Molecular Immunology 46 (2009) 1935–1941

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Molecular characterization of a large cohort of patients with Chronic Granulomatous Disease and identification of novel *CYBB* mutations: An Italian multicenter study

Gigliola Di Matteo[a,1], Lucia Giordani[b,1], Andrea Finocchi[a,c], Annamaria Ventura[b], Maria Chiriaco[a], Jan Blancato[d], Cecilia Sinibaldi[e], Alessandro Plebani[f], Annarosa Soresina[f], Claudio Pignata[g], Rosa Maria Dellepiane[h], Antonino Trizzino[i], Fausto Cossu[j], Roberto Rondelli[k], Paolo Rossi[a,c], Domenico De Mattia[b,*], Baldassarre Martire[b], with IPINET (Italian Network for Primary Immunodeficiencies)

[a] *Department of Public Health and Cellular Biology, Tor Vergata University, Rome, Italy*
[b] *Department of Biomedicine of Development Age, University of Bari, Italy*
[c] *Division of Immunology and Infectious Diseases, Children's Hospital Bambino Gesù, Rome, Italy*
[d] *Department of Oncology, Georgetown University, Washington, DC, USA*
[e] *Department of Biopathology, Human Genetics Section, University of Rome Tor Vergata, Italy*
[f] *Department of Pediatrics, University of Brescia, Italy*
[g] *Department of Pediatrics, University of Naples, Italy*
[h] *Department of Pediatrics, IRCSS-Fondazione Policlinico University of Milano, Italy*
[i] *Pediatric Oncohematology "G. Di Cristina" Hospital, Palermo, Italy*
[j] *BMT Unit Ospedale Microcitemico, Cagliari, Italy*
[k] *Department of Pediatrics, University of Bologna, Italy*

ARTICLE INFO

Article history:
Received 19 March 2009
Accepted 20 March 2009
Available online 1 May 2009

Keywords:
CYBB
Chronic Granulomatous Disease
NADPH oxidase
Mutation analysis
Genotype–phenotype correlation

ABSTRACT

Chronic Granulomatous Disease (CGD) is a rare inherited disorder in which phagocytes fail to produce antimicrobial superoxide because NADPH oxidase activity is absent. In about 65% of the cases, the disease is due to mutations affecting the X-linked *CYBB* gene, encoding the gp91^{phox} subunit of NADPH oxidase. We investigated 34 CGD male patients by DHPLC and direct sequencing. A mutation was found in the *CYBB* gene of 33 patients and 9 of these were novel: one non-sense mutation (c.1123 G>T), three missense mutations (c.58G>A; c.1076 G>C; c.1357 T>A), two splice site mutations (c.141+5G>T; c.142-1G>A), one duplication (c.42_45dupCATT), one deletion (c.184delT), and one rare deletion of two non-contiguous nucleotides (c.1287delT + c.1290delC). One patient had the most frequent GT homozygous deletion in exon2 of the NCF-1 gene encoding the p47^{phox} subunit of NADPH oxidase. The carrier analysis was performed in 23 patients' mothers and 16 female relatives through molecular and FISH studies. No clear correlation between the severity of clinical symptoms and the type of mutation could be demonstrated. This study further supports the great heterogeneity of the disease and the notion that genetic analysis is a critical step in obtaining a definitive diagnosis for CGD.

1. Introduction

Chronic Granulomatous Disease (CGD; MIM# 306400) is a primary immunodeficiency that affects phagocytes of the innate immune system caused by defects in the NADPH oxidase system. CGD patients are susceptible to recurrent life-threatening infections, particularly those caused by catalase positive bacteria and fungi. In addition, CGD patients often have poor wound healing and exaggerated inflammatory responses, leading to granuloma formation in subcutaneous tissues, lungs, lymph nodes, liver and bones (Segal et al., 2000; Winkelstein et al., 2000; Martire et al., 2008). The NADPH oxidase complex acts mainly by transferring electrons from NADPH to molecular oxygen in order to form superoxide indispensable for killing of phagocyted microorganisms. This complex is composed of a heterodimeric membrane-bound flavocytochrome b558 (formed by gp91^{phox} and p22^{phox}) and four cytoplasmatic subunits: p47^{phox}, p67^{phox}, p40^{phox} and Rac2. Mutations in any of the genes encoding NADPH oxidase subunits can cause CGD.

* Corresponding author at: Dipartimento di Biomedicina dell'Età Evolutiva, Università di Bari, Piazza Giulio Cesare 11, 70124 Bari, Italy. Fax: +39 080 5592290.
E-mail address: dematti a@bioetaev.uniba.it (D. De Mattia).
[1] These authors equally contributed to the study.

0161-5890/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.molimm.2009.03.016

Approximately two thirds of CGD cases result from mutations in the X-linked *CYBB* gene, encoding the gp91^{phox} subunit. The remaining patients with CGD have an autosomal recessive form (AR-CGD), the most frequent of which, is due to a mutation of *NCF1* gene that encodes the p47^{phox} subunit (Dinauer, 2005). Herein we report the results of *CYBB* and *NCF-1* gene sequence analysis in a cohort constituted by 34 CGD patients from 29 unrelated families and their relatives and we describe nine novel CYBB mutations. Furthermore, since the clinical severity of the CGD varies widely, we investigated the correlation between the rate of severe infections per patient-year and the mutation type and we did not find a statistically significant variation, although in patients with deletion and nonsense mutations a higher number of severe infections were found. Polymorphisms in oxygen independent antimicrobial systems or environmental factors should be investigated to find a correlation with the clinical severity of the disease.

