Novel Diagnostic Tool for p47\(^{\text{phox}}\)-Deficient Chronic Granulomatous Disease Patient and Carrier Detection

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Chronic granulomatous disease (CGD) is a primary immunodeficiency caused by mutations of the phagocytic nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Autosomal recessive p47\(^{\text{phox}}\)-deficient CGD (p47\(^{\text{phox}}\) CGD) is the second most frequent form of the disease in western countries, and more than 94% of patients have a disease-causing dinucleotide deletion (\(\Delta\text{GT}\)) in the neutrophil cytosolic factor 1 (NCF1) gene. The \(\Delta\text{GT}\) mutation is most likely transferred onto the NCF1 from one of its two pseudogenes co-localized on the same chromosome. The presence of NCF1 pseudogenes in healthy individuals makes the genetic diagnostics of \(\Delta\text{GT}\) p47\(^{\text{phox}}\) CGD challenging, as it requires the distinction between \(\Delta\text{GT}\) in NCF1 and in the two pseudogenes. We have developed a diagnostic tool for the identification of p47\(^{\text{phox}}\) CGD based on PCR co-amplification of NCF1 and its pseudogenes, followed by band intensity quantification of restriction fragment length polymorphism products. The single-day, reliable p47\(^{\text{phox}}\) CGD diagnostics allow for robust discrimination of homozygous \(\Delta\text{GT}\) p47\(^{\text{phox}}\) CGD patients from heterozygous carriers and healthy individuals, as well as for monitoring gene therapy efficacy.

INTRODUCTION
Chronic granulomatous disease (CGD) is a primary immunodeficiency of phagocytes, leading to recurrent severe bacterial and fungal infections due to impaired reactive oxygen species (ROS) production by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex.1 Disease-causing mutations are found in all NADPH oxidase subunits gp91\(^{\text{phox}}\), p47\(^{\text{phox}}\), p67\(^{\text{phox}}\), p22\(^{\text{phox}}\), and p40\(^{\text{phox}}\). In western countries, p47\(^{\text{phox}}\) deficiency (p47\(^{\text{phox}}\) CGD) is the second most frequent form of CGD. p47\(^{\text{phox}}\) CGD is genetically exceptional, as 97% of patients share the same mutation, a dinucleotide deletion (\(\Delta\text{GT}\)) within the GTGT sequence in exon 2 of the neutrophil cytosolic factor 1 (NCF1) gene.2 On chromosome 7, the NCF1 gene is accompanied by two pseudogenes (NCF1B and NCF1C) with 99.5% sequence homology to NCF1 (Figures 1A–1C).

The \(\Delta\text{GT}\) mutation is also present in both pseudogenes, in healthy individuals, in CGD patients, and in carriers. NCF1 \(\Delta\text{GT}\) results from unequal cross-over events between NCF1 and one of its pseudogenes during DNA replication or repair, leading to partial pseudogene sequence transfer, including \(\Delta\text{GT}\), onto the NCF1 gene.3–5 Genetic diagnostics of \(\Delta\text{GT}\) p47\(^{\text{phox}}\) CGD are challenging, as it requires the distinction between \(\Delta\text{GT}\) in NCF1 and in the pseudogenes. Currently, diagnosis of \(\Delta\text{GT}\) p47\(^{\text{phox}}\) CGD relies on the gene-scan method,6 which is based on the comparison of fluorescence intensities of short co-amplified sequences of NCF1 and its pseudogenes, which differ in size by the 2-nt of the \(\Delta\text{GT}\) mutation. Other diagnostic methods are allele-specific hybridization7 and determination of the \(\Delta\text{GT}\)-GTGT ratio by the TaqMan copy number variation (CNV) assay.7

RESULTS AND DISCUSSION
We have developed a novel diagnostic tool for the identification of \(\Delta\text{GT}\) p47\(^{\text{phox}}\) CGD based on PCR co-amplification of NCF1 and its pseudogenes, followed by band intensity quantification of restriction fragment length polymorphism products (Figures 1B–1G). This 1-day method determines the NCF1 gene CNV by quantification of GTGT content in the NCF1 gene and pseudogene loci, and thus it detects the presence or absence of the \(\Delta\text{GT}\) mutation within NCF1 gene and pseudogene alleles. It can be established in any molecular biology laboratory, and it allows for the robust discrimination of homozygous \(\Delta\text{GT}\) p47\(^{\text{phox}}\) CGD patients from heterozygous carriers and healthy individuals for rapid diagnostic purposes, as well as for the monitoring of NCF1 genome-editing-based gene therapy.8

