The use of reverse transcription-PCR for the diagnosis of X-linked chronic granulomatous disease

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Abstract

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Chronic granulomatous disease (CGD) is an inherited disorder of the innate immune system characterized by a defective oxidative burst of phagocytes and subsequent impairment of their microbicidal activity. Mutations in one of the NADPH-oxidase components affect gene expression or function of this system, leading to the phenotype of CGD. Defects in gp91-*phox* lead to X-linked CGD, responsible for approximately 70% of CGD cases. Investigation of the highly heterogeneous genotype of CGD patients includes mutation analysis, Northern blot or Western blot assays according to the particular case. The aim of the present study was to use reverse transcription (RT)-PCR for the analysis of molecular defects responsible for X-linked CGD in eight Brazilian patients and to assess its potential for broader application to molecular screening in CGD. Total RNA was prepared from Epstein B virus-transformed B-lymphocytes and reverse transcribed using random hexamers. The resulting cDNA was PCR-amplified by specific and overlapping pairs of primers designed to amplify three regions of the gp91-*phox* gene: exons 1-5, 3-9, and 7-13. This strategy detected defective gp91-*phox* expression in seven patients. The RT-PCR results matched clinical history, biochemical data (nitroblue tetrazolium or superoxide release assay) and available mutation analysis in four cases. In three additional cases, RT-PCR results matched clinical history and biochemical data. In another case, RT-PCR was normal despite a clinical history compatible with CGD and defective respiratory burst. We conclude that this new application of RT-PCR analysis - a simple, economical and rapid method - was appropriate for screening molecular defects in 7 of 8 X-linked CGD patients.

Introduction

Chronic granulomatous disease (CGD) is a primary immunodeficiency originally described in 1957 as a clinical entity affecting male infants and named, at the time, fatal granulomatous disease of childhood. The main characteristics of CGD are recurrent and severe infections involving the natural barriers of the organism such as the respiratory tract and lymph nodes, and eventually inner structures such as the liver, spleen, bones, and brain (1-3). The estimated incidence of this rare disease is 1/250,000 live births per year. The infections are generally caused by catalase-negative bacteria such as *Staphylococcus aureus*, Gram-negative bacilli, and fungal species such as *Aspergillus*, *Candida* and *Nocardia* (4,5).

The NADPH-oxidase system generates superoxide and other reactive oxygen intermediates, crucial for the microbicidal activity of phagocytes. The biochemical defect in CGD is an impairment of NADPH-oxidase activity and subsequent inability to destroy microorganisms (6). The main components of the NADPH-oxidase system are gp91-, p22-, p47-, p67-, and p40-*phox*. Molecular defects causing CGD are generally due to absence, low expression or malfunctioning of one of the NADPH-oxidase components. The X-linked form of this disease is caused by defects in gp91-*phox*, the heavy chain of cytochrome b_{588}, and accounts for approximately 70% of all cases (7,8). The autosomal recessive forms are caused by defects in one of the cytosolic components of NADPH oxidase (p47- or p67-*phox*, respectively in 20 and 5% of cases), or the cytochrome b_{588} light chain component (p22-*phox*, 5% of cases) (9,10). CGD is a highly heterogeneous condition: over 300 mutations have been registered in an internationally maintained X-CGD database (8). The mutations have been distributed largely within the 13 exons or at the exon/intron boundaries of the gp91-*phox* (*CYBB*) gene and almost 200 of these mutations are unique.

The diagnosis of CGD is generally based on the clinical characteristics of the disease plus defective NADPH-oxidase activity as demonstrated by abnormal nitroblue tetrazolium (NBT), dihydrorhodamine 123, or superoxide release assays (11,12). In the stimulated NBT test, normal individuals display nearly 100% positive cells, while in CGD patients fewer than 5% of the cells are positive (13). In addition, cells from patients with variant CGD are positive, but show only very low activity (6). The NBT test also detects the carriers of X-linked CGD (mothers and sisters). A definitive molecular CGD diagnosis is established in patients with an abnormal NBT test or respiratory burst activity who have one of the following characteristics: a mutation in gp91-, p22-, p47-, or p67-*phox*; absent mRNA for one of these genes detected by Northern blot analysis; and/or absent protein for one of these oxidase components by Western blot. A genetic, but not molecular, diagnosis can be established by demonstration of maternal cousins, uncles, or nephews with an abnormal NBT test or respiratory burst (14). Thus, the establishment of a definitive diagnosis of this rare and highly heterogeneous disease requires complex and expensive methodologies such as a combination of Northern blot or Western blot, and single-strand conformation polymorphism analysis (SSCP) followed by DNA sequencing of several family members, all performed in high complexity research laboratories.

