Hyper-IgE syndrome with a novel STAT3 mutation-a single center study from India

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Summary

Background: **Hyper IgE syndrome (HIES) is a rare primary immunodeficiency disorder characterized by the triad of elevated IgE and eosinophilia, eczema and recurrent skin and pulmonary infections. Mutation in the STAT3 gene accounts for majority of the autosomal dominant and sporadic forms of HIES.**

***Objective*:** **To report clinical and molecular analyses of patients with Hyper IgE syndrome from a single tertiary care center in India.**

***Methods*:** **Four patients with suspected HIES were studied. Flowcytometry for T_H17 cell numbers and phosphoSTAT3, and STAT3 gene sequencing were performed.**

***Results*:** **T_H17 cells were significantly reduced. Mutations were found in the DNA-binding domain in three and a mutation in the transactivation domain in one patient. One of the mutations detected was a novel mutation (g54792 c.1018A>C p.K340Q) in the DNA binding domain. *Mycobacterial* infection, which is usually not commonly associated with HIES was found in two of our cases, one with a cutaneous abscess in the shoulder, and the other with BCG site reactivation.**

Conclusions*:** **A novel mutation in the STAT3 is reported. *Mycobacterial* infections can be seen in the spectrum of HIES related infections. *(Asian Pac J Allergy Immunol 2014;32:321-7)

Keywords: *Hyper-IgE Syndrome, Mycobacterial infection, novel STAT3 mutation, phosphoSTAT3, T_H17 cells*

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Introduction

Davis, Schaller, and Wedgwood first coined the term Job's syndrome[1] for this entity in 1966. They reported two red-haired, fair-skinned girls with frequent sinopulmonary infections, severe dermatitis, and recurrent staphylococcal cold abscesses in the skin. The syndrome was further defined and clarified by Buckley et al. in 1972, who noted similar manifestations in two boys with severe dermatitis, characteristic facies, and elevated IgE levels, leading to the term Buckley's syndrome.[2]

The pathogenesis of Hyper IgE Syndrome (HIES) remained unknown till 2007 when mutations in STAT3 (Signal Transducer and Activator of Transcription 3) were documented in patients with autosomal dominant (AD) and sporadic forms of HIES by two investigating groups simultaneously.[3,4] HIES is clinically a triad of high serum levels of IgE (>2000 IU/ml), recurring staphylococcal skin abscesses, and pneumonia with *pneumatocele* formation. Chronic eczematoid dermatitis, coarse facies, mild eosinophilia, and mucocutaneous candidiasis, are variable features. Dental abnormalities (retained primary teeth, non-eruption of permanent teeth, double rows of teeth), anomalies in midline facial development, and skeletal abnormalities (bone fractures, hyperextensible joints, scoliosis) reflect the multisystem nature with the involvement of seemingly unrelated systems and dissociated manifestations. Both autosomal dominant (AD-HIES) and recessive (AR-HIES) modes of inheritance are seen, with skeletal manifestations being a feature of the former, whereas viral infections and neurologic manifestations are a feature of the AR-HIES.

STAT 3 mutations are the predominant cause of sporadic and familial HIES,[3,4] although other genomic loci may also be involved. STAT3 is involved in the signal transduction of many cytokines, including but not limited to IL-6, IL-10, IL-21, IL-22, and IL-23, and also plays an integral role in wound healing, angiogenesis, cancer, and immunity.[5-10] STAT3 is implicated in the differentiation of IL-17 producing $CD4^+$ T lymphocytes, important for immunity to extracellular bacterial infections and fungi.[11-14] STAT3-related HIES have been consistently shown to have impaired development of T_H17 cells and IL-17 production[15,16] and this defect in the STAT3-IL-17 axis have been proposed as a major pathogenic mechanism of the defective immunity against extracellular bacterial pathogens.[15-17] The other genes that have been implicated in HIES include TYK2[18,19] and DOCK8.[20-23]

Methods

Four cases of STAT-3 related Hyper-IgE Syndrome, from three kindreds, diagnosed at the Postgraduate Institute of Medical Education and Research, Chandigarh, India, were included. Written informed consents were obtained from the patients or their families. The study was approved by the Institute Ethics Committee. The clinical presentation and laboratory findings are summarised in table 1.

T_H17 cell assessment

PBMCs were isolated with Ficoll-Hypaque density centrifugation (Sigma Aldrich, St Louis, Mo). T_H17 cells were identified by means of intracellular staining of $CD4^+$ T cells for the production of IL-17. Briefly, 1×10^6 cells from patients and control subjects were stimulated for 6 hrs with 10 ng/ml phorbol 12-myristate 13-acetate and 1 ug/ml ionomycin (Sigma-Aldrich, St Louis, Mo) in the presence of GolgiPlug (BD Biosciences, San Jose, CA). After cell-surface staining with PerCP-conjugated anti-CD4 (BD Biosciences, San Jose, CA), cells were fixed, permeabilized (Cytofix/Cytoperm, BD Biosciences, San Jose, CA), and stained with Alexa Fluor 647-conjugated anti-IL-17A (BD Biosciences, San Jose, CA). An immunoglobulin isotype control was used as a background control. Since a subset of T_H17 cells also produce IFN-γ, $CD4^+$, T cells were also evaluated for IFN-γ production (Fluorescein isothiocyanate-conjugated anti-IFN-γ; BD Biosciences, San Jose, CA). Flow cytometric studies were performed on a FACS ARIA III instrument (BD Biosciences) and analyzed with Cell Quest Pro software (BD Biosciences).

Evaluation of STAT3 phosphorylation

Tyrosine phosphorylation of STAT3 was assessed by flow cytometry using the BD Phosflow reagents as per the manufacturer's instructions (BD Biosciences, San Jose, CA). Briefly, 100 µl of whole blood was stimulated with 100 ng of IL-6 (Peprotech, USA) for 15 minutes at 37°C. Cells were simultaneously fixed and lysed in lyse/fix buffer (BD Biosciences, San Jose, CA). After washing with staining buffer, cells were permeabilized with Perm Buffer III (BD Biosciences, San Jose, Calif) and stained with Alexa Fluor 647 conjugated mouse anti-tyrosine 705 phosphorylated STAT3 (pY705-STAT3) mAb (BD Biosciences, San Jose, CA). Samples were acquired on FACS ARIA III and results were analyzed using Cell Quest Pro software (BD Biosciences).

Mutation analysis

The STAT3 gene was amplified by PCR from genomic DNA (gDNA) using specific oligonucleotide primers. Briefly, gDNA was prepared from venous blood by using the AxyPrep Blood Genomic DNA Miniprep Kit (Axygen BioSciences, Union City, USA). The amplified gene fragments were sequenced from Euroffin Genomics India Pvt Ltd, Bangalore,using the ABI Big Dye Terminator mix (Applied Biosystem, Carlsbad CA) and analyzed with a 3730xl DNA Analyzer, BDT version 3.1 (Applied Biosystems, Carlsbad CA). The sequencing data were analysed using Codon Code Aligner software. A polymorphism Phenotyping program (PolyPhen, http://genetics.bwh.harvard.edu/pph) and a SIFT (Sorting Intolerant from Tolerant) program were used to predict the effect of the identified STAT3 mutations. This program predicts whether an amino acid substitution affects protein function, based on sequence homology and the physical properties of amino acids.

Results

Serum IgE levels and absolute eosinophil counts (AEC):

Three of the four patients showed IgE levels above 2000 IU/ml at presentation (45,935 IU/ml, 22,400 IU/ml, and 4300 IU/ml). One patient (case 3) had a lower initial serum IgE of 822 IU/ml but this subsequently increased to 54,0000 IU/ml. The AECs were 295, 856, 603, and 948/mm^3 respectively (normal range 50-350/mm^3).

Table 1. Table showing clinical profiles and laboratory findings of the four cases.

Case No.	Age at presentation	Sex	Clinical presentation	NIH Score	AEC (cells/µl)	Serum IgE (IU/ml)
1	35 years	Male	Repeated episodes of pneumonia, skin rashes, and skin abscesses since age 15 days. At 13 years, had widespread skin infection. At 18 had cough & hemoptysis treated as TB. At 30 had *Staph* lung abscess. Facial features present (Fig. 1a, 1b). Family history: A son had recurrent pneumonia, pneumothorax and pyoderma at the age of 14 months. Facial features present (Fig 1c). Serum IgE was 2449 IU/ml, NIH Score 42. Expired, genetic studies not done.	52	295	45,935
2	5 years	Male	5 years old son of case No. 1. History of recurrent upper respiratory tract infections and itching. No history of pulmonary infections and no characteristic facial features.	25	856	22,400
3	1 year 10 months	Male	Recurrent skin infections, ear discharge, empyema, recurrent pneumonia, with pneumatocele formation. First episode of pneumonia at 11 months of age. Facial features present (Fig 1d). Swelling over right elbow and shoulder (antibioma). Showed granulomatous inflammation with numerous AFB.	45	603	54,000
4	4 months	Male	Rash since day 2 of life, with anal and oral ulcerations. Recurrent oral and tongue lesions, resulted in destruction of the right lateral border of the tongue (Fig 1d, 1f), scrapings showed fungus. Nodular swelling at BCG site, FNAC showed presence of AFB.	42	948	4300

AFB: Acid fast bacilli. ATT: Anti-tubercular therapy. BCG: Bacillus Calmette-Guerin. FNAC: Fine needle aspiration cytology.