For quantification of the GTGT content, corresponding NCF1, NCF1B, and NCF1C sequences were co-amplified in one PCR reaction, digested, and visualized by PAGE or agarose electrophoresis (Figures 2A and 2B; detailed characterization of bands in polyacrylamide gel in Figures S2–S6). PCR-restriction fragment length polymorphism (RFLP) analysis was performed for 60 healthy individuals, 10 conventionally diagnosed \(\Delta\text{GT}\) p47\(^{\text{phox}}\) CGD patients, and 8 conventionally diagnosed \(\Delta\text{GT}\) p47\(^{\text{phox}}\) CGD carriers (see Table S1).

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Received 28 August 2018; accepted 4 February 2019;
https://doi.org/10.1016/j.omtm.2019.02.001.
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274 Molecular Therapy: Methods & Clinical Development Vol. 13 June 2019 © 2019 The Authors.
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Figure 1. PCR-RFLP Analysis of NCF1 Loci

(A) Co-amplified fragments of the NCF1 gene and pseudogenes. Positions of the GT-dinucleotide, 20-nt repeat, BsrG1 and Pst1 restriction sites, and primer-binding sites (forward and reverse) are shown. (B) In healthy individuals and (C) in patients with the ΔGT mutation in NCF1, the PCR co-amplification of NCF1 (correct, blue; mutated, red) (legend continued on next page)

GTGT-content = \[ \frac{\text{Int}_{169bp}/169}{(\text{Int}_{181bp}/181)+\text{Int}_{201bp}/201} \]
The co-amplification PCR products spanned the NCF1 gene and pseudogene GTGT locus within exon 2, as well as the surrounding intronic regions containing one or two repeats of a 20-nt sequence (1 × 20 nt or 2 × 20 nt; Figure 1A). Figure 1 displays possible DNA sequence variations of these loci, their configuration on chromosome 7, corresponding PCR co-amplification products, as well as PCR-RFLP results. CGD patients with homozygous ΔGT mutation in NCF1 can be identified by electrophoresis based on the absence of the 169-bp band (Figures 1C, 1F, and 2A, and 2B). The intensity of the 169-bp band was substantially weaker in heterozygous carriers. The absence of the 181-bp band (Figure 2, control 3) was observed in individuals who had two copies of the 20-nt repeat in intron 2 in all NCF1 gene and pseudogene alleles, a genotype that may be observed in healthy individuals. Calculation of the GTGT content (Figure 1G) allowed for the differentiation between NCF1 ΔGT mutation carriers and healthy individuals. Representative PCR-RFLP samples developed by PAGE and agarose electrophoresis (Figures 2A–2C) compare controls (healthy individuals with GTGT in two NCF1 alleles and different 20-nt intronic repeat numbers), X-CGD and autosomal recessive CGD NCF2 (gp91phox and p67phox deficiency, respectively), two NCF1 ΔGT carriers (Carrier ΔGT #1 and Carrier ΔGT #2), an induced pluripotent stem cell (iPSC) NCF1 AGT cell line, a human acute myeloid leukemia cell line PLB-985 (wild-type), and a PLB-985 NCF1 ΔGT cell line.

The median GTGT content in 55 healthy individuals who carried two NCF1 gene alleles with the GTGT sequence and the ΔGT mutation in all four pseudogene alleles (Control ratio 1:2; Figure S1A) was 0.29 in the polyacrylamide gel and 0.20 in the agarose gel. With NCF1 allele PCR co-amplification, followed by single-molecule real-time (SMRT) sequencing (Table S1), we identified five healthy individuals with two functional NCF1 alleles with GTGT sequence plus one of four pseudogene alleles with GTGT sequence (Control ratio 1:1; Figure S1B). In these individuals, the determined GTGT content values were 0.49 and 0.29 in polyacrylamide and in agarose gel, respectively. The observed results in agarose gels were lower than theoretical values expected by the genetic background, which may be explained by a partial loss of signal intensity in the thick agarose gels. GTGT content values established for 8 ΔGT p47phox CGD carriers, in which one NCF1 allele carries the ΔGT mutation, was 0.10 in polyacrylamide gel (Figure 2D) and 0.08 in agarose gel (Figure 2E; data for individual samples and statistics in Table S1).

