Molecular Analysis of Four Cases of Chronic Granulomatous Disease Caused by Defects in NCF-2: The Gene Encoding the p67-phox

Mohsen Badalzadeh, Fatemeh Fattahi, Mohammad Reza Fazollahi, Shaghayegh Tajik, Mohammad Hassan Bemanian, Fatemeh Behmanesh, Massoud Movahedi, Massoud Houshmand, and Zahra Pourpak

Immunology, Asthma and Allergy Research Institute, Tehran University of Medical Sciences, Tehran, Iran
Department of Pediatrics, Division of Allergy and Immunology, Shahid Sadoughi Hospital, School of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
Allergy Research Center, Ghaem Hospital, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
Department of Immunology and Allergy, Children Medical Center, Tehran University of Medical Sciences, Tehran, Iran
Department of Medical Genetics, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran

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ABSTRACT

Chronic granulomatous disease (CGD), a rare inherited primary immunodeficiency disorder, is caused by mutation in any one of the genes encoding components of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase enzyme. NCF2 gene (encoding P67-phox component) is one of them and its mutation is less common to cause CGD (around 5-6%). Here, we assessed mutation analysis of NCF2 in 4 CGD patients with p67-phox defect in Iran.

These patients showed classical CGD symptoms. NCF2 sequence analyses revealed two different homozygous mutations including a nonsense mutation in exon 4, c.304C>T (Arg102X) in one case and a CA deletion in exon 13 (Leu346fsX380) in one brother and sister; the latter is a new mutation which has not been reported in previous studies.

In another patient in whom the attempts to amplify exon 2 individually from genomic DNA were unsuccessful, PCR amplification of exon 2 revealed no band of this exon on agarose gel. A PCR amplification mix of exon 2 and exon 7, with an internal control, confirmed the lack of exon 2 in this patient. Although a gross deletion in other exons of NCF2 has been previously reported, a large deletion encompassing exon 2 has been not reported yet. This abstract was also presented in ESID 2012, Florence, Italy.

Keywords: Chronic Granulomatous Disease, P67-phox, NADPH oxidase, NCF2

INTRODUCTION

Chronic granulomatous disease (CGD) is a rare primary immunodeficiency disorder in which the
phagocytic cells have impairment in bactricidal activity. This disease is caused by mutation in any one of the genes encoding the components of nicotinamide adenine dinucleotide phosphate (NADPH)- oxidase enzyme including NCF1 (encoding p47-phox protein), NCF2 (encoding P67-phox protein), CYBA (encoding p22-phox protein), CYBB (encoding gp91-phox). The latter is resulted in X-linked CGD type and the defect of one of the rest subunits results in autosomal recessive (AR) CGD type. Defect in fifth subunit of NADPH (p40-phox encoded by NCF4), defined in 2009, also causes another subtype of AR-CGD. In CGD, the phagocytes fail to kill the catalase positive microbes which leads to life-threatening and recurrent bacterial and fungal infections in the affected patients. In contrast to the report from European countries3-8 and USA9 showing the predominance of XL-CGD over AR-CGD, most CGD patients from Middle East countries (like Iran, Turkey, Jordan, and Israel)11 have a mutation in one of the genes encoding the AR components of NADPH oxidase. Among the subtypes of AR-CGD, the defect in p67-phox caused by mutation of NCF2 was reported to be less common (around 5-6% of all cases). The NCF2 gene is localized on chromosome 1q25 containing 16 exons with a size of around 40 kb. Up to now, not including our study, totally 56 different mutations in the NCF2 gene in patients with CGD have been published in HGMD Professional (Human Gene Mutation Database 2011.3: http://www.hgmd.cf.ac.uk/ac/all.php). Here, we presented mutation analysis of NCF2 in four patients with CGD resulting in p67 component defect in Iran.