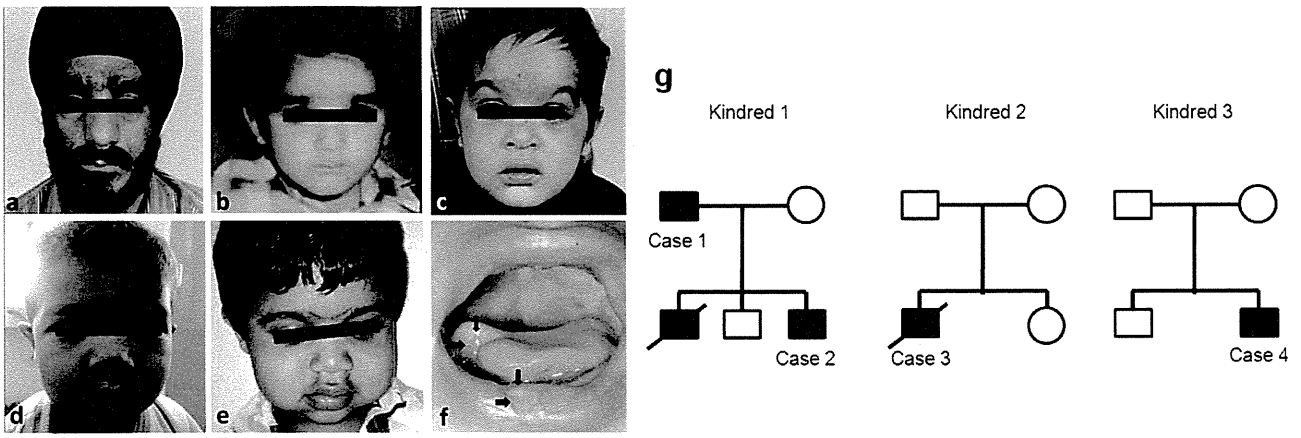

Figure 1. (a) Case 1 showing coarse facial features with broad forehead, bushy eyebrows and bulbous nose. (b) The patient, in his childhood photograph showed the presence of the facial features. (c) Facial appearance of the 14 months old child, elder sibling of case 2 with widely spaced eyebrows, wide forehead, and a broad nasal bridge. This child expired before he could be investigated. Mutation studies could not be done, and hence are not included in this case series (d) Case 3, showing relatively milder facial features. (e) Case 4, despite a cushingoid appearance because of therapy with steroids, a gradual coarsening of facial features could be appreciated. (f) Ulcers in the tongue and lower lip (arrows) with punched out distortion of the right lateral border of the tongue in case 4, photographed at a stage when the acute lesions were healing after steroids. (g) Family trees representing the three kindred.

Percentage of T_H17 cells

All the four cases showed a reduction in the T_H17 cell numbers, which ranged from 0.09 to 0.2% (0.2, 0.09, 0.1 and 0.2% respectively). The data are represented in Table 1 and Figure 3.

Evaluation of Phospho-STAT3 status

Flowcytometry for pSTAT3 could be performed in two patients (case 1 and case 4). A normal pSTAT3 was observed in case 1 with 46.9% of the cells showing pSTAT3 against a control of 52.4 % respectively, while in case 4, only 2.5% of the cells showed phosphorylation. The test could not be performed in cases 2 and 3. Figure 3 shows pSTAT3 activity in terms of change in mean fluorescence intensity (MFI) between the stimulated and un-stimulated cells of cases 1 and 4.

Mutation analysis:

The various mutations observed are shown in Table 2. Case 1 showed an A to C transversion in exon 10 at the nucleotide g54792 position (c.1018 A>C), leading to substitution of lysine at amino acid position 340 for glutamine (p.K340Q) in the DNA binding domain (Figure 4a). This mutation was not found in the single nucleotide polymorphism database (dbSNP; www.ncbi.nlm.nih.gov/projects/SNP) and hence is considered to be a novel mutation. The mutation had a SIFT score of 0.01 (damaging) and a polyphen score of 0.99 (damaging). The same mutation was demonstrated in Case 2. One hundred and two alleles from 51 healthy volunteers were sequenced for exon 10, and no mutations were found.

Case 3 showed a G to A transition in exon 13 at nucleotide position g.58854 (c.1145 G>A), leading to substitution of arginine at amino acid position 382 for glutamine (p.R382Q) in the DNA binding domain (Figure 4b). The mutation had a SIFT score of 0.12 (tolerated) and a polyphen score of 1.00 (probably damaging).

Case 4 showed a C to T transition in exon 22 at nucleotide position g.71311 (c.2141 C>T), leading to substitution of threonine at amino acid position 714 for isoleucine (p.T714I) in the transactivation domain (Figure 4c). The mutation had a SIFT score of 0.43 (tolerated) and a polyphen score of 0.99 (probably damaging).

Discussion

HIES is a relatively rare disorder and diagnosis requires a high index of clinical suspicion. Though definitive clinical scoring systems have been devised, the diagnosis can be challenging, especially in young children. This is exemplified in case number 4, where the clinician suspected the diagnosis, in spite of a very atypical presentation, based solely on the observation of a gradually coarsening facies over a very short follow-up.

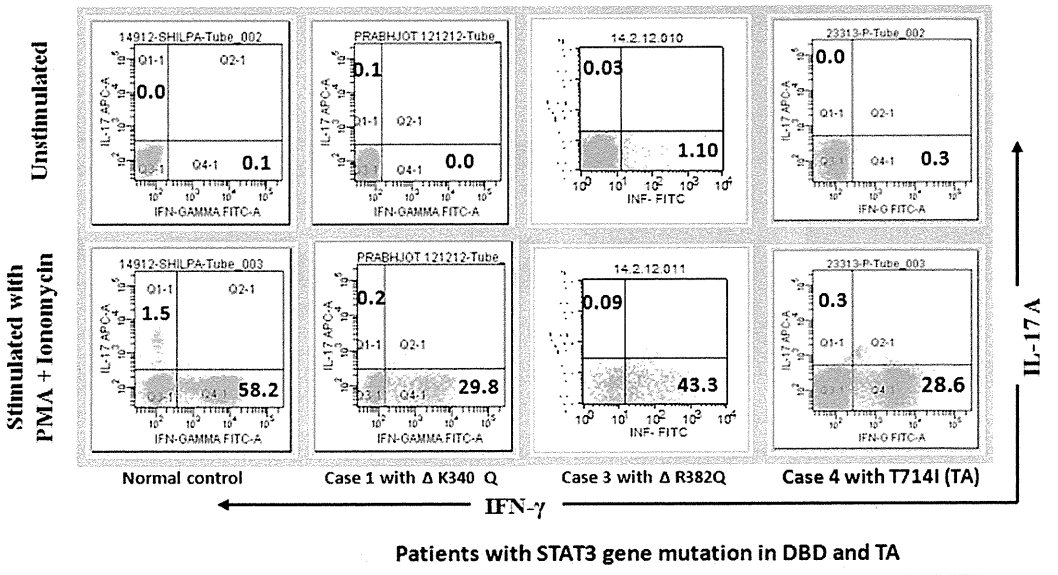

Figure 2. Flowcytometric analysis of $CD4^+$ $IL\text{-}17A^+$ T cells (T_H17 cells) from healthy control and patients with HIES.

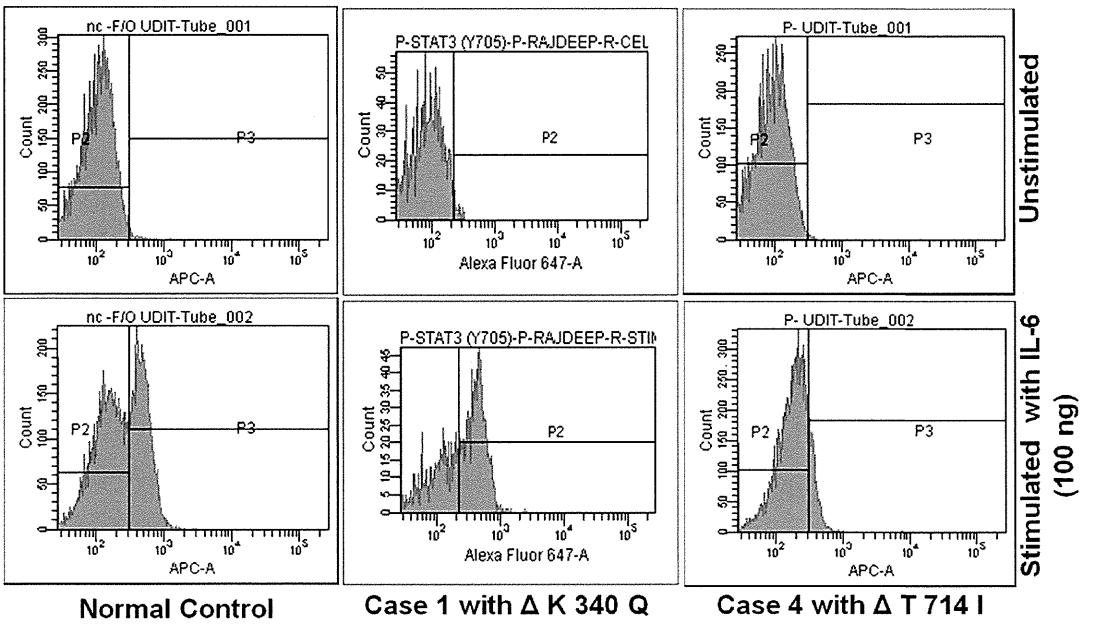

Figure 3. Flowcytometric analysis of STAT3 phosphorylation (Y705) from healthy controls and two patients with mutations in the DBD (ΔK340Q) and TA (ΔT714I) domains.

Pneumonias in HIES are mainly caused by *Staphylococcus aureus*, *Streptococcus pneumoniae*, or *Haemophilus influenzae*. The *pneumatocele* that typically follow the resolution of cured bacterial pneumonias are frequently superinfected by *Pseudomonas aeruginosa* and *Aspergillus fumigatus*.[1,2,25,26] Mycobacterial infections, though reported,[27,30] are not a common feature observed in HIES; however, Case 3 had an abscess in the arm which was positive for Mycobacterium tuberculosis. Case 4 showed BCG site infection. It seems likely that the occurrence of Mycobacterial infections in HIES might not be uncommon in countries where mycobacterium tuberculosis is endemic. BCG vaccination in cases where the diagnosis of HIES is made early enough, should be undertaken cautiously and a drug prophylaxis should be considered.