The results of the PCR-RFLP analysis were confirmed by SMRT sequencing: undigested pools of barcoded co-amplification PCR products (Figures 1A and 1B) were sequenced, and the frequencies of GTGT signature-containing reads were calculated (Figure 2F; Table S1). GTGT sequence was identified in 27% of reads from healthy individuals with two GTGT-carrying NCF1 alleles (Control ratio 1:2), in 37% of reads from healthy individuals with GTGT within two NCF1 alleles and one of four pseudogene alleles (Control ratio 1:1), and in 16% of reads from NCF1 ΔGT carriers. Percentage differences in the GTGT signature-containing reads were statistically significant, and they allowed for the discrimination of healthy individuals from NCF1 ΔGT carriers and CGD patients.

In conclusion, we propose a package of complementary methods to be used for single-day reliable p47phox CGD diagnostics, based on PCR-RFLP, giving comparable results to SMRT sequencing. Furthermore, the PCR-RFLP diagnostic method represents an attractive alternative to the existing methods used in CGD diagnostics in terms of appliance requirements and costs per tested sample (Table 1). In addition to diagnostics, both methods can be also effectively applied for the assessment of correction of the ΔGT mutation upon genome-editing-based gene therapy.

**MATERIALS AND METHODS**

**DNA Isolation and PCR Amplification**

Sample processing was covered by ethical vote KEK ZH 2015/0135, BASEC-Nr. PB_2016-02202. Genomic DNA from healthy individuals, diagnosed ΔGT p47phox CGD patients, and their family members were isolated using DNeasy Blood & Tissue Kit (Qiagen, Hombrechtikon, Switzerland). The 411- to 433-bp fragments of NCF1, NCF1B, and NCF1C genes were PCR co-amplified using published PCR primers (Microsynth, Balgach, Switzerland). Phusion High-Fidelity DNA Polymerase and deoxyribonucleotides (dNTPs) were from Thermo Fisher Scientific (Reinach, Switzerland). PCR reaction included GC 10× buffer, dNTPs (200 μM each), primers (240 nM each), 0.04 U/μL Phusion High-Fidelity DNA Polymerase, and 2.5 ng/μL DNA. Initial 3-min denaturation (95°C) was followed by 36–40 cycles of denaturation (95°C, 30 s), annealing (65°C, 30 s), and elongation (72°C, 15 s) and final elongation (72°C, 3 min).

**Determination of the GTGT Content by RFLP**

The PCR co-amplification products of NCF1, NCF1B, and NCF1C were digested with BsrGI and PstI (New England Biolabs, Frankfurt am Main, Germany) (37°C, 180 min), followed by enzyme inactivation (80°C, 20 min) (Figures 1A–1C). The digestion fragments were developed in a 7.5% polyacrylamide (ratio 29:1) gel or a 5% (w/v) acrylamide gel stained with GelRed Nucleic Acid Gel Stain (Biotum, Fremont, CA, USA) and visualized using Gel Logic 100 Imaging System (Kodak, Eysins, Switzerland). Band intensities were quantified with ImageJ software. For determination of the GTGT content and its pseudogenes (red) results in a mixture of PCR products with a defined stoichiometry. In the majority of individuals, co-amplified PCR products differ by 2-nt of the GTT dinucleotide locus, and in 20-nt of the intron 20-nt repeat sequence. A significant fraction of the mixture comprises cross-hybridized PCR products derived from NCF1 and the pseudogenes (marked with an asterisk) (Figures S2–S6). BsrGI and Pst1 restriction digestion leads to the appearance of up to seven different restriction fragments in healthy individuals (A) and up to five fragments in patients with ΔGT deletion in NCF1 (B) (Figure 2A). (D–F) Typical densitometry images of digested fragments in a polyacrylamide gel of (D) a healthy individual with NCF1 gene to pseudogene ratio 1:2 (Control 1:2), (E) a carrier of the ΔGT mutation with NCF1 gene to pseudogene ratio 1:5 (Carrier ΔGT), and (F) a ΔGT p47phox CGD patient (CGD NCF1 ΔGT). (G) Size-normalized band intensities of 169-, 181-, and 201-bp fragments (B–F, blue) are used for calculation of the GTGT content and for identification of ΔGT p47phox CGD patients and ΔGT mutation carriers.
RFLP band intensities of 169-, 181-, and 201-bp BsrG1/Pst1 digestion products (Figures 1D–1F, 2A, and 2B) were size normalized by dividing band intensities by their length (number of base pairs). The size-normalized band intensity of the 169-bp band was divided by the sum of normalized band intensities of the 181- and 201-bp bands (Figure 1G).