The reverse transcription-PCR (RT-PCR) method involves the amplification of cDNA by PCR. This technique can be easily standardized in less sophisticated laboratories. It provides information about gene expression and preliminary data about the structure or size of the mRNA of the defective component. RT-PCR has seldom been used in CGD research, being limited to pathophysiology studies (15-29). To date, the potential use of this useful tool for establishing the definitive

diagnosis of X-linked CGD, the most frequent form, has not been extensively investigated. The aim of the present study was to evaluate the use of RT-PCR for screening molecular defects responsible for X-linked CGD, a rare and possibly misdiagnosed immunodeficiency, in eight Brazilian patients.

Patients and Methods

Patients

The study included 8 unrelated male Brazilian patients with probable X-linked CGD (2 Blacks and 6 Caucasians; age 2-8 years; height 88-108 cm; weight 11-19 kg). The patients presented clinical histories of recurrent severe infections such as pneumonia, lymphadenitis, liver abscess, pyodermitis, and adverse reactions to BCG immunization. They were referred to our laboratory for biochemical and molecular diagnostic evaluation. Written informed consent was obtained from the participants prior to the study. The Medical School Ethics Committee approved the protocol in accordance to the Helsinki Convention and Brazil Ministry of Health, Resolution 196/96.

Biochemical diagnosis of CGD

The biochemical diagnosis of CGD was established according to the Pan American Group for Immunodeficiencies criteria. An impairment of NADPH-oxidase activity was demonstrated by the NBT slide test and/or the superoxide anion release assay by peripheral blood neutrophils and mononuclear leukocytes (14,30,31). Neutrophils and mononuclear leukocytes were obtained by centrifugation of blood samples over a Ficoll-Hypaque density gradient (32).

The NBT slide test was based on the reduction of NBT to formazan by activated leukocytes (31). The assay was performed as previously described (30). More than 95% of 200 normal neutrophils stimulated with

30 nM phorbol 12-myristate 13-acetate (PMA) should be able to reduce NBT. Absent reaction or <5% positive cells was considered to indicate a diagnosis of CGD (14).

Quantitative superoxide release by neutrophils and mononuclear leukocytes was assessed by a modified superoxide dismutase-inhibitable cytochrome c reduction assay (33-35). The amount of superoxide released was calculated using an extinction coefficient of 0.21 nM/cm for cytochrome c. The results are reported as nmol superoxide released by 10^6 cells per hour. Patients with CGD showed less than 10% of control values.

Screening molecular defects responsible for X-linked CGD

B-lymphocytes from X-linked CGD patients were transformed *in vitro* with Epstein B virus (EBV) (15,16) in order to provide an abundant source of nucleic acids for molecular studies. The EBV-transformed B cell lines reproduce the biochemical and molecular defects of CGD patients (15,16,36), and eliminate the need for repeated blood collections. Briefly, peripheral blood leukocytes from X-linked CGD patients were cultured with supernatants from B95-8, an EBV-producer cell line (15,16,36,37), in RPMI 1640 medium supplemented with heat-inactivated fetal bovine serum (10%), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml of streptomycin, at 37ºC, in a humid atmosphere with 5% CO_2. Cell viability was monitored and the cultures were maintained throughout the study period.

