stained tube (dark blue). (II) GeneScan

analysis of the *NCF1* gene in **a** control, **b** patient, **c** mother, and **d** father, where the ratio of *NCF1*/pseudo-*NCF1* genes is calculated by measuring peak height of the *NCF1* gene at 207 bp and pseudogene at 205 bp. Patient **b** shows homozygous del GT mutation; parents show a carrier status with altered ratio of 1:6 and 1:7

pulmonary tuberculosis being the major cause of death. This observation was supported by previous studies [5, 24]. In the

present study, *Aspergillus* sp. was isolated in only 19 % cases as against 35 % reported previously [5]. Forty percent of

**Table 4** p47<sup>phox</sup> antibody expression on neutrophils by flow cytometry measured in median fluorescent intensity (MFI)

Pt. no.	p47 <sup>phox</sup> antibody expression on neutrophils by flow cytometry (MFI)											
	Control			Patient			Mother			Father		
	Unstained	Stained	S/N	Unstained	Stained	S/N	Unstained	Stained	S/N	Unstained	Stained	S/N
57	145	1553	10.71	64	125	1.95	123	734	5.96	156	652	4.17
62	272	1562	5.74	610	185	0.30	270	765	2.83	266	658	2.47
63	86	1215	14.12	463	241	0.52	NA	NA	NA	NA	NA	NA
64	145	1553	10.71	513	511	0.99	123	734	5.96	156	652	4.17
65	145	1553	10.71	585	542	0.92	123	734	5.96	156	652	4.17
71	210	1105	5.26	267	179	0.67	225	368	1.63	161	454	2.81
73	613	2300	3.75	463	207	0.44	737	1666	2.26	693	2338	3.37
75	164	1363	8.31	224	184	0.82	483	628	1.30	375	627	1.67
78	89	1383	15.53	242	139	0.57	93	1271	13.66	107	1041	9.72
79	89	1383	15.53	178	122	0.68	126	1074	8.52	NA	NA	NA



patients received antitubercular therapy (ATT). Thus, morbidity due to mycobacterial tuberculosis infection is higher in the Indian cohort of patients with CGD which may be due to high endemicity of tuberculosis in India. This also highlights the need for investigating patients with recurrent tuberculosis for underlying CGD. All patients in this group had already received Bacillus Calmette-Guerin (BCG) immunization, as it is routinely administered at birth in India [5]. However, only three patients had left axillary and cervical lymphadenopathy following BCG vaccination; none of them had disseminated BCGiosis which is observed in XL-CGD patients. These three patients responded well to ATT.

Non-infectious inflammatory complications involving gastrointestinal tract, genitourinary tract, vasculitis, etc., is the major concern in patients with CGD. Inflammatory bowel disorder (IBD) is commonly seen in patients with CGD. Seven to 11 % of the patients with *NCF1* defect showed IBD as a primary symptom of CGD [32, 33]. In our cohort of patients, 34 % showed history of chronic diarrhea at the time of diagnosis. Majority of the patients received antimicrobial therapy and showed response to the same. None of the patients were further evaluated for biopsy-proven IBD or colitis. These complications need to be kept in mind if the patient has persistent non-infectious diarrhea as they may require additional immunomodulatory or immunosuppressive therapy [33].

AR-CGD often has milder clinical presentation compared to XL-CGD [34]. Moreover, the clinical manifestations, age of onset, and condition of survival depend on the nature of the underlying genetic defect. However, our cohort of patients had a clinical heterogeneity despite having an identical genetic defect. Of the 21 patients, 52 % presented clinical manifestation within the first year of life. Twenty-four percent of patients (5 out of 21) expired due to severe infections. Two patients were transplanted at the age of 4.5 and 8 years, respectively, for severe clinical manifestations. Sixty-seven percent of patients had a relatively milder course and were significantly stable on antibacterial and antifungal prophylaxis. Interestingly, one of the family had three children who were affected, but only one child required frequent medications (pt. no. 57) whereas the other two (pt. nos. 64, 65) were relatively asymptomatic with normal growth and mild clinical manifestations in the form of URTI and could easily be treated with oral antibiotics. We tried to understand their clinical heterogeneity by looking at their other immunological parameters like lymphocyte subset analysis and immunoglobulin profile. However, no significant variation was observed between the patients with severe and milder phenotype.

It may be interesting to study the residual NADPH oxidase activity in these patients. Intact residual NADPH oxidase enzyme activity depends on the underlying genetic defect and the type of mutation which affects the severity of the disease, chances of survival, and clinical manifestation in the patient. It can be used as a tool for

predicting the severity of the defect in CGD patients [13, 18]. Unfortunately, this was not done in the present study.

Despite of the advances in antimicrobial and antifungal prophylaxis and in the supportive management, CGD is still associated with significant morbidity and mortality. Average life span of CGD patients is 30 years with yearly mortality of 2 % [35]. Only hematopoietic stem cell transplantation (HSCT) currently offers the definitive therapy for CGD. The success of HSCT for CGD has improved significantly over the last few years even with matched unrelated donor (MUD), making it an important therapeutic option offered at diagnosis to all the patients especially in experienced centers [36, 37]. Two patients in our cohort with severe disease were successfully treated with matched sibling bone marrow transplantation, one for severe recurrent infections and one for chronic inflammatory complication. However, marked clinical heterogeneity seen in the present cohort clearly suggests that HSCT is not indicated in all the patients with CGD. Recurrent severe infections and chronic inflammatory or autoimmune complication requiring long-term steroid therapy are indicators of poor prognosis. Elevated alkaline phosphatase, a history of liver abscesses, and decline in platelet count reflecting portal hypertension are some other adverse prognostic indicators [36]. All these patients can be considered for early HSCT.

A study by Kuhns et al. has shown worse long-term survival with markedly reduced NADPH oxidase activity suggesting that these patients might be considered for early HSCT [18, 36]. The NADPH activity depends on the underlying genetic defect; however, it is not clear from the studies if differential residual NADPH oxidase activity is seen with identical genetic defect and it can be used as a predictive tool in patients with Del GT mutation. The effect of other genes and of epigenetic and environmental factors also plays an important role in having such wide heterogeneity [19].

In conclusion, early detection of *NCF1* defect is important for timely management of CGD patients. Flow cytometry offers a rapid screening method for detection of p47<sup>phox</sup> defect from other forms of AR-CGD. Wide heterogeneity is seen in clinical manifestations of patients with Del GT mutation. Further studies are required to understand the underlying mechanisms for this phenotypic heterogeneity.

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#### Compliance with Ethical Standards

**Conflict of Interest** The authors declare that there is no conflict of interest.

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