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Detection of NCF-2 Gene Expression in Egyptian Children with Chronic Granulomatous Disease

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ABSTRACT

Background: An uncommon inherited primary immunodeficiency illness (PID) known as chronic granulomatous disease (CGD) is characterised by an increased vulnerability to serious bacterial and fungal infections. It results from defects in one of the five polypeptide subunits of the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase complex, CYBB (gp91-phox), NCF1 (p47-phox), CYBA (p22-phox), NCF2 (p67-phox), or NCF4 (p40-phox) with a resulting failure of the phagocytes in generating a variety of microbicidal reactive oxygen radicals during respiratory burst. **Objective:** In the present study we aimed to diagnose AR-type of CGD by detection of NCF-2 gene expression using real time RT-PCR as a cheaper diagnostic method for CGD subtypes categorization among CGD children. **Patients and methods:** This case-control study was conducted on 15 children provisionally diagnosed as CGD patients with dihydrorhodamine (DHR) stimulation index (Group I). The study also included 12 mothers and 8 fathers of the studied patients (Group IIa and IIb respectively) to detect the genetic mutations in carriers, if any, and 14 apparently healthy children as a control group (Group III). **Results:** In the present study, cases with a fold change of NCF-2 gene expression less than 0.67 were considered defective for NCF-2 gene expression. At this cut-off value, from our molecularly studied subjects, 2 CGD cases and one mother showed under-expression of NCF-2 gene.

Conclusion: We could establish the diagnosis of 2 out of 15 CGD cases as AR- CGD form, derived from defects in the NCF-2 gene, which encodes gp67-phox of the oxidase, without the need to use complex and expensive methodologies such as northern blot, or genomic DNA sequencing.

Keywords: CGD, PID, NADPH, NCF-2 gene, DHR, RT-PCR.

INTRODUCTION

A class of hereditary diseases known as primary immunodeficiency disorders (PIDs) prevent normally developing and functioning immune systems. PIDs are still underdiagnosed despite numerous improvements in diagnosis, treatment, and research in this area, particularly in less developed nations [(1)].

The nicotinamide adenosine dinucleotide phosphate (NADPH) oxidase complex in neutrophils and macrophages is defective in chronic granulomatous disease (CGD), which is an inherited immunodeficiency disease, causing these cells to ineffectively create superoxide anions and their metabolites to kill peroxidase-positive bacteria and fungi. These flaws put patients at risk for repeated, serious infections and overactive inflammation at an early age [(2)].

NADPH oxidase enzyme consists of five subunits: gp91phox, p22phox, p47phox, p67phox, and p40phox encoded by the genes cytochrome b-245 beta subunit (CYBB), cytochrome b-245 alpha subunit (CYBA), Neutrophil Cytosolic Factor-1(NCF-1), Neutrophil Cytosolic Factor-2 (NCF-2), and Neutrophil Cytosolic Factor-4 (NCF-4) respectively. They are dormant in resting cells and become activated in response to pro-inflammatory mediators, the presence of microbes, phagocytosis, and/or the activation of pattern recognition receptors (PRRs)[(3)]. Chronic granulomatous disease is caused by pathogenic variants in one of the five genes that encode the subunits of phagocyte NADPH oxidase: biallelic pathogenic variants in CYBA, NCF-1, NCF-2, and NCF4 cause autosomal recessive CGD (AR-CGD); mutation of CYBB causes X-linked CGD (XL-CGD)[(4)]. Of all mutations associated with CGD, NCF2 gene mutations are the rarest and are previously reported as clinically milder than others[(5)].

Patients with CGD typically have symptoms in the first two years of life, at a young age. However, some people may also show up later in life due to the disease's varied hereditary causes. Because a mutation in an X-chromosome-linked gene is the primary cause of CGD, the majority of patients (around 80%) are men [(6)].

In the present study we aimed to diagnose the AR type of CGD in a group of Egyptian children by detection of NCF-2 gene expression using real time RT-PCR as an aiding tool in the molecular diagnosis of gene expression of NCF-2 among CGD patients and correlate it to patients' clinical and laboratory data.

