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X-linked Chronic Granulomatous Disease secondary to skewed X chromosome inactivation in a female with a novel CYBB mutation and late presentation

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Abstract

Chronic Granulomatous Disease (CGD) is characterized by defects in the superoxide producing enzyme NADPH oxidase causing phagocytes to improperly clear invading pathogens. Here we report findings of a late presenting 16 year old female with X-linked CGD. The patient presented with community-acquired pneumonia, but symptoms persisted for 2 weeks during triple antimicrobial coverage. Cultures revealed *Aspergillus fumigatus* which was resolved through aggressive voriconazole treatment. Neutrophil studies revealed NADPH oxidase activity and flavocytochrome *b*₅₅₈ levels that were 4–8% of controls and suggested carrier status of the mother. We found a null mutation in the CYBB gene (c.252insAG) predicting an aberrant gp91^{phox} protein (p.Cys85fsX23) in the heterozygous state. Methylation analysis demonstrated extremely skewed X chromosome inactivation favoring the maternally inherited defective gene. In conclusion, a novel mutation in the CYBB gene and an extremely skewed X-inactivation event resulted in the rare expression of the CGD phenotype in a carrier female.

Keywords

CGD; X-inactivation; flavocytochrome *b*₅₅₈; neutrophil; carrier; gp91^{phox}; CYBB; NADPH oxidase; primary immunodeficiency

Introduction

Chronic granulomatous disease (CGD) is a genetic syndrome characterized by a dysfunction of the respiratory burst, which is necessary to kill certain phagocytized pathogens [1;2]. This disease occurs in approximately 1 in 250,000 people worldwide [3]. The neutrophils of CGD patients fail to adequately generate oxygen-dependent mechanisms to kill microorganisms. The respiratory burst response is mediated by a variety of reactive oxygen species that both

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directly and indirectly kill phagocytized bacteria and fungi [4;5]. Some organisms take advantage of the compromised host, including catalase-positive organisms such as *Staphylococcus aureus*, as well as *Burkholderia cepacia*, *Serratia* species, *Pseudomonas* species, *Nocardia* species, *Candida* species and perhaps most fatally, *Aspergillus* species [3]. Classically, CGD is associated with recurrent, severe infections of the skin, lungs, liver, spleen and lymph nodes. As the name implies, granulomas are another feature of this disease. These granulomas occur principally in the skin, gastrointestinal and genitourinary tracts, leading to obstructive lesions that may need to be treated with steroids or surgery [1;3;6].

The fundamental defect in CGD lies in the NADPH oxidase, the enzyme complex responsible for initiating the respiratory burst. Defects in four of the NADPH oxidase components allow for the various phenotypic expressions of CGD. The four components of the NADPH complex implicated in CGD are gp91^{phox}, p22^{phox}, p47^{phox} and p67^{phox}. Two of these proteins, gp91^{phox} and p22^{phox}, make up the membrane-associated flavocytochrome b₅₅₈. The gp91^{phox} subunit is thought to contain all the electron transport machinery of the oxidase as well as the NADPH and FAD binding sites [7]. The p22^{phox} subunit promotes gp91^{phox} maturation through heterodimer formation, and acts as a binding site for the cytosolic components of the NADPH oxidase. The p47^{phox} and p67^{phox} proteins are located in the cytosol [8] and translocate to the membrane and bind the flavocytochrome upon neutrophil stimulation. This causes a conformational change in the latter allowing for the production of superoxide anions through the electron transport from NADPH to molecular oxygen [9]. Defects in any of the four subunits can manifest as CGD. Thus, CGD patients can be phenotypically similar but genetically heterogeneous depending on which NADPH oxidase component is defective. The most common form of CGD is X-linked recessive resulting from a mutation of the CYBB gene encoding for gp91^{phox}. CYBB is located within the Xp21.1 band region and accounts for approximately 70% of all cases of CGD. The remaining 30% of cases are caused by defects in the other three proteins, all of which are inherited in an autosomal recessive manner [3;10].

The membrane bound flavocytochrome forms a stable heterodimer of gp91^{phox} and p22^{phox} during its maturation and a defect in one membrane subunit leading to a loss of expression is reflected in the loss of expression of the other. Thus, patients with CGD caused by a gene defect leading to the lack of protein expression in either the X-linked CYBB gene (gp91^{phox}) or the CYBA gene (p22^{phox}) will not express either protein [2;11;12]. In contrast, a deficiency resulting in the loss of expression of any one of the cytosolic subunits p47^{phox} or p67^{phox} can be identified directly by Western blot and/or flow cytometry [13;14;15].

Other components of the NADPH complex include p40^{phox} and Rac 1 or 2. There have been no reported cases of CGD caused by p40^{phox} gene defects. There is a single reported case of a child with a Rac2 mutation leading to clinically significant phagocytic cell dysfunction. The child's phenotype more closely resembled Leukocyte Adhesion Defect in its clinical picture than it did CGD [16;17].

The X-linked recessive form of CGD is by far the most common, resulting in a greater number of affected males. However, there have been reports of affected females with the diagnosis of X-linked CGD [18;19;20;21;22;23;24;25] attributed to skewed X-inactivation. According to the Lyon hypothesis of X chromosome inactivation, "either one of the two X's may be inactivated in different cells of the same animal, and the inactivation occurs early in development" [26]. Generally this is a random process of inactivation, with an expected ratio of 50:50, although in reality the ratio follows a Gaussian distribution [27]. Thus, female carriers of the X-linked form of CGD should be mosaics, with approximately half of their cells expressing the healthy normal X chromosome, and the remaining cells expressing the mutated, disease carrying X chromosome. However, certain X chromosome mutations seem to influence the ratio to favor the inactivation of the disease carrying X chromosome: a nonrandom X-

inactivation with selective advantage. There are numerous hypotheses as to why nonrandom X-inactivation occurs, however there is no one prevailing theory [28].