SMRT Sequencing

PCR co-amplification products of the NCF1 gene and its pseudogenes were produced using individually barcoded Fwd1 primers, utilizing PCR conditions described above. PCR products were purified using the QIAquick Gel Purification Kit (QIAGEN). 20 ng gel-purified bar-coded PCR products of individual subjects were pooled and analyzed with SMRT sequencing by Functional Genomics Center Zurich, ETH/University of Zurich, Switzerland, as described.

Statistical Analysis

The Kruskal-Wallis tests with post hoc Dunn’s multiple comparison tests were performed using IBM SPSS Statistics version 23.0 (IBM, Armonk, NY, USA).

| Method | Time (Days) | Primer Labeling | Equipment | Cost |
|--------|-------------|-----------------|-----------|------|
| PCR-RFLP (new method described in this article) | 1* | no* | PAGE or agarose electrophoresis system* | * |
| Gene scan | 1* | yes (fluorescently labeled)** | DNA sequencer*** | *** |
| Allele-specific hybridization | 2** | yes (32P oligonucleotides)** | autoradiography equipment* | ** |
| TaqMan CNV | 1* | yes (fluorescently and MGB-labeled probe)*** | qPCR instrument*** | *** |

Asterisks indicate time and resource requirements (*lowest, ***highest). MGB, minor groove binder.
SUPPLEMENTAL INFORMATION
Supplemental Information includes seven figures and two tables and can be found with this article online at https://doi.org/10.1016/j.omtm.2019.02.001.

AUTHOR CONTRIBUTIONS
D.W. conducted experiments. D.W., U.S., and J.R. designed experiments, analyzed data, made figures, and wrote the manuscript.

CONFLICTS OF INTEREST
The described diagnostic tool has been submitted for intellectual property (IP) filing.

ACKNOWLEDGMENTS
This study was supported by the CGD Society (grant CGDS16/01) and Hochspezialisierte Medizin Schwerpunkt Immunologie (HSM-2-Immunologie). D.W. received a research grant from the University of Zurich (Forschungskredit, grant FK-17-041). J.R. is supported by the Uniscientia Foundation and the Clinical Research Priority Program ImmuGene of the University of Zurich. We thank the Functional Genomics Center Zurich for performing SMRT sequencing and data analysis.

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Supplemental Information

Novel Diagnostic Tool
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The scheme of the genetic configuration of GTGT and ΔGT sequences located in NCF1 and pseudogene alleles on chromosome 7

(A) A majority of healthy individuals exhibit the GTGT sequence in both alleles of the NCF1 gene, and the ΔGT mutation in all four alleles of the NCF1 pseudogenes (NCF1B and NCF1C), resulting in a ratio of GTGT to ΔGT carrying NCF1 alleles of 1:2. (B) A minority of healthy individuals exhibit three GTGT carrying NCF1 alleles, two located on both NCF1 gene alleles and one present on one of the pseudogenes, while the ΔGT mutation is shared by the remaining three NCF1 pseudogene alleles. Hence, in these healthy individuals the ratio of GTGT to ΔGT carrying NCF1 alleles is 1:1. (C) In carriers of the ΔGT mutation, GTGT is present in only one of the NCF1 gene alleles, while (D) in ΔGT p47phox CGD patients, all NCF1 loci carry the ΔGT mutation, resulting in a ratio of 1:5 and 0:6, respectively.

Introduction to Figures S2 to S6

The following figures describe the origin of heteroduplex PCR products that result from simultaneous PCR co-amplification of the NCF1, NCF1B and NCF1C sequences.

Supplemental Figure S2

PCR co-amplification and RFLP products of the NCF1 gene and its pseudogenes developed in agarose and polyacrylamide gels.

