X-linked chronic granulomatous disease in a male child with an X-CGD carrier, Klinefelter brother

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Summary

Background: **Chronic granulomatous disease (CGD) is a rare primary immunodeficiency (PID) caused by a dysfunctional respiratory burst enzyme NADPH-oxidase. The concurrence of Klinefelter's Syndrome (KS) and CGD would be extremely rare.**

Objective: **We describe the study of a family where the youngest male child had X-linked CGD (X-CGD) while his older brother was both an X-CGD carrier and a Klinefelter.**

Methods**: Flow cytometry was used to study respiratory burst and gp91-phox expression, while genetic investigation was done by RT-PCR, PCR and X-chromosome short tandem repeat (X-STR) analysis.**

Results: **The Dihydrorhodamine (DHR) assay showed the patient's neutrophils failed to produce a respiratory burst, while both the mother and an older brother showed a bimodal response. gp91-phox expression was absent in the patient's neutrophils, and bimodal in the mother's and brother's neutrophils. The patient's cDNA showed a C>T change at nucleotide 676 of the *CYBB* gene. The same change was seen in the patient's gDNA, while the brother and mother were heterozygous, with C and T, in this position. The c.676C>T is a nonsense mutation that leads to premature termination of the gp91-phox protein. The brother karyotyped as 47, XXY and X chromosome analysis showed that he had inherited both his mother's X chromosomes.**

Conclusions: **This study showed that the patient had gp91-phox deficient CGD while his older brother was a CGD carrier and a Klinefelter, who had inherited both his mother's X chromosomes. This is the first report of such a concurrence in an individual, and argues for family members to be included in PID studies.** ***(Asian Pac J Allergy Immunol 2013;31:167-72)***

Key words: *Chronic granulomatous disease, CYBB, gp91-phox, Klinefelter's syndrome NADPH-oxidase*

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Introduction

In chronic granulomatous disease (CGD) the neutrophil, a mediator of the innate response, fails to control infections as the enzyme Nicotinamide Adenine Dinucleotide Phosphate (NADPH) -oxidase is unable to generate the reactive oxygen intermediates needed to kill phagocytosed pathogens[1]. NADPH-oxidase is a multi-protein complex making CGD a heterogeneous disease, as a reconfiguration or absence of any component may result in the characteristic enhanced susceptibility to infection. Each CGD type, whilst clinically similar,

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has a unique basis; X-linked CGD is due to a mutation in the *CYBB* gene that codes for gp91-phox, while autosomal CGD is caused by mutations in genes that code for the other components *ie*. *CYBA* for p22-phox, *NCF-1* for p47-phox, *NCF-2* for p67-phox,[1] *NCF-4* for p40-phox[2] and the *RAC2* gene for Rac2.[3]

X-linked CGD predominates in the USA[4] and Europe[5] while a higher frequency of consanguineous marriages contributes to predominantly autosomal CGD in Israel[6] and Tunisia.[7] Regardless of the predominant type, CGD is a rare disease with a global frequency of about 1/250,000 individuals.[1] Hence, the concurrence of an X-linked PID such as CGD, with another X-linked syndrome or with a sex chromosome abnormality would be extremely rare. Nevertheless, an extreme example of the former with concurrence of several X-linked syndromes *ie.* CGD, Duchenne Muscular Dystrophy, retinitis pigmentosa, and McLeod syndrome due to a contiguous deletion has been reported in one individual.[8] An example of the latter *ie*. concurrence of CGD and Klinefelter's or XXY syndrome (KS) has also been reported by Sanders et al. in 1974.[9] This concurrence is remarkable as the child inherited two copies of his mother's disease carrying X chromosome in order to have gp91-phox deficient CGD.

This paper describes a case of gp91-phox deficient CGD in a family where the proband has gp91-phox deficient CGD. His mother and, most unusually, his brother were both CGD carriers. The carrier status of the brother was explained by the fact that he also had Klinefelter's syndrome and, more importantly, had inherited both his mother's X chromosomes.