Here we report a unique patient with CGD having the following features: a female with the X-linked form of the disease secondary to extremely skewed X chromosome inactivation; a novel, previously unreported mutation of the *CYBB* gene that codes for gp91^{phox} protein; and a late diagnosis at age sixteen. There is no family history of CGD; however the patient's mother and sister are confirmed carriers of the disease, exhibiting the classic mosaic of flavocytochrome expressing neutrophils.

Materials and Methods

Patient

The patient is a 16 year old female who presented to our institution with a four week history of cough and high fevers. She initially presented to her primary care physician with fever, chills, myalgia and cough. Prior to referral, chest radiograph revealed diffuse bilateral infiltrates; the diagnosis of community acquired pneumonia was made and she was discharged on oral antibiotics. Her symptoms persisted for two weeks, at which time she was admitted to a local hospital, with computed tomography of chest demonstrating diffuse bilateral infiltrates, significantly worse than previous imaging. Bronchoscopy was performed and coverage broadened to include antifungal and anti-tuberculosis agents, and the patient was transferred to our institution. Bronchoalveolar lavage fluid and sputum grew colonies of *Aspergillus fumigatus*, despite ten days of antifungal therapy. The patient was treated aggressively for six weeks with voriconazole and improved rapidly. Follow up computed tomography of the chest demonstrated significant improvement of her infiltrates. Initial nitroblue tetrazolium (NBT) test was inconclusive because of technical issues and a dihydrorhodamine-123 (DHR) assay showed impaired oxidative burst capacity (9.68% DHR-positive cells in the unstimulated condition vs. 13.8% DHR-positive cells in the PMA stimulated condition). Subsequently, the patient has been placed on daily oral prophylaxis with itraconazole and trimethoprim/sulfamethoxazole. She also receives interferon gamma - 1b (ACTIMMUNE, InterMune, Inc.) injected subcutaneously three times a week.

Past history was significant for no major illnesses or hospitalization, but did include recurrent axillary "boils," beginning at 7 months of age, with worsening over the past two years. The site of her lesions did express purulent material and she would occasionally develop fever. The lesions required lancing and oral antibiotics about once a year. She denied any history of previous pneumonia, other deep/soft tissue, urinary tract, central nervous system, hematologic, gastrointestinal, sinopulmonary or mucocutaneous infections. Her immunizations are all current for age.

Family medical history reveals no history of primary immunodeficiency. The patient's mother developed rectal cancer at about age 40 and was diagnosed with Crohn's disease sometime later. Two older half siblings (different fathers), one male and one female, are both healthy. The mother has six full siblings, and their histories are significant for diabetes mellitus type two and rectal cancer. A maternal female first cousin has systemic lupus erythematosus, requiring a renal transplant.

Materials

Phosphate buffered saline solution (PBS) without Ca²⁺ + Mg²⁺ and Hanks' Balanced Salt Solution (HBSS) were obtained from Life Technologies (Carlsbad, CA). Sterile H₂O was obtained from Baxter Healthcare Corporation (Deerfield, IL). Luminol was obtained from SERVA (Heidelberg, Germany). Nicotinamide adenine dinucleotide phosphate (NADPH) was

obtained from Boehringer-Ingelheim (Petersburg, VA). 1,2-Didecanoyl-*sn*-Glycero-3-Phosphate (10:0 PA) and 1-Oleoyl-2-Acetoxy-*sn*-Glycerol (OAG) were obtained from Avanti® Polar Lipids Inc (Alabaster, AL). Arachidonic acid (AA) was obtained from NuChekPrep (Elysian, MN). All other chemicals were obtained from Sigma® (St. Louis, MO). Antibodies were generously donated by Dr. Algirdas Jesaitis (Montana State University (Bozeman, MT); 54.1-gp91^{phox}, 44.1-p22^{phox} [29]), Dr. Tom Leto (NIH (Bethesda, MD); Anti-p47^{phox}, p67^{phox}, and p40^{phox} [30]), and Prof. Nakamura (Nagasaki University (Nagasaki, Japan); 7D5 [31]). The anti-Rac antibody (Cat# 05-389) was purchased from Upstate Biotechnology (Lake Placid, NY). Secondary antibodies were from BD Biosciences and Pierce.

Low Endotoxin Neutrophil Isolation

Neutrophils were isolated as previously described [32;33] with modifications. All reagents used in this procedure were sterile and of the lowest endotoxin content commercially available. In brief, heparinized venous blood was obtained from the patient, the patient's mother and sister, and normal subjects following informed consent, using a protocol approved by the Wake Forest Institutional Review Board. Erythrocytes were removed by gravity sedimentation over Isolymp (Gallard-Schlesinger, Plainview, NY). The leukocyte-rich suspension was centrifuged over Isolymp and contaminating erythrocytes were removed by hypotonic lysis. Neutrophils were resuspended in HBSS at 1×10^8 cells/mL.