Mucocutaneous fungal disease can be seen in up to 43%-85% of HIES, including 64% of patients with oral candidiasis during the neonatal period.[31-33] Along with a low T_H17 cell count, HIES can thus have features overlapping with chronic mucocutaneous candidiasis. Patients with HIES however also present with other predominant features, such as eczema, skin abscesses and pneumonias, while isolated significant and destructive mucocutaneous candidiasis is not a common presenting feature. Case 4 in our report presented predominantly with oral and anal ulcerations very early in life, i.e. at two months of age, which prompted the initial diagnosis of neonatal Behcet's disease. This is a rare presenting feature in HIES.

Table 2. Table showing the various mutations detected.

Case No.	T_H 17 cells (%)	pSTAT3	Exon	Mutation	Type	Domain
1	0.2%	Normal	10	g.54792, c.1018 A>C, p.K340Q	Heterozygous point mutation, novel	DBD*
2	0.1%	Not Done	10	g.54792, c.1018 A>C, p.K340Q	Heterozygous point mutation, novel	DBD*
3	0.09%	Not Done	13	g.58854, c.1145 G>A, p.R382Q	Heterozygous point mutation	DBD*
4	0.2%.	Reduced	22	g.71311, c.2141 C >T, p.T714I	Heterozygous point mutation	TA†

*DBD- DNA binding domain, †TA- Trans-activation domain.

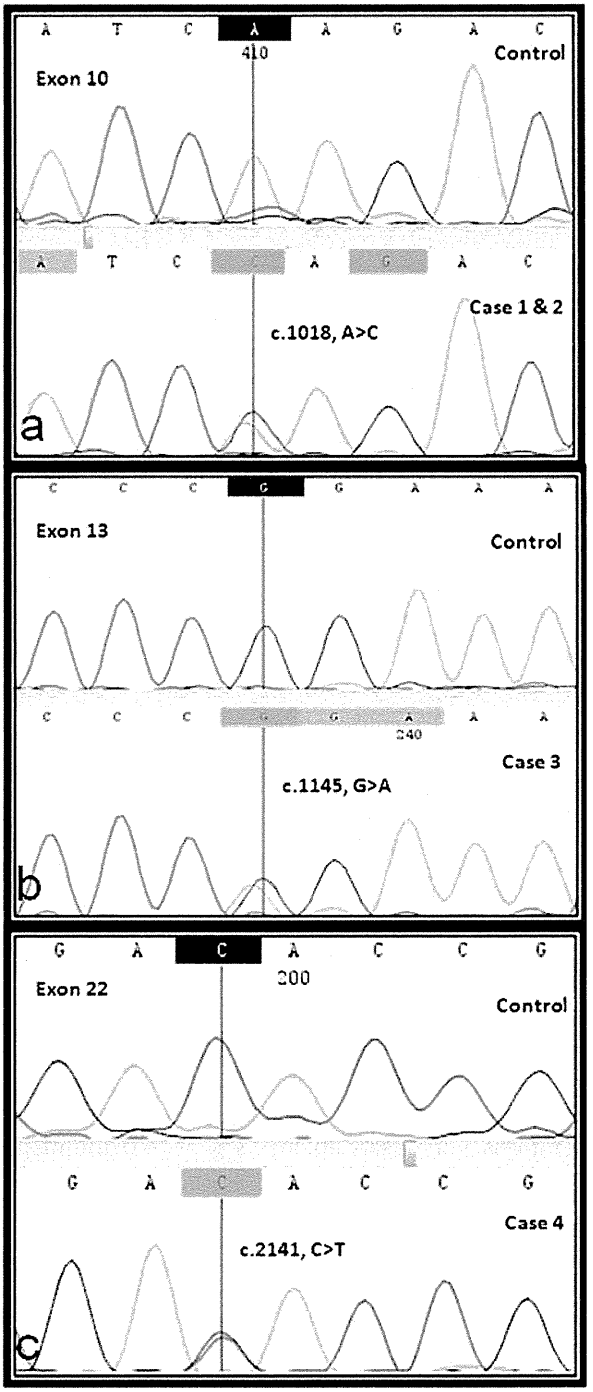

Figure 4. (a) Sequencing results for case 1 and case 2 showing a novel heterozygous mutation (Exon 10, c.1018 A>C, p.K340Q) in DNA binding domain of STAT3 gene. (b) Case 3 showing a heterozygous peak (Exon 13, c.1145 G>A, p.R382Q) in DNA binding domain of STAT3 gene. (c) Case 4 showing a heterozygous peak (Exon 22, c.2141C >T, p.T714I) in transactivation domain of STAT3 gene.

The mutation demonstrated in the first kindred, with a clear cut autosomal dominant phenotype, is a novel mutation and hasn't yet been reported in literature. The mutations in case 3[3,4] and case 4[29] have been previously reported and were sporadic mutations.

Mutations involving the DNA binding domain along with the SH2 domain comprise the mutation hotspots in patients with HIES. STAT3 phosphorylation has been shown to be reduced in SH2 domain mutations, whereas with mutations in the DNA binding domain, STAT3 phosphorylation is often normal. [34,35] Mutations in the transactivation domain, which primarily affect the phosphorylation site, are likely to affect phosphorylation of STAT3 to a greater extent. Hence, pSTAT3 status in our patients correlated with the mutations.

This is the first single centre based report of STAT3 mutation analysis from the Indian subcontinent. More cases need to be screened on a multi-institutional nation-wide collaborative basis.

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ORIGINAL RESEARCH

Chronic Granulomatous Disease: Two Decades of Experience From a Tertiary Care Centre in North West India

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Abstract Chronic granulomatous disease (CGD) results from an inherited defect in the phagocytic cells of the immune system. It is a genetically heterogenous disease caused by defects in one of the five major subunits of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex. There is a paucity of data from India on CGD. We herein describe the clinical features in 17 children with CGD from a single tertiary referral center in India. A detailed analysis of the clinical features, laboratory investigations and outcome of 17 children 7 with X-linked (XL) and 10 with autosomal recessive (AR) form was performed. Diagnosis of CGD was based on an abnormal granulocyte oxidative burst evaluated by either Nitroblue Tetrazolium (NBT) test or flow cytometry based Dihyrorhodamine 123 assay or both. The molecular diagnosis was confirmed by genetic mutation analysis in 13 cases. The mean age at diagnosis and the age at onset of symptoms was significantly lower in children diagnosed with XL- CGD compared those with AR disease. Mutations were detected in *CYBB* gene in 6 patients with XL-CGD and *NCF-1* gene mutations were observed in 7 cases of AR- CGD. The course and outcome of the disease was much worse in children diagnosed with X-linked form of disease compared to AR forms of the disease; 4/7 (57 %) children with X-CGD were dead at the time of data analysis. This is one of the largest series on chronic granulomatous disease from any developing country.

Keywords Chronic granulomatous disease · NADPH oxidase · CYBB gene · NCF-1 gene · dihydrorhodamine

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Introduction

Chronic granulomatous disease (CGD) is an inherited and genetically heterogenous immunodeficiency disorder resulting from defects of one of the subunits of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme complex in phagocytic cells. It is a rare disease affecting between 1in 2,00,000 and 1 in 2,50,000 live births [1]. The actual incidence is likely to be higher due to underdiagnosis of patients presenting with milder disease phenotype. CGD was initially described in 1954 [2] and 1957 [3], but it was not well characterized as a distinct clinical entity until 1959 [4].

NADPH oxidase complex is composed of five major subunits. Two of these *gp91phox* (cytochrome b-245 β

polypeptide) and *p22phox* (cytochrome b-245 α polypeptide) are membrane bound components encoded by the *CYBB* gene and the *CYBA* gene respectively. The remaining three components of the complex include *p47phox, p67phox and p40phox* encoded by the corresponding genes namely, *NCF1* (neutrophil cytosolic factor 1), *NCF2* (neutrophil cytosolic factor 2) and *NCF4* (neutrophil cytosolic factor 4) [5]. All the components of the NADPH oxidase except for the gp91phox are not phagocyte specific and expressed in other tissues as well [6]. Therefore defects in the components other than gp91phox may have subtle effects on other tissues.

The NADPH oxidase complex catalyzes the conversion of molecular oxygen O_2 to superoxide anion (O_2^-) and other reactive oxygen intermediates. Therefore, defects in any of the components of the NADPH oxidase complex results in impaired killing of intracellular microorganisms and renders patients with CGD susceptible to recurrent and often life threatening infections with bacteria and fungi. X-linked recessive form of the disease due to mutations in the *CYBB* gene encoding for gp91phox accounts for approximately 65 % of patients with CGD. Mutations in the *NCF1* gene encoding for the *p47phox* account for 30 % of the cases whereas *CYBA* and *NCF2* mutations are detected in <5 % patients each. Only one patient with mutation in *NCF4* has been reported thus far [7]. The risk of mortality in CGD is estimated to be 1–5 % annually and is likely dependent on the mode of inheritance i.e. X-linked or AR.

There is a paucity of data on chronic granulomatous disease from developing countries although large series have been published from Europe and USA. The clinical spectrum of disease including the type of infections, frequency of breakthrough infections, morbidity and mortality are likely to be different in the context of a developing country. Hence we embarked to perform a retrospective analysis of our cohort of chronic granulomatous disease diagnosed and managed over the last 2 decades.

Patients and methods

A detailed data analysis of 17 children diagnosed with chronic granulomatous disease from August 1993 to April 2013 was performed. The study was conducted in the Pediatric Allergy and Immunology, Unit, Advanced Pediatrics Centre and the Department of Immunopatholgy, Postgraduate Institute of Medical education and Research (PGIMER), Chandigarh. Our Institute serves as tertiary level referral centre for North West India . The study was approved by the Department Review Board in consonance with the existing practice at our institution. Data were retrieved from the case records and files of the Pediatric Immunodeficiency Clinic at the Advanced Pediatrics Centre, Postgraduate Institute of Medical Education and Research (PGIMER). Evaluation of the clinical manifestations included the age at presentation and diagnosis, presenting complaints and detailed laboratory parameters.