PATIENTS AND METHODS

Study Subjects:

This case-control study was conducted on 15 children diagnosed with CGD recruited from children's hospitals of different universities in Egypt. The laboratory work was conducted in the Clinical Pathology Department, Immunology Unit, Ain Shams University Hospitals from October 2018 to October 2020. The study included three groups; Group (I) included 15 children (9 males and 6 females) provisionally diagnosed as CGD patients based on DHR testing using phorbol myristate acetate (PMA) with stimulation index < 30%. Patients with known positive HIV serology or HIV nucleic acid testing, active malignancy and known myelodysplasia of the bone marrow or abnormal bone marrow cytogenetics, all were excluded from the study. Group (II) included 20 subjects from the parents of the enrolled CGD cases,

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divided into 12 mothers (Group IIa) and 8 fathers (Group IIb) to detect the genetic mutations in carriers, if any. Group (III) included 14 apparently normal age- and sex-matched children (8 males and 6 females) as a control group.

All study participants were subjected to the following: Complete history taking including consanguinity history of the parents, Full clinical examination, Laboratory workup including: complete blood picture (group I and III only) on three part cell counter Mindray (Shenzhen Mindray Bio-Medical Electronics Co., Ltd., High-tec Industrial Park, Nanshan 518057 Shenzhen China), estimation of phagocytic and lytic indices, dihydrorhodamine (DHR) testing (group I only), and real time reverse transcription polymerase chain reaction (RT-PCR) for expression of NCF-2 gene in the three included groups.

Blood sampling:

Six ml of venous blood were collected under complete aseptic conditions. Withdrawn samples were divided onto three tubes [two sterile EDTA (1.2mg/ml) (Greiner) vacutainer tubes; one for the CBC and the second was for PCR, while the 3rd one was lithium heparin (17 IU/mL of blood) containing tubes for phagocytic and lytic indices estimation. Samples were transported in ice box in order to avoid any possible RNA degradation.

Phagocytic lytic index estimation:

This assay was used to evaluate the ability of the neutrophils to phagocytose and lyse opsonized candida. It evaluates the ability of the neutrophils to generate reactive oxygen species by which it kills the foreign invaders. Phagocytosed candida albicans were counted within 100 polymorphonuclear leukocytes (PMNs). The intracellular candida that intensely stained were counted (phagocytosed but not lysed candida) and the intracellular unstained candida appearing as halo oval bodies were also counted (phagocytosed and lysed candida). The phagocytic and lytic indices were calculated from the following formulae:

$$\text{Phagocytic index} = \frac{\text{Total number of phagocytosed candida/100 PMNs}}{100}$$

$$\text{Lytic index} = \frac{\text{Total number of lysed candida /100PMNs}}{100}$$

NCF-2 gene expression by reverse transcription PCR which included 3 steps:

i- RNA extraction: RNA was extracted using QIAamp® whole blood RNA extraction kit supplied by (Qiagen str.1, 40724 Hilden, Germany).

ii. Reverse Transciption: using QuantiTect Reverse Transcription Kit supplied by (Qiagen str.1, 40724 Hilden, Germany).

iii. Amplification and detection: using QuantiTect SYBR Green PCR Kit, QuantiTect NCF-2 primer assay using rotor-GeneQ MDX, and housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer. All reagents and rotor gene cycler were supplied by (Qiagen, 40724 Hilden, Germany). The cycling condition included initial activation step at 95ºC for 5 minutes, then, denaturation follows at 95ºC for 5 seconds, thereafter, combined annealing/extension at 60ºC for 10 seconds for a total of 35 to 40 cycles. This was followed by analysis of the experiment and interpretation of results by viewing the amplification plots and setting the baseline and threshold values.

Interpretation was done using relative quantification (Calculation of fold change of gene expression using delta threshold cycle (CT), delta-delta CT and fold change formulae) as follows[7]:

Delta Ct(ΔCT) (eq.1), delta-delta Ct(ΔΔ CT) (eq.2) and fold change ($2^{-\Delta\Delta CT}$) (eq.3) were used.

- eq1: ΔCT =CT target gene – CT GAPDH (housekeeping gene).
- eq.2: ΔΔ CT= ΔCT of patient or control sample – mean of ΔCT of control samples.
- eq.3: fold change= $2^{-\Delta\Delta CT}$.

Ethical consent:

An approval of the study was obtained from Ain Shams University Academic and Ethical Committee. Every parent of participating children signed an informed written consent for acceptance of participation in the study. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Statistical analysis

IBM SPSS statistics (V. 26.0, IBM Corp., USA, 2019) was used for data analysis. Data were expressed as median, range and percentiles for quantitative non-parametric measures in addition to both number and percentage for categorized data. Mann Whitney Test was used to assess the statistical significance of the difference of a non-parametric variable between two study groups. The probability of error at 0.05 was considered significant, while at 0.01 and 0.001 were highly significant.