Luminol-Enhanced Chemiluminescence

The neutrophil respiratory burst was monitored by luminol-dependent chemiluminescence (CL) [34]. Isolated neutrophils (5×10^4 cells/mL) in HBSS were incubated with 20 μ M luminol in a white 96-well plate (Greiner, Lake Mary, FL). Stimulus [1 μ M *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), 10 μ M dihydrocytochalasin B (dCB) followed by 1 μ M fMLP, 0.05 mg/mL opsonized zymosan (OPZ) prepared as previously described [35], or 100 nM phorbol myristate acetate (PMA)] was added and light emission was monitored continuously at 25°C for the indicated time period in a MicroLumat Plus LB 96 V luminometer (Berthold Technologies, Oak Ridge, TN). Data are expressed as relative light units (RLUs).

Subcellular Fractionation of Neutrophils

Isolated neutrophils in HBSS were treated with 1.71 mM diisopropyl fluorophosphate, resuspended at 2×10^8 cells/mL in ice-cold sonication buffer (50 mM Tris, pH 7.4, 11% sucrose, 100 mM NaCl, 2 mM EGTA, 2 mM EDTA, 25 mM NaF, 10 μ g/mL leupeptin, 10 μ g/mL pepstatin, 1 μ g/mL aprotinin, and 1 mM PMSF) and sonicated on ice to ~90% cell breakage. Membrane and cytosolic fraction proteins were separated on a 15/40% discontinuous sucrose gradient and stored at -70°C until needed [36]. Protein concentrations were determined by the Bradford assay [37].

Cell-Free NADPH Oxidase Assay

Cell-free NADPH oxidase assays were performed as previously described [38]. In brief, reaction mixtures (50 mM Na_xPO_4 , pH 7.0, 1 mM EGTA, 5 mM MgCl_2 , 10 μ M FAD, 0.1 mM cytochrome *c*, 1 μ M guanosine 5'-*o*-(3-thiotriphosphate), 0.5 μ g membrane protein, 12.5 μ g cytosolic protein) were incubated with or without 0.05 mg/mL superoxide dismutase and activated with the addition of lipid (30 μ M OAG + 30 μ M 10:0 PA or 25 μ M AA) for 60 min at 25°C. NADPH (0.2 mM) was added to induce superoxide production and the rate of superoxide dismutase-inhibitable cytochrome *c* reduction was measured continuously at 550 nm on a UV-2401 PC spectrophotometer (Shimadzu, Kyoto, Japan). NADPH oxidase activity was determined from linear slopes using the cytochrome *c* extinction coefficient of

$21\text{mM}^{-1}\text{cm}^{-1}$ [39]. Data are expressed as nmol of superoxide produced/min/mg of membrane protein.

Western Blot Analysis

Protein samples were prepared in Laemmli sample buffer and separated using SDS-PAGE on 7%, 10%, or 14% polyacrylamide gels [40]. Proteins were transferred to nitrocellulose and Western blot analysis was performed as described [41]. In brief, blots were blocked for 1 hr with 5% milk in TBST (10mM Tris, 100mM NaCl, and 0.1% Tween 20), washed, and then incubated with appropriate primary antibody for 2 hr at room temperature. Blots were then washed in TBST followed by HRP-conjugated secondary antibody incubation (~1 hr) and detection by CL.

Flow Cytometry Analysis

Flow cytometric analysis was performed as previously described [42] but with modifications. Isolated neutrophils (5×10^6 cells/mL) were suspended in cold PBS with 0.1% gelatin and incubated with mouse IgG or mAb 7D5 (0.03mg/mL) for 30 min on ice. Cells were washed 3x with cold PBS/gelatin before adding FITC-GAM (1:100) to all conditions. Cells were incubated for 30 min on ice under foil, then washed 3 times and resuspended in 1mL of PBS/gelatin. Flavocytochrome *b₅₅₈* expressing cells were counted using a BD FACSCalibur flow cytometer (BD Biosciences, San Diego, CA). Data are expressed as number of events versus fluorescent intensity.

X Chromosome Inactivation Analysis

DNA was extracted from peripheral blood and isolated neutrophils from the patient and her mother using a standard commercial protocol (Qiagen Inc., Valencia, CA). X chromosome inactivation analysis using the HUMARA gene followed a previously published protocol with slight modifications [43]. Briefly, 1 μ g of DNA from peripheral blood and neutrophils from the patient and mother, along with appropriate controls, were digested with *Hha*I (20U/ul) and *Hpa*II (10U/ul) (New England BioLabs Inc., Ipswich, MA) at 37°C overnight followed by enzymatic inactivation by heating at 95°C for 10 min. Separate PCR amplification was performed using 100ng of digested and undigested DNA from all samples using primers specific for the methylation regions of the HUMARA gene. Fluorescently labeled PCR products were denatured for 97°C for 3 min then analyzed by capillary gel electrophoresis using an ABI 3100 Genetic Analyzer and ABI GeneScan software (Applied Biosystems, Foster City, CA). X chromosome inactivation ratios were determined using the peak heights for the various PCR products using the following formula:

$$\%X_{(A/B)} \text{ Inactivation} = \frac{\text{Allele B}_{\text{digested}}}{[(\text{Allele B}_{\text{undigested}} \times \text{Allele A}_{\text{digested}}) / \text{Allele A}_{\text{undigested}}] + \text{Allele B}_{\text{digested}}} \times 100$$

CYBB Genetic Analysis

Bi-directional sequence of all 13 coding exons of the X-linked CYBB gene and their intron/exon boundaries were obtained and analyzed by GeneDx DNA diagnostic services (Gaithersburg, MD).