2. Patients and methods

2.1. Patients

Thirty-four male patients, from the Italian registry of Chronic Granulomatous Disease which is part of the Italian Primary Immunodeficiency Network (IPINET), were tested for mutations in the *CYBB* gene. Patients were diagnosed as having CGD on the basis of their clinical history and the inability of their phagocytes to generate reactive oxygen species (ROS) by conventional granulocyte functional tests as previously described (Martire et al., 2008).

Age at diagnosis ranged from 1 month to 49 years (mean 7.16 years, median 3 years). Carrier detection was performed in 23 patients' mothers and 16 female relatives by means of genetic analysis and through fluorescent *in situ* hybridization (FISH) of metaphase chromosomes from lymphoblastoid cell lines when needed.

All relevant clinical features, immunological and therapeutic data have been already described in a previous report by the IPINET (Martire et al., 2008). All patients were put on antiinfective prophylaxis with trimethoprim/sulphomethoxazole and itraconazole early after diagnosis. Some subjects (patients 2, 4, 6, 8, 17, 20, 23, 24, 26–28, 32) received discontinuous administration of γ-interferon.

Informed consent was obtained from each studied subject or parents of each child and all procedures used were in accordance with the guidelines of the Helsinki Declaration on Human Experimentation.

All studies were performed at the Paediatric Immunology and Biotechnology Laboratory, Tor Vergata University of Rome and at the Department of Biomedicine of Development Age, University of Bari.

FISH studies were performed in the Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC, USA.

2.2. Cell lines

Neutrophils were purified by discontinuous Percoll density gradient centrifugation from peripheral blood of patients 17, 33 and 34 as previously described (Giudicelli et al., 1982). EBV-transformed lymphoblastoid cell lines (LCL) were obtained by incubating approximately 5×10^6 PBMC, with 2 ml of supernatant from the EBV-secreting cell line B95-8 for 1 h at 37 °C. Infected cells were cultured at 1×10^6 cells/ml in RPMI 1640 containing 20% fetal bovine serum, 2 mM L-glutamine and 1 μg/ml of cyclosporin A in 24-well culture plates. Clumps of cells were usually observed at the end of the first or second week and stable EBV-lymphoblastoid cell lines were established in 3–4 weeks.

2.3. Molecular studies

Genomic DNA isolated from peripheral blood of patients and healthy controls (QIAamp DNA Blood kit by QIAGEN GmbH, Hilden, Germany) was amplified using primers (available upon request) flanking the 13 coding exons and the promoter region of the *CYBB* gene (NG_009065). PCR reactions were carried out in a volume of 50 μl containing 100 ng of genomic DNA, 200 μM of each dNTP, 0.4 μM of each primer and 0.5 U of GoTaq DNA polymerase (Promega, Madison, WI). The samples were denatured at 95 °C for 5 min followed by 30–40 cycles at 95 °C for 30 s, annealing at exon specific temperature (available upon request) for 45 s, 72 °C for 30 s with a final 5 min extension at 72 °C.

DHPLC was performed on a WAVE analysis system (Transgenomic, Omaha, NE) using different temperature conditions (available upon request). Briefly, PCR products were mixed in approximately equimolar proportions with an amplified sample known to contain a wild-type *CYBB* fragment, then denatured at 94 °C for 5 min and then cooled at room temperature, to allow heteroduplex formation. Each sample were run on a DnaSept.M column (Transgenomics) and monitored by ultraviolet light (260 nm). Optimum DHPLC temperatures were detected using software-predicted melting profile as a starting point. Samples were run at a mean of three temperatures due to the heterogeneity in the distribution of GC-rich regions (Xiao and Oefner, 2001). Each elution chromatographic profile was compared with profiles associated with homozygous wild-type sequence. Samples with an altered chromatographic profile were sequenced.

Direct sequencing was performed after purifying PCR products with QIAquick PCR purification kit (QIAGEN, Hilden, Germany). Both strands were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and analyzed on an ABI PRISM 3130 and 310 automated sequencers (Applied Biosystems, Foster City, CA).

For the detection of GT homozygous deletion in exon 2 of *NCF-1* gene we used primers pair and PCR conditions already described (Noack et al., 2001), which do not differentiate between *NCF*-1 and *φNCF1*. DHPLC analysis revealed the homozygous profile of the patient 20 and the data was confirmed by sequence.