RESULTS

This study is a case control study, conducted on fifteen patients (9 males, 6 females), provisionally diagnosed as CGD (Group I), with their age ranging from (3-15 years) with the median age (5 years), their parents (12 mothers - Group IIa and 8 fathers - Group IIb), and 14 healthy children as a control group (Group III). All study participants were assessed for their phagocytic index (PI), lytic index (LI) and fold change of NCF-2 gene expression by RT-PCR. Total leucocytic count (TLC) and absolute neutrophil count (ANC) were done for group I and III only**.** The descriptive statistics of the different parameters studied in the three groups are shown in table 1.

Table (1): Descriptive statistics of all parameters studied in three groups

Parameter	Group(I) Patients N=15 Median (25^{th}-75^{th})	Group(IIa) Mothers N=12 Median (25^{th}-75^{th})	Group(IIb) Fathers N=8 Median (25^{th}-75^{th})	Group(III) Control N=14 Median (25^{th}-75^{th})
Age(year)	5 (4-8)	30 (25-35)	34 (28-40)	6 (4-10.25)
TLC(10^3/ul)	5.20 (3.9-8.7)	----------	----------	8.95 (6-12.47)
ANC(10^3/ul)	2.40 (1.60-5)	----------	----------	4.80 (2.37-7.27)
PI	6.80 (5.64-8.60)	9.55 (7.30-10.70)	10.90 (9.35-11.28)	8.97 (7.86-10.13)
LI	0.80 (0.51-1.10)	1.24 (0.59-2.52)	1.35 (0.49-2.08)	1.30 (1.04-1.43)
Fold Change	1.55 (0.73-2.10)	1.48 (0.77-1.94)	2.05 (1.02-3.03)	1.01 (0.67-1.83)

TLC: total leucocytic count, ANC: absolute neutrophil count, PI: phagocytic index, LI: lytic index.

Comparative statistics between group I (CGD patients) and group III (healthy control) showed significant statistical decrease of PI% and LI% in group I when compared to group III. While there were no statistically significant differences between the 2 groups regarding age, TLC and ANC and fold change of NCF-2 gene expression (Table 2).

Table (2): Comparative statistics between CGD patients (Group I) and control group (group III) regarding different studied parameter

	Group(I) Patients N=15 Median (25^{th}-75^{th})	Group(III) Control N=14 Median (25^{th}-75^{th})	P value	Sig.
Age(year)	5 (4-8)	6 (4-10.25)	0.52	NS
TLC(10^3/ul)	5.2 (3.90-8.70)	8.95 (6.02-12.47)	0.06	NS
ANC(10^3/ul)	2.4 (1.6-5)	4.8 (2.37-7.27)	0.11	NS
PI	6.80 (5.64-8.60)	8.97 (7.86-10.13)	0.005	HS
LI	0.80 (0.51-1.10)	1.30 (1.04-1.43)	0.026	S
Fold change NCF-2 expression	1.55 (0.73-2.10)	1.01 (0.67-1.83)	0.407	NS

(HS = Highly Significant, S = Significant, NS = Non-significant).

Comparative statistics between group I (CGD patients) and group IIa (Mothers group) showed no statistically significant difference between the 2 groups regarding the fold change (Table 3)**.**

Table (3): Comparative statistics between CGD patients (group I) and mothers (group IIa) regarding the fold change of NCF-2 gene expression

	Group(I) Patients N=15 Median (25^{th}-75^{th})	Group(IIa) Mothers N=12 Median (25^{th}-75^{th})	P value	Sig.
Fold change of NCF-2 gene expression	1.55 (0.73-2.10)	1.48 (0.77-1.94)	0.83	NS

NS = Non-significant

Comparative statistics between group I and group IIb (Fathers group) showed no statistically significant difference between the 2 groups as regarding the fold change (Table 4)**.**

Table (4): Comparative statistics between CGD patients (group I) and fathers' group (group IIb) regarding the fold change of NCF-2 gene expression

	Group (I) Patients N=15 Median (25^{th}-75^{th}	Group (IIb) Fathers N=8 Median (25^{th}-75^{th})	P value	Sig.
Fold Change of NCF-2 gene expression	1.55 (0.73-2.10)	2.046 (1.02-3.029)	0.20	NS