Results

Evidence for neutrophil dysfunction

To determine if the patient had neutrophils capable of producing a measurable respiratory burst, the luminol-enhanced CL assay was performed on isolated neutrophils from both the patient and a control volunteer. We used three different stimuli (PMA, OPZ, fMLP) because each one is known to result in NADPH oxidase activation through different pathways [44]. As shown

in Fig. 1, the neutrophils of the patient showed a markedly reduced respiratory burst response in response to all stimuli tested compared to control cells. By using the area under the curve for the patient's respiratory burst response to each of the stimuli tested, we determined the percentage of functional response compared to that of the control subject. The patient's functional response as compared to that of control cells was as follows: PMA = 7.94%, OPZ = 7.78%, fMLP = 7.58% and dCB/fMLP = 4.72%. This low response was confirmed using a whole cell cytochrome *c* reduction assay (data not shown). This small, but measurable respiratory burst response suggests some NADPH oxidase activity persists in the patient's cells, but that this activity is insufficient for effective resistance to *Aspergillus* infection.

In the intact cell, it is difficult to discern which component of the oxidase is functionally deficient because of the numerous subunits that are needed for proper complex formation. By using a cell-free system [45], we can distinguish whether the defect is in the membrane-associated or cytosolic components of NADPH oxidase. Membrane and cytosolic fractions were isolated from patient and control neutrophils and mixed in the presence of cell-free activators (10:0 PA+OAG, AA) to induce NADPH oxidase activity. As shown in Fig. 2, the mixture of control membrane and cytosolic fractions resulted in normal superoxide production. Mixing only patient fractions resulted in low oxidase activity, indicating that the patient has an NADPH oxidase defect. Mixing the patient's cytosolic fraction with the control membrane fraction showed normal superoxide production in the system, indicating that NADPH oxidase components in the cytosol were not defective. In contrast, only low levels of oxidase activity were obtained when the control cytosolic fraction was mixed with the patient's membrane fraction. This reduction indicates a deficiency in the patient's membrane-bound oxidase components, e.g. flavocytochrome *b*₅₅₈.

Flavocytochrome *b*₅₅₈ expression is diminished in the neutrophils of the patient

In a complementary approach, the levels of NADPH oxidase components in the isolated cytosolic and membrane fractions were assessed by Western blot analysis. Fig. 3 shows normal expression of the cytosolic oxidase components p47^{phox}, p67^{phox}, p40^{phox} and Rac in the patient's neutrophils. In contrast, the amounts of flavocytochrome *b*₅₅₈ subunits p22^{phox} and gp91^{phox} were markedly diminished, but detectable. This low level of flavocytochrome *b*₅₅₈ likely accounts for the markedly decreased respiratory burst in the patient's neutrophils.

The patient has two distinct populations of neutrophils expressing different levels of flavocytochrome *b*₅₅₈

The previous experiments do not identify which of the flavocytochrome subunits is affected, since each subunit requires expression of the other subunit for stability. If the defect is in the CYBA gene, this gene would be expressed in all of the patient's neutrophils and assessment of flavocytochrome *b*₅₅₈ expression by flow cytometry would reveal a single population of cells. In contrast, if the defect is in the CYBB gene, the defective gene would be expressed in a sub-population of the patient's neutrophils. We assessed the level of flavocytochrome *b*₅₅₈ expression in individual cells by flow cytometry, using the monoclonal antibody 7D5, which recognizes an extracellular epitope of native gp91^{phox} [31]. Fig. 4 shows that a single population of flavocytochrome *b*₅₅₈ – expressing neutrophils was observed for the normal control donors. In contrast, neutrophils from the patient, her mother, and her sister exhibited two populations of neutrophils with respect to flavocytochrome *b*₅₅₈ expression. The flavocytochrome *b*₅₅₈-negative neutrophils (92.9% of total) in the patient's sample (Figure 4a) greatly outnumbered the flavocytochrome *b*₅₅₈-positive (7.1% of total) cells. In contrast, the distribution of cells between these two populations in the mother and sister showed a higher (61.9% and 52.1% respectively) percentage of flavocytochrome *b*₅₅₈ positive neutrophils. The intact cell respiratory burst activity elicited in the neutrophils of the mother and sister were not different from that of normal, healthy control cells (data not shown) despite the presence of a

population of flavocytochrome *b*₅₅₈-negative cells. The dual populations in the neutrophils from the patient, her mother, and her sister are indicative of an XCGD carrier [46;47], suggesting the presence of a coding defect in the *CYBB* gene in one population of cells.

The patient demonstrates extremely skewed X-inactivation

The flow cytometry results suggest the possibility that the patient has skewed X-inactivation. We used the HUMARA assay to assess the extent of X chromosome inactivation in the patient and the mother. This assay is based on methylation-sensitive, *HhaI* and *HpaII* restriction sites located on the HUMARA (human androgen-receptor gene) locus on the X chromosome. In DNA obtained from whole blood and isolated neutrophils, both the normal control and the patient's mother demonstrated normal X-inactivation while the patient's DNA showed a markedly skewed X-inactivation event (Table 1). These results are in general agreement with the results from the flow cytometric assay (Figure 4). If we assume that the methylation of the HUMARA allele is indicative for the methylation of *CYBB* on the same X chromosome, then an extremely skewed X-inactivation event (normal range 50 to 89%; skewed 90 to 100% [48]), favoring expression of the defective *CYBB* gene, would explain why this patient displays the X-CGD phenotype when she appears to be an XCGD carrier.