2.4. RT-PCR

When required, total RNA was isolated from either mononuclear cells or EBV-transformed B lymphocytes of both CGD patients and healthy individuals by using Trizol procedure (Invitrogen, Life Technologies, Milano, Italy). Reverse transcription was performed using a single reaction kit (Superscript, Invitrogen, Life Technologies, Milano, Italy) according to the manufacturer's instructions. Three overlapping following primer pairs were used for amplification of the cDNA (NM_000397.3): 1 for: 5′-ACTGGGCTGTGAATGAGGG-3′; 5 rev: 5′-CCATTCCACATTAAATAGATGTGC-3′; 3 for: 5′-GAAATCTGCTGTCCTTCCTCAG-3′; 8 rev: 5′-CCTTCTGTTGAGATCGCCA-3′; 7 for: 5′-GGAATGCCCAATCCCTCAG-3′; 13 rev: 5′-GGGCCAGACTCAGAGTTGG-3′. For the XK gene we used the following primer pairs: XK for: 5′-CAACATGTTCTGCTGGTCTGCTGT-3′; XK rev: 5′-AGAGAATGGCTGTGCAGTACCCAA-3′.

2.5. Immunoblot analysis

Lymphoblastoid cell lines or neutrophils from patients and healthy controls were lysed on ice cold JS lysis buffer (50 mM Tris/HCl pH 8, 150 mM NaCl, 1.5 mM $MgCl_2$, 5 mM EGTA, 1% Triton-X, 10% glycerol, 1 mM PMSF, aprotinin 1 mg/ml, leupeptin 1 mg/ml, pepstatin 1 mg/ml, 1 mM DTT) for 20 min on ice and then clarified by centrifugation at 12,000 rpm for 10 min at 4 °C. 25 μg of

total cell lysate was size-fractionated by SDS-PAGE gel and then transferred to nitrocellulose membrane (Protran by Schleicher & Schuell-Bioscience, Dassel, Germany). Membranes were blocked in 5% milk for 1 h at room temperature and then incubated, for 1 h at room temperature, with primary antibody (mAb48 kindly provided by Dr. D. Roos, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam). The specific binding of the antibody to protein band was detected using goat anti-mouse IgG conjugated with horseradish peroxidase and visualized by ECL (LiteAblot by Euroclone SpA, Switzerland).

2.6. FISH

FISH was performed on metaphase lymphoblastoid cell lines preparations using the CGD region probe directly labelled with Spectrum Orange and an alpha X chromosome control probe (Qbiogene) labelled with fluoroscein. The *CYBB* probe was DNA prepared by a long PCR method using from exons 2–4, 4–8, 8–13 of the *CYBB* region as already described (Simon et al., 2005). DNAs were directly labelled with Spectrum Orange (Vysis, Downer's Grove, IL) by nick translation and mixed with COT-1 and all PCR products were mixed as a probe cocktail for hybridizations. Ten metaphase chromosome preparations with two positive X chromosome control probes were scored for the presence of the test CGD probe. Images were analyzed with a Zeiss Axioskop (New York, NY) microscope equipped with a Cytovision imaging system (Applied Imaging, Pittsburgh, PA). The imaging system allows for separate exposures of the fluorochromes which assists in visualization of smaller red signal relative to the larger control green fluorochrome.

2.7. Statistical analysis

Participating centers were required to register all consecutive CGD cases adopting a web-base data-base created by Oracle and protected by IANUS® Technology, implemented at Interuniversity Computing Center (CINECA), in Bologna. Data were stored in a central database, organized at the Associazione Italiana Ematologia Oncologia Pediatrica (AIEOP) Operation Office.

Standard statistical descriptions of parameters were used to characterize the data (mean, median, and range). Fisher's exact test was used to compare differences in percentages for categorical variables, whereas Student's *t*-test was adopted to compare means for continuous variables.

The infection's incidence rate per patient-year of follow-up for main infection categories was calculated overall and for each group of mutation.

The measure of association used was the relative risk as estimated by the odds ratio. Summary relative risks were computed by the maximum likelihood method; a 95% confidence interval was calculated by use of a test-based estimation procedure. A two-tailed significance test for trend was computed by the Mantel extension of the Mantel–Haenszel procedure.

All *P* values were two-sided and values less than 0.05 were considered statistically significant. Analysis used December 31, 2007 as the reference date, i.e., the day at which all centers locked data on patient outcomes. Computations were performed using STATA package (StataCorp LP, College Station, TX, USA).

3. Results and discussion

Thirty-three out of the 34 CGD patients included in the present study had mutations in the *CYBB* gene (NG_009065), the remaining CYBB negative-CGD patient, had a GT homozygous deletion in exon 2 of *NCF-1* gene encoding the p47phox protein, due to recombination events between *NCF-1* and its two pseudogenes (φNCF1) that contain GT deletion (Noack et al., 2001). All mutations are detailed in Table 1. Mutations are described using the HGVS gene numbering system where nucleotide +1 corresponds to the A of the ATG initiation codon. The level of protein expression was documented in the most of patients and indicated in Table 1.

