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LETTER TO EDITOR

Myriad Faces of Chronic Granulomatous Disease: All in an Indian Family with Novel *CYBB* Defect

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To the Editor,

Chronic granulomatous disease (CGD) is a phagocytic disorder affecting the NADPH oxidase complex [1]. Mutations in the *CYBB* gene result in X-linked form of CGD (XL-CGD). Female carriers of XL-CGD are known to have autoimmune phenomenon, and extreme lyonization of the X chromosome in female carriers could also result in deficient gp91phox protein and manifestations of CGD [2]. We report a family with X-linked CGD due to a novel *CYBB* defect where the index patient had a severe form of CGD, sister had manifestations of CGD, and carrier mother had manifestations of lupus.

Case Details

Index male patient presented at the age of 16 months with a history of recurrent episodes of fever and cervical adenitis that started from 4 months of age. Two maternal uncles had died at first year due to infective illness (Fig. 1a). Acid-fast bacilli were identified from fine-needle aspiration of his left axillary lymph node at initial presentation. He was treated with isoniazid, rifampicin, and ethambutol for 6 months for the BCG adenitis. Nitroblue tetrazolium (NBT) test showed an absent reduction in stimulated neutrophils, suggestive of CGD. He was started on cotrimoxazole and itraconazole prophylaxis. We could not use interferon-gamma therapy because of the lack of availability of the same in our country. He developed cervical adenitis due to *Burkholderiacepacia*, severe pneumonia with left-sided pleural effusion at 18 months. The combination of levofloxacin and cotrimoxazole was given for 3 months. At 2 years, he developed impetigo and cervical adenitis due to *Klebsiella pneumoniae*. He developed pneumonia at 3 years and the computerized tomography (CT) of the chest showed bilateral diffuse reticulonodular lung infiltrates [3]. Dihydrorhodamine (DHR) test showed an absent right shift of neutrophils with a markedly decreased stimulation index (Fig. 1b, d, and e). He subsequently developed osteitis of the left fourth and fifth ribs and intramuscular collection in the left posterior chest wall. He underwent surgical debridement of the infected lung and partial resection of the left fourth and fifth ribs. Biopsy of the resected specimens showed granulomas and fungal profiles consistent with *Aspergillus* spp. He continued to have recurrent fever spikes and dyspnea despite 6 weeks of intravenous amphotericin B and 3 months of oral voriconazole. Fungal invasion extended to the eighth rib and he also developed 11 × 7 cm abscess in the posterior chest wall that warranted surgical excision of the rib and partial decortication. He also developed a central line–related bloodstream infection. Hematopoietic stem cell transplantation (HSCT) was contexmplated. However, the procedure was not possible because of the high infection burden, financial constraints, and lack of easy access to HSCT. He was subsequently tried on oral posaconazole and oral pioglitazone (1 mg/kg/day) which was hiked to 3 mg/kg after 15 days. Pioglitazone was added as a desperate measure to improve the oxidative capacity and for clearance of fungal infection. Despite 3 months of oral pioglitazone therapy, no change in stimulation index (1.0) was noted in

