



Detection of the *NQO1* C609T polymorphism by a simple one step Tri-primer Amplification Refractory Mutation System-PCR method

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Abstract

NAD(P)H:quinone oxidoreductase 1 (NQO1) is an enzyme with antioxidant functions. It catalyzes the two-electron reduction of quinones and nitrogen-oxides, reducing the formation of reactive oxygen species by decreasing one-electron reductions. A common polymorphism in the *NQO1* gene involves the substitution of cytosine with thymine (609 C→T), giving rise to a proline to serine substitution, which decreases NQO1 enzymatic activity. The C609T polymorphism was shown to be important in a broad range of biomedical fields, especially cancer research. In this paper, we describe a new method for the detection of C609T based on the Amplification Refractory Mutation System – Polymerase Chain Reaction method (ARMS-PCR). Two specific forward primers differing in length and in the 3' base, and one common reverse primer were combined in a single PCR reaction. Genotype adscription was based on the amplification of either one or both of two specific amplicons (75 bp for the C allele and 90 bp for the T allele) as shown following agarose gel electrophoresis. The method was validated using a PCR-RFLP method. The proposed method was used to characterize the genotype distribution of the C609T polymorphism in a sample population from Aleppo, Syria, where 2.8% of the sample population were found to be homozygous for the T allele, and 78.8% were found to be homozygous for the C allele.

Keywords: NQO1; C609T; single nucleotide polymorphism (SNP); Pro187Ser; RFLP; Amplification refractory mutation system.

1. Introduction

NQO1, also referred to as DT-diaphorase, is a 274-amino acid protein that catalyzes a two-

electron reduction of various quinones including their respective quinonoid derivatives into stable non-toxic hydroquinones. NQO1 is the dominant intracellular two-electron transfer quinone reductase. The enzymatic activity of that protein

blocks the one electron reduction of quinones that causes the formation of radical species. The human representative *NQO1* gene is located on chromosome 16q22. The replacement of cytosine with thymine (609 C→T) is accompanied by a proline to serine substitution, which decreases the NQO1 enzymatic activity [1-3]. *NQO1* genotypes from the C609T polymorphism were classified as wild-type (C/C), heterozygous genotype (C/T), and homozygous genotype (T/T).

NQO1 was found to play an important role in apoptosis and oxidative stress, and the effects of C609T were thoroughly investigated in several types of cancers [4-8], and neurologic disorders [9-11], with positive association being found in several reports. The C609T polymorphism was confirmed to play an important role in the susceptibility to chronic benzene poisoning among industrial workers, with TT homozygotes having greatly elevated risk [12-15]. The polymorphism was also recently investigated with respect to the effects of air pollution [16].

Several methods were reported for the detection of C609T with PCR-RFLP as the most common method [17 -19]. PCR-CTPP and the TaqMan method were also utilized [10,20,21].

The aim of the present work was to develop a simple, rapid, and cost-effective method for the detection of *NQO1* C609T polymorphism that overcomes several disadvantages associated with the commonly used methods, such as low detection throughput and high cost.

2. Methods and Materials

2.1 Subjects

The population studied consisted of 71 white healthy unrelated subjects living in Aleppo, Syria

(40 males, 31 females, Mean age 41 ± 10). All subjects were native Syrians and of the same ethnicity (Arabs). Informed consent was obtained from each subject. Blood samples were collected in EDTA tubes and anonymously coded and stored.

2.2 DNA isolation

Genomic DNA was isolated from 200 μ l venous blood using a spin column format kit (EuroGold™ Blood DNA mini kit, Euroclone, Italy). The procedure was carried out according to the manufacturer instructions.

2.3 Quantification of genomic DNA

Quantification of DNA was performed using V-650 UV-VIS spectrophotometer (Jasco, Japan). A260/A280 ratios were in the range of 1.8-1.9 indicating high purity genomic DNA extract. Extraction yield was in the range of 4-8 μ g/200 μ l blood.

2.4 Tri-primer ARMS-PCR analysis

Three primers were used in a single PCR reaction (Fig. 1a). Two specific forward primers, P1 and P2 (Table 1), differing in length, were designed with complementary 3'- terminal nucleotide to the corresponding polymorphism. A common reverse primer P3 is used to amplify a 75 bp C allele-specific fragment or 90 bp T allele-specific fragment with P1 or P2, respectively. To enhance specificity, a destabilizing mismatch was incorporated at the 3rd nucleotide from the 3'-terminus in each specific primer. A mismatch at the terminal 5' nucleotide of the short specific primer was introduced in order to minimize megapriming.

Table 1 Primers used in C609T *NQO1* analysis by Tri-primer ARMS-PCR.

Primer	Sequence (5'→3')
P1	<u>C</u> TGGCT TCCAAGTCT TAG <u>C</u> AC
P2	TTATTTTTTATTTCAGTGGCTTCCAAGTCTTAGT <u>A</u> T
P3	GGATTGAATTCGGGCGTCT

Underlined bases indicate the introduced mismatches.