Diagnosis of CGD was based on an abnormal granulocyte oxidative burst evaluated by either Nitroblue tetrazolium test [8, 9] (NBT, $n=10$) or flow cytometry based Dihydrorhodmaine (DHR)123 assay [10, 11] ($n=1$) or both ($n=6$). These tests were also performed on the parents and siblings where available ($n=9$) to determine the mode of inheritance. The NBT test was performed using leucocyte rich plasma whereas heparin anticoagulated whole blood was used for the DHR assay . Phorbol Myristate acetate (PMA) was used for stimulation of neutrophils in the DHR assay and yeast cells and/or PMA where used for stimulation in the NBT dye reduction test.

Complete blood count including a total and differential leucocyte count, mean platelet volume, absolute lymphocyte count, eosinophil and neutrophil counts were determined in each case using a five part automated hematology analyzer.

Serum Immunoglobulins IgG, A and M were estimated by end-point nephelometry using a semi-automated nephelometer MININeph (The Binding Site, Birmingham, UK). Lymphocyte subset analysis was done using BD Tritest™ CD45PerCP, CD3-FITC and CD19- PE antibody cocktail from BD Biosciences (San Jose, USA) following a lyse no wash protocol. Briefly 50 μl of EDTA anticoagulated blood was added to 20 μl of the antibody cocktail in a FACS tube, mixed, vortexed and solution. After 10 min the tubes were vortexed and acquisition was performed on a BD FACSCalibur flow cytometer.

Investigations also included X-rays and CT scan when indicated.

Genetic mutation analysis results was performed in 13 cases. The mutation analysis was not done in four cases because some of the children were diagnosed when facilities for mutation studies were not available or the children had died before these studies could be performed. The mutation analysis was conducted at the National Defense Medical College, Saitama, Japan and at the Department of Pediatrics and Adolescent Medicine, The University of Hong Kong, Hong Kong after obtaining written informed consent from the parents.

Results

Seventeen patients (15 males and 2 females) from 15 families with a diagnosis of CGD were included in the analysis. 7 children (41 %) children were diagnosed with X-linked form of CGD and 10 (59 %) were found to have an AR form of disease (Table I). Definite mutation analysis was available in 13/17 patients. Mutations were detected in CYBB gene was detected in 6 patients classified as definite X-CGD. Similarly mutations in NCF-1 gene were detected in 8 patients (definite

Table I Clinical phenotype and genotype of CGD patients at PGIMER Chandigarh

Family number	Patient	Clinical diagnosis	NBT or DHR122	Age at onset	Age at diagnosis	Follow up and prognosis	Gene involved (Protein encoded)	Exon/ Intron	Domain	Nucleotide change	Codon change
1	Patient 1	X-CGD	Both	1 month	2 years	1 year 8 months alive	CYBB (gp91phox)	Exon 4	Trans-memb domain III	271 C>T	p.Arg91X
2	Patient 2	X-CGD	NBT	10 days	7 months	7 days Dead	CYBB (gp91phox)	Exon 8	FAD binding domain	868 C>T	p.Arg290X
3	Patient 3	X-CGD	NBT	2 months	8 months	12 days Dead	CYBB (gp91phox)	Exon 5	Trans-memb domain IV	469 C>T	p.Arg157X
4	Patient 4	X-CGD	NBT	9 months	5 years	6 years alive	CYBB (gp91phox)	Exon 5	Extracellular domain	383 C>T	p.Gly145Arg
5	Patient 5	X-CGD	NBT	1 year	1 year	1 month Dead	CYBB (gp91phox)	Exon 11	NADP binding domain	1449 G>A	p.Trp483X
6	Patient 6	X-CGD	NBT	12 years	16 years	3 years 2 months alive	CYBB (gp91phox)	Exon 10	NADPH binding	1291 G>A	p.Ala431Thr
7	Patient 7	Presumed AR-CGD[a]	Both	1 1/2 months	2 years	2 months dead	CYBB, NCF-1, CYBA wild type	–	–	–	–
8	Patient 8	Presumed X-CGD[b]	NBT	3 months	9 months	1 month dead	N.D.	N.D.	N.D.	N.D.	N.D.
9	Patient 9	Presumed AR-CGD[a]	Both	5 months	1 year	1 years 6 months	CYBB, NCF-1, CYBA wild type	–	–	–	–
10	Patient 10	AR-CGD	NBT	10 months	10 years	3 years alive	NCF-1 (p47phox)	Exon 2	PX domain	73_74delGT	p.Tyr26HisfsX25
11	Patient 11	AR-CGD	Both	5 years	7 years	6 years alive	NCF 1 (p47phox)	Exon 2	PX domain	73_74delGT	p.Tyr26HisfsX25
12	Patient 12	AR-CGD	NBT	1 year	9 years	3 years alive	NCF-1 (p47phox)	Exon 2	PX domain	73_74delGT	p.Tyr26HisfsX25
13	Patient 13	AR-CGD	NBT	2 years	3 years	19 years alive	NCF 1 (p47phox)	Exon 2	PX domain	73_74delGT	p.Tyr26HisfsX25
13	Patient* 14	Presumed AR-CGD	NBT	5 years	9 years	5 years dead	N.D.	N.D.	N.D.	N.D.	N.D.
14	Patient 15	AR-CGD	Both	1 year 6 months	2 years	2 months alive	NCF-1 (p47phox)	Exon 2	PX domain	73_74delGT	p.Tyr26HisfsX25
14	Patient 16	AR-CGD	Both	2 years	4 years	2 months alive	NCF-1 (p47phox)	Exon 2	PX domain	73_74delGT	p.Tyr26HisfsX25
15	Patient 17	AR-CGD	DHR 123	4 months	3 years	4 years alive	NCF-1 (p47phox)	Exon 2	PX domain	73_74delGT	p.Tyr26HisfsX25

[a] Patients 8 and 10 were found to have no mutations in CYBB, CYBA and NCF-1

[b] Patient 9 has history of death of 3 elder male siblings *Patient 15 is the deceased sister of Patient 14

AR-CGD). One case had a history of loss of 3 elder male siblings and this was classified as presumed X-CGD. Two cases with no mutation in CYBB, NCF-1 and CYBA genes were presumed to be AR-CGD cases. The mean age for onset of symptoms was 1 year 8 months (median 10 months, range 1 month - 8 years) and a diagnosis of CGD was made at a mean age of 4 years 6 months (median 3.0 years, range 7 months -16 years). The mean age at diagnosis was significantly lower in children diagnosed with X-linked CGD compared those with autosomal recessive disease: mean 3 years 8 1/2 months (median 1 year, range 7 months–16 years) versus mean 5 years (median 3 years 6 months, range 1 year–10 years) respectively. Similarly the age at onset of symptoms was also different in two groups mean 2 years ½months (median 3 months, range 1 month–12 years) versus mean 1 year 10 months (median 1 year 3 months, range 1 1/2 months–5 years) (Table II).

Infectious Complications

All patients in the present cohort received prophylactic antimicrobials in the form of co-trimoxazole and Itraconazole or Ketoconazole after diagnosis. None of the patients received therapy with recombinant IFN-γ, because of financial constraints. Infectious episodes were managed with appropriate antimicrobials and antifungal agents including the newer antifungal agents such as voriconazole often for prolonged periods.

Pneumonia and recurrent lymphadenitis were the commonest clinical manifestations, present in 82.3 % (14/17) (Figure 1 and Table III). Recurrent skin and/or subcutaneous abscesses were found in 47 % (8/17) children. Hepatomegaly was detected in 59 % (10/17). In addition, liver abscesses were observed in 23.5 % (4/17) of children, in the absence of significant hepatomegaly in two children. Gastrointestinal symptoms in the form of loose stools, abdominal pain and distention were present in 35 % (6/17). Septicemia was detected in 25 % (4/16) children, albeit pre-terminally in 3 children. Bone and joint involvement was detected in 3 children. Perianal/ischiorectal abscess was also seen in 2 children. Other less common manifestations included recurrent otitis media and renal involvement, found in two children and one child respectively . Recurrent ulcerative stomatitis was also observed in one child (Table III).

Microorganims Isolated From Patients

An infectious etiology could not be established in most infectious episodes. Culture reports were often negative despite repeated blood and urine culture, culture of bronchoalveolar lavage whenever required on clinical grounds, fine needle aspirates and biopsy specimens probably because most of these children had received antimicrobials, including antifungals in some cases, prior to culture. Polymerase chain reaction based tests were not performed for etiological diagnosis because of lack of availability of the same. Aspergillus species was the most frequently isolated microorganism, being isolated from lung, synovium and blood in 6 patients. Apart from the common species i.e. *A fumigatus*, *A flavus,* some rarer species such as *A terreus* were also isolated. Candida species were also isolated in a significant proportion of cases (3/17). Fungal serology for *Aspergillus* was positive in four children (Table IV).

Staphyloccocus aureus was isolated from culture of pus in Patient 6 who preented with recurrent and multiple liver abscesses. *Burkholderia cepacia* was isolated from blood culture in one child with fulminant septicemia (Patient 2) and in one case *Mucor* was identified along with *Mycobacterium tuberculosis* from a resected segment of lung (Figure 2) (Patient 5). In addition *Fusarium dimerum* was cultured from the sputum in a child with autosomal recessive form of disease (Patient 16).

