High throughput genotyping for the detection of a single nucleotide polymorphism in NAD(P)H quinone oxidoreductase (DT diaphorase) using TaqMan probes

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Abstract

***Aims*—The two electron reduction of quinones to hydroquinones by NAD(P)H quinone oxidoreductase (NQO1) plays an important role in both activation and detoxification of quinone and similarly reactive compounds. A single nucleotide polymorphism at exon 6 leads to an amino acid change at codon 187 from proline to serine. The variant allele has been associated with decreased NQO1 enzyme activity and increased cancer risks. The aim of this study was to develop a rapid genotyping procedure for epidemiological and clinical research into the potential biological and toxicological implications associated with this genetic polymorphism.**

***Methods*—A high throughput genotyping method using fluorogenic probes has been developed to screen this single nucleotide polymorphism. This assay utilises the 5' nuclease activity of Taq polymerase in conjunction with fluorogenic TaqMan probes. The TaqMan genotyping procedure was validated by a restriction fragment length polymorphism method and direct sequencing.**

***Results*—This method can be used for the rapid screening of known polymorphisms in large populations. In a population of 143 unrelated individuals, Pro/Pro (wild-type), Pro/Ser (heterozygous), and Ser/Ser (mutant) genotypes were 69.2%, 26.6%, and 4.2%, respectively.**

***Conclusions*—This genotyping method is highly accurate and could be applied to automated large scale genotyping studies.**

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Keywords: NAD(P)H quinone oxidoreductase; genotyping; TaqMan

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NAD(P)H quinone oxidoreductase (NQO1; EC1.6.99.2; DT diaphorase) is a cytosolic flavin containing enzyme that catalyses the two electron reduction of quinone substrates.[1] Reduction of quinone to hydroquinone prevents the generation of reactive oxygen species, thus protecting cells from quinone induced oxidative damage.[2 3] The enzyme also catalyses the reductive biotransformation of α-tocopherol derived quinones to hydroquinones, which have significant antioxidant potential.[4] In contrast, the same enzyme activates certain compounds through reduction reactions, including the chemotherapeutic agents mitomycin C,[5] porofiromycin and mitoxantrone,[3] and the environmental carcinogens, dinitropyrenes[6] and cigarette smoke condensate.[7] A single nucleotide polymorphism of NQO1 was identified with a nucleotide substitution (C to T) at base pair 609 of exon 6.[5] Recent genotyping studies revealed that the allelic frequency of this polymorphism is highly variable among different ethnic groups.[8] This polymorphism causes an amino acid substitution of codon 187 from proline to serine.[9] Individuals who are homozygous and heterozygous for this mutation have ~ 2% and 33% of the normal NQO1 activity, respectively.[10] The mutant allele has been associated with increased resistance to mitomycin C resulting from a deficiency in converting the drug to a toxic reactive metabolite.[5 10 11] An increased mutant allele frequency has also been seen in patients with lung cancer and urothelial carcinoma,[7 11 12] suggesting that an NQO1 deficiency in metabolising carcinogens might be a risk factor for developing certain cancers. Because several clinically important anticancer drugs and environmental toxins are substrates of NQO1, a ready tool would be valuable for the study of potential biological and toxicological implications associated with this genetic polymorphism.

The objective of our study was to establish a high throughput genotyping method for detecting NQO1 polymorphism. Genotyping is a technique that is being used increasingly in biomedical research and clinical molecular diagnostics. Simple, reliable, high throughput automated DNA analyses are highly desirable for large scale genotyping projects such as disease association studies and pharmacogenetic applications. Most commonly applied genotyping assays require gel electrophoresis based procedures, restriction fragment length polymorphism (RFLP) analysis, and allele specific polymerase chain reaction (PCR) analyses. These methods are labour intensive and require the participation of highly skilled technical staff for the final analysis of results. A commonly applied method for the detection of NQO1 polymorphism is PCR amplification of genomic DNA followed by restriction digestion with HinfI.[13] Our goal was to develop a protocol that would allow rapid screening of single nucleotide polymorphisms (SNPs) in a single PCR tube and eliminate the need for gel based product separation.