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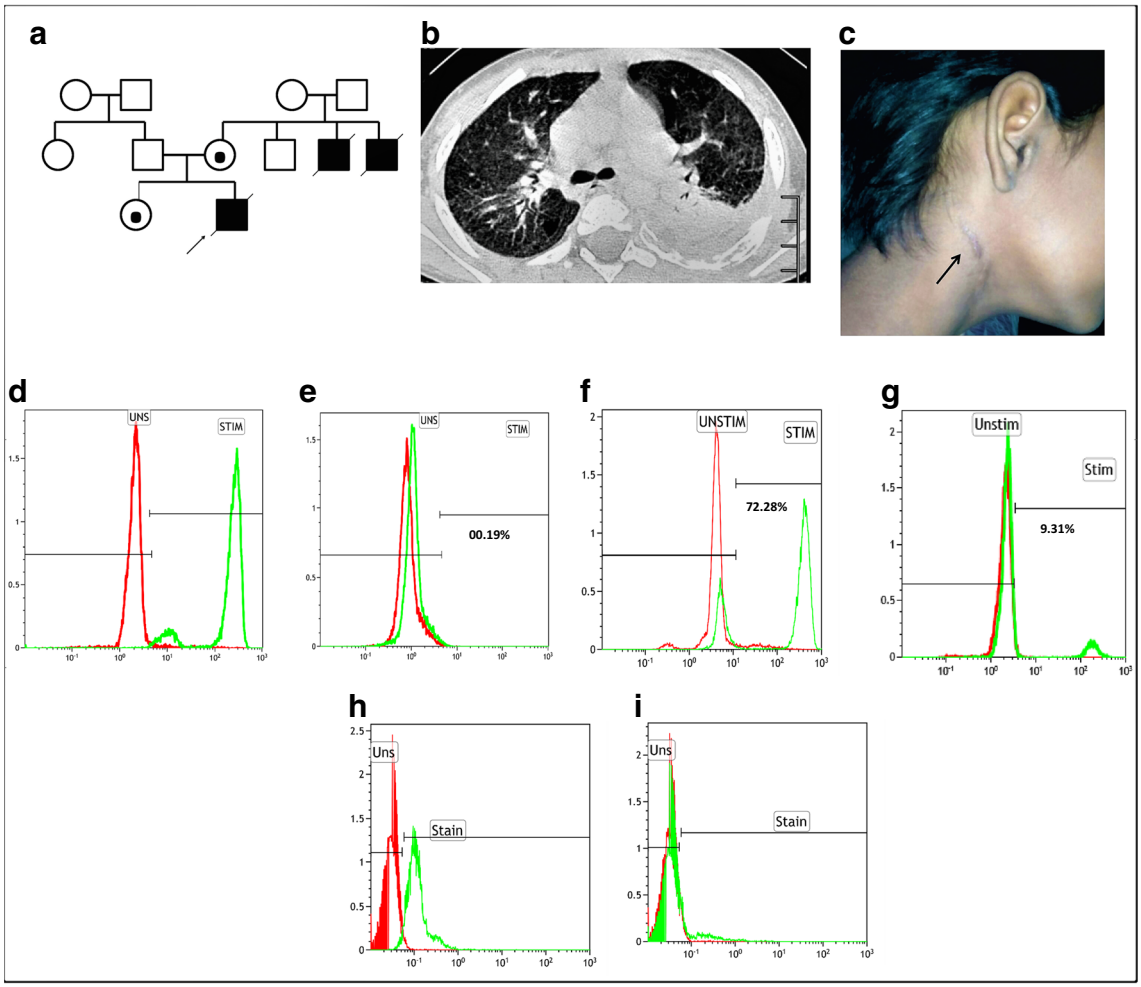

Fig. 1 Clinical and laboratory features of the family with X-linked CGD. (**a**) Pedigree chart; (**b**) CT chest image of affected proband-consolidation of bilateral lungs and infiltration of adjacent ribs in left side; (**c**) incision scar of right cervical suppurative adenitis in sister; (**d**) DHR of healthy control-normal right shift of the neutrophils after PMA stimulation (SI*-117.8); (**e**) DHR of affected proband-absent right shift of stimulated neutrophils (SI*-1.34); (**f**) DHR of mother showing mosaic pattern; 72.3% DHR+ (**g**) DHR of the sister showing absent right shift of stimulated neutrophils (SI*-1.00) 9.3% DHR+; (**h**) normal gp91phox expression in healthy control (89.7%); (**i**) markedly decreased expression of gp91phox (11.67%) in the neutrophils of the sister. *Stimulation index is derived from the median fluorescence intensity (MFI) of stimulated neutrophils divided by MFI of unstimulated neutrophils. CT, computerized tomography; DHR, dihydrorhodamine; PMA, phorbol 12-myristate 13-acetate; SI, stimulation index

DHR assay, and he continued to have progressive worsening in his respiratory status. He was subsequently tried on combination antifungal therapy-voriconazole and IV amphotericin B. However, he did not improve and finally succumbed to his illness at the age of 4 years.

Patiient's mother developed a malar rash and symmetric polyarthritis of metacarpophalangeal and proximal interphalangeal joints of hands and ankles at 30 years of age. Antinuclear antibodies by indirect immunofluorescence showed 4+ fine speckled positivity. Anti-double-stranded DNA antibodies were also elevated (69 IU/mL, normal < 40). Immunoblot showed positivity for anti-ribonuclear protein. DHR showed dual peaks suggestive of a carrier state (Fig. 1f). She improved with oral corticosteroids and weekly oral methotrexate.

His sister developed suppurative anterior cervical adenitis at the age of 6 years that required incision and drainage (Fig. 1c). DHR showed only 9.38% right shift of PMA-stimulated neutrophils and the stimulation index was 1.0. Flow cytometric analysis of gp91phox expression was performed using FITC-conjugated anti-human flavocytochrome b_{558} antibody (Clone 7D5 from MBL Life Sciences, Japan). This was found to be markedly decreased in the sister (Fig. 1g–i). She was started on oral cotrimoxazole and itraconazole prophylaxis. The sister was doing well with septran and itraconazole

prophylaxis without any complications. Hence, we did not consider pioglitazone or HSCT in the sister.