The patient has a heterozygous single base change in intron 3 of the *CYBB* gene

The identification of the patient as a CGD carrier with a skewed X-inactivation event localized the gene defect to the *CYBB* gene. In order to determine the precise mutation in *CYBB*, blood samples were sent to GeneDx, Inc. (Gaithersburg, MD) for analysis. Bi-directional sequencing of all 13 coding exons of the X-linked *CYBB* gene and their intron/exon boundaries revealed a heterozygous single base change of A→G at the 3' end of intron 3. A schematic representation of the projected effects of this mutation is shown in Fig. 5. The mutation analysis suggests a potential disruption of the wild type splice site between intron 3 and exon 4, which would result in a more favorable splice site two bases upstream. This alternate splice site allows for a 2bp insertion of intronic sequence into the mRNA coding for gp91^{phox}. The intronic insertion is predicted to result in a frame shift and a new, premature stop signal 23 codons downstream of the mutation. Consequently, a non-functional, severely truncated (~107 amino acids) form of the protein would be expressed, which is likely to be unstable, accounting for the lack of expression of flavocytochrome *b*₅₅₈ in one population of the neutrophils from the patient, her mother and her sister.

Discussion

Based on this patient's presentation with persistent and recurring *Aspergillus* infections and suspicious laboratory results for the neutrophil respiratory burst, we used a comprehensive series of laboratory assays to explore the possible presence of CGD. We demonstrated that the patient's neutrophils showed a pronounced decrease in the respiratory burst in response to physiological stimuli that are known to activate the respiratory burst response through a variety of signaling pathways [44]. This was the first indication that the patient has a defect within the NADPH oxidase components themselves and not in the signaling pathways leading to activation of the functional response. Subsequent experiments localized the defect first to the membrane fraction and then to a deficiency of flavocytochrome *b*₅₅₈. Flow cytometry results revealed that the patient is an X-CGD carrier, with only 7–8% of her cells expressing flavocytochrome *b*₅₅₈.

X-CGD consists of three subtypes; X91^o (gp91^{phox} not expressed), X91⁻ (low gp91^{phox} expression), and X91⁺ (gp91^{phox} expressed but not functional) [49]. Our results indicate that this patient is the X91⁻ genotype, caused by extremely skewed X-inactivation of the chromosome carrying the normal gene. The mutation we identified predicts expression of a

truncated protein, which appears to be unstable. Previous studies indicate that proper gp91^{phox} expression is needed for p22^{phox} expression [50]. This explains why the cells expressing the defective gene do not express either gp91^{phox} or p22^{phox}.

Carrier-status with a CYBB gene defect being secondary to an extremely skewed X-inactivation event is rare (<2% of all reported CGD cases), but has been observed [18;25;51;52;53;54]. Initial presentation of the CGD phenotype typically occurs in childhood, however several cases involving CGD carriers appear later in life, suggesting the possibility of a shift in X-inactivation with age [18;53;54]. While our patient has had a history of chronic infections, her latest infection was more severe, consistent with the possibility that her skewing might have shifted. As with other reported cases of X-inactivation within CGD, our patient exhibits a small but significant respiratory burst response (Fig 1). While this was not enough to overcome her infection alone, it may be possible to enhance this response in the future if breakthroughs in controlling the regulation of X-inactivation can be accomplished, allowing for a purposeful shift favoring the expression of her paternal X chromosome. Consequently, an analysis of X-inactivation over time can be useful in the assessment of females with atypical presentations of CGD.

X-inactivation is controlled through DNA methylation. DNA methylation has been linked to numerous disease states such as cancer, imprinting disorders and birth defects, as well as autoimmune disease [55]. Regulation of DNA methylation is poorly understood, but is thought to involve the expression of the *Xist* locus as well as long interspersed nuclear elements (LINEs) clustered around this loci [28]. While these factors were not explored in our case, they could play a part in the disease development of our patient and would be useful to examine in the future.

The unique mutation in the CYBB gene found in this patient is a single base change of (A→G) two bases upstream of the wild type splice site at the intron 3/exon 4 border. This mutation is thought to create a more favorable splice site. The resulting exon is predicted to not only translate incorrectly but lead to a prematurely truncated gene product due to the creation of a new stop codon downstream (Fig 5). Splice site mutations in the CYBB gene leading to X-CGD consist of less than 20% of all the known CYBB mutations [56]. Furthermore, this particular mutation has not been reported in any known CGD database.

In conclusion, we have described the manifestation of Chronic Granulomatous Disease in a 16 year old female XCGD carrier secondary to an extremely skewed X-inactivation event. To our knowledge, this is the first report of a patient carrying a splice site mutation in the CYBB gene in conjunction with skewing X-inactivation. The patient is on prophylactic antibiotics and doing well.

