Clinical, functional, and genetic characterization of chronic granulomatous disease in 89 Turkish patients

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Background: Chronic granulomatous disease (CGD) is a rare primary immunodeficiency disorder of phagocytes resulting in impaired killing of bacteria and fungi. A mutation in one of the 4 genes encoding the components $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ of the leukocyte nicotinamide dinucleotide phosphate reduced (NADPH) oxidase leads to autosomal recessive (AR) CGD. A mutation in the *CYBB* gene encoding $gp91^{phox}$ leads to X-linked recessive CGD.

Objective: The aim of this study is to show the correlation between clinical, functional, and genetic data of patients with CGD from Turkey.

Methods: We report here the results of 89 patients with CGD from 73 Turkish families in a multicenter study.

Results: Most of the families (55%) have an AR genotype, and 38% have an X-linked genotype; patients from 5 families with a suspected AR genotype (7%) were not fully characterized. We compared patients with CGD according to the severity of NADPH oxidase deficiency of neutrophils. Patients with $A22^0$, $A67^0$ or $X91^0$ phenotypes with a stimulation index of 1.5 or less have early clinical presentation and younger age at diagnosis (mean, 3.2 years). However, in $p47^{phox}$-deficient cases and in 5 other AR cases with high residual oxidase activity (stimulation index ≥ 3), later and less severe clinical presentation and older age at diagnosis (mean, 7.1 years) were found. Pulmonary involvement was the most common clinical feature, followed by lymphadenitis and abscesses.

Conclusion: Later and less severe clinical presentation and older age at diagnosis are related to the residual NADPH oxidase activity of neutrophils and not to the mode of inheritance. CGD caused by $A22^0$ and $A67^0$ subtypes manifests as severe as the $X91^0$ subtype. (J Allergy Clin Immunol 2013;132:1156-63.)

Key words: *Chronic granulomatous disease, dihydrorhodamine-1,2,3 assay,* CYBB, CYBA, NCF1, NCF2, *nicotinamide dinucleotide phosphate reduced oxidase, mean fluorescence intensity, stimulation index*

Chronic granulomatous disease (CGD; OMIM no. 306400) is a rare (approximately 1 case in 200,000-250,000 newborns) genetic disorder of the innate immune system. The disease is characterized by a severe susceptibility to bacterial and fungal infections caused by the failure of phagocytic leukocytes to produce microbicidal reactive oxygen intermediates (ROIs). The source of these radicals is superoxide generated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a multicomponent enzyme complex expressed in phagocytic leukocytes (neutrophils, monocytes, eosinophils, and macrophages).[1-3]

The disease is classified by mutations in specific subunits of the NADPH oxidase enzyme. A defect in membrane $gp91^{phox}$ (phagocyte oxidase [phox]), which is encoded by the *CYBB* gene on the X chromosome, leads to X-linked recessive chronic granulomatous disease (X-CGD; approximately 70% of patients worldwide).[1-3] A defect in any one of the other 4 components of the NADPH oxidase (ie, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ encoded by the autosomal *CYBA*, *NCF1*, *NCF2*, and *NCF4* genes, respectively) leads to autosomal recessive (AR) CGD (AR-CGD).[1,4,5] The majority of patients with X-linked recessive CGD (X-CGD) are given a diagnosis before the age of 2 years, whereas patients with $p47^{phox}$ deficiency might have their condition undiagnosed until adulthood.[1,3,4]

X-linked CGD is caused by mutations in the *CYBB* gene, which encodes $gp91^{phox}$, a key membrane protein in the phagocyte NADPH oxidase system.[2] This protein is the large subunit of the membrane-bound flavocytochrome b_{558} complex; the small subunit is $p22^{phox}$. Heterodimer formation between $gp91^{phox}$ and $p22^{phox}$ is important for stable expression of each subunit in phagocytes. Immunoblotting or flow cytometric analysis with specific antibodies for individual components of NADPH oxidase helps to identify the deficient protein in the majority of cases.[6,7]

Both the positive family history for CGD and granulocyte function tests in parents and siblings are critical for differentiation

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Abbreviations used

AR: Autosomal recessive
$A22^R$: A22 CGD with residual oxidase activity
CGD: Chronic granulomatous disease
DHR: Dihydrorhodamine-1,2,3
MFI: Mean fluorescence intensity
NADPH: Nicotinamide dinucleotide phosphate reduced
NBT: Nitroblue tetrazolium
phox: Phagocyte oxidase
PMA: Phorbol 12-myristate 13-acetate
ROI: Reactive oxygen intermediate
SI: Stimulation index
X-CGD: X-linked recessive chronic granulomatous disease

of the mode of inheritance (X-CGD or AR-CGD).[3] The nitroblue tetrazolium (NBT) microscope test is a simple test for diagnosis of CGD but has been largely replaced by flow cytometry. The dihydrorhodamine-1,2,3 (DHR) assay, in addition to being a rapid and sensitive assay for the diagnosis of CGD, can also easily detect carriers of X-linked disease by using flow cytometry.[6,8] A mosaic of oxidase-positive and oxidase-negative neutrophils in a female member of a family with CGD strongly suggests X-CGD carrier status caused by random inactivation of the paternal or maternal X chromosome in each female cell.[6,7,9] Carriers in families with autosomal CGD can only be detected by means of mutation analysis.[7,10] Consanguineous marriage is a risk factor for AR-CGD.[9-11]

In the present study we have applied the DHR assay for the diagnosis of CGD and flow cytometric and Western blot analysis with specific antibodies for subgroup detection. On this basis, the *CYBB*, *CYBA*, *NCF1*, and *NCF2* genes were sequenced, and the clinical and molecular characterization of 89 patients with CGD from 73 Turkish families are reported. We compared patients with CGD with regard to the NADPH oxidase activity of the patients' neutrophils and also evaluated the functional, clinical, and molecular spectrum of the disease.