The mutations were widely distributed over the *CYBB* gene and affected both coding and non-coding regions. Nine out of 26 unique mutations found, were novel and submitted to XCGD database (http://www.uta.fi/imt/bioinfo/CYBBbase/), the other 17 had been previously published. All known mutations appear in the X-CGD database with the relative accession number referred to each detected patient and indicated in Table 1. The following nine novel mutations were identified:

c.58G>A: missense mutation in exon 2, resulting in the already described p.G20R amino acid change in the N-term domain, was detected in the patient 12. Of interest this patient displayed an extremely severe phenotype characterized by 2 episodes of brain abscess and pneumonia, 1 liver and 1 gluteal abscess and a high recurrence of dermatitis and gingivitis (21 and 20 episodes, respectively). Furthermore at the age of 12 years he was diagnosed having celiac disease.

c.1123G>T: non-sense mutation in exon 9, resulting in the p.E375X amino acid change, introducing a premature stop codon in the FADBD functional domain, was found in two unrelated patients (pts. 9 and 10).

c.1357T>A: missense mutation in exon 11, resulting in the already described W453R amino acid change in the NADPHBD (caused by c.1357T>C), was detected in two brothers (pts. 18 and 19). This mutation causes the substitution of a non-polar hydrophobic amino acid with a positively charge and hydrophilic one. Interestingly, the younger brother (pt. 19) was diagnosed at the age of 3 years because of severe and atypical infections while his older brother (pt. 18) was diagnosed later on, only on the basis of biochemical and molecular analysis but without symptoms of CGD (Finocchi et al., 2008).

c.1076G>C: missense mutation in exon 9, causing the amino acid change p.G359A in the FADBD functional domain, was found in the patient 17. This mutation was found to be *de novo*. Phylogenetic comparison underlined that G359 is a highly conserved residue (*Rattus norvegicus*, *Gallus gallus*, *Xenopus tropicalis*, *Ciona intestinalis*, *Danio rerio*) reinforcing its involvement in the disease. In this patient, the expression of gp91 subunit, tested in neutrophils and lymphoblastoid cell lines, was highly reduced but not absent, although residual ROS production was not observed.

c.184delT in exon 3, resulting in a frameshift at F62 with a premature stop codon at L66 (p.F62SfsX66), was found in patient 21. The patient's twin sisters were carriers of the same mutation. In one of them the prenatal diagnosis allowed the identification of a newborn female carrier.

c.1287delT and c.1290delC: deletions of two non-contiguous nucleotides was found in the patient 25. His mother was heterozygous for both deletions in DNA isolated from peripheral leucocytes. The analysis of the affected family pedigree revealed that the maternal grandmother, grandfather and maternal aunt were not carriers. These two non-contiguous base pairs, are located within a region with no evidence of repeated or palindromic sequences. Furthermore, neither the T deletion nor the A deletion have been reported in CYBBbase. This evidence, excludes the possibility of a mutational hot spot in this site. We hypothesized that a *de novo* mutation in the patient's mother, or a single or double deletion in healthy maternal grandfather germ line might have occurred (Lázaro et al., 1994; Crow, 2000; Dobrovolny et al., 2005). Paternity was confirmed using commercial STRs kit (AMPF1STR®IdentifilterTM, Applied Biosystem). Microsatellite analysis indicated that affected allele is present in proband's mother, maternal aunt and maternal grandfather (data not shown). However, the analysis of the DNA from maternal grandfather's spermatozoa failed to detect the presence of the single or

Table 1
Genotype and classification of CGD patients.

