Defining p47-phox deficient Chronic Granulomatous Disease in a Malay family

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Summary

Background: **The most common autosomal form of Chronic Granulomatous Disease, p47-phox deficient CGD, generally features a GT (ΔGT) deletion in the GTGT sequence at the start of exon 2 on the *NCF-1* gene. This consistency is due to the coexistence of and the recombination between 2 homologous pseudogenes (ψs) and *NCF-1.* The GTGT: ΔGT ratio mirrors the *NCF-1*: *NCF-1ψ* ratio and is 2:4 in normal individuals.**

Objective: **To determine the molecular basis of the Autosomal-CGD in a family with 2 children, a male and female, affected by the disease. The female patient suffered recurrent infection, retinitis pigmentosa and discoid lupus.**

Methods: **Chemiluminescence (CL) was used to study the respiratory burst, while genetic analysis was done by RT-PCR, PCR, ΔGT and the 20bp gene scans.**

Results: **The CL response of the patient was profoundly low. The patient's p47-phox band was absent in the RT-PCR for NADPH-oxidase component mRNAs. The ΔGT scan showed that the patient's GTGT: ΔGT ratio was 0:6, the parents' and the younger brother's was 1:5 and the younger sister's was 2:4. Examination of other *NCF-1*/ *NCF-1*ψs differences showed that the father had a compound ΔGT allele *ie*. ΔGT-20bp, inherited by the patient, and that both parents had compound GTGT alleles with a single 30bp segment in intron 1.**

Conclusions: **The patient was a classic, homozygous ΔGT p47-phox deficient CGD with one allele harbouring a compound ΔGT-20bp gene. The ΔGT and 20bp gene scans offer a relatively simple and efficient means of defining a p47-phox deficient CGD patient.** ***(Asian Pac J Allergy Immunol 2012;30:313-20)***

Key words: *Chronic Granulomatous Disease, Primary Immunodeficiency, NCF-1, p47-phox, NADPH-oxidase*

Introduction

Patients with Chronic Granulomatous Disease (CGD), a primary immunodeficiency, suffer an enhanced susceptibility to bacterial and fungal infection.[1] The breach lies in the neutrophil where a functionally compromised enzyme fails to convert molecular oxygen to superoxide and other potent reactive oxygen intermediates that kill ingested pathogens.[1] The enzyme, nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase, is a complex comprising two membrane bound proteins gp91-phox and p22-phox and several cytosolic proteins *ie*. p47-phox, p67-phox, p40-phox and Rac2 that translocate to the membrane when the neutrophil is activated.[1] A defect in any one of these proteins may result in CGD, making it a heterogeneous disease; about 65% of cases, due to a defect in gp91-phox, are X-linked, while the rest are autosomal with some 25% arising from a defective p47-phox, 5% from a defective p67-phox and 5% from a defective p22-phox.[1] In addition, a case each of p40-phox[2]- and Rac2[3]- defective CGD have also been described.

Each NADPH-oxidase component is encoded by its own gene; the *CYBA* gene codes for gp91-phox,[4]

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Submitted date: 9/4/2012
Accepted date: 7/6/2012

CYBB for p22-phox,[5] *NCF-2* for p67-phox,[6] while the gene for p47-phox is *NCF-1* at 7q11.23.[6] *NCF-1* is unique because two highly homologous pseudogenes (ψs), *NCF-1B* and *NCF-1C*[7] co-localize with and flank the wild type gene; *NCF-1B* lies 5' and in the same orientation, while *NCF-1C* lies 3' but in the reverse orientation to the *NCF-1* gene.[8] As a result of the recombination between them, p47-deficient CGD generally features a consistent homozygous mutation with most patients missing a GT (ΔGT) in the GTGT tandem repeat found at the start of exon 2.[7,9] This genetic consistency stands in marked contrast to the diversity of mutations seen in the other types of CGD.[1]

Interestingly, in addition to other differences, this one at the start of exon 2 distinguishes *NCF-1* from both ψs.[10,11] Thus the GTGT:ΔGT ratio mirrors the *NCF-1*: *NCF-1*ψ ratio and tends to be characteristic, in that it is generally 2:4 in normal individuals, 1:5 in carriers and 0:6 in patients.[11] As it often happens, a small minority of patients have been found to have an unusual range of GTGT: ΔGT ratios, such as 1:5, 2:4 or 1:1.[12] This again, in general, is caused by recombination between *NCF-1* and its pseudogenes, but in particular, by the occurrence of non-ΔGT mutations, so that a patient may be homozygous for a non-GT mutation, or heterozygous with either a combination of non-ΔGT and ΔGT mutations, or with 2 different non-ΔGT mutations.[12,13] There are some 9 exonic and 140 intronic[13,14] differences that allow us to distinguish between the *NCF-1* and *NCF-1*ψs. The three most common differences used, in this regard, are all intronic: the 30bp sequence in intron 1 is present as a tandem repeat in *NCF-1* and singly in *NCF-1*ψs; the 20bp sequence in intron 2 is found singly in *NCF-1* and as a tandem repeat in *NCF-1*ψs; and, the intron 1 nucleotide 122bp upstream of the start of exon 2, that is C in *NCF-1* and T in the *NCF-1*ψs.[12,14]