**PATIENTS AND METHODS**

This study was performed on patients who were diagnosed to have CGD in Immunology, Asthma and Allergy Research Institute (IAARI); the main referral center for immunodeficiency disorders in Iran related to Tehran University of Medical Sciences. They had been diagnosed by defect in Neutrophil functional assay of NBT and their diagnosis had been approved by DHR assay. Patients with confirmed diagnosis were registered in IPIDR (Iranian Primary Immunodeficiency Registry). Among the patients, those with the defects of p67-phox, determined by western immunoblotting, were included in this study for molecular investigation. Totally, five CGD patients have been diagnosed to have p67-phox defect and among them, DNA samples were from four patients was available for molecular investigation. The protocol of the study was approved by the ethics committee of IAARI. The clinical information of these patients is briefly given as follows:

**Patient 1**

A 6.5-year-old girl, born in 2005 from first-cousin parents, diagnosed as having CGD in the age of 20 months old. She was the first kid in her family. Her vaccination was complete. She presented with axillary lymphadenopathy because of BCGitis when she was 4 months old and anti-tuberculosis medicine was prescribed for her. She received the drug regimen for 6 months and finally her lymphadenopathy was drained when she was 2 years old. From 20th months of age, she was hospitalized due to pneumonia for 6 times. Hepatomegaly, hepatitis and meningitis were reported once during her disease course. She was receiving prophylaxis with trimethoprim/sulfamethoxazole (TMP-SMX) and IFN-γ when included in the study.

**Patient 2**

A 9-year-old boy, born in 2003 from non-consanguineous parents presented with liver abscess and fever. He was the first kid in his family. His problems started when he was 2 years old. He used TMP-SMX for one year. His vaccination was complete without any side effects. At 3 years of age, he developed abscessified lymphadenopathies in his neck. CGD was diagnosed for him when he was 4 years old. He was receiving prophylaxis with TMP-SMZ, IFN-γ and itraconazole when included in the study.

**Patient 3**

A 4.5-year-old girl who was sister of Patient number 2 was included with the presentation of hand swelling. She was admitted at hospital at 4.5 months old for 2 weeks because of wrist arthritis. She received her vaccination completely without any side effect. She was hospitalized another time with the same problem at 4.5 months of age. She was diagnosed with CGD at 4.5 months when she was screened for CGD because of her affected brother. She experienced severe diarrhea at 5 months of age. The next episode of her gastrointestinal problem occurred as diarrhea and vomiting when she was 1.5 years old. She was on prophylaxis with TMP-SMZ and IFN-γ at the time of inclusion to the study.
Patient 4

A 5.5-year-old boy from second-cousin parents, presented for the first time with oral thrush at birth time and diarrhea at 7 months of age. He was not vaccinated and he developed frequent infections in different sites including pneumonia, pulmonary abscess, genitalia and perianal abscess resulting in his hospitalization for several times. He was diagnosed at 8 months of age. Other remarkable points in his disease course were lymphadenopathy in his right inguinal region and hepatosplenomegaly. Bone marrow transplantation was performed for him and was unfortunately failed. He was receiving prophylaxis with TMP-SMZ, IFN-γ and itraconazole at the time of inclusion in the study.

Neutrophil Functional Assays and Western Blot Analysis

The functional ability of the neutrophils to produce reactive oxygen species (ROS) was assessed by the NBT (nitroblue tetrazolium) slide test, and flow cytometric dihydrorhodamine-1,2,3 (DHR) assay as stated elsewhere. Anti-coagulated fresh blood samples from the patients and the controls were used to identify the affected subunit of the NADPH oxidase. Protein immunoblotting with polyclonal antibodies against four subunits of the NADPH oxidase complex (gp91phox, p22phox, p47phox and p67phox) was performed as described before.

DNA Analysis of NCF2 Gene

Genomic DNA was extracted from peripheral blood leucocytes by standard procedures. The PCR primers for 16 exons of NCF2 gene were designed using Primer3, http://frodo.wi.mit.edu/primer3 (Table 1). The exons were amplified in independent PCR runs. PCR was performed in a final volume of 30 µl containing 100 µM dNTP, 1x Taq DNA polymerase buffer, various concentrations of MgCl2, 2 U Taq DNA polymerase (Cinagene, Tehran, Iran), 200 ng genomic DNA and 10 pmol of each primer. After initial denaturation for 2 min at 95°C, 30 cycles of amplification were performed as follows: 30 s at 95°C, 30 s at 59-65°C and 20 s at 72°C. PCR products were evaluated on 1.5% agarose gel. Finally, sequence analysis of PCR products was performed.