Table II Clinical course of children with CGD at PGIMER

	Overall (months/year)	X-linked CGD (months/year)	Autosomal recessive CGD (months/year)
Median age at onset (Range)	10 months (1 month–8 years)	3 months (1 month–12 years)	1 year 3 months (1 1/2 months–5 years)
Mean age of onset of symptoms (Mean ± SD)	1 year 8 months±2.4	2 years 1/2 months±4.4	1 years 10 months±1.8
Median age at diagnosis (Range)	3 years (7 months–16 years)	1 year (7 months–16 years)	3 years 6 months (1–10 years)
Mean age at diagnosis (Mean ± SD)	4 years 6 months±4.6	3 years 8 1/2 months±5.6	5 years±3.4
Mean delay in diagnosis (Mean ± SD)	2 years 10 months±2.9	1 year 8 months±1.8	3 years 2 months±3.0
Median delay in diagnosis	2 years (0–9 years 2 months)	6 months (0–4 years 3 months)	2 years (6 months–9 years 2 months)
Deceased	6/17 (35 %)	4/7 (57 %)	2/10 (20 %)
Median age at death (Range)	1 year 5 months (7 months–14 years)	10 months (7 months–2 years)	9 years

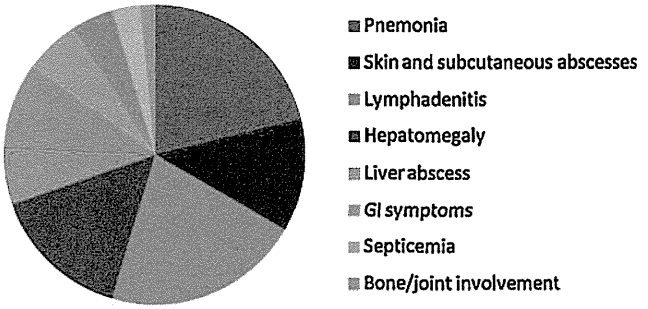

Fig. 1 Predominant clinical manifestations in children with CGD

Non Infectious Complications

Two X-CGD patients presented with abdominal pain and distention without any evidence of an infectious etiology. Patient 8 presented with features of intestinal obstruction with abdominal distention and pain. This was initially attributed to sepsis, the antimicrobials were changed twice. However when he did not respond to this regimen, steroids were given to which he responded dramatically with improvement in the gastrointestinal symptoms. However subsequently he developed respiratory distress and succumbed to his illness.

Patient 1 with X-linked form of the disease presented with fever, hepatosplenomegaly and persistent anemia, thrombocytopenia, hyperferritinemia (1,013 ng/ml) and mild hypertriglyceridemia (301 mg/dl) on follow-up. Bone marrow examination showed evidence of hemophagocytosis. His fibrinogen level was however normal at 2.4 g/l. A diagnosis of hemophagocytic lymphohistiocytosis (HLH) was made based on the clinical features and laboratory parameters. He responded well to immunosuppressive therapy in combination with intravenous vancomycin, meripenem and voriconazole.

Patient 4 presented with renal involvement in the form of mildly deranged renal function tests and hydronephrotic changes in the right kidney.

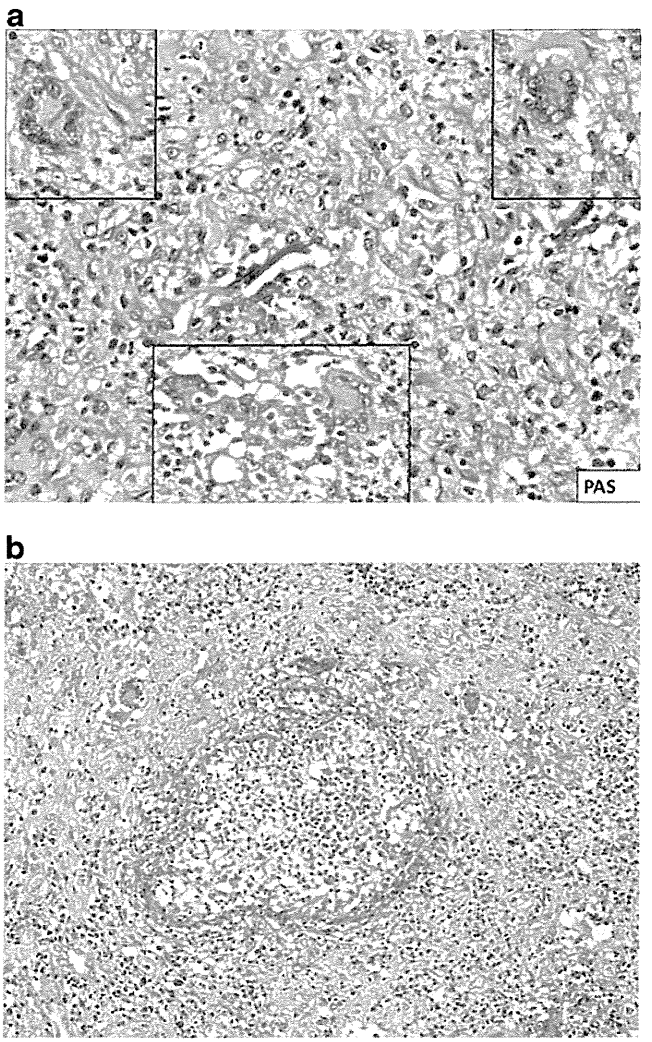

Fig. 2 **a** Photomicrograph showing epithelioid granulomas with multinucleate giant cells. **b** Photomicrograph depicting angioinvasion (H&E) ×40

Table III Infectious complications in the patients

Site of disease	No. of patients (%)	No of episodes	No of patients with ≥1 episode	% of patients with ≥1 episode
Lung	14 (82.3 %)	40	12	71 %
Skin/subcutis	8 (47 %)	28	8	47 %
Lymph node	14 (82.3 %)	32	11	65 %
Gastrointestinal	4 (23.5 %)	10	3	18 %
Liver	4 (23.5 %)	11	2	12 %
Kidney/Urinary Tract	1 (6 %)	1	0	0 %
Septicemia	4 (23.5 %)	6	2	12 %
Ear	2 (11.8 %)	4	2	12 %
Bone/Joint	3 (11.8 %)	5	2	12 %

Investigations

Most of the children had panhypergammaglobulinemia with mean IgG level of 1571.5±450 g/dl, mean IgM level

Table IV Microorganisms isolated from the patients

Microorganisms	Number of patients	Percentage
Staph aureus	2	12
Aspergillus flavus	3	18
Aspergillus fumigatus	2	12
Aspergillus terreus	1	6
Burkholderia cepacia	1	6
Mucor	1	6
Mycobacterium tuberculosis	1	6
Fusarium dimerum	1	6
Candida sp.	3	18

of 232.9 ±101.2 g/dl and a mean IgA level of 252.8± 179 g/dl. Lymphocyte subsets were within normal range in most children.

Nitroblue Dye Reduction Test [8, 9]

Leucocyte rich plasma was separated from heparin anticoagulated blood from both test and control subjects. Two drops (100 μl) each of leucocyte rich plasma was placed on 2 different glass slides labeled as "stimulated" and "unstimulated" respectively. Two drops of (100 μl) of a 0.2 % Nitroblue tetrazolium dye was added to each slide. One drop of a 20 % suspension of Baker's yeast was added to the slide labeled as "stimulated". Both the slides were then placed in a humified chamber at 37 °C for 30 min. The slides were taken out after 30 min, cover slips were placed on both them and they were examined under a light microscope.

Neutrophils showing blue black cytoplasmic clumps of formazan were counted in both stimulated and unstimulated tests. At least 100 neutrophils were counted and the results were expressed as a percentage of cells showing the blue black granules. The procedure was repeated similarly with the control sample. Normally 85–95 % of stimulated neutrophils showed reduction of nitroblue tetrazolium to blue black formazan. Tests in which less than 30 % of stimulated neutrophils showed no reduction were interpreted as abnormally low and suggestive of chronic granulomatous disease. In some cases 2 μl of (100 μg/ml) of Phorbol myristate acetate was used to stimulate the neutrophils.

Dihydrorhodamine Assay [10, 11]

Staining Procedure

Four tubes, two each for normal control and patient sample were labeled as unstimulated and stimulated. Hundred (100 μl) of heparin anticoagulated blood was added to each tube. 1 μl of 1 mM Dihydrorhodamine (Sigma Cat No D1054) in DMSO was added to each tube and incubated for 15 min at 37^0C in a water bath. 2 μl of Phorbol-12- myristate-13- acetate (100 μg/ml) was added to the tubes labeled stimulated for both test and control samples. The tubes were further incubated for further 15 min at 37^0C and then lysed using BD FACS lysing solution for 10 min. The tubes were then centrifuged at 1,500 rpm for 5 min. Wash the cells with Phosphate buffered saline and spun again at 1,500 rpm for 5 min and decant supernatant. This procedure was repeated twice.

Acquisition and Data Analysis

Samples were acquired on FACS Calibur or FACS CANTO flow cytometer. Threshold was adjusted to exclude debris and neutrophils were gated by Forward Scatter(FSC)/Side scatter (SSC) gating. At least 10,000 gated events were acquired and recorded for each tube. Mean fluorescence intensities (MFIs) of stimulated and unstimulated samples and fold change in MFIs for both test and control samples was recorded.

Interpretation

The assay was performed in 7/17 patients. Four (4) of these patients were AR-CGD cases with a mutation in NCF-1 gene. Two of these were presumed AR-CGD (Patient 7 and 9) and one patient was a case of X-CGD with a mutation in the CYBB gene. The mean of mean fluorescence intensities in the unstimulated state in test was 52.6±59.2 and in the stimulated state was 68.9±78 and mean fold change was 1.23± 0.2. In the control the mean MFI in the unstimulated tube was 39.82±52 and in the stimulated tube was 627. 16±880 and the mean fold change was 21.75±18.1 A diagnosis of CGD was considered when there was no shift in the histograms after stimulation with PMA and minimal or no change in MFIs in unstimulated vs stimulated states (Table V) (Fig. 3a–d).

We could not estimate resdiual NADPH oxidase activity as superoxide production was not estimated in any of the cases.