Materials and Methods

Patient and Family members

Blood was obtained, with informed consent, from the CGD patient and his family members. The study was approved by the Institutional Review Board, Institute for Medical Research, Kuala Lumpur and the Medical Research Ethics Committee, Ministry of Health, Malaysia.

Family B. The Index patient is a male child of non-consanguineous Chinese parents, born in 2010. He suffered an episode of meningitis at day 27 of life, followed by BCG lymphadenitis at 4 months and at 6 months was admitted to hospital for prolonged fever with recurrence of lymphadenitis in the axillary lymph node. His older brother had pneumonia at birth, recovered and has been well since. The pedigree of the family is shown in Figure 1.

Functional Assays

Respiratory burst activity was determined by the Dihydrorhodamine (DHR) test and processed according to the manufacturer's instructions (Orpegen, Germany). The samples were acquired and analysed in a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson, San Jose, CA). Results are expressed in terms of the Stimulation Index (SI) = Geometric Mean Channel Fluorescence Intensity of PMA stimulated granulocytes/Geometric Mean Channel Fluorescence Intensity of unstimulated granulocytes.

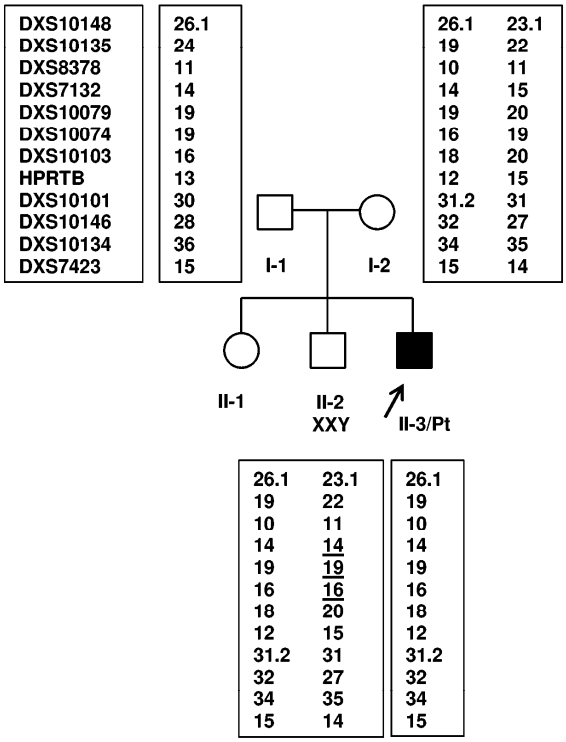

Figure 1. Family B Pedigree and X-STR Genotypes. Arrow indicates proband. The mother/I-2 and brother/II-2 are carriers and the brother also has KS. STR genotypes are depicted in the locus order shown in the top left corner. The KS brother inherited both his mother's X chromosomes, with recombination in one maternal X chromosome at DSX7132, DXS10074 and DXS10079 (underlined), so that both X chromosomes were homozygous at these loci

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gp91 expression was determined with a labeled anti-flavocytochrome monoclonal antibody 7D5 (MBL, Nagoya, Japan) and a modified 2-tube flow cytometry protocol recommended by the manufacturer. Whole blood was added to the tubes which held either CD45-PerCP or CD19-FITC/7D5-PE/CD45-PerCP monoclonal antibodies. Using CellQuest, both neutrophils and B cells were gated and analysed in the FACSCalibur (Becton Dickinson, San Jose, CA).

RNA and DNA Extraction

Total RNA was extracted from blood using the RNeasy Mini Blood Kit (Qiagen, GmbH) as recommended by the manufacturer. First strand cDNA was synthesized with 1µg total RNA using Superscript II Rnase H^- Reverse Transcriptase (Invitrogen, USA) and random hexamers (Promega, Madison, WI) incubated at 42^0C for 1 hr. The QIAamp DNA Blood Mini Kit (Qiagen, GmbH, D-40724, Hilden) was used for DNA extraction, as recommended by the manufacturer.