pt.	Age at diagnosis	Carrier's status	Western blot analysis	Exon intron	cDNA change	Accession nrs. on CYBBbase	Protein change
Non-sense mutations							
1	3 years	m carrier	X91^0	Ex 2	c.83 G>A	(A0563)	p.W28X
2	7 years	–	X91^0	Ex 2	c.127A>T	(A0261)	p.R43X
3	3 years	m carrier	X91^0	Ex 5	c.388C>T	(A0065, A0113, A0267-272, A0427-431, A0587, A0596)	p.R130X
4	10 years	–	X91^0	Ex 5	c.388C>T		p.R130X
5	8 months	m carrier	X91^0	Ex 5	c.469C>T	(A0074-75, A0095, A0098, A0152, A0177, A0274-277, A0433-434, A0565-568)	p.R157X
6	3 years	–	X91^0	Ex 7	c.691C>T	(A0421-422)	p.Q231X
7	10 months	m carrier + st carrier	X91^0	Ex 8	c.868C>T	(A0045-46, A0145, A0159, A0194, A0198,A0288-289,A0443-453, A0639)	p.R290X
8	13 years	–	X91^0	Ex 9	c.1006G>T	(A0087-88, A0291)	p.E336X
9	**3 years**	**m + a carriers**	**n.d.**	**Ex 9**	**c.1123G>T**	**A0610**	**p.E375X**
10	**2 years**	**m carrier**	**X91^0**	**Ex 9**	**c.1123G>T**	**A0610**	**p.E375X**
Missense mutations							
11	6 years	m carrier + st carrier	X91^0	Ex 1	c.1 A>G	(A0242)	p.M1V
12	**2 years**	**–**	**X91^0**	**Ex 2**	**c.58 G>A**	**A0647**	**p.G20R**
13^s	6 years	m carrier	X91$^-$	Ex 9	c.925G>A	(A0101-102, A0368-372)	p.E309K
14^s	1 years	m carrier	X91$^-$	Ex 9	c.925G>A	(A0101-102, A0368-372)	p.E309K
15^a	49 years	m carrier	X91$^-$	Ex 9	c.925G>A	(A0101-102, A0368-372)	p.E309K
16^a	12 years	m + 1st + 3 nieces carrier	X91$^-$	Ex 9	c.925G>A	(A0101-102, A0368-372)	p.E309K
17	**2 years**	**m not carrier**	**X91$^-$**	**Ex 9**	**c.1076G>C**	**A0611**	**p.G359A**
18	**6 years**	**m carrier + st not carrier**	**X91^0**	**Ex 11**	**c.1357T>A**	**A0613**	**p.W453R**
19	**3 years**	**m carrier + st not carrier**	**X91^0**	**Ex 11**	**c.1357T>A**	**A0613**	**p.W453R**
Deletion–duplication							
20*	5 years		A47^0	Ex2	c.73_74del	56 patients reported on ncf1base	p.V25VfsX51
21*	**16 years**	**m carrier + st1 + st2 carrier**	**X91^0**	**Ex 3**	**c.184delT**	**A0614**	**p.F62SfsX66**
22	4 years	m carrier	n.d.	Ex 6	c.484-100_674+291del		p.N162_E225del
23	4 years	–	X91^0	Ex 7	c.675+?_804+?del		p.R226_M268del
24	2 years	–	X91^0	Ex 7	c.755delG	(A0219-220, A0195)	p.G252EfsX254
25	**3 years**	**m carrier**	**X91^0**	**Ex10**	**c.1287delT + c.1290delC**	**A0617**	**p.C428fs**
26^c	1 year	–	n.d.	Ex 1-3	c.1+?_252+?del		p.M1_A84del
27^c	1 month	–	n.d.	Ex 1-3	c.1+?_252+?del		p.M1_A84del
28	2 years	m carrier + st carrier	X91^0	Ex 1–13	Del ≥550 kb+XK gene		–
29	4 years	m not carrier	X91^0	Ex 1–13	Del∼500 kb		–
30	**2 years**	**m carrier**	**n.d.**	**Ex 1**	**c.42_45dupCATT**	**A0619**	**p.L16HfsX35**
Splice site mutations							
31	**10 months**	**m carrier**	**X91^0**	**Int 2**	**c.141+5G>T**	**A0615**	**r.46_141del**
32	**6 years**	**m carrier**	**n.d.**	**Int 2**	**c.142-1G>A**	**A0616**	**r.142_252del+wt**
33	38 years	m carrier; st1 + st2 + st3 not carriers; st4 carrier	X91^0	Ex 3	c.252G>A	(A0022, A0127, A0063, A0100,A0193, A0226-228, A0230-231)	r.142_252del
34	23 years		X91^0	Ex 3	c.252G>A	(A0022, A0127, A0063, A0100,A0193, A0226-228, A0230-231)	r.142_252del

In bold patients with novel mutations are shown. m: mother; s: siblings; c: cousins, a: aunt; st: sister and *: dead.

double deletion, thus allowing possible a *de novo* mutation in the patient's mother.

c.141+5G>T: splice-site mutation in intron 2, resulting in the skipping of exon 2, was found in the patient 31. In this patient, the expression of the gp91 subunit in lymphoblastoid cell lines was absent.

c.142-1G>A: splice-site mutation in intron 2 was found in the patient 32. This mutation causes the skipping of exon 3 that does not generate coding frameshift. cDNA analysis revealed the presence of the exon 3 deleted cDNA form and a small amount of wild-type cDNA deriving from a residual normal mRNA splicing. However, the patient's family history revealed that two brothers deceased at the age of 2 months and 3 years, due to pulmonary disease and a third affected brother died at the age of 28 years due to lung aspergillosis. This mutation was excluded in 100 healthy donors.

c.42_45 dupCATT: mutation in the 3′ end of exon 1 was detected in the patient 30. His mother was heterozygous for the same mutation. cDNA analysis demonstrated that this duplication did not affect the splicing of exon 1 but was conserved in the transcript causing a frameshift at L16 with a premature stop codon at I35.

The remaining mutations have been previously reported and indicated in the X-CGD mutation database (Roos, 1996; Heyworth et al., 2001).

Particularly, we found six cases of *deletions*: two unrelated cases (pts. 28 and 29) harbouring a deletion encompassing the whole gene, two cousins (pts. 26 and 27) with a deletion including the exons 1–3 (the upstream deletion breakpoint is under investigation), one case (pt. 22) with a 581 bp deletion including the exon 6, one case where the deletion c.755delG resulted in a frameshift with a premature stop codon at I254 (pt. 24) and the last one that included exon 7 (pt. 23). The deletion breakpoints in the patients 28 and 29 have not been characterized, however in patient 28 the deletion was large enough to affect from the adjacent XK gene (OMIM +314850) whose lack is responsible of the Mc Leod syndrome,

until the SRPX gene (OMIM 300187), hypothetically involved in the pathogenesis of retinitis pigmentosa. The length of the deletions was deduced amplifying specific genomic regions of the genes included in the short arm of X chromosome at Xp21 where *CYBB*, XK and SRPX genes reside (Peng et al., 2007). Of note, the patient 28 did not present signs or symptoms suggestive of retinitis pigmentosa neither of Mc Leod phenotype. He only displayed weak expression of Kell erythrocyte antigens, differently from its normal expression in pt. 29, and red cell acanthocytosis (Redman et al., 1999; Peng et al., 2007).