RNA samples from EBV-transformed B-cell lines were prepared by the guanidine HCl method, followed by ethanol precipitation and quantification by standard methods (38,39). The cDNA samples were obtained by reverse transcription of 2 µg of total RNA with SuperScript II RT (GIBCO BRL) and random hexamers (15). The quality of the mRNA samples was checked by PCR amplification of ß-actin, a constitutive gene con-

trol (bp 920-943 and bp 1494-1471) (Gen Bank accession No. NM001101).

gp91-*phox* gene expression was assessed by RT-PCR. Specific and overlapping pairs of primers (Gen Bank accession No. NM_000397, Table 1) were used to amplify (30 cycles) three gp91-*phox* exonic regions: 1-5, 3-9, and 7-13. This strategy permitted us to screen all gp91-*phox* exons. PCR products were analyzed by 2% agarose gel elec-

trophoresis and stained with ethidium bromide.

Relative gp91-*phox* gene expression was analyzed with the Image Master Software (Pharmacia-Biotech). The densitometry result for target samples was divided by the densitometry result of ß-actin, a constitutive gene control, normalizing the level considered for analysis. The results of RT-PCR assays were compared to patient clinical his-

Table 1. Oligonucleotide primers used for gp91-*phox* RT-PCR analysis.

Region	Sequence of gp91-*phox* primers	
	Forward	Reverse
Exons 1-5	5'-GCT CTA GAG CAT GAG GGG CTC TCC ATT TTT GTC A-3'	5'-CGG GAT CCC GAG TTC AGA GAG TGC TAC TGA ATA A-3'
Exons 3-9	5'-GCC TGC CTG AAT TTC AAC-3'	5'-TCA TCT GTA GCT CGA TG-3'
Exons 7-13	5'-GGA ATG CCC AAT CCC TCA G-3'	5'-GGG CCA GAC TCA GAG TTG G-3'

Table 2. Stimulated respiratory burst activity of granulocytes (GRA) and mononuclear leukocytes (MON) as assessed by the nitroblue tetrazolium (NBT) test or superoxide release assay, and gp91-*phox* gene expression in Epstein B virus-transformed B lymphocytes from patients with chronic granulomatous disease as assessed by RT-PCR analysis.

Patient	Respiratory burst activity		Relative gp91-*phox* expression		
	NBT	Superoxide	Exons 1-5	Exons 3-9	Exons 7-13
1. G.M.		MON 1.0	Reduced	Reduced	Absent
2. R.B.	<5%		Reduced	Reduced	Reduced
3. G.G.	<5%		Possible splicing defect	Absent	Absent
4. T.P.B.	<5%	MON 0.27 GRA 0.22	Normal expression	Normal expression	Normal expression
5. M.F.	<5%	GRA 0.22	Normal expression	Reduced	Reduced
6. T.P.	<5%	MON 0.25 GRA 0.46	Normal expression	Reduced	Absent
7. R.S.		MON -0.03 GRA 0.58	Possible splicing defect	Absent	Reduced
8. J.E.M.	<5%	MON 0.0 GRA 0.3	Absent	Absent	Absent

NBT is reported as percent of positive cells. Normal individuals display nearly 100% positive cells. GRA and MON from healthy controls released superoxide in the range of 6-14 and 5-7 nmol O_2^- 10^6 cells^{-1} 60 min^{-1}, respectively.

tory, biochemical assays (NBT and/or super-oxide release assay), and available mutation analysis data (http://www.sbi.org.br/Sbi2003/index.htm).

Results

We studied 8 male patients with clinical histories of recurrent severe infections, referred to our laboratory for biochemical and molecular diagnosis of CGD. The results of the NBT slide tests and the superoxide release assays are shown in Table 2. Six of the patients presented less than 5% positive leukocytes in the NBT slide test. Six patients showed impaired superoxide release by granulocytes and/or mononuclear leukocytes (less than 10% compared to healthy controls). Four patients had abnormal results in both tests. All patients presented at least one abnormal test and received the diagnosis of probable X-linked CGD. The mother and the sister of patient T.B.P. presented an NBT slide test compatible with the carrier status of X-linked CGD. During the study period

one patient (G.M.) died from pneumonia.

We next investigated the definitive diagnosis of X-linked CGD by RT-PCR analysis of gp91-*phox* gene expression. The results of RT-PCR amplification with three sets of overlapping primers are presented in Table 2. The quality of the mRNA sample was checked by PCR amplification of ß-actin, a constitutive gene control. The expected normal products were obtained in this case (Figures 1, 2 and 3).