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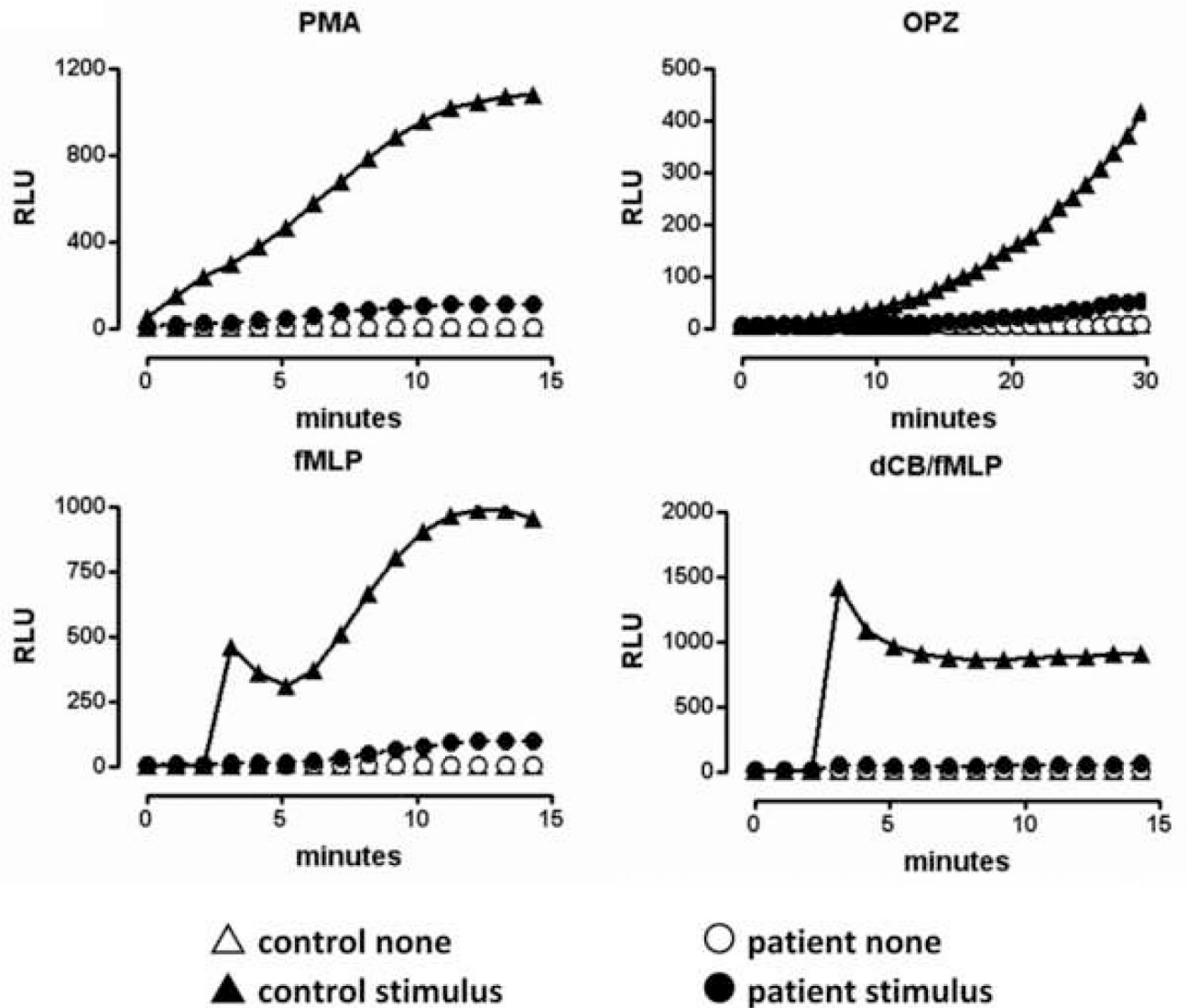


Figure 1. Evidence for neutrophil dysfunction

Isolated neutrophils in HBSS/0.1% gelatin from the patient (circles) and a control donor (triangles) were incubated in the presence of 20 μ M luminol. The indicated stimulus (solid symbols) or buffer (None, open symbols) was added either directly (100nM PMA or 0.05mg/ml OPZ) or after established baseline readings (1 μ M fMLP or 10 μ M dCB + 1 μ M fMLP). Relative light units (RLU) were recorded continuously for 15 or 60 min depending on the stimulus. All stimuli were tested in duplicate wells and the patient's cells were examined on two different occasions. Data shown are from one experiment.