With respect to the *point mutations*, we described a c.925G>A missense mutation (p.E309K) in exon 9 in a large family with 4 affected members (pts. 13–16). This mutation caused an X91$^-$ phenotype with reduced gp91 expression. We also detected a missense mutation at the start codon of the gene (pt. 11). We found seven *non-sense mutations* all reported in Table 1. We described a total of seven CG → TG and CG → CA (pts. 3, 4, 5, 7, 13–16 related, 33, 34) transitions out of 19 single nucleotide substitutions (36.8%). These mutations are among the most frequent causes of human genetic disease phenotypes due to the well known mechanism, restricted to CpG dinucleotides, of methylation–deamination of cytosine which leads to formation of thymidine. In our cohort, the mutations c.388C>T, c.469C>T, c.868C>T, c.925G>A that involve CpG nucleotides, are respectively, already found in 16, 16, 20 and 7 patients reported in the CYBBbase. Moreover the mutations c.388C>T and c.252G>A are recurrent in our relatively little cohort, thus reinforcing the probability of a common molecular mechanism in the onset of these mutations (Roos et al., 1996; Kannengiesser et al., 2008). The *splice-site mutation* c.252G>A, in exon 3 adjacent to intron 3 of the *CYBB* gene, was found in two unrelated patients (pts. 33 and 34). This mutation, leading to exon 3 skipping in the most of transcripts, was already described (Roos et al., 1996; Brunner et al., 2007; Ishibashi et al., 2001; Kannengiesser et al., 2008).

In 20 families, after the identification of the patient's mutation, we analyzed the X-CGD carrier status in mothers and female family members. Carrier detection revealed the mutated gene in 90% of the mothers with two *de novo* mutations representing 10% of the studied cases, similarly as seen in previous report (Rae et al., 1998). The identification of carrier status in mothers of large deletion patients 28 and 29, was not carried out by sequencing because of amplification of normal copy of the gene in the homologous X chromosome. This issue was addressed by FISH. As shown in Fig. 1, FISH analysis revealed that the mother of patient 28 was carrier of the large deletion and microsatellites study confirmed the same status for his sister (data not shown).

Beside the heterogeneity of the mutations identified, a wide variability of the clinical phenotype related to the same mutation was also found; i.e., siblings 13–14 and 18–19: onset of severe infections shortly after birth in patients 14 and 19 and absence of clinical manifestations in their older brothers (pts. 13 and 18) who were diagnosed later on, only once patients 14 and 19 were found affected.

Of interest, the only patient with AR-CGD who might be expected to have fewer infections and a mild phenotype (Winkelstein et al., 2000), showed instead a severe clinical history and died regardless the early institution of appropriate long-term prophylaxis. Furthermore, in patient 29 the deletion encompassing the whole gene results in a milder disease phenotype than expected.

Conversely, it is interesting the clinical similarity observed in two of the oldest unrelated patients (pts. 33 and 34) of our cohort, who presented the same splice site mutation c.252G>A. Both patients showed a similar clinical phenotype (good clinical conditions still in adult age in absence of appropriate anti-infectious prophylaxis) suggesting a correlation, probably due to residual ROS production (Brunner et al., 2007), between this specific mutation and a leaky phenotype. However the analysis of the cDNA from these patients failed to detect wild-type cDNA deriving from a residual normal mRNA splicing.

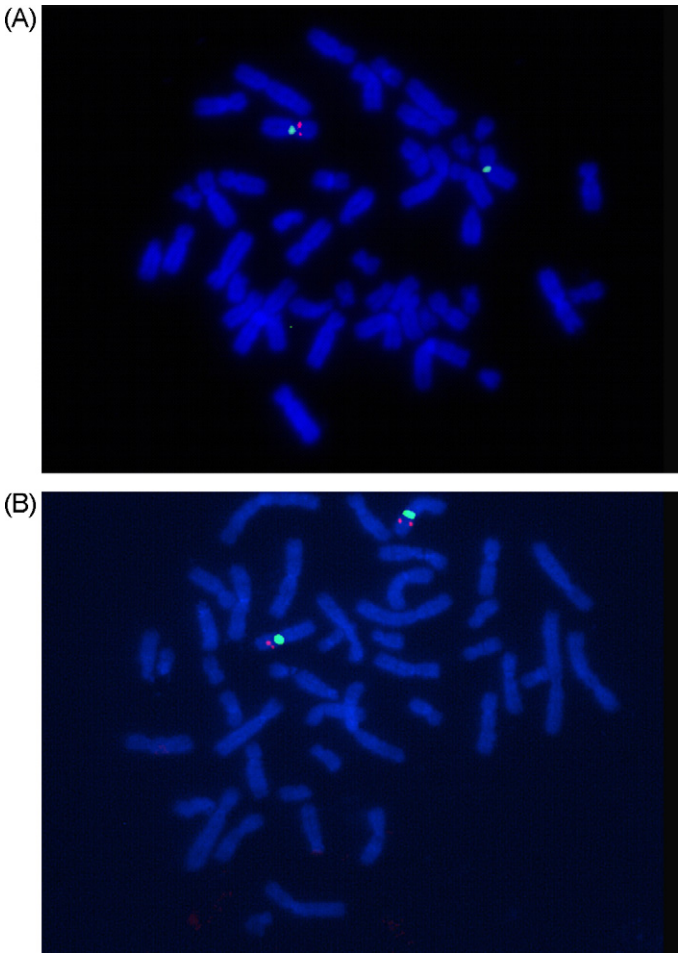

Fig. 1. (A) Mother of the CGD patient 28: carrier. (B) Mother of the CGD patient 29: non-carrier.