The PCR reaction was carried out in a total volume of 30 μ l, containing 100–200 ng of genomic DNA as template, 0.4 μ M of each primer, 0.2 mM of each dNTP, 2.3mM of MgCl₂, Taq buffer (20mM Tris-HCl pH 8.4, 50mM KCl), and 1 U of a HotStart Taq DNA polymerase (JumpStart™ Taq DNA Polymerase, Sigma-Aldrich, USA). PCR amplification was carried out in a MasterCycler® thermal cycler (Eppendorf, Germany), with an initial denaturation at 94 °C for 5 min, followed by 33 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 10 s, and a final extension at 72 °C for 5 min. Six microliter of loading buffer was added to the PCR reaction volume and a 20-30 μ L aliquot was subjected to agarose gel (3%) electrophoresis.

2.5 Validation of genotypes assigned by tri-primer ARMS-PCR

Genotype assignment was validated for 10 samples using the PCR-RFLP method which was carried out as previously reported [17]. The PCR product was digested with *Hinf*I restriction enzyme (Fermentas, Lithuania) according to the manufacturer's directions. The digested PCR fragments were separated on 2% agarose gels. Genotypes were scored as homozygous for the C allele if the 172 bp PCR fragment after digestion remained uncut, or homozygous for the T allele if two bands were observed following enzyme digestion (131 and 41 bp). Gels with three DNA fragments (172, 131, and 41 bp) were scored as heterozygotes.

3. Results and discussion

3.1 Tri-primer ARMS-PCR analysis

The procedure rendered 2 bands in heterozygotes (75 and 90 bp), and a single band at 75 bp or 90 bp in CC or TT homozygotes, respectively (Fig. 1b).

PCR components concentrations and reaction conditions were thoroughly optimized in order to ensure high specificity and sensitivity. The introduction of a mismatch at the 3rd in the 3' termini of the specific primers and the use of a high melting temperature ensured maximum specificity.

A second mismatch at the 5' ultimate nucleotide of the short C allele specific primer greatly reduced the addition of a single-stranded 5'-tail to the 75 bp fragments through megapriming which can be a result of the annealing of the T allele specific primer, producing an artifact that interferes with separation by gel electrophoresis.

Limiting the distance between the forward primers and the reverse primer, and the addition of a long AT-rich 5'-tail to the T allele specific primer were shown to be effective approaches in order to facilitate the separation of the two specific amplicons within a reasonable amount of time by agarose gel electrophoresis.

The proposed method was validated by a PCR-RFLP method for 10 samples. There was a 100% accordance between both methods for genotype adscription.

The basis of ARMS is that oligonucleotides with a mismatched 3'-residue do not function as primers in the PCR upon the use of a DNA polymerase without 3'-exonuclease activity. Classically, the technique requires that only the terminal 3'-nucleotide of a PCR primer be allele specific. Thus, the primer is synthesized in two forms: the wild form is refractory to PCR on mutant template DNA, and conversely. Then, two different PCR reactions must be performed to determine the zygosity of any sequence change [28].

In contrast to the most commonly used PCR-RFLP method [17-19], the proposed method does not involve the time consuming incubation step with costly restriction enzymes, resulting in significant improvement in detection time and cost.

TaqMan and direct sequencing methods require costly reagents, dedicated instruments, and relatively high level of expertise [20, 21]. On the contrary, the proposed method involves a simple PCR step followed by gel electrophoresis.

The PCR-CCTP [6, 22], which is similar to the tetra-primer ARMS-PCR [29], is a simple one-step method that does not involve the use of restriction enzymes. However, the proposed Tri-primer method involves the use of only three primers and does not lead into the production of a third common non-indicative PCR amplicon,

which can compete with the amplification of the indicative specific amplicons and/or lead into the production of false positive results, rendering the

method more difficult to optimize, and harder to be applied for the simultaneous detection of multiple SNPs in a single PCR reaction.