The GTGT: ΔGT ratios were among the parameters examined in a Malay family with two affected children, a female and male, after it was ascertained that the former had p47-phox deficient CGD. The younger male sibling died before this study was done. The GTGT: ΔGT ratios of the patient and her family members were characteristic but further investigation showed a more complex picture and this paper describes the first comprehensive investigation of a p47-phox deficient CGD case in Malaysia.

Methods

Patient and Family members

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

Family A. The index patient is a Malay female born in 1987 to non-consanguineous parents. Her clinical history records meningococcaemia, retinitis pigmentosa in the right eye, and a protracted episode that included herpes simplex, cellulitis in her left foot which grew *Chromobacterium violaceum,* hepatitis, a gastrointestinal haemorrhage, septicaemia and oral candidiasis. She now also has discoid lupus erythematosus. CGD was diagnosed in the patient and her younger brother born in 1993 by a negative NBT and a depressed CL response (1994). The affected younger brother, II-3, who had a history of abscesses (Zarina L et al., manuscript in prep) succumbed to a *Chromobacterium violaceum* septicaemia complicated by intracranial bleeding just before this study was initiated. The family pedigree is shown in Figure 1A.

Functional Assays

Respiratory burst activity was determined by chemiluminescence as described by Kaever, Robitzsch, Stangel et al.,[15] with minor modification. Two hundered and fifty µl of reaction mixture (pre-diluted blood + 0.4 mmol/l luminol + 8 g/l opsonised zymosan in 1 % gelatin in M-HBSS) was placed in the well of an opaque cliniplate and the chemiluminescence was measured in a luminometer (Luminoskan, Thermo) at 37^0C with measurements taken every minute for 50 minutes. The blood leukocyte count was determined in a Blood Counter (A^C.T diff, Beckman Coulter) to calculate CL/granulocyte and results were expressed as a percentage of control's response.

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.