In order to define a possible correlation between molecular defect and clinical course of the disease, a retrospective survey of patients from the time of diagnosis to December 2007 was carried out. Clinical data were available for 29 patients: 3 with splice mutation (pts. 31–33), 8 with missense (pts. 11–17, 19), 10 with deletion (pts. 20–24, 26–30), and 8 with non-sense mutations (pts. 2–7, 8, 10), respectively. The infections were described according to the site of infection and the infective agent when available. An infection was considered severe when hospitalization or treatment with parenteral antibiotics or antifungals was required. The frequency and type of severe infections are shown in Table 2. At last follow-up, the patients' mean age was 16.1 years (median 15, range 1–42), mean duration of follow-up was 9.3 years while the total period of observation was 271 years. The mean ages at first infection and at diagnosis were not statistically different in the four groups of patients. The rate of severe infections per patient-year, did not significantly vary in the four groups of mutations ($P > 0.05$) (Table 3) and no evidence of genotype–phenotype correlation was detected, although all the deaths occurred in patients having deletions. The molecular analysis of our series of patients with X-linked CGD underlines the breadth and the heterogeneity of mutations responsible for the disease.

It is likely that other factors may affect the clinical severity of CGD such as polymorphisms in oxygen-independent antimicrobial systems or other components of the immune system. However systematic characterization of molecular defect in larger cohorts of

Table 2
Localization and type of severe infections in 29 CGD patients.

Infection	Mutation			
	Splice *N patients* = 3	Missense *N patients* = 8	Deletion *N patients* = 10	Non-sense *N patients* = 8
Pneumonia	2	7	20	12
Lung abscess	0	0	1	0
Dermatitis	0	1	1	1
Subcutaneous abscess	0	0	1	4
Lymphadenitis	1	2	7	7
Septicemia	1	0	3	0
Colitis	0	0	0	1
Enteritis	1	0	0	1
Brain abscess	0	2	2	0
Liver abscess	1	1	4	3
Otitis	0	3	0	0
Kidney abscess	0	1	0	0
Osteomyelitis	0	0	3	1
Perianal abscess	1	1	0	1
Gluteal abscess	0	0	0	0
Luti	0	0	0	1
Total infections	7	18	42	32

Table 3
Patients characteristics and incidence of infections in the different groups of mutations.

	Splice (*N patients* 3)	Missense (*N patients* 8)	Deletion (*N patients* 10)	Non-sense (*N patients* 8)	*P*					
					SvsM	SvsD	SvsN	MvsD	MvsN	DvsN
N of total infections	11	79	52	43						
N of severe infections	7	18	42	32						
N of patient-years of observation	15	46	125	85						
Mean age at 1° infection (months)	2.3	7.5	13.8	7.7	0.439	0.082	0.278	0.393	0.958	0.353
Mean age at diagnosis (years)	14.3	9.7	4.5	4.4	0.700	0.171	0.200	0.397	0.384	0.960
Rate of severe infections per patient-year	0.46	0.39	0.33	0.37	0.677	0.417	0.589	0.581	0.886	0.626

P-value < 0.05 was considered statistically significant. The infection rate per patient-year of follow-up was calculated as: number of infections/number of years of observation.

patients might help us to better relate clinical form and functional data to the type of the mutation found. In any case mutation analysis remains an essential tool for genetic counselling and prenatal diagnosis of CGD.

Acknowledgments

We thank all the families for their participation in the study and for providing us the written consent for publication on their clinical and molecular data.

We thank Dr. D. Roos for critical reading of the manuscript and for the generous gift of monoclonal antibodies to gp91-phox. The authors wish to thank Dr. A. Filareto for technical assistance, Dr. F. Angelini and Dr. E. Giardina for their helpful suggestions.

References

Brunner, J., Dockter, G., Rösen-Wolff, A., Roesler, J., 2007. X-linked chronic granulomatous disease (CGD) caused by an intra-exonic splice mutation (CYBB exon 3, c.262G → A) is mimicking juvenile sarcoidosis. Clin. Exp. Rheumatol. 25 (2), 336–338.

Crow, J.F., 2000. The origins, patterns and implications of human spontaneous mutation. Nat. Rev. Genet. 1, 40–47.

Dinauer, M.C., 2005. Chronic granulomatous disease and other disorders of phagocyte function. Hematol. Am. Soc. Hematol. Educ. Program 1, 89–95.

Dobrovolny, R., Dvoráková, L., Ledvinová, J., Magage, S., Bultas, J., Lubanda, J.C., Poupetová, H., Elleder, M., Karetová, D., Hrebícek, M., 2005. Recurrence of Fabry disease as a result of paternal germline mosaicism for alpha-galactosidase a gene mutation. Am. J. Med. Genet. A 134, 84–87.