NS = Non-significant

The descriptive statistics for this case-control study revealed that 100% of patients had positive history of consanguinity (Table 5)./

Table (5): Disease characteristics of the studied CGD patients

Character	CGD patients (n=15)	
	No.	%
Males	9	60
Females	6	40
Consanguinity	15	100
Age of disease onset before 1.5 year	8	53
Age of disease onset after 1.5 year	7	47
Pneumonia	9	60
Skin Infection	4	26.7
Pneumonia + skin infection	2	13.3
No/Partial response to treatment	7	47
Good response to treatment	8	53

In the present study, the 25th percentile fold change of NCF-2 gene expression of the control group was 0.67 and was considered the cut-off. Cases with a fold change less than 0.67 were considered defective for NCF-2 gene expression for further confirmation with gene sequencing. Only 3 subjects (2 patients and 1 mother) showed lower values than 0.67 for fold change of NCF-2 gene expression.

Case (1): A 9 years old male patient who had a history of recurrent pneumonia and skin infection since he was two and half years old, repeated hospitalization and partial response to the frequently used antibiotics and antifungal. His lab results showed that TLC was (5.2x10^3/ul), ANC was (3.4x10^3/ul), PI was (8.6), LI was (2.41), DHR was (3.8), and the fold change of NCF-2gene expression was (0.06).

Case (2): A 6 years old male patient who had a history of recurrent pneumonia since he was 6 months old, repeated hospitalization and partial response to the frequently used antibiotics. His lab results showed that TLC was (4.7x10^3/ul), ANC was (1.5x10^3/ul), PI was (5.6), LI was (0.42), DHR was (2.65), and the fold change of NCF-2 gene expression was (0.59).

Case (3): A mother of a 6 months old CGD female patient had a Down expression of NCF-2 gene with fold change of 0.38. Her PI was (10.89) and LI was (2.8). However, her daughter who was presented by recurrent pneumonia showed normal level of NCF-2 gene expression with fold change of (2.5). In addition, the lab results of the daughter showed that TLC was (6.3x10^3/ul), ANC was (2 x10^3/ul), PI was (5.64), LI was (1.1).

DISCUSSION

The molecular diagnosis of CGD involves measuring NADPH oxidase activity in phagocytes, measuring protein expression of NADPH oxidase components and mutation analysis of genes encoding these components. It was found that flow cytometry-generated patterns lack specificity in different mutations of the disease. This limits its role in predicting individual's underlying genotype and highlights the importance of molecular testing in confirming diagnosis of CGD [8].

In our study, we retrieved the results of the DHR from the patients' files and all of them were having decreased stimulation index (<30%) and therefore provisionally diagnosed as CGD. This is in accordance with **Blancas-Galicia *et al.*** [9] who studied 93 patients in Mexico City for clinically suspected CGD. Based on lab results, they diagnosed CGD in 89 out of their studied 93 patients as follows; using NBT assay (n = 6), DHR (n = 27), and by using NBT plus DHR (n = 56).

In the present study, provisionally diagnosed CGD patients underwent examination of phagocytic and lytic indices. In our study, the median PI of the cases was (6.80%) while that of the control group was (8.97%) with a highly statistically significant difference (P=0.005). These results don't agree with other research groups. **Gaither *et al.*** [10] found a mean PI value for their studied cases to be (2.13%) and for the control group to be (1.48%). Also, by using flow cytometry, **Hasui *et al.*** [11] found that the ingestion of fluorescent bacteria by PMNs of CGD patients was significantly increased, that is about 1.5 times that of normal controls. This unexplained decrease in the phagocytic activity of the cases included in the present study may be attributed to subjectivity of the test or other factors decreasing the phagocytic function in some of our patients such as any infection occurring at the time of sample withdrawal as periodontitis [12] or oral mucositis [13]. As regards LI, the median of the cases in the present study was (0.80%) and that of the control group was (1.30%) with a significant statistical decrease (P=0.026). This finding is in accordance with the fact that in patients with CGD the lytic activity of neutrophils is decreased [14]. Also, these results agreed with **Leijh *et al.*** [15], **Gazendam *et al.*** [16] and **Gazendam *et al.*** [17] who showed in their experiments that CGD neutrophils were severely impaired in killing of opsonized Candida albicans and Aspergillus conidia respectively.