The amplification of exons 1-5 detected two patients (G.M. and R.B.) with reduced gp91-*phox* gene expression (Figure 1, lanes 1 and 2, respectively). One patient (J.E.M., lane 8) showed absent gp91-*phox* gene expression. Two patients (G.G. and R.S.; Figure 1, lanes 3 and 7, respectively) presented a smaller PCR product (between 300 and 400 bp), suggesting a splicing defect or small deletion. Three patients presented normal gp91-*phox* expression (T.P.B., M.F. and T.P.; Figure 1, lanes 4, 5, and 6, respectively).

The results of RT-PCR amplification of gp91-*phox* exons 3-9 are presented in Figure
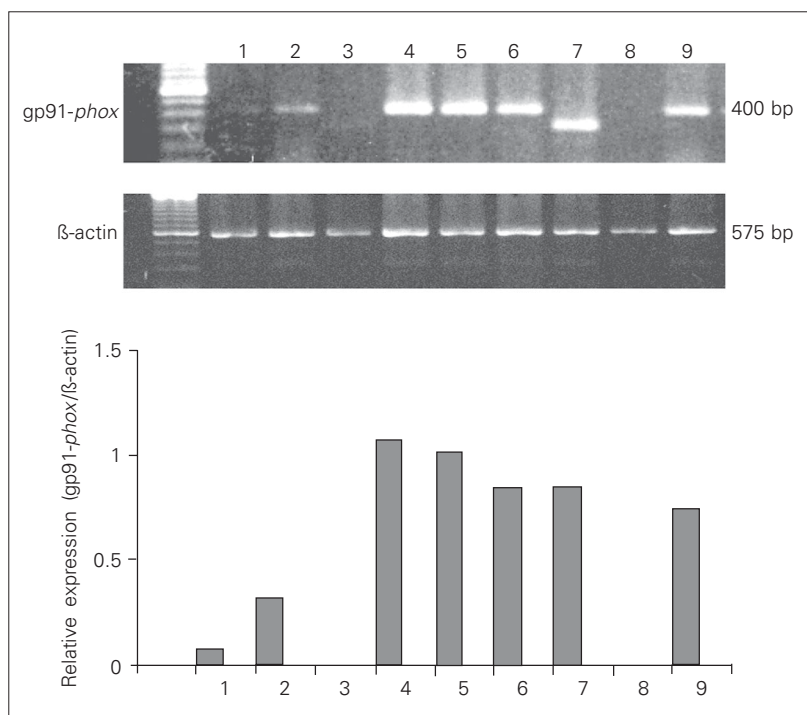

Figure 1. RT-PCR analysis of gp91-*phox* gene expression (exons 1-5) in Epstein B virus-transformed B cells from patients with X-linked chronic granulomatous disease. Representative 2% agarose gel electrophoresis of PCR products stained with ethidium bromide. Size standards (100-bp ladder); *Lane 1*, patient G.M.; *lane 2*, patient R.B.; *lane 3*, patient G.G.; *lane 4*, patient T.P.B.; *lane 5*, patient M.F.; *lane 6*, patient T.P.; *lane 7*, patient R.S.; *lane 8*, patient J.E.M., and *lane 9*, healthy control. ß-actin was used as an internal control for RT-PCR in all samples. The lower panel shows the mean band densitometry of gp91-*phox*/ß-actin relative expression (N = 3).