Molecular Basis of the Defects

Molecular defects could be identified in 13 of the 17 patients with CGD. The mutation analysis could not done in 4 cases because some of the children were diagnosed when facilities for mutation studies were not available or the children had died before these studies could be performed. A mutation in the *CYBB* gene was detected in 6 children with X-linked CGD in which the mutation analysis was performed. All the six mutations detected in the CYBB gene were point mutations. Four of these six point mutations were nonsense mutations and 2 were missense mutations. The two missense mutations in the *CYBB* gene were novel and both mutations were deleterious using the SIFT program for the prediction of protein change. Same type of mutation in *NCF1* gene encoding for *p47phox* protein (c. 73_74delGT, p.Tyr26HisfsX25) were detected in 7 of the 8 children with AR- CGD in whom mutation analysis was performed (Table I).

Mortality and Cause of Death

Six of the 17 patients (35 %) had succumbed at the time of the analysis. Four of these were boys who had X-linked CGD (Patients 2, 3, 5, and 8) and Patient 15 who was a sister of

Table V Details of dihydrorhodamine assay in patients with CGD at PGIMER

Patient	MFI unstim pt	MFI stim pt	Fold change [SI]	MFI unstim cont	MFI stim cont	Fold change [SI] cont
Patient 1	8.5	8.69	1.02	7.79	174.1	22.35
Patient 7	11.25	12.7	1.12	23	380.91	16.56
Patient 9	8.8	9.9	1.12	2.8	173	61.78
Patient 11	175	223	1.8	155	2612	16.85
Patient 15	37.46	50.29	1.34	28.82	301.7	10.47
Patient 16	59.98	62.44	1.04	28.82	301.7	10.47
Patient 17	67.34	114.98	1.7	32.51	446.72	13.74
Mean ± SD	52.6±59.2	68.9±78	1.23±0.2	39.82±52	627. 16±880	21.75±18.1

MFI unstim pt: Mean fluorescence intensity unstimulated patient, MFI stim pt: Mean fluorescence intensity stimulated (PMA) patient

MFI unstim cont: Mean fluorescence intensity unstimulated control, MFI stim pt: Mean fluorescence intensity stimulated (PMA) control, SI : Stimulation index

patient 14 with AR-CGD with mutation in *NCF1* gene had also died. Patient 7 with presumed AR-CGD had also succumbed to his illness at time of analysis. Median age at death was 1 year 5 months with a range of 7 months–9 years. The median age at death in children with XL-CGD was 10 months with a range of 7 months–2 years. Three children

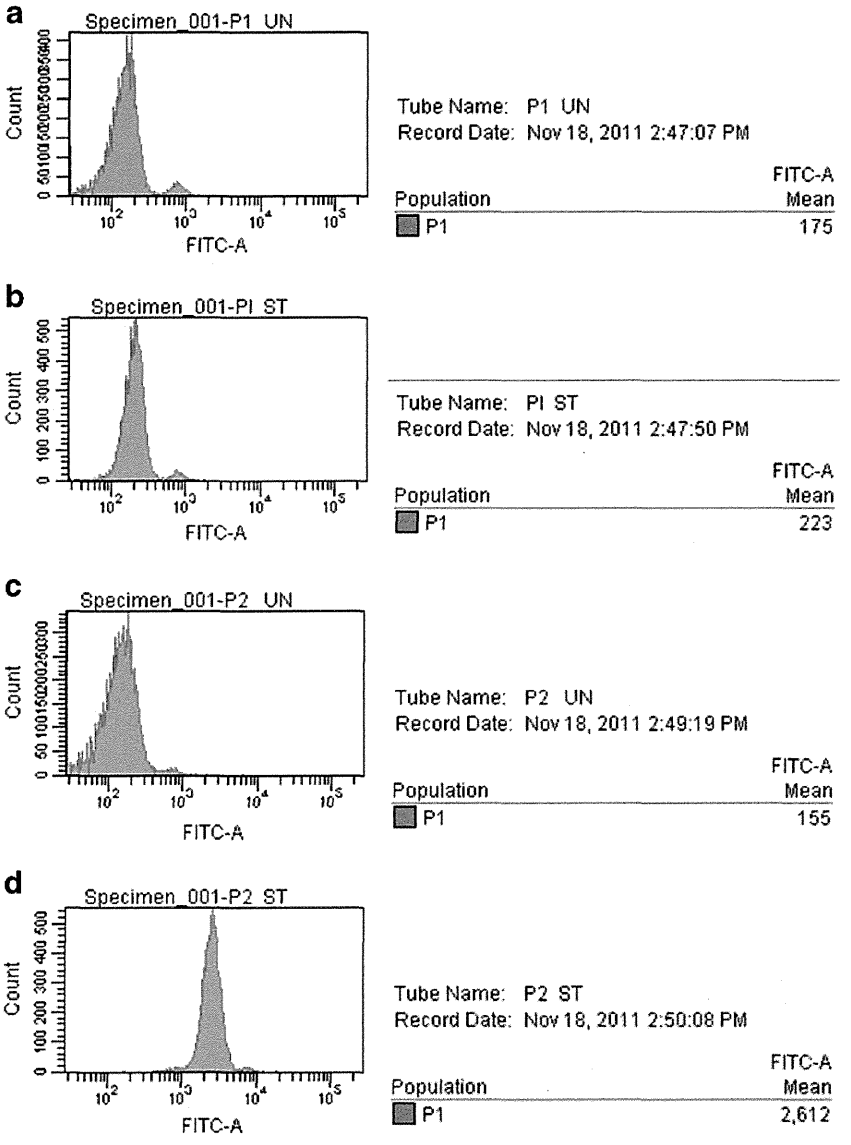

Fig. 3 Histograms from a DHR assay showing MFIs in gated neutrophils (**a**) Unstimulated patient (**b**) Post stimulation with PMA patient (**c**) Unstimulated normal control (**d**) Post stimulation with PMA normal control

with X-linked CGD died of fulminant septicemia. Another child (Patient 5) with X-linked CGD died of a massive pulmonary bleed following decortication to remove a segment of the lung for Mucormycosis. One girl (Patient 15) with autosomal recessive CGD and another child with presumed AR-CGD (Patient 7) died due to fulminant pneumonia at the age of 14 years and 2 years respectively. Mean survival in X-CGD was estimated to be 31.23 months (95 % CI: 5.1, 57.8 months) while the mean survival in AR-CGD was 176.6 months (95 % CI : 72.2, 281 months). This difference in survival was found to be close to level of statistical significance by Log Rank (Mantel-Cox) test ($p=0.065$) (Fig. 4).

Discussion

Chronic granulomatous disease has been infrequently been reported from India with only occasional case reports [12–15]. However in these case reports a presumptive diagnosis of CGD was made on the basis of NBT dye reduction test without a confirmed diagnosis by genetic analysis. The present study is to best of our knowledge the largest case series of CGD from India with a well-characterized molecular defect in 13 of the 17 patients. Thirteen cases of CGD have been reported from another tertiary care centre in North India in a period of 2 years between July 2004–August 2006 [16]. However all the cases in this study where diagnosed on the basis of NBT dye reduction test, DHR assay was not performed and the diagnosis was not confirmed by a genetic analysis for the putative genes in CGD in any of these patients.

AR-CGD was more common in the present series detected in 58 % of children compared to X-linked form of disease which was detected in 42 % of children. This is in contrast to previous studies from Europe and USA in which X-CGD. [1, 17, 18] and similar to a large series reported from Turkey [19] and another series from Tunisia [20]. Consanguinity is not frequent in North India as has been reported from the series reported from Turkey and Tunisia. Consanguinity was found in 2/17 patients in our cohort and both had AR-CGD with mutation in the NCF-1 gene. However the pattern of marriages is largely endogamous, with marriages being restricted within closed knit communities and this may result in a higher incidence of different autosomal recessive diseases in different population groups [21]. The mean age at diagnosis was 4 years 6 months whereas the mean age of onset of symptoms was 1 year 8 months and children with X-linked CGD were diagnosed earlier than AR- CGD, similar to cohorts reported earlier[22, 23]. Clinical manifestations were also similar to what has been reported earlier with rare and interesting findings in two cases [22–24].

Hemophagocytic lymphohistiocytosis (HLH) is a rare and potentially fatal complication of CGD. Only few cases of HLH complicating the course in CGD have been reported thus far. Apart from an increased susceptibility to recurrent infections by intracellular microorganisms, CGD is also associated with hyperinflammation and proinflammatory cytokine milieu, which could predispose these children to HLH. Most cases of HLH in children with CGD are secondary to infections mainly with Burkholderia cepacia and Leishmania. An isolated case with a perforin gene variant has also been reported [25–27].

One of the children had pulmonary mucormycosis (Fig. 2a and b) along with a co-infection with *Mycobacterium tuberculosis*. This child presented with a mass in the right hemithorax eroding the ribs and extending into the mediastinum. Mucormycosis has rarely been reported in patients with CGD, almost exclusively in those receiving prolonged and significant immunosuppression [26, 27]. However this child had not received any form of immunosuppressive therapy,

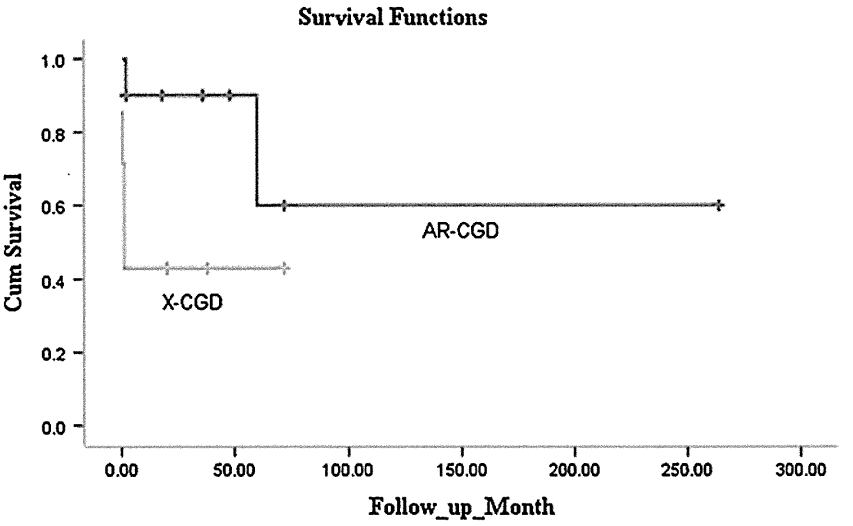

Fig. 4 Survival and follow-up in AR-CGD vs X-CGD

highlighting that mucormycosis can occur in CGD even without immunosuppression.