RT-PCR and PCR

RT-PCR covering most of the *CYBB* gene was carried out using 3 overlapping primer sets ie. 1L/1R, 2L/2R and 3L/3R, and the cycling conditions described in Hui et al.[10]

PCR of gDNA to study exon 7 of the CYBB gene was done with the 7L/7R primer set and the cycling conditions described in Hui et al.[10]

Sequencing

PCR products amplified from cDNA or gDNA were sent to First Base Laboratories (M) Pte Ltd and sequenced on an automated fluorescent sequencer (ABI 3730XL, Applied Biosystems) using Big Dye Terminator (V3.1) chemistry. GenBank reference sequences NG_009065.1 (DNA) and NM_000397.3 (mRNA) were used for sequence comparison. In the numbering of cDNA, the A of the ATG translation initiation codon was taken as +1, and this codon was taken as codon 1.[14]

X chromosome Typing

The origin of the X chromosome(s) in the carrier KS sibling and patient were determined by X-STR analysis using the Investigator Argus X-12 Kit (Qiagen GmbH, D-40724, Hilden) according to the manufacturer's instructions and analysed using GeneMapper (v3.2.1) software.

Results

Figure 1 depicts the pedigree of the family and shows that the brother's karyotype was 47, XXY, while the patient was a normal 46, XY (not shown). The X-STR data, shown in the same figure, corroborated the karyotypes and revealed that the carrier sibling had inherited both his mother's X chromosomes. Furthermore, one maternal X chromosome appeared to have undergone recombination in the Linkage II group XSTRs ie. DSX7132, DXS10079 and DXS10074, so that both X chromosomes were homozygous at these loci.

The DHR results in Figure 2A show the patient's neutrophils were unable to produce a respiratory burst upon PMA stimulation with 97 % of his cell population at an SI = 1. The histogram of the control and patient's sister show 99.8 % and 96% of their neutrophils generating a respiratory burst of SI 48.6 and 23 respectively. The mother's histogram shows a bimodal response pattern to PMA stimulation with 51% of the neutrophils unable to produce a respiratory burst, SI = 3, and 45 % producing a respiratory burst, SI = 35. The brother's histogram also showed a bimodal distribution with 58 % of the neutrophils producing a respiratory burst, SI = 54, and 40% unable to produce a respiratory burst, SI = 5.

Flow cytometric assessment of gp91 expression in Figure 2B showed that this closely resembled the fluorescence pattern seen in the DHR assay. The mother's histogram showed two populations of neutrophils with 44% expressing gp91-phox at a mean fluorescence intensity (MFI) of 25, while 56% were negative, MFI = 7. The brother's histogram also displayed two neutrophil populations, with 70% positive for gp91, MFI = 85 and 30% negative for gp91, MFI = 6. The patient's histogram showed that 99.8 % of his neutrophils were negative for gp91, MFI = 7. In addition, the patient's histogram indicates that there was no difference in the position of the peaks between his unstained neutrophils, *ie.* control, and his neutrophils stained with gp91 antibody.

To identify the mutation cDNA of the *CYBB* gene was amplified in three fragments and sequenced. Figure 3A shows a single nucleotide base change in position 676 from C to T in the second fragment in the patient. In the cDNA of the mother, sister and normal, position 676 was occupied by a C. In Figure 3B, the gDNA amplification of exon 7, sequenced by a reverse primer, shows that the patient had G in position 23936 and the control and sister had A in that position .The carrier mother and brother had both G and A in this position.