Figure 2. RT-PCR analysis of gp91-*phox* gene expression (exons 3-9) in Epstein B virus-transformed B cell lines from patients with X-linked chronic granulomatous disease. Representative 2% agarose gel electrophoresis of PCR products stained with ethidium bromide. Size standards (100-bp ladder); *Lane 1*, patient G.M.; *lane 2*, patient R.B.; *lane 3*, patient G.G.; *lane 4*, patient T.P.B.; *lane 5*, patient M.F.; *lane 6*, patient T.P.; *lane 7*, patient R.S.; *lane 8*, patient J.E.M., and *lane 9*, healthy control. ß-actin was used as an internal control for RT-PCR in all samples. The lower panel shows the mean band densitometry of gp91-*phox*/ß-actin relative expression (N = 3).
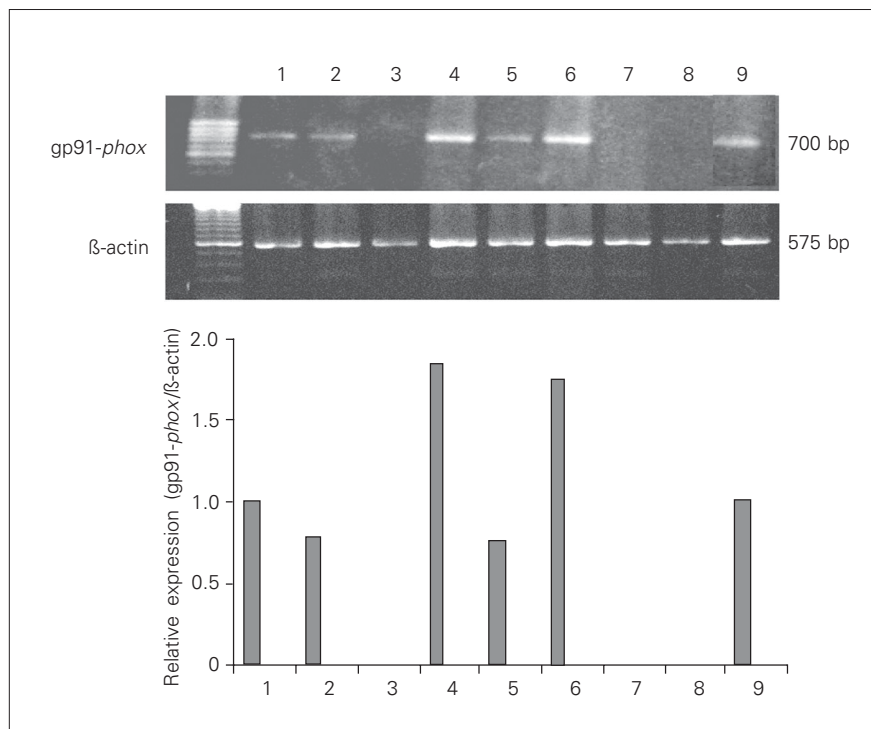

Figure 3. RT-PCR analysis of gp91-*phox* gene expression (exons 7-13) in Epstein B virus-transformed B cell lines from X-linked CGD patients. Representative 2% agarose gel electrophoresis of PCR products stained with ethidium bromide. Size standards (100-bp ladder); *Lane 1*, patient G.M.; *lane 2*, patient R.B.; *lane 3*, patient G.G.; *lane 4*, patient T.P.B.; *lane 5*, patient M.F.; *lane 6*, patient T.P.; *lane 7*, patient R.S.; *lane 8*, patient J.E.M., and *lane 9*, healthy control. ß-actin was used as an internal control for RT-PCR in all samples. The lower panel shows the mean band densitometry of gp91-*phox*/ß-actin relative expression (N = 3).
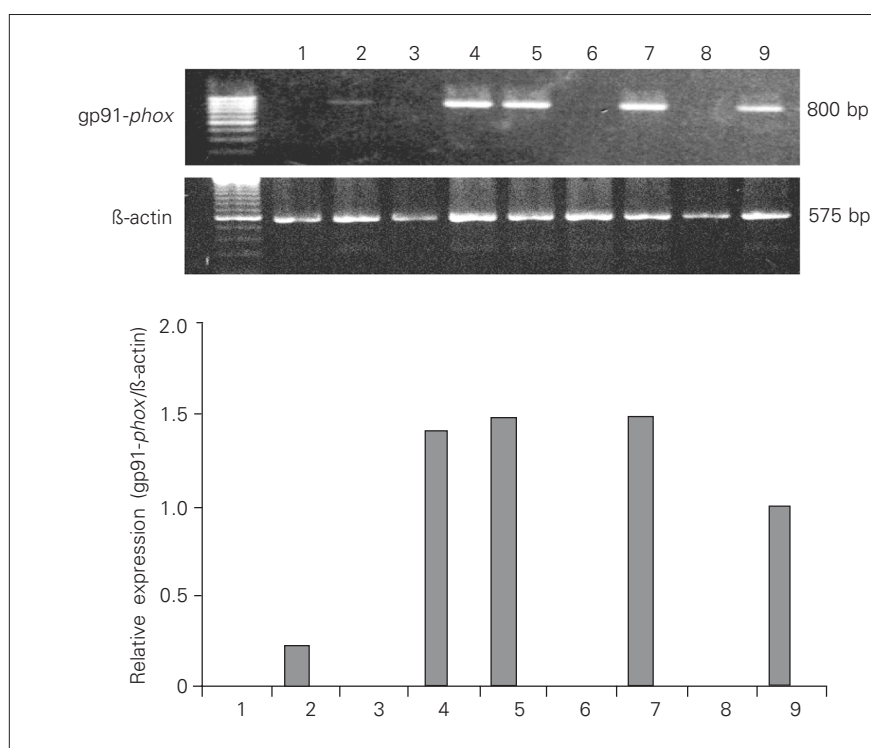
2. Two patients (R.B. and M.F.) presented reduced gp91-*phox* gene expression (Figure 2, lanes 2 and 5, respectively). Three patients (G.G., R.S., and J.E.M.; lanes 3, 7, and 8, respectively) presented absent gp91-*phox* gene expression. Three patients presented normal gp91-*phox* expression (G.M., T.P.B. and T.P.; Figure 2, lanes 1, 4 and 6, respectively).