METHODS

Patients

Between 2002 and 2012, heparinized venous blood samples from 89 patients with CGD and 310 relatives and from 100 healthy control subjects were sent by their physicians from different university hospitals throughout Turkey to the Immunology Laboratory of the University of Erciyes, following their procedures and appropriate informed consent protocols. The study protocol was approved by the Ethical Committee of the University of Erciyes and financially supported by TÜBİTAK (the Scientific and Technological Research Council of Turkey) within Euro-CGD project number 110S252.

Functional analysis of neutrophils

Total leukocytes were isolated from 100 to 200 μL of blood from healthy control subjects, patients, and relatives by means of lysis of the erythrocytes in the pellet fraction with a nonfixing lysis solution of 155 mmol/L NH_4Cl, 10 mmol/L $NaHCO_3$, and 0.1 mmol/L EDTA. Neutrophils were purified from white cells by using standard procedures.[12] The capacity of the neutrophils to generate ROIs was tested both with the NBT slide test and the DHR assay.[7]

The DHR assay was performed (with slight modifications) with total leukocytes, as described by Köker et al.[6-8] In this test isolated neutrophils were incubated with DHR, stimulated with phorbol 12-myristate 13-acetate (PMA), and analyzed by means of flow cytometry. The results are shown as the stimulation index (SI; ie, the ratio of the mean fluorescence intensity [MFI] of the stimulated cells and that of the unstimulated cells). For more details, see the Methods section in this article's Online Repository at www.jacionline.org. In cases in which 2 distinct fluorescent populations were observed, the SI for each neutrophil population was calculated.[6,9]

Subgroup analysis of patients with CGD by using flow cytometry and Western blot analysis

Expression of NADPH oxidase components was analyzed by means of flow cytometry in the Immunology Laboratory of the University of Erciyes, and the results were compared with those after Western blot analysis carried out in the Sanquin Laboratory (Amsterdam, The Netherlands). The tests were performed by using previously defined methods with small modifications of antibodies.[6,9] We used antibodies specific for $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$ from Santa Cruz Biotechnology (Santa Cruz, Calif) for subgroup detection of samples in flow cytometry.

DNA analysis

Genomic DNA was isolated from total blood leukocytes by using standard procedures and analyzed for mutations in exons and exon/intron boundaries of *CYBB*, *CYBA*, *NCF1*, and *NCF2* by means of PCR amplification of each exon with its intronic boundaries, followed by sequence analysis.[11,13]

Statistical analysis

Statistical analysis was accomplished with the SPSS statistical software package, version 16 (SPSS, Chicago, Ill). Both χ^2 and Fisher exact tests were used to compare differences in percentages for categorical variables. *P* values of less than .05 were considered statistically significant. The Student *t* test (or Mann-Whitney *U* test in nonparametric conditions) was used to compare means for continuous variables. Kaplan-Meier graphs were used to show survival rates.

RESULTS

Eighty-nine patients with clinically suspected CGD (25 female and 64 male patients; median age, 10 years) were enrolled in the study (Table I). The diagnosis of CGD was confirmed by using the NBT test, the DHR assay, or both. Thirty-four (38.2%) patients from 28 families were given a diagnosis of X-CGD because the DHR assay performed in their mothers showed nonfunctional and functional PMN subpopulations indicative of the X-linked recessive carrier state. The remaining 55 (61.8%) patients from 45 families were suspected to have AR-CGD.

In all patients with X-CGD and 20 of the patients with suspected AR-CGD, no expression of either $gp91^{phox}$ or $p22^{phox}$ protein in the leukocytes was detected. Subsequent genetic analysis revealed mutations in *CYBB* in 31 of the 34 patients suspected of having X-CGD (the remaining 3 are still under investigation). Mutations in *CYBA* were found in all 20 patients with suspected AR-CGD without $gp91^{phox}$/$p22^{phox}$ expression. In 17 of the patients with suspected AR-CGD, there was no $p47^{phox}$ expression (confirmed by means of mutation analysis of *NCF1*). In 13 patients from the suspected AR-CGD group, we found a lack of $p67^{phox}$ expression (confirmed by means of mutation analysis of *NCF2*). Five patients with suspected AR-CGD are still under investigation.

Altogether, in our cohort 31 (34.8%) patients (from 25 families) had X-CGD ($gp91^{phox}$ deficiency), 20 (22.5%) patients (18 families) had $p22^{phox}$ deficiency, 17 (19.1%) patients (9 families) had $p47^{phox}$ deficiency, and 13 (14.6%) patients (13 families) had $p67^{phox}$ deficiency (Table I). Eight (9.0%) patients (8 families) are still under investigation for complete characterization.

The 81 fully characterized patients with CGD belong to 65 families. In 39 of 40 families with proved AR, the parents were