Mycobacterial infections both due to BCG and M. tuberculosis have been reported in patients with CGD from China, Iran and Latin America [28, 29]. Mycobacterium was detected in only one of our patients, diagnosed by detection of acid fast bacilli in a lung biopsy specimen. Although BCG vaccine is administered at birth in India only one child in our cohort developed suppuration at site of BCG administration followed by swelling at the root of the neck. The apparently low incidence of BCG and Mycobacterium tuberculosis infection in our cohort of CGD patients could be due to under detection. Moreover, some of the children presenting pneumonic consolidation did receive antitubercular therapy based on a presumptive diagnosis of tuberculosis. Two children in our cohort were diagnosed as tuberculosis based on the finding of granulomatous inflammation in lung biopsy specimens despite no isolation of Mycobacterium on culture or demonstration of acid-fast bacilli in the biopsy specimens. Recently germline mutations in the *CYBB* gene that selectively impair the activity of the NADPH oxidase complex in the monocyte-derived macrophages but not monocytes and neutrophils and predisposition to infection by *Mycobacteria tuberculosis* have been reported [30].

The spectrum of microorganisms isolated in the present series were similar to those reported in earlier studies [24, 31, 32]. Aspergillus was the most common organism isolated in our cohort followed by Staphylococcus *aureus*, Burkholderia *cepacia* and Candida. Several previous studies have shown that Aspergillus is emerging as the single most significant pathogen for infectious complications and mortality in patients with CGD. In North America, majority of infections in CGD are caused by five microorganisms namely *Staphylococcus aureus*, Burkholderia cepacia, *Serratia marcescens*, Nocardia and Aspergillus species [1].

All the presently known mutations in the *CYBB* gene have been reported in a large series recently [33]. It comprised of 1,267 unrelated kindreds with 1,415 patients. Six hundred and eighty one different mutations were identified in the patients and 487 (73.1 %) of these mutations were unique for one kindred. Single nucleotide substitutions were the commonest mutations reported. The missense mutations, splice site mutations and nonsense mutations were observed in 21.3 %, 17.6 % and 14.1 % respectively. Deletions contributed to 35.6 % of all mutations and insertions to 8 % of the mutations. All the mutations in *CYBB* gene in our cohort were single nucleotide substitutions; 66.7 % were nonsense mutations and 33.3 % were missense mutations. Two novel deleterious missense mutations were detected in our cohort.

Similarly a large series of all known mutations in the genes implicated in AR-CGD have also been published [34]. All the patients with AR- CGD in our cohort were found to have a same mutation in the *NCF1* gene encoding for *p47phox* protein although they were unrelated. Mutation in *NCF1* gene is the commonest cause of AR- CGD and accounts for approximately 20–25 % of all cases. All the patients also had the same mutation in the *NCF1* gene i.e. GT dinucleotide deletion in exon 2. This particular mutation has been reported in more than 60 patients worldwide with AR-CGD due to mutation in NCF1 gene and 97 % of the alleles [35]. There are 2 pseudo *NCF1* genes with a GT deletion in each of them. The preponderance of this mutation in patients can be explained by multiple recombination events between the functional *NCF1* gene and these closely linked pseudogenes, each gene having multiple recombination hot spots such as Alu repeats, Chi sequence and human mini-satellite repeats [36].

The overall mortality (35 %) was higher than what has been reported previously. The mortality in a large cohort of 429 patients from Europe was 20 % [23] whereas in an Italian cohort the mortality was 13 % [22]. This high mortality could be attributed to several factors. None of our patients received recombinant interferon γ. All patients were managed on cotrimoxazole and itraconazole prophylaxis along with management of breakthrough infections. The possibility of increased exposure to infectious agents in a developing, tropical country might have also contributed to a higher incidence of infections.

A higher frequency as well as greater severity of breakthrough infections compared to developed countries could have contributed to the increased mortality. Delay in initiating therapy for intercurrent infections due to the parents having to travel long distances to reach medical facilities and poor economic conditions are likely to have contributed significantly to this higher mortality. Haemotopoietic stem cell transplantation was not performed in any of our patients.

Finally although the total number of patients in the present cohort is very small to draw definite conclusions, it was observed that the course and outcome of the disease was much worse in children diagnosed with XL-CGD compared to AR forms of the disease. Four of the seven children (4/7) with X-CGD were dead at the time of data analysis. Three of these four children had nonsense mutations in the *CYBB* gene resulting in a stop codon as has been reported previously [1, 23, 29]. However it has also been conclusively shown that residual NADPH oxidase activity determined largely by the specific mutation in any of genes responsible for CGD rather than the gene itself is a useful predictor of outcome and survival [37]. It can also be concluded from this cohort that it is possible to provide a reasonable quality of life to patients with prophylactic antimicrobials even in a developing country like ours with all its constraints.

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RESEARCH ARTICLE

Generating a transgenic mouse line stably expressing human MHC surface antigen from a HAC carrying multiple genomic BACs

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Abstract The human artificial chromosome (HAC) vector is a promising tool to improve the problematic suppression and position effects of transgene expression frequently seen in transgenic cells and animals produced by conventional plasmid or viral vectors. We generated transgenic mice maintaining a single HAC vector carrying two genomic bacterial artificial chromosomes (BACs) from human HLA-DR loci (DRA and DRB1). Both transgenes on the HAC in transgenic mice exhibited tissue-specific expression in kidney, liver, lung, spleen, lymph node, bone marrow, and thymus cells in RT-PCR analysis. Stable functional expression of a cell surface HLA-DR marker from both transgenes, DRA and DRB1 on the HAC, was detected by flow cytometric analysis of splenocytes and maintained through at least eight filial generations. These results indicate that the de novo HAC system can allow us to manipulate multiple BAC transgenes with coordinated expression as a surface antigen through the generation of transgenic animals.

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Introduction

Transgenic animals have provided tools for investigating many biological problems. Genomic fragments cloned by bacterial artificial chromosomes (BACs) have been utilized to generate transgenic animals when tissue-specific or temporally controlled expression of transgenes is desired. Due to the large insert capacity (~350 kb) of a BAC vector, the genomic fragments can often possess the complete promoters and control elements of the gene of interest (Asami et al. 2011). In addition, BAC transgenes seem to be more resistant to position effects than smaller transgenes, such as artificial expression cassettes with complementary DNA (cDNA) (Gong et al. 2003). Typically, BAC transgenic mice are generated by microinjection of the BAC DNA into the pronucleus of fertilized mouse eggs (Vintersten et al. 2008). However, in principle, this method causes random integration (non-specific insertion) of BAC DNAs into the mouse genome, and the number of insertion copies is variable. Increased copy number of a BAC transgene correlates with increased expression of the BAC transgene (Chandler et al. 2007). When investigating the cooperation of two transgenes in a transgenic mouse, generally two characterized transgenic mouse lines are crossed, but this is a time-consuming method and maintaining an appropriate level of gene expression is difficult.

A de novo human artificial chromosome (HAC) was constructed with naked human centromeric repetitive DNA (Harrington et al. 1997; Ikeno et al. 1998) and a HAC vector system developed in which one copy of a DNA fragment can be handled by Cre/lox insertion and transferred into a variety

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of vertebrate cell lines (Ikeno et al. 2009; Iida et al. 2010). A HAC is an episomal vector that can harbor a large DNA and is exploitable for generating transgenic animals using embryonic stem (ES) cell technology (Kazuki and Oshimura 2011; Ikeno et al. 2012). Thus, the HAC system can avoid the copy number problem and/or position effects caused by non-specific insertion of the BAC transgene. The HAC vector is expected to be available for the production of transgenic mice carrying two or more single-copy genes with a large control region over tens of kilobases. Recently, a transgenic mouse harboring a single copy of a HAC, termed a trans-mini-chromosomal (TMC) mouse, carrying three continuous non-correlated genes from the human genome was generated by inserting a single BAC DNA from chromosome 21 (Miyamoto et al. 2014). However, whether two or multiple independent BAC transgenes can be gathered onto a single HAC and cooperatively function in a transgenic mouse has not been investigated.

Here, we describe a transgenic mouse using a HAC vector carrying two single-copy human HLA-DR genomic genes. HLA-DR is a major histocompatibility complex (MHC) class II cell surface receptor consisting of an αβ heterodimer. We introduced a DR α-chain (HLA-DRA locus) and DR β-chain (HLA-DRB1*0405 locus) into a single HAC vector (HLA-HAC). In transgenic mice harboring HLA-HAC (carrying HLA-DRA and DRB1 genes), tissue-specific expression of human MHC class II cell surface receptor in spleen cells was detected by flow cytometric analysis through at least eight filial generations.

Materials and methods

Cell culture

Chinese hamster ovary (CHO) cells were cultured in Ham's F-12 nutrient mixture (Wako) supplemented with 10 % fetal bovine serum (FBS) at 37 °C and 5 % CO_2. The mouse ES cells were maintained on feeder cells in an ES cell medium consisting of Dulbeccos' modified Eagle's medium (DMEM) (Kohjin Bio) supplemented with 20 % FBS, 0.1 mM non-essential amino acids (Gibco), 2 mM glutamine (Gibco), 1000 U/ml ESGRO (Chemicon), and 0.1 mM β-mercaptoethanol (Sigma).