The heteroduplex PCR products (marked with asterisks) form during the PCR reaction by annealing of single-stranded PCR products derived from the NCF1 gene and its pseudogenes. These heteroduplexes migrate differently in polyacrylamide and agarose gels, as compared to the homoduplex PCR products, even after restriction digestion. PCR-RFLP product of size 63 bp is not shown in the gels.
**Figure S3**

PCR products of co-amplified *NCF1*, *NCF1B*, and *NCF1C* sequences in agarose (A) and polyacrylamide (B) gels. Sequences around the *NCF1* exon 2 were PCR co-amplified utilizing one pair of primers, and representative PCR products from eleven individuals (C) were developed in a 5 % agarose (A) or a 7.5 % polyacrylamide (B) gel. Migration of bands “α” and “β” indicate PCR product sizes between 400 and 450 bp in both, the agarose gel (A) and the polyacrylamide gel (B). The band “γ” migrates differently: in the agarose gel it is located below the 500 bp band of the marker, whereas in the polyacrylamide gel it is located between 500 bp and 600 bp.

In healthy controls 1 and 2 (Control 1 and 2; lanes 1 and 2 in A and B) restriction digestion of PCR products with BsrG1 and Pst1 generates a 201 bp fragment derived from the pseudogenes and a 181 bp fragment (see also: Figure 1B and C, as well as Figure 2A and B) derived from the *NCF1* gene. These fragments differ from one another only by the presence or absence of the second copy of the 20-nucleotide (20-nt) sequence repeat within intron 2. Interestingly, PCR products of healthy control 3 (Control 3; lane 3 in A and B) do not include PCR products “α” and “γ”. Restriction digestion of the PCR products of this control with BsrG1 and Pst1 results in a band pattern where the 181 bp band is absent (see: Figure 2A, B). The lack of this band indicates the presence of a double 20-nt repeat sequence within the intron 2 of all six *NCF1* loci. This configuration of the *NCF1* intronic 20-nt repeat has not been linked to the CGD phenotype.
The restriction fragment length polymorphism analysis of upper (“γ”) and lower fractions (“α” and “β”) of PCR co-amplification products of the NCF1 loci

PCR co-amplification products of the NCF1 loci from a healthy control, and from a ΔGT p47phox CGD patient were separated in an agarose gel (see: Figure S3A), followed by gel purification of upper and lower fractions (gel not shown). The lower fraction consisted of bands “α” and “β” (see: Figure S3A) and the upper fraction corresponded to the band “γ” (see: Figure S3A). To avoid changes in the composition of isolated cross-hybridized PCR heteroduplexes, the temperature has not exceeded 46 °C during purification. The upper (band “γ”) and lower (bands “α” and “β”) fractions of PCR co-amplification products were subjected to restriction digestion with BsrG1 and Pst1 restriction enzymes. Digestion was performed as described in Methods, without the final denaturation of DNA and without inactivation of the enzymes at 80 °C. The digestion products were visualized in a 7.5 % polyacrylamide gel. In healthy controls the lower fraction consisting of bands “α” and “β” (Figure S4A and B, lanes 5 to 8) can be digested by BsrG1 (Figure S4A, lane 6) and by Pst1 (Figure S4A, lane 7), whereas double digestion results in the expected band pattern for healthy individuals, with four bands in total (Figure S4A, lane 8). In ΔGT p47phox CGD, however, the ΔGT abolishes the BsrG1 restriction site in the lower fraction of PCR products (Figure S4B, lane 6), resulting only in 3 bands after digestion with BsrG1 and Pst1 (Figure S4B, lane 8). As expected, the smallest 169 bp band, which is the result of BsrG1 digestion of the PCR co-amplification products from the healthy control (Figure S4A, lanes 6 and 8), is absent in digestion samples from the ΔGT p47phox CGD patient (Figure S4B, lane 6). The 181 bp and 201 bp bands were produced solely by Pst1 digestion of the lower fraction of PCR products of both, the healthy control and the ΔGT p47phox CGD patient (Figure S4A and B, lanes 7 and 8).