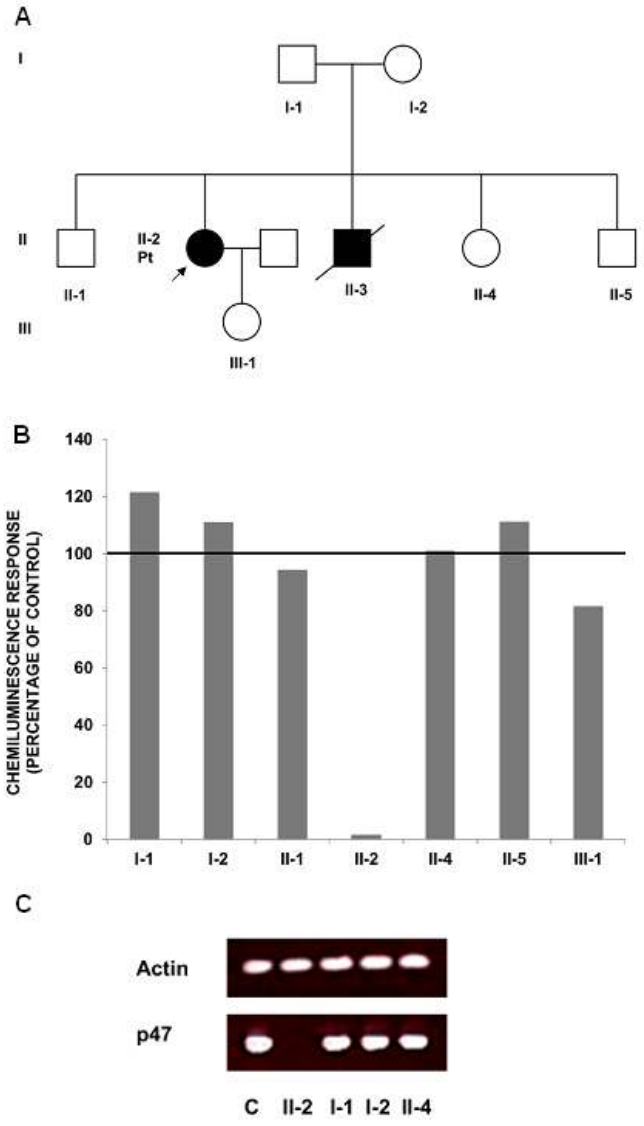

Figure 1. Family A: Data. (A) Family A pedigree. (B) Chemiluminesence response of patient and family members expressed as percentage of control's response, shown as dark horizontal line at 100%. Patient/II-2's response is profoundly low. (C) RT-PCR using primers for p47-phox and actin with cDNA from Control (lane 1), II-2/Patient (lane 2), I-1/Father (lane 3), II-2/Mother (lane 4) and II-4/sister (lane 5). Patient/II-2's p47-phox band is absent

RT-PCR detection of NADPH oxidase subunits and the NCF-1 gene

Amplification of gp91-phox, p22-phox, p47-phox and p67-phox transcripts was carried out using primers and cycling conditions described by Li and Shah.[16]

The amplification of the *NCF-1* gene cDNA was done in two overlapping RT-PCRs; the first, using primers cDNA1F and GTGT-R[12] to amplify an exon 1 to exon 2 fragment, and the second fragment, exon 2 to exon 11, was amplified using primers cDNAGTGT and cDNA11R[12]. The patient's cDNA was amplified with allele non-specific cDNA primers cDNA1F and cDNA4R[13] to confirm the ΔGT mutation.

PCR

The GTGT: ΔGT ratio was first determined by calculation of the mean *NCF-1*/*NCF-1*ψ nucleotide peak height ratios for an exon 2 fragment, nt 81 to 107, amplified from gDNA with the 2LB2/2RB2 allele non-specific, intronic primers described by Heyworth et al.[10] The GTGT: ΔGT ratio was also determined by the ΔGT gene-scan using the 6-Fam-labeled P47-ΔGT-fwd and P47-ΔGT-rev primers for the 198/196 fragment. This ratio was further refined with the 20-bp gene-scan using the P47-ΔGT-fwd and Hex-labeled P47-20bp-rev primers. The cycling conditions used for both gene scans were described in Dekker, de Boer and Roos.[11]

The allele-specific long PCR to capture the 30bp sequence in intron 1 was done with primers 1L and GTGT-R, and sequenced with the latter primer, using cycling conditions described by Noack, Rae, Cross et al.[12] The NCF-1 4-10 exons were amplified both by allele-specific and allele non-specific PCR using primers and cycling conditions described by Noack, Rae, Cross et al.[13]

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 NM_000265.4 (mRNA) and U57833.1, U57834.1 and U57835.1 (DNA) 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.

Results

The chemiluminescence response of each clinically normal family member was close to the response of the unrelated, normal control. The patient, on the other hand, had a profoundly depressed respiratory burst (Figure 1B) that was 1.6% of the control's response.

The RT-PCR of mRNA fragments coding the four main proteins *ie.* gp91-phox, p22-phox, p47-phox and p67-phox, detected normal bands for these proteins from all the normal individuals in family A. However, as shown in Figure 1C, the patient's p47-phox band was absent.

The electropherograms of the 2LB2/2RB2 amplified exon 2 products showed multiple traces for 5 family A individuals and the control. In contrast, the patient showed a clean, single trace sequence. The *NCF-1*: *NCF-1*ψ ratio of each subject calculated from the average peak height ratio of each *NCF-1* to *NCF-1*ψ nt over a defined stretch in exon 2 is shown in Table 1. The patient's ratio was 0:6; the parents, patient's younger brother and daughter gave ratios of 1:5, while the control and the patient's younger sister displayed a normal ratio of 2:4. In addition, the patient's PCR product sequenced as –cagGTACATGT-, 3 nt 5' and 8 nt of exon 2, shows that she was missing a ΔGT in exon 2. The ΔGT gene-scan results in Figure 2B show that the patient's product resolved into a single peak at 196bp indicating a GTGT: ΔGT ratio of 0:6. Both the parent's PCR products showed 2 peaks, with a 198:196 peak height ratio of 1:5. The ratio in the patient's younger sister was 2:4, while both the younger brother and patient's child gave a ratio of 1:5, as summarized in Table 1.

The results of the 20-bp gene-scan, in Figure 3, corroborated those of the ΔGT gene-scan. The additional finding was that the father had a third peak at 411 bp comprising a compound (cpd) *NCF-1*ψ allele *ie*. GT-20bp. That is, the PCR product amplified from the father's gDNA resolved into 3 peaks at 411bp, 413bp and 431bp at a ratio of 1:1:4 and the patient inherited this compound allele so that her ratio of 411:413:431 bp peaks or cpd*NCF-1*ψ: *NCF-1*: *NCF-1*ψ is 1:0:5. The patient's daughter, on the other hand, displayed a ratio of 0:1:5, with no compound allele.