Microcell-mediated chromosome transfer

Microcell-mediated chromosome transfer (MMCT) from CHO cells to mouse ES cells was carried out as described previously (Suzuki et al. 2006). Briefly, twenty 10-cm dishes of CHO cells were grown to 70 % confluency and Colcemid (Wako) added to 0.05 μg/ml. The cells cultured for 72 h were harvested by trypsinization and resuspended in pre-warmed serum-free DMEM (Wako) containing Cytochalasin B (Calbiochem) at a final concentration of 20 μg/ml. The suspension was incubated, and then an equal volume of Percoll (Amersham Biosciences) was added. The suspension was centrifuged in a Hitachi R20A2 rotor at 15,000 rpm for 90 min at 37 °C. The microcell fraction containing the HLA-HAC was mixed with ES cells. After centrifugation at 2000 rpm for 5 min, the pellet was suspended in 1 ml 50 % PEG1500 (Roche). The fusion product was washed and plated onto three 10-cm dishes layered with feeder cells. ES cells containing the HLA-HAC were selected with 150 μg/ml G418 (Sigma).

Generation of chimeric mice

Chimeric mice were produced from mouse ES cell lines. The ES cells were aggregated with eight-cell embryos derived from BDF2 mice and then transferred into pseudopregnant ICR females. Almost 100 % coat color chimeric mice were mated with C57BL/6 (B6) mice (Jackson Laboratory) to obtain transgenic mice. All animal experiments were approved by the Institutional Animal Care and Use Committee of RIKEN RCAI and Kazusa DNA Research Institute.

HLA-DR gene constructs

We used a whole CTD-2052L14 BAC clone (100 kb) as the HLA-DRA gene because this BAC contains only the HLA-DRA gene (referenced on NCBI CloneDB browser; http://www.ncbi.nlm.nih.gov/clone/79827/). The DRA-BAC used in this study contains an upstream 22-kb region and the HLA-DRA gene (Fig. 1a). Only a 268-base pair region of the 5′-flanking region of the HLA-DRA gene has been reported to be sufficient for the cell type-specific expression on the transgenic mouse as a multi-copy in this case (Fukui et al. 1993). So, we included the longer upstream region. On the other hand, for the *cis*-elements of the DRB1 gene, consensus has not yet been reached; therefore, we used the maximum length of the upstream region, close to the border of the next pseudo gene. A HLA-DRB1*0405 BAC was constructed from a 28.5-kb upstream region of the HLA-DRB1*0103 gene in a RP11-379F19 BAC clone (referenced on NCBI CloneDB browser; http://www.ncbi.nlm.nih.gov/clone/312446/) and genomic DNA (IHW09415) containing all exons of HLA-DRB1*0405 by using a Red recombination with pBADTcTypeG plasmids whose mutant Redα and mutant Redβ proteins increase the recombination efficiency (Nakayama and Ohara 2005). Consequently, the 50-kb BAC had 28.5 kb upstream of the translational start site and all exons and introns of the HLA-DRB1*0405 gene (Fig. 1a). The HLA-DRA and DRB1 BACs were modified by addition of the lox66/puromycin resistance cassette or lox66/blasticidin S resistance cassette, respectively (Fig. 1), in place

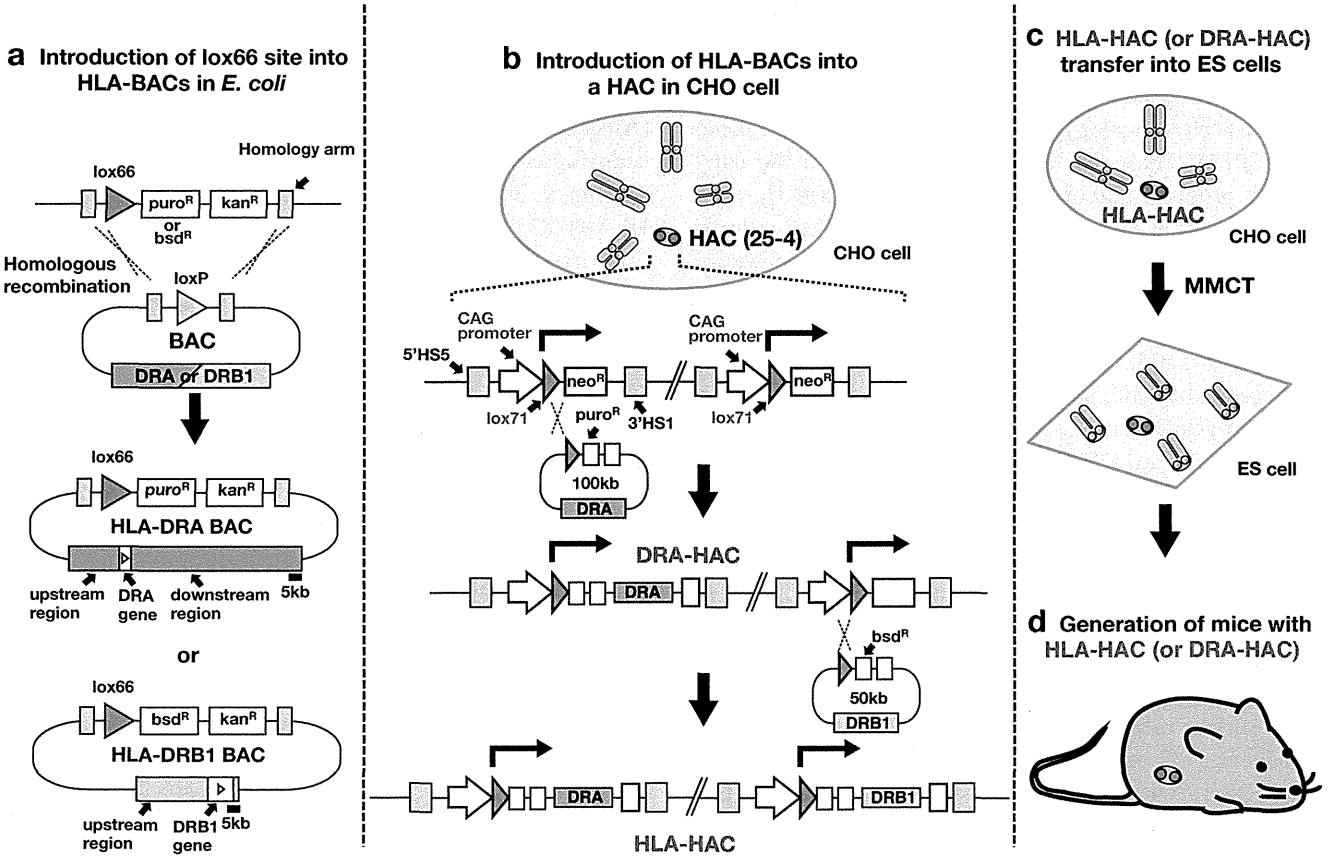

Fig. 1 Generation of mice with HACs containing human genes. **a** Introduction of the lox66 site into the HLA-BAC by Red recombination in *E. coli*. **b** Schematic representation of two consecutive introductions of HLA-BACs into a HAC vector using Cre/lox recombination. The gene in the entry vector was inserted into a HAC vector in CHO cells by Cre/lox recombination. Successful recombinants were selected by puromycin (*puro*) or blasticidin (*bsd*) resistance. **c** HLA-HAC transfer from CHO cells to mouse ES cells was achieved by MMCT. **d** Chimeric mice with HLA-HAC were created by aggregation of the ES cells with BDF2 eight-cell embryos

of loxP by the Red recombination in *Escherichia coli* according to a previously described method (Ikeno et al. 2009).

DNA transfection

For the insertion of the HLA-DR BACs into the HAC vector, 1 μg of the HLA-DR BAC DNA was co-transfected with 0.5 μg of CAGGS-Cre into CHO cells (5×10^5) retaining the HAC vector (Ikeno et al. 2009) with FuGENE HD (Promega) according to the manufacturer's instructions. DRA cell lines were selected with 6 μg/ml puromycin (Sigma) and DRB1 cell lines with 3 μg/ml blasticidin S (Wako).

Real-time quantitative PCR analysis

Total RNA was isolated using the RNeasy Micro Kit (Qiagen). cDNA was synthesized using the Verso cDNA Synthesis Kit (Thermo Scientific), and 25-ng aliquots were used for PCR. Real-time PCR was carried out using the ABI 7500 Real-Time PCR System (Applied Biosystems). PCR reactions were carried out using the Luminaris Probe Low ROX qPCR Master Mix (Thermo Scientific) and each assay mix: Hs00219578_m1 (HLA-DRA), Hs00830030_sH (HLA-DRB1), or Mm99999915_g1 (mouse GAPDH). The PCR protocol was 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 60 s.

Genomic PCR analysis

Genomic DNA was extracted from mouse tails using a Wizard Genomic DNA Purification Kit (Promega) and PCR performed as follows. The amplification conditions were 98 °C for 1 min, followed by 35 cycles of 98 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s. The following primers were used: HLA-DRA-1, 5′-CACGAACAGCCCTGTGGAAC-3′ and 5′-CTCAGTTGAGGGCAGGAAGG-3′; HLA-DRA-2, 5′-TCTCCCAGAGACTACAGAGAACG-3′ and 5′-CCTGCG TTCTGCTGCATTG-3′; HLA-DRB1-1, 5′-TCATTTCTTC AACGGGACGGAG-3′ and 5′-TGCACTGTGAAGCTCT CACC-3′; HLA-DRB1-2, 5′-TGGTCTGCTCTGTGAA TGG-3′ and 5′-TCCACTGTGAGAGGGCTCATC-3′; HLA-DRB1-3, 5′-GTGGGAGATGCAGACTTGTGG-3′ and 5′-