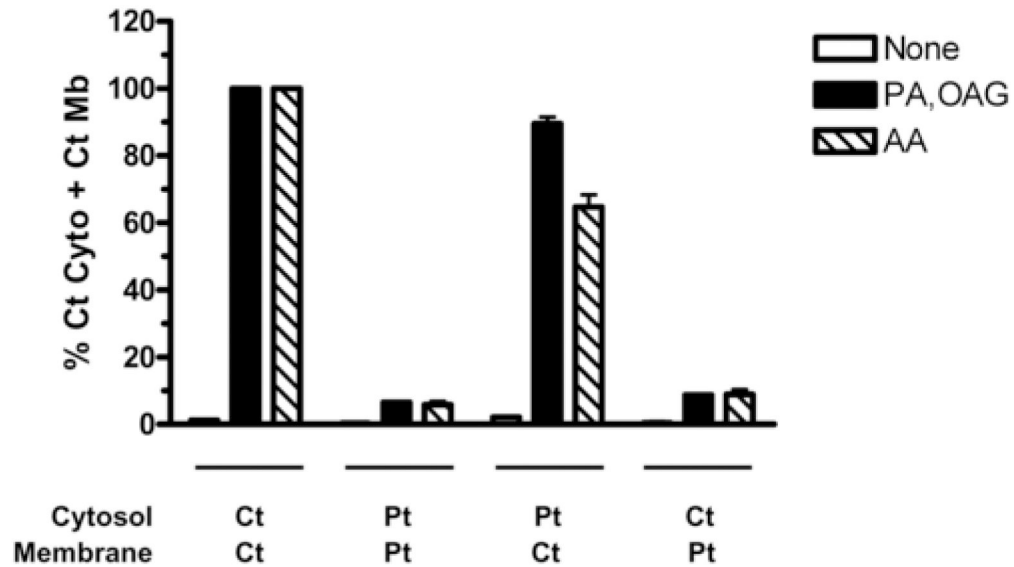


Figure 2. Membrane-bound components of the patient's NADPH oxidase are functionally deficient Cytosolic and membrane fractions were prepared from the neutrophils of the patient (Pt) and a normal healthy control donor (Ct). The fractions (12.5 μ g cytosol and 0.5 μ g membrane) were mixed in the indicated combinations and incubated as described in the Methods with either water (empty bars), 100 μ M 10:0 PA + 100 μ M OAG (solid bars) or 25 μ M AA (striped bars) for 60min at 25°C. NADPH (0.2mM) was added and the reduction of cytochrome *c* was measured at 550nm. Data are expressed as a percentage of control cytosol plus control membrane (Ct Cyto + Ct Mb) for each activator. Specific activities for the control fractions (Ct Cyto + Ct Mb) expressed as nmol O₂⁻/min/mg memb were: None = 43 \pm 9, PA/OAG = 3397 \pm 23, AA = 1745 \pm 196 (Mean \pm SEM, n=3).

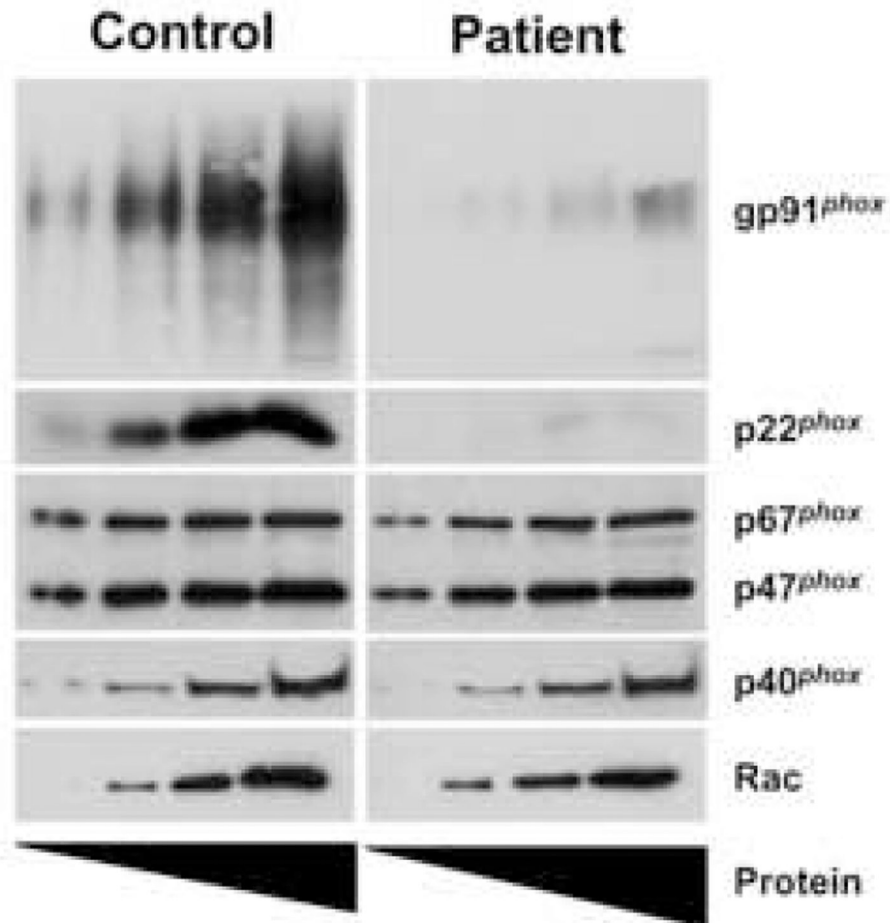


Figure 3. The patient expresses low levels of gp91^{phox} and p22^{phox}

Increasing amounts of isolated neutrophil cytosolic and membrane fractions (1, 2.5, 5, 10 μ g for gp91^{phox}; 5, 10, 20, 40 μ g for all other components) from both a control subject (left) and the patient (right) were prepared in Laemmli sample buffer, subjected to SDS-PAGE and transferred to nitrocellulose. Western blot analysis was performed on the membrane fractions for gp91^{phox} and p22^{phox}. Cytosolic fractions were probed for p67^{phox}, p47^{phox}, p40^{phox} and Rac. The blots are representative of two experiments.