The allele–specific PCR sequence results of the exon 1- intron 1 fragments are shown in Figure 4. The PCR product of the control showed 2 x 30bp sequences, while the patient's mother, father and sister had a single 30bp sequence. All subjects showed variations in the 5 nucleotides preceding the 30bp sequence and the father's 30bp sequence was altered, in that, there was an additional C in the -tcccct- sequence at the start of the fragment. This PCR was repeated for all individuals with the same results as for the first assay.

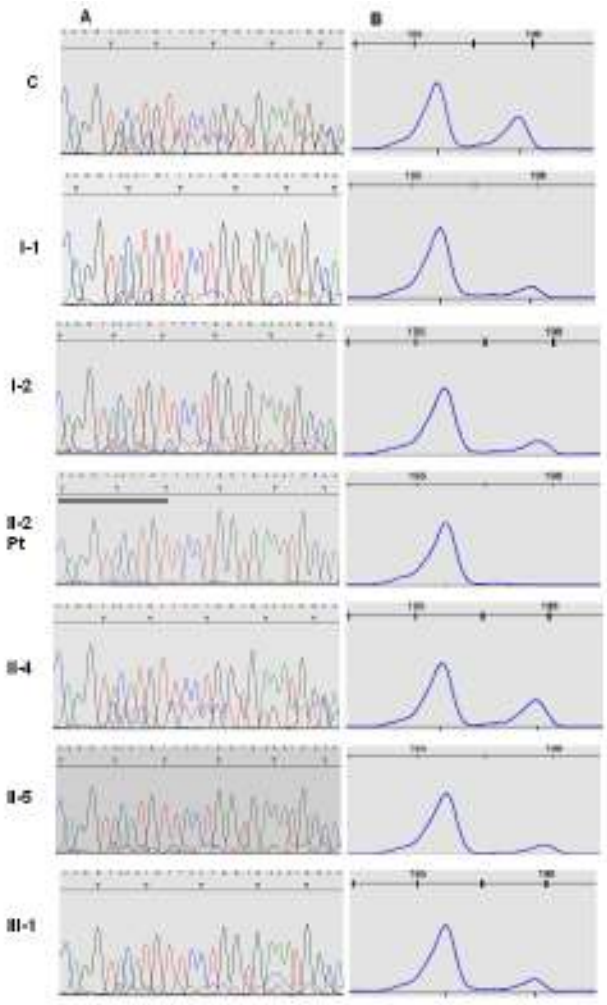

Figure 2. gDNA Exon 2 Analysis. (A) Sequence electropherograms of *NCF1-1*/ *NCF-1* ψ exon 2 amplicons of Family A members. Only the patient/II-2's product shows a clean trace *ie.* only *NCF-1* ψ genes. *NCF1-1*/ *NCF-1*ψ nucleotide peak heights over a 27-bp stretch from were calculated to determine GTGT: ΔGT ratios of the study subjects (See Table 1). Patient's underlined sequence shows missing ΔGT at the start of exon 2. (B) ΔGT gene scan results of Family A. The left peak is the *NCF-1*/198bp product and the right peak is the *NCF-1*ψ/196bp product. Patient/II-2's product resolves into a single peak at 196bp *ie*. *NCF-1*: *NCF-1*ψ ratio of 0:6, parents and younger brothers' products display 2 peaks at a 198:196 ratio of 1:5. The control and sister's products resolve into 2 peaks at a 198:196 ratio of 2:4.

Table 1. GTGT: ΔGT ratio in control and family A members

	C	I-1	I-2	II-2	II-4	II-5	III-1
GTGT:ΔGT Ratio by eletropherogram*	2:4 (0.5)#	1:5 (0.17)	1:5 (0.21)	0:6 (0)	2:4 (0.44)	1:5 (0.17)	1:5 (0.22)
GTGT:ΔGT Ratio by ΔGT Gene Scan Ratio	2:4 (0.5)*	1:5 (0.15)	1:5 (0.20)	0:6 (0)	2:4 (0.42)	1:5 (0.15)	1:5 (0.18)

Mean Peak height *NCF-1* nucleotide/ Mean Peak height *NCF-1ψ* nucleotide (nt 81 to nt 107 in exon 2)
* Height 198bp peak/ Height 196bp peak

Figure 5 illustrates the hypothetical *NCF-1*/*NCF-1ψ* haplotypes of the mother, father and patient constructed from the ΔGT and 20bp scans, as well as the exon 1- intron 1 allele specific PCR. The father is heterozygous with one allele (FI) consisting of a compound *NCF-1* gene flanked by two *NCF-1ψ* genes, and the second allele (FII) comprises a compound *NCF-1ψ* gene flanked by two *NCF-1ψ* genes. The mother is similarly heterozygous; her first allele (MI) is identical to the father's FI allele, and the second allele (MII) comprises three *NCF-1ψ* genes. The patient inherited the second allele of each parent; whilst homozygous for ΔGT, she acquired her father's compound *NCF-1ψ*.