In the present study, all included subjects (patients, controls, fathers and mothers) were tested for NCF-2 gene expression by real time RT-PCR. We estimated our cut-off value for NCF-2 gene expression to be 0.67-fold change (calculated by the 25th percentile of the control group). By using this cut-off in our study, NCF-2 gene under-expression was found in only two patients out of 15 patients (13.3%) and one mother. This percentage of CGD patients with under expression of NCF-2 gene agrees with other research groups such as

Köker *et al.* [14], **Rawat *et al.* [18]**, **Akar *et al.* [19]** and **Wolach *et al.* [20]** who found in their studies the percentages of CGD cases with NCF-2 mutations to be 14.6%, 14.9%,12% and 19% respectively. On contrast to our results, **El Hawary *et al.* [21]** studied 28 Egyptian CGD patients and only 2 out of 28 patients (7.1 %) were defective for P67-phox protein. Similarly, **Blancas-Galicia *et al.* [9]**, **Fattahi *et al.* [22]**, **van den Berg *et al.* [23]** and **Zhou *et al.* [24]** studied CGD cases from different countries around the world and found the percentages of NCF-2 mutations to be 6.2%, 8.3%, 8% and 7.7% respectively. The discrepancy of results could be due to the high frequency of consanguineous marriage in Egypt. Chronic granulomatous disease associated with NCF2 is inherited in an autosomal recessive manner.

High rates of consanguineous marriage in Egypt result in high rates of autosomal recessive inherited disorders as documented in other diseases studied in consanguineous populations **[25].** In our study, 100% of CGD patients had first degree consanguineous parents. This agrees with **Tajik *et al.* [26]** who reported that 32 patients (84%) out of 38 Iranian CGD patients were from consanguineous marriage and the most common form of CGD in Iran was AR-CGD. Also, many studies done by **Wolach *et al.* [20], El Hawary *et al.* [21]** and **Fattahi *et al.* [22]** reported consanguineous marriages in 82.1%, 77.4% and 64% respectively. Similarly, the study of **Bakri *et al.* [27]** investigated the clinical and molecular features of 31 CGD patients, 22 Jordanian, 7 Libyan, and 2 Iraqi from 21 different families. All patients except 9 were children of consanguineous parents (70.9%).

Patients with CGD usually manifest their symptoms at an early age, mostly in the first 2 years of life. Our patients were found to present shortly after birth with median age of disease onset 1.5 years (range from 1 month to 3 years). This was in accordance with **El Hawary *et al.* [21]** study, which reported that the median age at onset of symptoms was 8 months (range from 0.1 month to 6 years). In contrast to our results, **Fattahi *et al.* [22]** enrolled 93 CGD patients with a median age of 11 years at the onset of symptoms (range from 7.2 months to 48 years).

Chronic granulomatous disease mostly presents with infections (bacterial and/or fungal) **[14]**. Infections due to CGD occur most commonly in organs in contact with the outside world **[6].** In the present study, pneumonia was the most common clinical presentation in our patients (73%) followed by skin abscesses (40%). Our results were in accordance with **Wang *et al.* [2]**, **Rawat *et al.* [18], El Hawary *et al.* [21], Marciano *et al.* [28] and Fernando *et al.* [29]** who found that the most common clinical presentation of CGD was pneumonia. In contrast to our results, **Fattahi *et al.* [22]** found that lymphoreticular system involvement as abscessified adenopathy or lymphadenopathy was the most common clinical feature (65.6%) followed by pulmonary involvement (57%). Although the results of this study do not match with our results, they still reported that the first three most common sites involved with infection in CGD are (lymphoreticular system, lung, and skin).

With regards to treatment response in our patients, only one patient underwent hematopoietic stem cell transplantation (HSCT) with good response. Among the rest of our patients, 53% of them showed good response to medical treatment and 47% experienced partial response.

CONCLUSION

In conclusion, our study was able to detect down expression of NCF-2 gene in 2 out of 15 CGD cases and one mother out of 12 mothers without the need to use complex and expensive methodologies such as genomic DNA sequencing. However, there are some limitations in our study, such as the small sample size studied due to the disease rarity, some missing data, and restriction to studying only one gene (NCF-2) among many others controlling the human NADPH oxidase system, which is because of the limited resources. Future studies on a larger sample size of CGD patients is highly recommended to draw definite conclusions about the clinical significance of real time RT-PCR technique as a diagnostic tool for this immunodeficiency disease. Also, the study of other subunit encoding genes of NADPH oxidase enzyme in these patients is recommended to reach the exact genotype of this genotypically heterogeneous disease.

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