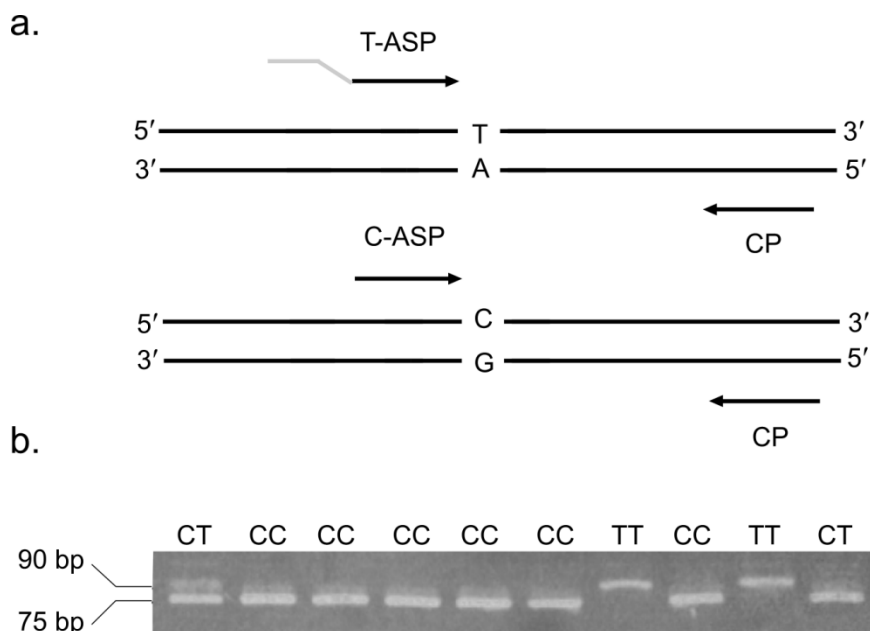


Fig. 1 a. The principle of Tri-primer ARMS. The T allele specific primer (T-ASP) is attached to a discriminative long 5'- tail amplifying a 90 bp fragment in conjunction with the common primer (CP). The C allele specific primer amplifies a 75 bp fragment in conjunction with the common primer. **b.** Agarose gel electrophoretogram of C609T *NQO1* polymorphism detection in 10 samples. The presence of a single 90 bp or 75 bp band indicates a TT homozygous or CC homozygous, respectively. The presence of the two aforementioned bands indicates a CT heterozygous.

3.2 Genotype distribution of C609T polymorphism in a sample population from Syria

A total of 71 healthy subjects living in Aleppo, Syria, were genotyped for the C609T polymorphism, which is known to be differentially distributed among different ethnic groups. Among the 71 subjects genotyped, 54 (76%) were found to be homozygous for the C allele, 2 (2.8%) were found to be homozygous for the T allele, and 15 (21.2%) were found to be heterozygous. Allele frequencies were found to be 87% and 13% for the T allele and C allele, respectively. The genotype distribution was found to be in Hardy-Weinberg equilibrium ($\chi^2=0.56$; $df=1$; $p=0.76$).

The genotype distribution of the C609T polymorphism in the sample population is

representative of the Arab population which is a distinct ethnic group. The obtained genotype distribution is close to that previously reported for the Caucasian, Hindu and African populations [23-26], and different from that previously reported for the East Asian and Hispanic populations [6,17,27], where the T allele is nearly as common as the C allele (Fig. 2). The genotype distribution and allele frequencies obtained can potentially serve as control values for multiple case-control association studies involving Arab populations.

In conclusion, a rapid, simple, and cost-effective method has been described for the detection of C609T, based on an uncommon variation of the ARMS method that simplifies detection and facilitates multiplexing.

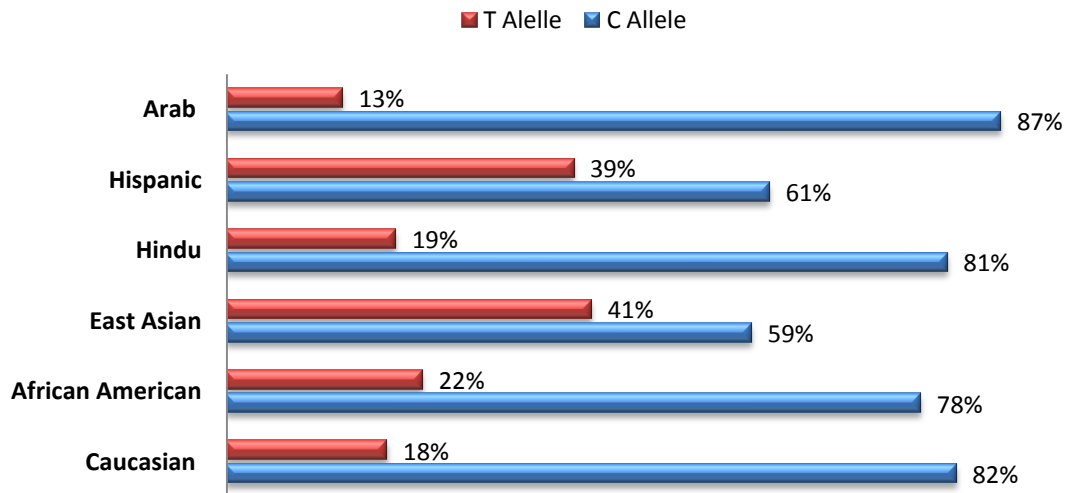


Fig 2. A comparison chart of C609T allele frequencies between different ethnic groups (Arab (present study), Hispanic [27], Hindu [25], East Asian [17], African American [26], and Caucasian[23]).

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