The TaqMan® allelic discrimination assay uses the 5' nuclease activity of Taq polymerase to detect a fluorescent reporter generated dur-

ing or after PCR reactions.[14] For genotyping this NQO1 single nucleotide polymorphism, one pair of TaqMan probes and one pair of PCR primers were used. Each TaqMan probe consisted of a 36 or 37 bp oligonucleotide complementary to the polymorphic region. The two TaqMan probes differ only at the polymorphic site, with one probe complementary to allele 1 (wild-type) and the other to allele 2 (mutant). A 5' reporter dye (6-carboxy-4,7,2',7'-tetrachlorofluorescenin; TET) and a 3' quencher dye (6-carboxy-N,N,N',N'-tetrachlorofluorescein; TAMRA) were linked covalently to the allele 1 probe. Similarly, the allele 2 probe was labelled with a 5' reporter dye (6-carboxyfluorescein; FAM) and the same 3' quencher dye (TAMRA). When the TaqMan probe was intact, fluorescence was quenched as a result of the physical proximity of the two dyes.[15] During the annealing step of the PCR reaction, the TaqMan probes hybridise to the targeted polymorphic site within the forward and reverse primer regions. During the extension phase of the PCR reaction, the 5' reporter dye is cleaved by the 5' nuclease activity of the Taq polymerase, leading to an increase of the reporter dye's characteristic fluorescence. By measuring the intensities of the TET and FAM signals immediately after the PCR reaction, using a fluorescent sequence detector, the specific genotype was determined.[16] In this report, we describe a recently developed and validated, highly robust, high throughput genotyping method to detect NQO1 polymorphism using TaqMan probes.

Materials and methods

REAGENTS

All reagents were purchase from Perkin-Elmer (Foster City, California, USA) unless otherwise stated.

STUDY POPULATIONS

Anonymous blood samples from 144 unrelated volunteers were collected in accordance with approved protocol. Subject identifications were stripped from the samples before starting the genotyping procedure to ensure confidentiality.

DNA PURIFICATION

DNA was extracted from peripheral blood leucocytes using a mini column method, following the manufacturer's instructions (Qiagen, Chartsworth, California, USA).

OLIGONUCLEOTIDES

PCR primers and TaqMan probes were designed with the primer express program (Perkin-Elmer/Applied Biosystems) and were custom synthesised by Perkin-Elmer. Two TaqMan probes were designed according to the NQO1 gene polymorphic site (bold and underlined). Allele 1: TET-5'-CTT CCA AGT CTT AGA A**C**C TCA ACT GAC ATA TAG CAT-3'-TAMRA; allele 2: 6FAM-5'-TTC CAA GTC TTA GAA **T**CT CAA CTG ACA TAT AGC ATT G-3'-TAMRA; forward PCR primer: 5'-CTT ACC TCT CTG TGC TTT CTG TAT CCT-3'; reverse PCR primer: 5'-GGG CGT CTG CTG GAG TGT-3'.

TaqMan GENOTYPING ASSAY

The PCR was set up in 96 well PCR plates with 20 µl in each well and a final concentration of 20 ng genomic DNA, 300 nM TET probe, 50 nM FAM probe, 50 nM forward primer, and 300 nM reverse primer in 1× universal master mix (Perkin-Elmer). The above ratio of forward/reverse primer and TET/FAM probe concentration was optimized by using a series of primer and probe combinations to reach a maximal signal and the balance of the two probes by reading in an ABI 7200 sequence detector. The TaqMan universal PCR master mix is a premix of all the components, except primers and probe, necessary to perform a 5' nulcease assay. The TaqMan PCR was also optimised by increasing the annealing temperature from 60°C to 65°C. The final optimised conditions were: 50°C for two minutes, 95°C for 10 minutes, 95°C for 15 seconds, and 64°C for one minute for 40 cycles. After PCR reactions were completed, the plates were cooled to room temperature and read in an ABI 7200 sequence detector, and the results analysed by allelic discrimination of the sequence detection software (Perkin-Elmer/Applied Biosystem). The FAM and TET signals were stable for up to three days after PCR if stored in the dark.

VALIDATION OF NQO1 GENOTYPE BY DIRECT SEQUENCING

For the purpose of validating this method, all sequencing was performed at the Parke-Davis DNA Sequencing Core Facility (Ann Arbor, Michigan, USA) using an ABI 377 sequencer with dye terminator chemistry. Genomic DNA was PCR amplified for 30 cycles at 95°C for 30 seconds, 68°C for 30 seconds, and 72°C for one minute in a 50 µl reaction volume. PCR products were purified using a Wizard PCR purification system (Promega, Madison, Wisconsin, USA) and 125 ng of purified PCR products and 1.625 pmol of oligonucleotide primer were used for each sequencing reaction. The DNA samples were sequenced with both the forward and reverse primers.