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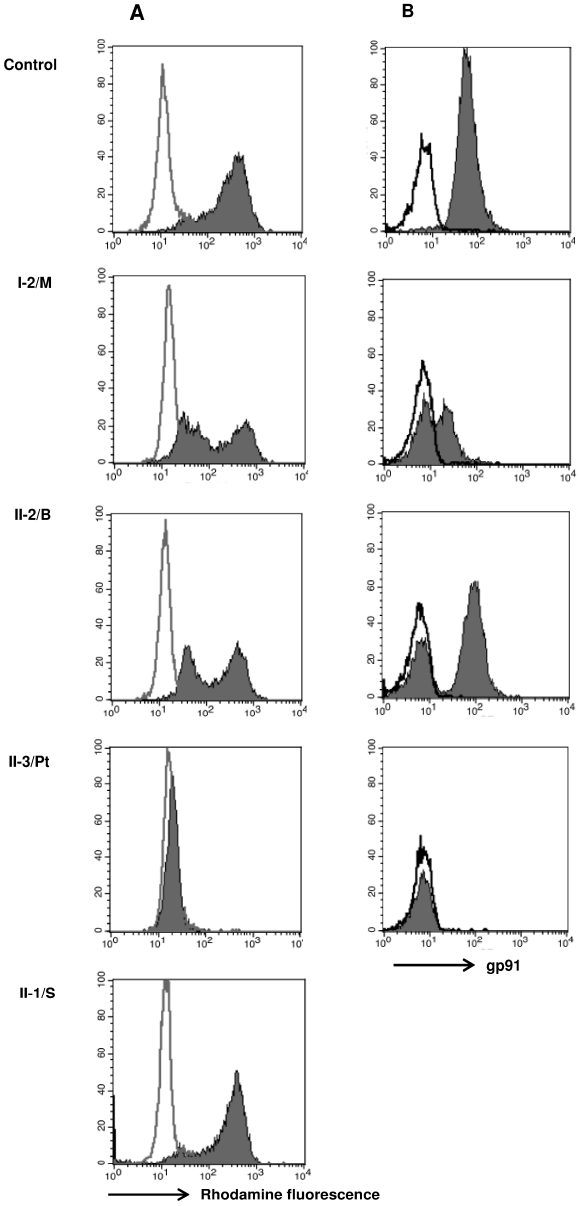

Figure 2. Assessment of neutrophils by Flow Cytometry. (A) Subjects' neutrophil DHR Response. The shaded peaks represent the stimulated neutrophil response, and non-shaded peaks, the non-stimulated response. PMA elicits a response in control's and sister's neutrophils, no response in Patient's neutrophils and a bimodal response in neutrophils from mother and brother. (B) Subjects' Neutrophil gp91 Expression. The shaded peaks depict the gp91-phox stained and non-shaded peaks depict the unstained neutrophils. The control's neutrophils express gp91, patient's neutrophils do not, mother's and brother's show a bimodal expression pattern

Discussion

The genetic abnormality in this CGD patient, c.676C>T is a nonsense mutation that results in the amino acid change p.Arg226X *ie*. premature termination of the gp91-phox protein at amino acid 226. This mutation has been reported in 17 cases with X-linked CGD.[11] All these cases do not express the gp91-phox protein, which generally signals a poor prognosis.[12]

The unusual finding in this study is the brother's DHR result indicating he was a carrier of X-linked CGD. The brother was karyotyped and found to have Klinefelter's syndrome. To the best of our knowledge this is the first report of a child who is a carrier of X–linked CGD with Klinefelter's syndrome. This is the essential difference between this case and the case described by Sanders et al.,[9] as their patient had both CGD and Klinefelter's syndrome. KS protects the sibling in this study from CGD.

This raises the issue of the frequency with which KS occurs in families with X-linked PIDs. KS has been reported in a case each of X-linked CGD[9] and X-linked lymhoproliferative disease (XLP).[13] However, KS does not protect against the X-linked syndrome in these cases as both patients inherited two copies of the maternal disease carrying X chromosome; in the CGD case this was inferred from the Xg blood group marker, while in the XLP case it was determined by X chromosome restriction fragment length polymorphism (RFLP) analysis. The protection conferred by KS is determined by two inter-related factors, the origin of the extra X chromosome and the non-disjunction event from which it arises; non-disjunction at Meiosis 1 (M1) delivers either an extra maternal or paternal chromosome, while Meiosis 2 (M2) usually delivers an extra maternal chromosome.[14] The latter mechanism explains the X-CGD and XLP cases, while the former, the case in this study. More importantly, these two examples of X-linked PID and KS concurrence are on record because they are cases; the frequency of co-segregation in unaffected siblings is not known as investigations may not include or report them. The KS, X-linked CGD carrier male sibling of this study and the KS, male properdin deficiency carrier,[15] in a study of a family with that disease, were discovered because of extended studies. Similarly the KS sibling of a Wiskott Aldrich patient was discovered because he was a candidate donor for his affected brother.[16]