In general, the cDNA allele non-specific and specific RT-PCR of the patient and her family members, respectively, showed that there were no other changes apart from the ΔGT and the known polymorphisms or variations that distinguish *NCF-1* from *NCF-1B* and *NCF-1C*. The amplification of exons 4-10 from gDNA and their sequences also corroborated this finding.

Discussion

Chronic Granulomatous Disease is a rare disease with a prevalence that varies in populations, ranging between 1 in 200,000 - 1 in 1,375,000 individuals.[17] Studies of CGD cohorts in the USA,[17] Europe[18] and Italy[19] report that X-linked CGD comprises 65-70% of all cases, while autosomal (AS) CGD accounts for the rest. There are reports of reversed frequencies in Iran,[20] Israel,[21] Latin America[22] and Tunisia,[23] where AS-CGD predominates and this is explained, in part, by a higher frequency of consanguineous marriages. Regardless of the predominant CGD type, p47-deficient CGD accounts for majority of the AS cases.

AS-CGD patients experience a much milder form of the disease as they present later, have fewer and shorter hospital stays, and live longer.[17,18,22] The conspicuous mildness of p47-deficient CGD is explained by the *in vitro* study that showed residual ROI generation by NADPH-oxidase in the absence of p47-phox.[24] Furthermore, a study of 287 CGD patients[25] revealed an association between residual ROI production and survival. The data showed, in fact, that disease course and survival were determined by the mutation and its effect on enzyme activity, regardless of the gene affected. Hence, residual ROI production constitutes important information in a case and would help to explain why some p47-deficient CGD patients suffer a particularly severe clinical course and succumb to their infections.

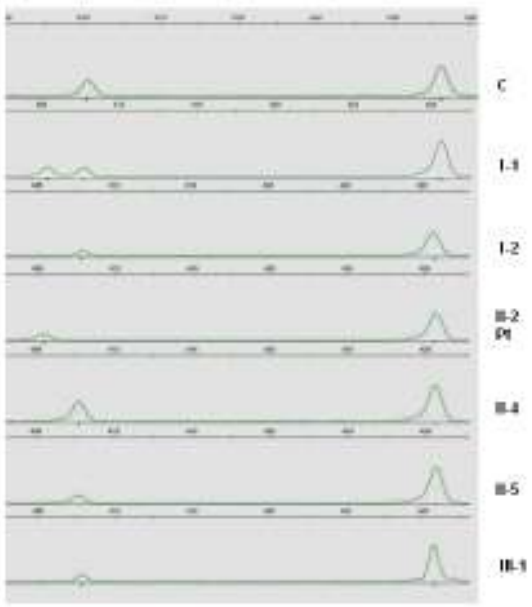

Figure 3. 20bp gene scan results of genomic DNA from Family A. gDNA from subjects was amplified and the product includes the GTGT sequence in exon 2 and the 20bp segment in intron 2. The *NCF-1* (413), *NCF-1ψ* (431) and compound *NCF-1ψ* (411) peaks are shown. The father's product resolves into 3 peaks cpd*NCF-1ψ*, *NCF*-1 and *NCF-1ψ* at a ratio of 1:1:4. The patient's product resolves into the cpd*NCF-1ψ* and *NCF-1ψ* peaks at a ratio of 1:5, and displays no *NCF-1* peak.