RT-PCR amplification of gp91-*phox* exons 7-13 is presented in Figure 3. One patient (R.B.) presented reduced gp91-*phox* gene expression (Figure 3, lane 2). Four patients (G.M., G.G., T.P. and J.E.M., lanes 1, 3, 6, and 8, respectively) presented absent gp91-*phox* gene expression. Three patients (T.P.B., M.F. and R.S.; Figure 3, lanes 4, 5 and 7, respectively) presented normal gp91-*phox* expression.

In four cases, it was possible to match RT-PCR data with available mutation analysis, presented elsewhere by Patiño et al. (30) or by our group (http: //www.sbi.org.br/Sbi2003/index.htm): J.E.M. presented a $C_{469} \rightarrow T$ transition in exon 5, predicting a nonsense mutation (R157X). M.F. presented a nonsense substitution in exon 3, R (arginine) 73-Stop. R.S. showed a 264 $G \rightarrow A$ substitution at the 3' splice junction of gp91-*phox* exon 3. The cDNA sequence showed a deletion of gp91-*phox* exon 3, giving rise to an unstable or nonfunctional mutant gp91-*phox*. G.G. presented a defective splicing of gp91-*phox* exon 3 (the underlying mutation has not yet been determined). The other patients continue to be under investigation.

As a whole, this strategy permitted the detection of defective gp91-*phox* expression in seven of eight patients. The RT-PCR results matched clinical history, biochemical data (NBT or superoxide release assay) and available mutation analysis in four cases. In three additional cases, RT-PCR results matched clinical history and biochemical data. In another case, RT-PCR was normal despite a clinical history compatible with CGD, a defective respiratory burst charac-terized by the NBT test and superoxide release assay and NBT tests of his mother and sister compatible with X-linked CGD carrier status.

Discussion

This paper reports on the biochemical and gene expression studies of 8 unrelated Brazilian male patients with a clinical history of CGD, who were referred to our laboratory for detailed investigation. CGD is a rare inherited disorder in which phagocytic cells are unable to generate superoxide anion and other reactive oxygen intermediates. We initially established the diagnosis of probable X-linked CGD by means of biochemical methods such as the NBT slide tests and/or superoxide anion release assays. Our results showed that all patients presented impaired NADPH-oxidase function and, in turn, probable X-linked CGD.