The upper fraction of the undigested PCR co-amplification products (see: Figure S3A and B, band “γ”) can be digested only by Pst1 for both samples, from the healthy individual (Figure S4A, lanes 1 to 4) and from ΔGT p47phox-deficient CGD patient (Figure S4B, lanes 1 to 4). The resulting two bands are also present in the polyacrylamide gel for samples derived from healthy controls and carriers of the ΔGT mutation (Figure 2A, marked with asterisks). Of these two, the smaller digestion product from the ΔGT p47phox CGD patient overlaps with the 230 bp band derived from digestion of the lower fraction with both BsrG1 and Pst1 enzymes (Figure 2A lanes 6, 9 and 11, and Figure S4A and B; a dashed line indicates the difference in the position of the smaller digestion products of the upper fraction in lanes 3, 4, 7 and 8).
The bending centers responsible for the altered curvature and electrophoresis motility of heteroduplex PCR products are presented in Figure S4C and D: the ΔGT mutation in one strand of the GTGT locus (Figure S4C) and the 20-nt repeat locus (Figure S4D). In healthy controls, the heteroduplexes contain one strand derived from the pseudogenes carrying the ΔGT mutation, and another from the NCF1 gene with the GTGT sequence, in which the restriction site for BsrG1 is disrupted (Figure S4C). These molecules retain their curvature upon restriction digestion with Pst1, as they still migrate slower (Figure S3A, lane 3 and 4). Conversely, the sequence of the 5’ Pst1 digested fragment for ΔGT p47\textsuperscript{phox} CGD patients is shared between the mutated NCF1 and the pseudogenes. Because of Pst1 digestion, the 5’ digestion fragment of the upper fraction loses its curvature and migrates with the same speed as the 230 bp fragment of the lower fraction (Figure S3B, lane 3 and 4). Additionally, the pattern of digested PCR products for healthy control 3, who exhibits two copies of the 20-nt intronic repeat in all NCF1 alleles (Figure 2A, lane 3), does not display the upper of the two additional bands marked with asterisks in Figure 2A, lane 3. The lack of this band again can be explained by lost DNA curvature upon Pst1 digestion. As already mentioned, this healthy control presents two copies of the 20-nt repeat sequence in all NCF1 loci, and Pst1 digestion releases the linear 3’ fragment of the heteroduplex that migrates as the regular 201 bp restriction fragment in Figure 2A, lane 3.

Figure S5

Denaturation and renaturation of the upper and lower fractions of the PCR co-amplification products

PCR co-amplification products of the NCF1 loci from a healthy control and a ΔGT p47\textsuperscript{phox} CGD patient were separated in an agarose gel, the lower fraction consisting of bands “α” and “β” (see: Figure S3), and the upper fraction corresponding to band “γ” (see: Figure S3) were purified (gel not shown). During isolation the temperature has not exceeded 46 °C. Thereafter, isolated upper and lower fractions were denatured at 95 °C for 3 minutes and renatured at room temperature. The denatured and renatured samples were developed in a 7.5 % polyacrylamide gel along the unprocessed samples. Denaturation of all fractions led to reappearance of the PCR heteroduplexes, indicating that the band “γ” (Figure S3) is not an unspecific PCR product, but a product of cross-hybridization of PCR co-amplification products derived from different NCF1 loci, the NCF1 gene and the NCF1 pseudogenes.
SMRT sequencing of digested PCR co-amplification

PCR co-amplification products of *NCF1* loci consisting of the upper fraction (band “γ”) and the lower fraction (bands “α” and “β”), as visualized in Figure S3, were digested with BsrG1 and Pst1 resulting in up to seven bands in a polyacrylamide gel (see: Figure 1B). All bands except for 63 bp were gel purified and analyzed by SMRT sequencing, as described in Methods. Size distribution of sequenced reads identified four distinct products that correspond to the lengths of expected digestion fragments of the PCR homoduplexes.

Altogether, the results presented in Supplemental Figures S2-S6 suggest that the upper fraction (Figure S3A and B, band “γ”) consists of cross-hybridized PCR heteroduplexes, where one strand of the DNA heteroduplex originates from the *NCF1* gene and another from one of its pseudogenes (see: Figure S2, marked with asterisks). These heteroduplexes are curved DNA molecules that contain two major bending centers: the GT-dinucleotide deletion in one strand in the GTGT locus (Figure S4C) and the 20-nt repeat locus (Figure S4D). Due to the curvature, the heteroduplexes migrate slower than expected by their size, as reported before for other curved DNA molecules.¹

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Figure S7

Uncropped PAGE and agarose gels shown in Figure 2A and B of the main text
Supplemental Tables

Table S1
The list of ΔGT p47phox-deficient CGD patients and carriers of the ΔGT mutation tested in the study.

The Table S1 is in the excel file: Supplemental Table S1.

Table S2
The GTGT-content calculated for individual tested in the study, CGD patients, carriers and controls, as determined by restriction length polymorphism visualized in polyacrylamide and in agarose gels, or determined by SMRT sequencing.

The Table S2 is in the excel file: Supplemental Table S2.

Supplemental References
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