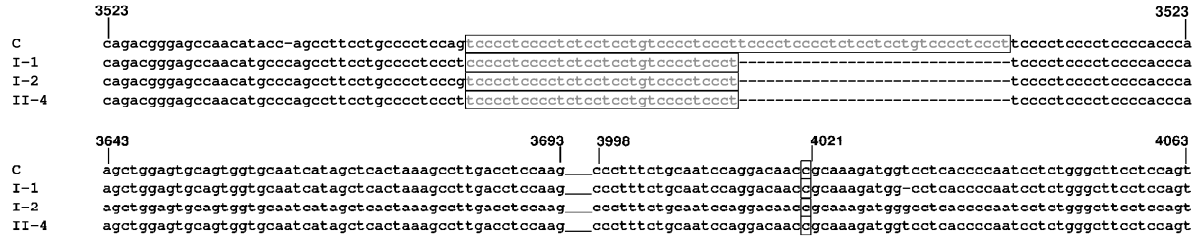

Figure 4. Sequence comparison of the *NCF-1* intron 1 in the study subjects. The sequence shown is from nt 3523 – nt 4063, and the numbering follows the GenBank reference sequence U57833.1. The control is the only subject with a 30bp tandem repeat in intron 1, shown in grey and bordered. The mother, father and sister of the patient carry a single 30bp segment in intron 1. All individuals carry 'c' at position 4021 (122 bp upstream of exon 2), which is characteristic of *NCF-1*.

While both the index case and her brother did not suffer particularly severe disease, the brother succumbed to a *Chromobacterium violaceum* infection, common in South East Asian CGD patients.[26] A limited immunoblot to detect the NADPH-oxidase proteins proved uninformative as the patient's protein yield was poor. Consequently, an RT-PCR was done to detect the mRNA of the major NADPH-oxidase proteins and it showed that the patient's cDNA failed to generate the p47-phox band. This is not surprising given that allele-specific primers were used. However, it must be noted that this RT-PCR may not always visually identify p47-phox deficient CGD particularly when non-ΔGT mutations are involved. It may take RT-PCR followed by sequencing, or preferably a western blot to identify the NADPH-oxidase protein affected.

We used both the average peak height ratio of a defined exon 2 segment[10] and the ΔGT gene-scan[11] methods to determine the GTGT: ΔGT ratios. The electropherogram of the amplified exon 2 product is effectively the first visual hint of the ratio. In this case, the patient's electropherogram showed a clean trace that sequenced as *NCF-1ψ ie.* she had no *NCF-1* alleles. The ΔGT gene scan concurred with a GTGT:ΔGT ratio of 0:6. The patient is a classic p47-defective CGD homozygous for the ΔGT deletion at the start of exon 2 ie. c.75_76delGT, which leads to a frame shift and premature termination at codon 51.

Although the GTGT: ΔGT ratios of the family members were characteristic, further examination revealed a more complex picture. The 20bp gene scan showed that one of the father's ΔGT alleles

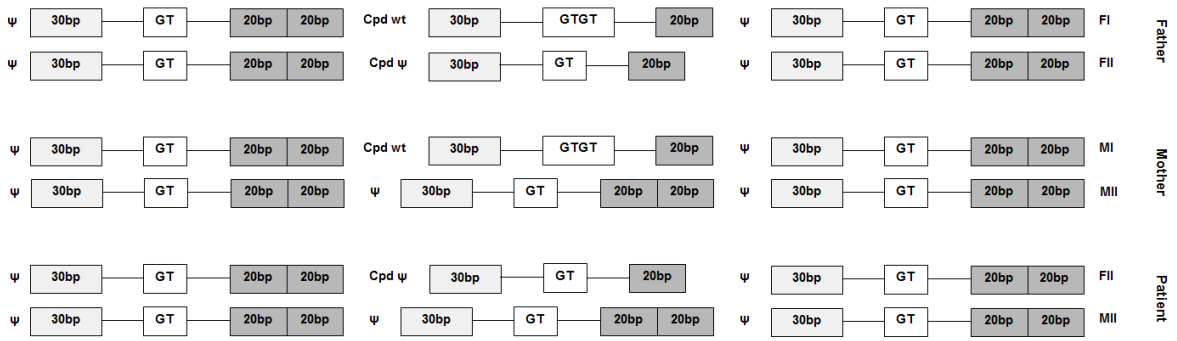

Figure 5. Hypothetical scheme of *NCF*-1/*NCF-1ψ* haplotypes in the mother, father and patient. The father has one allele with a compound *NCF-1*gene attached to 2 successive *NCF-1ψ genes ie. NCF-1ψ*-cmpd*NCF-1-NCF-1ψ* (FI), while the second allele comprises a compound *NCF-1ψ* attached to 2 *NCF-1ψs* genes(FII). The mother's MI allele is identical to the father's FI allele, while the MII allele comprises 3 successive *NCF-1ψs.* The patient inherits the father's FII allele with a compound *NCF*-1ψ, and the mother's MII allele.

was a compound allele comprising ΔGT-20bp, suggesting that recombination occurred somewhere between exon 2 and intron 2. This compound allele was inherited by the patient, but not by her daughter. The 30 bp sequence data revealed that both parents had only one GTGT allele and that this was a compound allele *ie*. 30bp-GTGT. Therefore, it may be extrapolated that the patient's sister, with a GTGT: ΔGT ratio of 2:4, has two compound GTGT alleles inherited from each parent. Furthermore, as no other mutations were found in the family and barring a *de novo* mutation, the affected brother was probably a classic homozygous p47-defective CGD, like his sister.