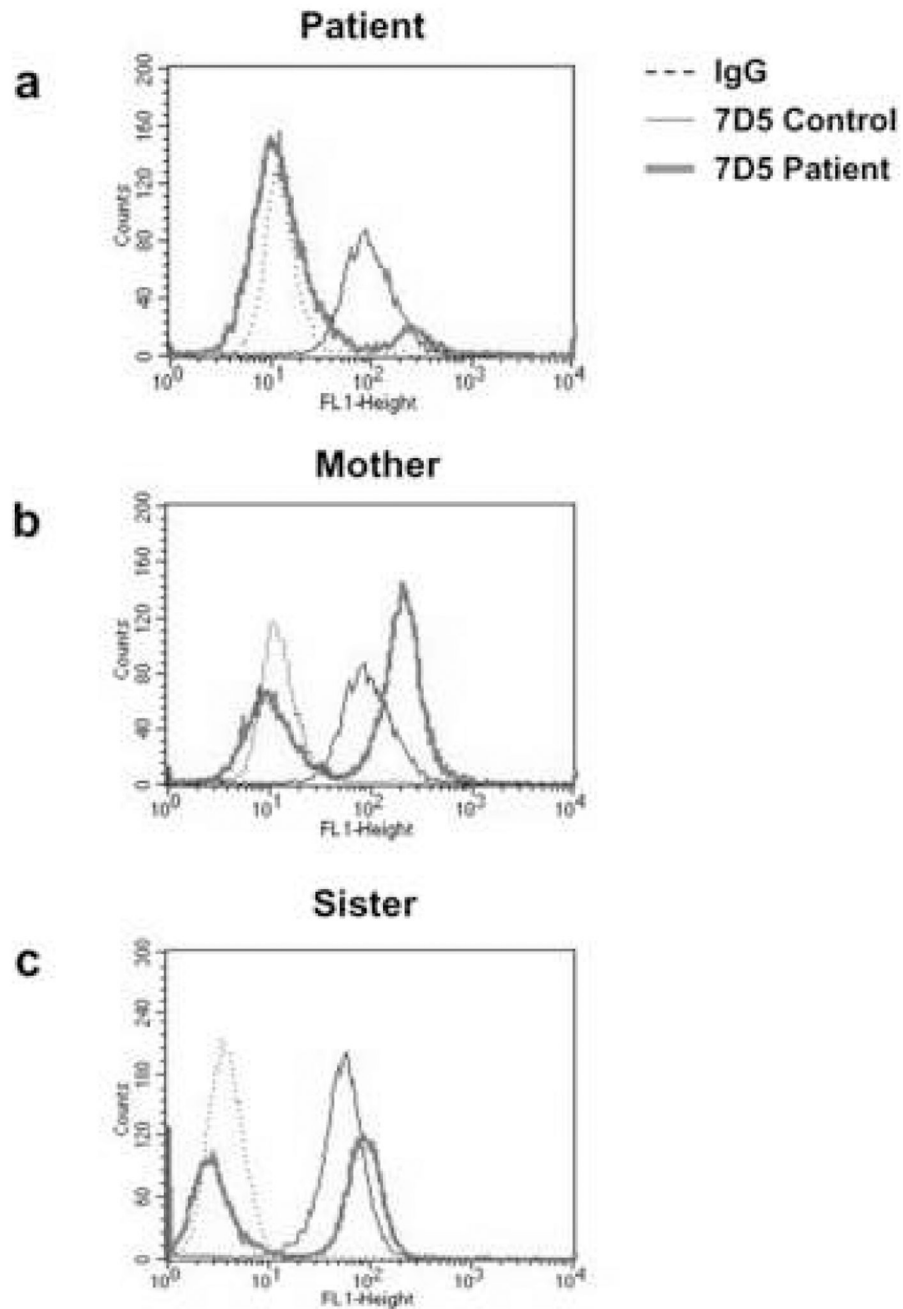


Figure 4. The patient, her mother, and sister are X-CGD carriers

Isolated neutrophils (5×10^6 cells/mL) from a control donor (thin solid line) or the indicated subject (thick solid line, 4a, Patient; 4b, Mother; 4c, Sister) were suspended in cold PBS/0.1% gelatin and stained with either irrelevant IgG (dashed lines) or mAb 7D5 (solid lines). Cells were then washed and incubated with FITC-goat anti-mouse secondary Ab and analyzed in a flow cytometer. Data are expressed as number of cells versus fluorescent intensity and are representative of at least three experiments.

Normal translation of Exon 4 of gp91^{phox}

aatccaaag TGCTGCTCAACA-TGGATGATTGCACTTCACTCT ggtaagttt
 Exon 4 coding →
 85-CysCysSerThr-TrpMetIleAlaLeuHisSer-112

Proposed Patient translation of Exon 4 of gp91^{phox}

Patient mutation → Intronic DNA insertion → Premature Stop ↓
 aatccag agTGCTGCTCAACAA-GGAT**TGA**TGCACTTCACTCT ggtaagttt
 85-SerAlaAlaGlnGln-Gly-107

Figure 5. The patient's mutation is predicted to result in the expression of a severely truncated and nonfunctional form of gp91^{phox}

The CYBB sequence results and the proposed translations of exon 4 in gp91^{phox} are shown for the wild type (upper panel) and the patient (lower panel). Wild type intronic DNA is in lower case and the wild type exonic DNA is in upper case lettering. The coding DNA for both wild type and patient sequence is boxed and its corresponding translation is shown below the sequence. The bolded lettering indicates the patient's variation from the wild type sequence. The 'ag' intronic insertion resulting from the patient's mutation leads to mistranslation and a premature stop signal 23 codons downstream of Ala 84.

X chromosome inactivation is skewed and mirrors the flavocytochrome *b₅₅₈* expression levels the patient and her mother

The HUMARA gene was used to determine X chromosome inactivation described in the Methods. Results are shown on the left. % $X_{A/B}$ represents the overall methylation of chromosome A or B for the indicated cell type (results shown from one blood draw). The percent of total neutrophils that express (%Pos) or do not express (%Neg) flavocytochrome *b₅₅₈* based on flow cytometric analysis are shown on the right ($n \geq 3$).

Table 1

	Methylated DNA			Flavocytochrome <i>b₅₅₈</i> Expression		
	Whole Blood			Neutrophils		
	% X_A	% X_B	% X_A	% X_B	% Neg	% Pos
Control	59.7	40.3	64.0	36.0	0.0	100.0
Mother	55.1	44.9	61.5	39.5	39.1	61.9
Patient	95.7	4.3	96.4	3.6	92.9	7.1