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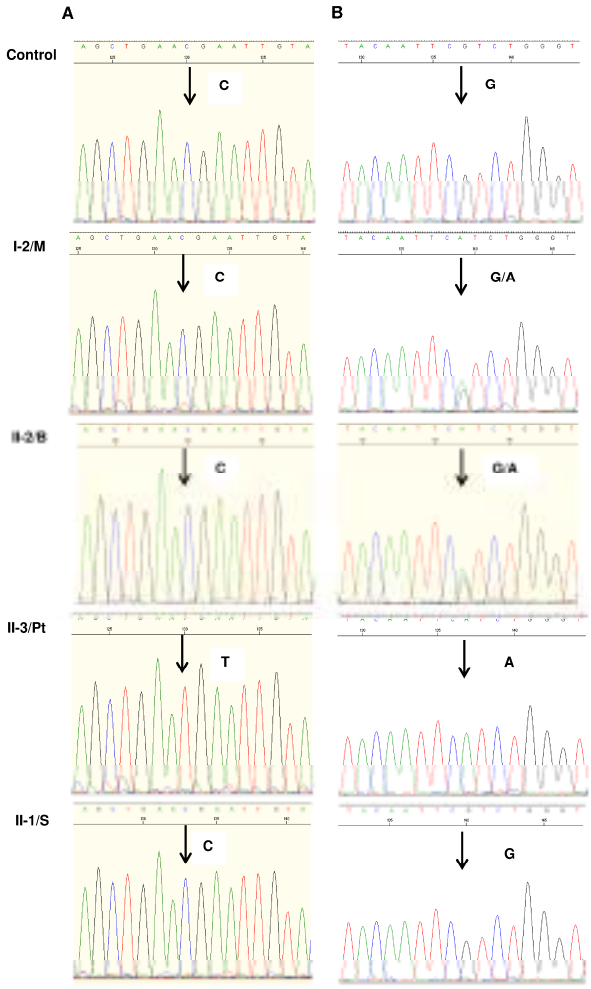

Figure 3. Sequence of Exon 7 cDNA and gDNA from Study Subjects. (A) Subjects' second Fragment cDNA Sequence. Control, mother, brother and sister have C in nt position 676, the patient has T at this position. (B) Subjects' gDNA Exon 7 Reverse Sequence. While control and sister have G in position 23936, patient has A and mother and brother are heterozygous at this position

The co-segregation of KS has also been observed at a low frequency in a number of X-linked syndromes.[17,18] One of these, Incontinentia pigmenti (IP) is distinctive in that it is normally fatal in males.[19] Seventy-two male IP patients have been reported and their survival has been attributed to concurrent KS in 8 cases. This suggests, subject to verification of the numbers, that KS occurs at a high frequency, *ie*. about 1/10 in this group as compared to between 1/500 to 1/1000 in the normal population.[20] It further suggests that KS is a minor pathway of mitigation against the disease. The survival of most male IP patients was attributed to somatic mosaicism or hypomorphic mutations.[19] Both mechanisms have been reported in PIDs,[21-26] and the latter are often described as 'leaky' syndromes. So, although IP represents an extreme end of a spectrum of X-linked diseases as it is fatal in males, it does reveal how an affected individual may survive such devastating diseases. While this may hint at KS's contribution to the survival of X-PIDs, we do not know its extent with any certainty.

This study brings the number of reported cases where KS is associated with X-PIDs to 5. These cases argue for family members to be included in investigations of PID patients,[9,13,15,16] for two main reasons. Firstly, as these studies suggest, other concurrent and potentially modifying, genetic abnormalities may surface. Secondly, the screening of entire families also identifies a) carriers with varying levels of functional capacity as in the family study of properdin deficiency,[15] b) cases before they become clinically apparent as illustrated by the Roos et al. study[27] of p47-phox deficient CGD, and c) conditions that may affect the selection of a bone marrow donor.

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