Both methods contribute to the probable diagnosis of CGD in different ways. The NBT slide test provides information about the number of cells that reduce NBT to formazan inside the cytoplasm and the intensity of this reduction. Thus, patients with variant forms of X-linked CGD show abnormal NBT slide tests, in which most of the cells weakly reduce NBT to formazan. The NBT slide test also detects female carriers of X-linked CGD. The superoxide release assay measures the reduction of cytochrome c by superoxide produced by activated leukocytes present in the reaction, permitting the diagnosis of variant forms of X-linked CGD. Both tests can be easily standardized in low complexity laboratories and neither requires expensive equipment. The dihydrorhodamine 123 test is a highly sensitive method for the biochemical diagnosis of CGD; however, it requires a flow cytometer, a very expensive instrument.

We evaluated gp91-*phox* gene expression in EBV-transformed B lymphocytes from CGD patients by RT-PCR analysis.

Specific and overlapping pairs of primers were used to amplify three regions of the gp91-*phox* gene by RT-PCR: exons 1-5, 3-9, and 7-13. This strategy permitted the detection of defective gp91-*phox* expression in 7 of 8 patients. The RT-PCR results matched clinical history, biochemical data (NBT or superoxide release assay) and available mutation analysis in four cases. In three additional cases, RT-PCR results matched clinical history and biochemical data.

gp91-*phox* gene expression was reduced in all exonic regions of patient R.B. This reduction can be the result of mutations leading to low RNA stability or altered transcriptional activity. Patients G.M. and T.P. showed absent expression of exons 7-13, suggesting that the decreased expression of these additional 3'-end exons can be the result of RNA instability or a splicing defect, e.g., partially correct splicing to produce a signal, but partially abnormal splicing to eliminate a primer binding site, a subject to be investigated by future genomic DNA mutation analyses.

Patient G.G. presented a diffuse, low abundance, smaller-sized product of exons 1-5 (Figure 1), and absent expression of other exonic regions. His mutation analysis showed a defect at the exon 3 splice site. Similarly, the mutation analysis of patient R.S. also revealed a defect at the exon 3 splice site. However, in this case, a less abundant PCR product could be detected.

Patient M.F. presented reduced expression of exonic regions 3-9 and 7-13, which can be the result of defective transcriptional activity in exon 3. J.E.M. presented a nonsense mutation in exon 5 that resulted in loss of gp91-*phox* expression, as evidenced by RT-PCR analysis, a possible consequence of nonsense-mediated mRNA instability.

In one case, patient T.P.B., gp91-*phox* gene expression was normal despite a family history compatible with CGD and a defective respiratory burst activity. In this case, the diagnosis of probable X-linked CGD was based on abnormal NBT tests in his mother and his sister, compatible with carrier status. We hypothesize that a point mutation, such as a single base substitution that does not change PCR fragment length or abundance, should be investigated in this particular case.

RT-PCR is a powerful tool to assess gene expression. This characteristic may partially explain the variability of gp91-*phox* gene expression among the patients included in this study, and the importance to combine overlapping pair of primers to screen the full length of the message. RT-PCR has been used in isolated case studies as part of the initial diagnosis of the different forms of CGD (17-22). RT-PCR analysis was also useful in the prenatal diagnosis of CGD, resulting from p47-*phox* deficiency (23). However, it has been more commonly used for the study of gene regulation of the NADPH-oxidase components in a variety of cells (15,16,24-29). To date, the potential use of this useful tool for establishing the definitive diagnosis of X-linked CGD, the most frequent form, has not been investigated extensively. Further studies should be performed to compare the sensitivity and specificity of this test compared to other complex assays such as Western or Northern blots.

Overall, we have demonstrated that RT-PCR, a simple and low cost methodology, established the definitive diagnosis of X-linked CGD in 7 of 8 cases, without the need to use complex and expensive methodologies such as Northern blot, slot blot, SSCP analysis, or genomic DNA sequencing. Thus, RT-PCR may be a suitable tool for diagnosing CGD in laboratories in developing countries. It is very important to determine the definitive molecular genetic defect in order to provide the appropriate genetic counseling and prognosis to kindreds with CGD. In addition, molecular genetic studies of the human NADPH-oxidase system will advance the knowledge about this crucial and ancient defense mechanism.

Acknowledgments

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