As the patient is a classic homozygous ΔGT p47-phox CGD routine/prenatal diagnosis and counselling for siblings in her generation is relatively simple and may be based solely on the GTGT: ΔGT ratio.[27] If, however, the patient had had a GTGT: ΔGT ratio other than 0:6 *ie*. 2:4, 1:5 or 1:1, found in about 15 % of p47-phox deficient CGD patients,[28] then routine / prenatal diagnosis is more complex, and will entail analysis of the *NCF-1* gene for non-ΔGT mutations as well.

Whilst defining the mutation in a p47-deficient CGD case may be complicated by the presence of the two highly homologous pseudogenes, determination of the GTGT: ΔGT ratio simplifies the task considerably. The ratio not only reveals the presence of the common mutation and its zygosity, it also determines how a patient's mutation(s) should be identified. Although the ratio may not always identify carriers or patients, it is pivotal to the process of defining a p47-phox deficient case. This may be relevant in Malaysia where, despite the fact that our first p47-deficient CGD case was not from such a union, consanguineous marriages are not uncommon.

Acknowledgements

The authors thank the Director-General of Health, Ministry of Health, Malaysia for permission to publish. We are grateful to the patient and her family for providing blood samples for this study; and to Koay BT, AIRC, IMR for his assistance with the figures. This study was funded by a grant, JPP-IMR 08-014, from the Ministry of Health, Malaysia.

References

1. Stasia MJ & Li XJ. Genetics and Immunopathology of chronic granulomatous disease. Semin Immunopathol. 2008;30:209-35.
2. Matute JD, Arias AA, Wright NAM, Wrobel I, Waterhouse CCM, Li XJ, et al. A new genetic subgroup of chronic granulomatous disease with autosomal recessive mutations in p40-phox and selective defects in neutrophil NADPH oxidase activity. Blood. 2009;114:3309-33.
3. Ambruso DR, Knall C, Abell AN, Panepinto J, Kurkchubasche A, Thurman G, et al. Human neutrophil immunodeficiency syndrome is associated with an inhibitory Rac2 mutation. Proc Natl Acad Sci USA. 2000;97:4654-59.
4. Royer-Pokora B, Kunkel LM, Monaco AP, Goff SC, Curnutte JT & Orkin SH. Cloning the gene for an inherited human disorder – chronic granulomatous disesase- on the basis of its chromosomal location. Nature. 1986;322:32-8.
5. Dinauer MC, Pierce EA, Bruns GA, Curnutte JT & Orkin SH. Human neutrophil cytochrome b light chain (p22-phox). Gene structure, chromosomal location, and mutations in cytochrome-negative autosomal recessive chronic granulomatous disease. J Clin Invest. 1990;86:1729-37.
6. Francke U, Hsieh C-L, Foellmer BE, Lomax KJ, Malech HL & Leto TL. Genes for Two Autosomal Recessive Forms of Chronic Granulomatous Disease to 1q25 (NCF2) and 7q11.23 (NCF1). Am J Hum Genet. 1990;47:483-92.
7. Casimir CM, Bu-Ghanim HA, Rodaway ARF, Bentley DL, Rowe P & Segal AW. Autosomal recessive chronic granulomatous disease caused by deletion at a dinucleotide repeat. Proc Natl Acad Sci USA. 1991;88:2753-7.
8. Roesler J, Curnutte JT, Rae J, Barrett D, Patino P, Chanock SJ & Goerlach A. Recombination events between the p47-phox gene and its highly homologous pseudogenes are the main cause of autosomal recessive chronic granulomatous disease. Blood. 2000;95:2150-6.
9. Brunson T, Wang Q, Chambers I, Song Q. A copy number variation in human *NCF-1* and its pseudogenes. BMC Genetics. 2010 Feb [cited 2012 April 7];11:13. Available from: http://www. Biomed central.com/14712156/11/13.
10. Heyworth PG, Noack DB & Cross AR. Identification of a novel *NCF-1* (p47-phox) pseudogene not containing the signature GT deletion for $A47^0$ chronic granulomatous disease carrier detection. Blood. 2002;100:1845-51.
11. Dekker J, de Boer M & Roos. Gene-scan method for the recognition of carriers and patients with $p47^{phox}$-deficient autosomal recessive chronic granulomatous disease. Experimental Hematology. 2001;29:1319-25.
12. Noack D, Rae J, Cross AR, Ellis BA, Newburger PE, Curnutte JT & Heyworth PG. Autosomal recessive chronic granulomatous disease caused by defects in *NCF-1*, the gene encoding the phagocyte p47-phox: mutations not arising in the *NCF-1* pseudogenes. Blood. 2001;97:305-11.
13. Roos D, de Boer M, Koker MY, Dekker J, Singh-Gupta V, Ahlin A, et al. Chronic Garnulomatous Disease Caused by Mutations Other Than the Common GT Delaetion in *NCF-1*, the Gene encoding the $p47^{phox}$Component of the Phagocyte NADPH-Oxidase. Hum Mutation. 2006;27:1218-29.