NQO1 GENOTYPING BY PCR–RFLP

To compare the throughput and validate the TaqMan genotyping, the PCR–RFLP method was also carried out according to the published protocol.[13] A 230 bp fragment was amplified from genomic DNA using the sense primer: 5'-TCC TCA GAG TGG CAT TCT GC-3' and antisense primer: 5'-TCT CCT CAT CCT GTA CCT CT-3'. PCRs were set up at a final concentration of 20 ng genomic DNA and 600 nM of each sense and antisense primer, 0.8 mM dNTP, and 0.5 U Amplitaq polymerase in 1× PCR buffer E (Invitrogen, La Jolla, California, USA). PCR conditions were: 94°C for four minutes, 94°C for 30 seconds, 60°C for one minute, and 72°C for one minute for 40 cycles, followed by one cycle at 72°C for seven minutes. PCR products were digested with 36 U of HinfI overnight at 37°C, and resolved

on a 4% agarose gel. When electrophoresis was completed, the gel was stained with ethidium bromide and visualised under UV light. Homozygous wild types exhibited two bands (195 and 35 bp). Homozygous mutants had three bands (151, 44, and 35 bp). Heterozygous individuals showed four bands (195, 151, 44, and 35 bp).

Results

DIRECT SCREENING OF HOMOZYGOUS NQO1 VARIANTS USING TaqMan PROBES

Seventy two genomic DNA samples were analysed using the TaqMan PCR protocol. Based on the raw fluorescence signals of the reporter dyes FAM and TET obtained from the "dye component" function in the sequence detection software, four distinct populations were identified (fig 1). According to the FAM to TET ratio, these four populations could be categorised as no amplification (low reading for both FAM and TET), allele 1 (wt/wt, homozygous wild type; FAM << TET), allele 2 (mut/mut, homozygous mutant; FAM >> TET), and alleles 1 and 2 (wt/mut, heterozygous; FAM ≈ TET) (fig 1). Two samples with the highest FAM to TET ratio were manually assigned as homozygous mutants.

GENOTYPING BY PCR–RFLP

To validate the specificity of TaqMan genotyping, the two homozygous variants (T/T), along with two samples from each of the other NQO1 genotype groups (C/C and C/T) identified by the TaqMan method in fig 1 were also genotyped by PCR–RFLP (fig 2). The genotype results produced by these two methods were in complete agreement.

VERIFICATION OF GENOTYPE BY DIRECT SEQUENCING

Two samples from each of the genotypic groups in fig 2 were also sequenced (data not shown). All the genotyping results were confirmed by sequence analysis.

ALLELE DISTRIBUTION OF NQO1 GENOTYPES IN A HUMAN POPULATION SAMPLE

After sequencing verification of the genotyping results, standard TaqMan allelic discrimination assays using control alleles were performed according to the manufacturer's protocol. Genomic DNA from one homozygous wild type and one homozygous mutant were used as control allele 1 and allele 2, respectively, to calibrate the assay for standard TaqMan allelic discrimination. These two control alleles were identified originally by TaqMan genotyping (fig 1) and subsequently verified by both PCR–RFLP (fig 2) and direct sequencing. Eight no template control samples, eight homozygous wild-type (allele 1), eight homozygous mutant (allele 2), and up to 72 genomic DNA samples were used for automatic genotype calling, utilising the sequence detection software (fig 3). These control sample numbers were chosen to achieve a 99.7% confidence level for automatic allele identification according to the manufacturer's protocol. Of the 144 samples analysed by this method, one sample did not amplify, and the number of samples is therefore reported as 143. The prevalence of the NQO1 genotypes was 99 of 143 (69.2%) homozygous wild type, 38 of 143 (26.6%) heterozygous, and six of 143 (4.2%) homozygous mutant. These values indicate allelic frequencies of 0.83 and 0.17 for homozygous wild type and mutant, respectively. Again, all the TaqMan genotyping results were also validated by PCR–RFLP and both methods gave identical genotyping results.

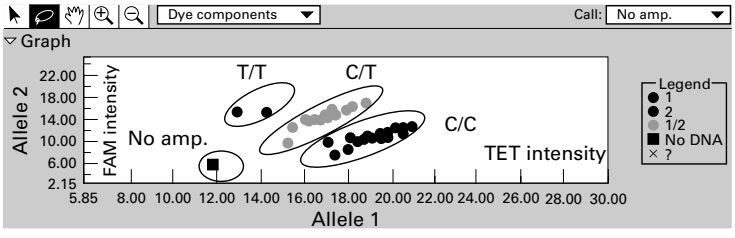

Figure 1 Detection of NAD(P)H quinone oxidoreductase (NQO1) polymorphisms using TaqMan allelic discrimination with manual selection of genotypes. Seventy two unrelated individuals were genotyped as described in the materials and methods section. The fluorescent signals viewed under the "dye component" field of the sequence detection software and the NQO1 genotypes were manually selected based on the ratio of the FAM and TET signals. Allele 1, 609 C/C (homozygous wild type; FAM << TET); allele 2, 609 T/T (homozygous mutant; FAM >> TET); alleles 1 and 2, 609 C/T (heterozygote; FAM ≈ TET).