RESULTS AND DISCUSSION

We studied four patients with CGD with the lack of p67-phox (A670), one rare subtype of CGD. All of these patients suffered from classical symptoms of CGD with abnormal neutrophil function tests (NBT slide test and DHR flow cytometric assay). These tests did not show any non-functional PMN subpopulations in patients’ mothers, indicative of autosomal recessive inheritance of the disease in included patients. We recently reported that among 67 cases with defined CGD, only 5 patients (7.5%) had p67-phox defect.

<table>
<thead>
<tr>
<th>Primer exon (L and R)</th>
<th>Sequence</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 2</td>
<td>TGGGTATGATGTCAGTTGC</td>
<td>544</td>
</tr>
<tr>
<td>Exon 3</td>
<td>CTAATGTGAGAGCGAAGGTG</td>
<td>597</td>
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<td>AACCATTTCACAAGGAGTT</td>
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<td>Exon 5</td>
<td>TGGAAAGGGAAGAGGCTA</td>
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<tr>
<td>Exon 6</td>
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</tr>
<tr>
<td>Exon 7</td>
<td>TTTCTCGCTCAGTTGGAAC</td>
<td>293</td>
</tr>
<tr>
<td>Exon 8</td>
<td>TGCTGTGCTAGTGAACCTG</td>
<td>494</td>
</tr>
</tbody>
</table>

Table 1. Oligonucleotide primers used for genomic DNA analysis of p67-phox.
Molecular Analysis of Chronic Granulomatous

Two patients described in our study were from consanguineous relative families and two patients from one family with non-consanguineous parents. These patients were tested for mutations in NCF2 by direct sequence analysis, except for patient 4 in whom the attempts to amplify exon 2 individually from genomic DNA were unsuccessful. Other exons in this patient were amplified normally, raising the possibility of a large deletion in exon 2 within the NCF2 gene. A PCR amplification mix of exon 2 and 7, including internal control, in this patient showed no band for exon 2 on PCR gel (Figure 1) indicating that he was homozygous for a genomic deletion of exon 2. Although a gross deletion in other NCF2 exons has been previously reported to the HGMD, a large deletion encompassing exon 2 has not been reported yet.

The mutation found in case 1 was a homozygous nonsense mutation in exon 4, c.304C>T, which changes CGA codon for arginine-102 into TGA stop codon (p.Arg102X). This mutation has been previously reported at the same position in the literature.\textsuperscript{13,16,17} This exon contributed in encoding one of the tandem repeat motifs in the tetra-tricopeptide (TPR) domain, TPR-3. Most reported mutations in p67-phox are located in the TPR domains which are small GTPase Rac-binding regions of p67-phox. This Rac p67-phox interaction is essential for the activation of the NADPH oxidase enzyme, consisting of translocation of the three cytosolic subunits p67-phox, p47-phox and p40-phox to flavocytochrome b558, binding of NADPH to gp91-phox and initiation of electron flow from NADPH to oxygen.\textsuperscript{16}

Remarkable finding in our study was a novel mutation detected in case 2 and 3 (brother and sister) which was homozygous CA deletion (frameshift in nucleotide of 1038-1039) detected in exon 13 which resulted in p.Leu346fsX380 product. This mutation has not been previously reported. Patino et al reported different mutation in exon 13 in an offspring of first-cousin parents native to Jordan.\textsuperscript{17} Our patients (case 2 and 3) were from non-consanguineous parents and they had mutation in different places of exon 13. Patino et al identified a homozygous deletion of 5 nucleotides, 1169-1173, at the 3-prime end of exon 13. The patient’s sister also showed the genetic defect without serious illness.\textsuperscript{17} They also found the same mutation in an unrelated affected Palestinian patient. This patient was also homozygous for the deletion. This mutation was also reported by Bakri et al.\textsuperscript{12} which resulted in a frameshift with a premature stop codon in the PB1 domain of p67-phox. This domain is involved in the binding of the PB1 domain of p40-phox leading to the stabilization and/or expression of both proteins and the correct recruitment of p67-phox to the plasma.
membrane during the oxidase assembly in phagosomes.\textsuperscript{12,18} Modifications in the PBI module of p67-\textit{phox} should reduce the binding interaction with p40-\textit{phox}, leading to a defect in the synthesis of both proteins. However, this is not always observed in AR67\textsuperscript{0}CGD, depending on the type and the location of mutations in the p67-\textit{phox} protein.\textsuperscript{12}

**REFERENCES**