14. Gorlach A, Lee PL, Roesler J, Hopkins PJ, Christenson B, Green ED, et al. A p47-*phox* Pseudogene Carries the Most Common Mutation Causing p47-*phox*-deficient Chronic Granulomatous Disease J Clin Invest. 1997;100:1907-18.
15. Kaever V, Robitzsch JT, Stangel W, Schleinkofer L & Resch K. Simultaneous Detection of Whole Blood Chemiluminescence in Microtitre Plates. Eur J Clin Chem Clin Biochem. 1992;30:209-16.
16. Li JM & Shah AM. Intracellular localization and preassembly of the NADPH oxidase complex in cultured endothelial cells. J Biol Chem. 2002;277:19952-60.
17. Winkelstein JA, Marino MC, Johnston RB Jr, Boyle J, Curnutte J, Gallin JI, et al. Chronic Granulomatous Disease. Report on a national registry of 368 patients. Medicine (Baltimore). 2000;79:155-69.
18. Van den Berg JM, van Koppen E, Ahlin A, Belohradsky BH, Bernatowska E, Corbeel L, et al. Chronic Granulomatous Disease: The European Experience. PLoS one 2009;4:e5234. Available from: http://www.plosone.org/article/info:doi/10.1371/journal.pone.0005234
19. Di Matteo G, Giordani L, Finocchi A, Ventura A, Chiriaco M, Blancato J, et al. with IPINET (Italian Network for Primary Immunodeficiencies). Molecular characterization of a large cohort of patients with Chronic Granulomatous Disease and identification of novel CYBB mutations: an Italian multicenter study. Mol Immunol. 2009;46:1935-41.
20. Teimourian S, de Boer M & Roos. Molecular Basis of Autosomal Recessive Chronic Granulomatous Disease in Iran. J Clin Immunol. 2010;30:587-92.
21. Wolach B, Gavrieli R, de Boer M, Gottesman G, Ben-Ari J, Rottem M, et al. Chronic granulomatous disease in Israel: Clinical, functional and molecular studies of 38 patients. Clin Immunol. 2008;129:103-14.
22. Agudelo-Florez P, Prando-Andrade CC, Lopez JA, Costa-Carvalho BT, Quezada A, Espinosa FJ, et al. Chronic granulomatous disease in Latin American patients: clinical spectrum and molecular genetics. Pediatr Blood Cancer. 2006; 46:243-52.
23. El Kares R, Barbouche MR, Elloumi-Zghal H, Bejaoui M, Chemli J, Mellouli F, et al. Genetic and mutational heterogeneity of autosomal recessive chronic granulomatous disease in Tunisia. J Hum Genet. 2006;51:887-95.
24. Freeman JL & Lambeth JD. NADPH-oxidase activity is independent of $p47^{phox}$ *in vitro*. J Biol Chem. 1996;271:22578-82.
25. Kuhns DB, Alvord WG, Heller T, Feld JJ, Pike KM, Marciano BE, et al. Residual NADPH Oxidase and Survival in Chronic Granulomatous Disease. N Engl J Med. 2010;363:2600-10.
26. Sirinavin S, Techasaensiri C, Benjaponpitak S, Pornkul R, Vorachit M. Invasive *Chromobacterium violaceum* infection in children: case report and review. Pediatr Infect Dis J. 2005;24:559-61.
27. De Boer M, Singh V, Dekker J, Di Rocco M, Goldblatt D & Roos D. Prenatal diagnosis in two families with autosomal $p47^{phox}$-deficient chronic granulomatous disease due to a novel point mutation in *NCF-1*. Prenat Diagn. 2002;22:235-40.
28. Roos D, Kuhns DB, Maddalena A, Bustamante J, Kannengiesser C, de Boer M, et al. Hematologically important mutations: The autosomal recessive forms of chronic granulomatous disease (second update). Blood Cells Mol Dis. 2010;44:291-9.