A novel hemizygous mutation in *CYBB* was identified (c.675-1G>T) in the affected proband. This variant was also evident in the heterozygous state in the mother and sister of the patient (Fig. 2a).The cDNA analysis of the affected proband revealed a decrease in the size of CYBB cDNA compared with the normal control probably as a result of skipping of exons 6 and 7 due to the splice-site defect (Fig. 2b). A similar splice-site defect (c.675-1G>A) has been reported to cause skipping of exons 6 and 7, resulting in a decrease of 321 bp from the mRNA [4]. In an attempt to explain observed differences in DHR and cytochrome B558 expression pattern, karyotyping of the sister was performed. Instead of expected 45X (Turner syndrome), sister's karyotype was found to be 47,XXX (Fig. 2c). With these findings, sister's physical features and development were reassessed, and were found to be consistent with that of triple X females (speech delay, epicanthal folds, and hypertelorism) (Fig. 2d) [5].

Discussion

We describe a family with X-linked CGD where the index patient had multiple infections since early infancy and recalcitrant fungal pneumonia. Pioglitazone is reported to induce mitochondrial reactive oxygen species production and was documented to enhance efferocytosis and reduce inflammatory and infective burden in patients with CGD [6, 7]. However, the index patient did not show any improvement in respiratory status following pioglitazone therapy. We also noted that the percentage of stimulated neutrophils determined by DHR did not improve after pioglitazone therapy. It seems that pioglitazone might not be effective in severe forms of CGD, especially in the presence of refractory

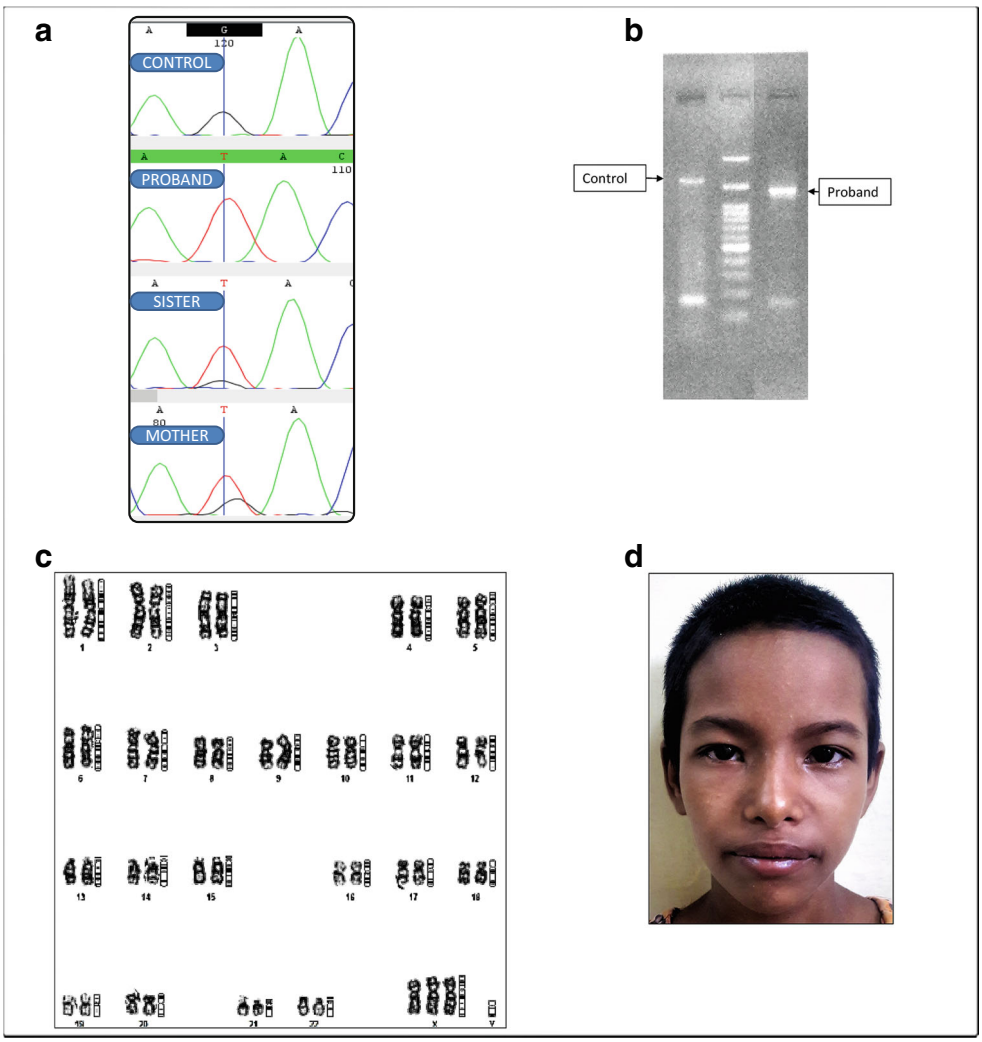

Fig. 2 Molecular tests in the family with X-linked CGD. (**a**) DNA electropherogram-*CYBB* c.675-1G>T in the affected proband and carrier state in the mother and sister; (**b**) left-panel-amplified product of 1713 bp using control cDNA as template, middle-panel-100 bp DNA ladder, and right-panel-product of lower size (1329 bp) using proband cDNA as template suggesting a probable skipping of exons 6 and 7 due to exon-skipping; (**c**) karyotyping of sister-47,XXX (estimated band resolution: 325); (**d**) facial features of sister-epicanthic folds and hypertelorism

Aspergillus infection. Higher doses of pioglitazone could have been tried in our child; however, the safety of higher doses has not been studied in the pediatric age group. We did not notice any adverse effects of pioglitazone (3 mg/kg/day).