Finocchi, A., Palma, P., Di Matteo, G., Chiriaco, M., Lancella, L., Simonetti, A., Rana, I., Livadiotti, S., Rossi, P., 2008. Visceral leishmaniasis revealing chronic granulomatous disease in one child. Int. J. Immunopathol. Pharmacol. 3, 739–743.

Giudicelli, J., Philip, P.J., Delque, P., Sudaka, P., 1982. A single-step centrifugation method for separation of granulocytes and mononuclear cells from blood using discontinuous density gradient of Percoll. J. Immunol. Methods 15, 43–46.

Heyworth, P.G., Curnutte, J.T., Rae, J., Noack, D., Roos, D., van Koppen, E., Cross, A.R., 2001. Hematologically important mutations: X-linked chronic granulomatous disease (second update). Blood Cells Mol. Dis. 27, 16–26.

Ishibashi, F., Mizukami, T., Kanegasaki, S., Motoda, L., Kakinuma, R., Endo, F., Nunoi, H., 2001. Improved superoxide-generating ability by interferon gamma due to splicing pattern change of transcripts in neutrophils from patients with a splice site mutation in *CYBB* gene. Blood 98, 436–441.

Kannengiesser, C., Gérard, B., El Benna, J., Henri, D., Kroviarski, Y., Chollet-Martin, S., Gougerot-Pocidalo, M.A., Elbim, C., Grandchamp, B., 2008. Molecular epidemiology of chronic granulomatous disease in a series of 80 kindreds: identification of 31 novel mutations. Hum. Mutat. 29 (9), 132–149.

Lázaro, C., Ravella, A., Gaona, A., Volpini, V., Estivill, X., 1994. Neurofibromatosis type 1 due to germ-line mosaicism in a clinically normal father. N. Engl. J. Med. 331, 1403–1407.

Martire, B., Rondelli, R., Soresina, A., Pignata, C., Broccoletti, T., Finocchi, A., Rossi, P., Gattorno, M., Rabusin, M., Azzari, C., Dellepiane, R.M., Pietrogrande, M.C., Trizzino, A., Di Bartolomeo, P., Martino, S., Carpino, L., Cossu, F., Locatelli, F., Maccario, R., Pierani, P., Putti, M.C., Stabile, A., Notarangelo, L.D., Ugazio, A.G., Plebani, A., De Mattia, D., IPINET, 2008. Clinical features, long-term follow-up and outcome of a large cohort of patients with Chronic Granulomatous Disease: an Italian multicenter study. Clin. Immunol. 126, 155–164.

Noack, D., Rae, J., Cross, A.R., Ellis, B.A., Newburger, P.E., Curnutte, J.T., Heyworth, P.G., 2001. 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 97, 305–311.

Peng, J., Redman, C.M., Wu, X., Song, X., Walker, R.H., Westhoff, C.M., Lee, S., 2007. Insights into extensive deletions around the XK locus associated with McLeod phenotype and characterization of two novel cases. Gene 392 (1–2), 142–150.

Rae, J., Newburger, P.E., Dinauer, M.C., Noack, D., Hopkins, P.J., Kuruto, R., Curnutte, J.T., 1998. X-Linked chronic granulomatous disease: mutations in the *CYBB* gene encoding the gp91-phox component of respiratory-burst oxidase. Am. J. Hum. Genet. 62, 1320–1331.

Redman, C.M., Russo, D., Lee, S., 1999. Kell, Kx and the McLeod syndrome. Baillieres Best Pract. Res. Clin. Haematol. 12, 621–635.

Roos, D., de Boer, M., Kuribayashi, F., Meischl, C., Weening, R.S., Segal, A.W., Ahlin, A., Nemet, K., Hossle, J.P., Bernatowska-Matuszkiewicz, E., Middleton-Price, H., 1996. Mutations in the X-linked and autosomal recessive forms of chronic granulomatous disease. Blood 87 (5), 1663–1681.

Roos, D., 1996. X-CGDbase: a database of X-CGD-causing mutations (CYBBbase). Immunol. Today 17 (11), 517–521.

Segal, B.H., Leto, T.L., Gallin, J.I., Malech, H.L., Holland, S.M., 2000. Genetic, biochemical, and clinical features of chronic granulomatous disease. Medicine 79, 170–200.

Simon, K.C., Noack, D., Rae, J., Curnutte, J., Sarraf, S., Kolev, V., Blancato, J.K., 2005. Long of X-polymerase chain reaction-based fluorescence in situ hybridization analysis of female carriers of X-linked chronic granulomatous disease deletions. J. Mol. Diagn. 7, 183–186.

Winkelstein, J.A., Marino, M.C., Johnston Jr., R.B., Boyle, J., Curnutte, J., Gallin, J.I., Malech, H.L., Holland, S.M., Ochs, H., Quie, P., et al., 2000. Chronic granulomatous disease. Report on a national registry of 368 patients. Medicine 79, 155–156.

Xiao, W., Oefner, P.J., 2001. Denaturing high-performance liquid chromatography: a review. Hum. Mut. 17, 439–474.