Female carriers of *CYBB* defect are usually asymptomatic. However, a recent series from the USA have documented a higher rate of autoimmune manifestations in the X-linked carriers and 25% of them met the ACR criteria for systemic lupus erythematosus (SLE) [2]. Positivity of two or more autoantibodies has been documented in only 2 of the 81 female carriers. The mother of index patient can be classified as SLE under SLICC 2012 criteria-malar rash, polyarthritis, elevated ANA, and anti-dsDNA titres [8]. STR analysis using X chromosome markers revealed that sister has inherited both X chromosomes from mother and one X chromosome from father, resulting in maternal isodisomy (Suppl. Fig. 1). Further, the analysis revealed that there was recombination in maternal X chromosome carrying the mutation and thus the sister got two X chromosomes with a mutation from mother. X inactivation assay revealed the absence of complete skewed X inactivation in sister but partial skewing cannot be ruled out (Suppl. Fig. 2).Progressive skewing of X chromosome inactivation occurs as age advances, and manifestations of CGD are reported to occur late in X-linked carriers [2]. However, the sister had the manifestations of CGD from the age of 6 years. Parental origin of the extra X chromosome in 47,XXX females is from maternal in 90% of cases [9]. Therefore, in daughter, percentage of cells with mutant allele (T) of *CYBB*on the active X chromosome is expected to be higher than in mother, thus contributing to a more severe phenotype in daughter as compared with the carrier mother.

To conclude, we describe a novel splice-site defect in *CYBB* in an Indian family with X-linked CGD. Triple X females who inherit 2 X chromosomes with the mutant allele from mothers who themselves are carriers can present with manifestations of CGD.

Methods

Flow Cytometry for gp91phox Using Anti-Human Flavocytochrome b558 Antibody

One hundred microliters of EDTA anticoagulated blood was taken in two FACS tubes labeled as tube A (cells only) and tube B (stained). Two microliters (1 μg/100 μL) of anti-flavocytochrome B558-FITC antibody (Clone 7D5; D162-4, MBL Inc., Japan) was added in tube B. Both tubes were then incubated at 37 °C for 30 min. After incubation, 1 mL of 1X RBC lysis buffer was added and incubated again for 15 min at room temperature. The tubes were centrifuged at 1500 rpm for 5 min and the supernatant was discarded. Two milliliters of phosphate-buffered saline (PBS) was added to the pellet and centrifuged again at 1500 rpm for 5 min. This step was performed twice. The final pellet was suspended in 500 μL of PBS and the sample was run on Beckman Coulter Navios flow cytometer. Neutrophils were gated on FSc vs SSc graph. Expression of cytochrome B558 was evaluated as median fluorescence intensity (MFI) as well as percentage positivity on the neutrophils.

DNA Extraction and Analysis

DNA was extracted from an anticoagulated blood sample using a Qiagen kit following the manufacturer's protocol. The quality and quantity of extracted DNA were determined by measuring the optical densities in a spectrometer (Nanodrop 2000, Thermoscientific). Polymerase chain reaction (PCR) was performed in Thermal Cycler (Biorad, Thermalcycler). Amplified products were electrophoresed on 1.2% agarose gel to check the size and quality. The PCR products were then cleaned with ExoSAP (Affymetrix, USA) before proceeding to downstream Sanger sequencing (Applied Biosystems, California, USA).

mRNA Extraction and cDNA Analysis

Total RNA was extracted from the control and the subject PBMCs. DNase was used to remove DNA traces. DNase-treated RNA was immediately converted to cDNA using AMV-reverse transcriptase and oligo dT primer. The cDNA of the case and control were then analyzed by PCR for checking the product (Fig. 2B).

Short Tandem Repeat and X Inactivation Analysis

Short tandem repeat (STR) analysis was done using markers on the X chromosome. The Investigator Argus X-12 Kit (Qiagen, USA) was used for amplification of 12 X-chromosomal short tandem repeat (STR) loci in the proband, mother, and sister. X inactivation assay was done using the human androgen receptor gene (HUMARA) assay as described before [10].

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Compliance with Ethical Standards

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

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study

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