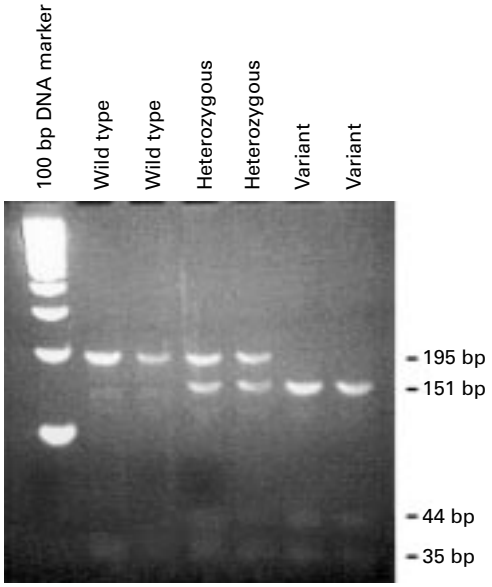

Figure 2 Detection of NAD(P)H quinone oxidoreductase (NQO1) polymorphism by PCR amplification and HinfI digestion. Wild type, homozygous wild type (609 C/C); variant, homozygous mutant (609 T/T); heterozygotous, heterozygote (609 C/T).

Discussion

Automated, rapid throughput, large scale genotyping for DNA polymorphism is highly desirable for population studies involving hundreds or thousands of individuals. These studies are also more intensive in those cases where multiple markers can be used for association analysis. Currently, PCR–RFLP is one of the most commonly used methods for genotyping single nucleotide polymorphisms. However, this approach is time consuming and labour intensive. The procedures involved are hard to automate and require highly skilled staff to

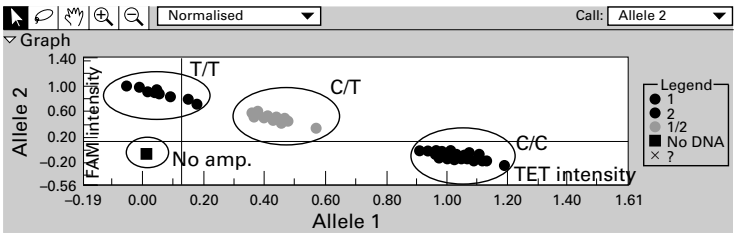

Figure 3 Scatter plot of NAD(P)H quinone oxidoreductase (NQO1) genotype using standard TaqMan allelic discrimination assays with automatic allele calling. Each reaction plate contains eight no template controls, eight homozygous allele 1, eight homozygous allele 2, and 72 unrelated individuals with respect to the polymorphic NQO1 genotype. The NQO1 genotypes were assigned automatically by the sequence detection software according to the ratio of FAM and TET signals. Allele 1, 609 C/C (homozygous wild type; FAM << TET); allele 2, 609 T/T (homozygous mutant; FAM >> TET); alleles 1 and 2, 609 C/T (heterozygote, FAM ≈ TET).

conduct and evaluate the tests. In addition, if the polymorphism does not involve a restriction site change, PCR–RFLP cannot be used. In contrast, TaqMan based allelic discrimination offers high sample throughput, with accurate detection of SNPs. This fluorescent based genotyping procedure greatly simplifies the assay protocol by eliminating the need for restriction enzyme digestion, gel electrophoresis, resolution of PCR products, and visual assessment of bands. In addition, the 96 well plate format and closed tube PCRs eliminate subsequent processing, thus greatly reducing the potential for contamination. Fluorescence reporter dye signals can be captured minutes after allelic discriminating reactions are performed and the genotypes can be identified automatically by the detection software used. The ability to automate data handling further enhances accuracy by eliminating operator bias. The simplicity of this method is that it is only necessary to prepare a master mix by adding appropriate primers and TaqMan probes to a universal master mix. We have integrated a robotic liquid handling system to measure out the unknown DNA into the PCR plates. Therefore, each PCR plate takes about 10 minutes to set up and six plates (432 samples) take one hour to set up. Each PCR assay takes two hours to complete. Reading each plate on ABI 7200 takes about 10 minutes or one hour for six plates. By using multiple PCR machines at the same time, one technician can routinely genotype 400 (six plates) to 500 samples (seven plates) in an eight hour day. By maximising PCR machine usage, it is possible to run two sets of PCR reactions or genotype 1000 samples/day, starting from genomic DNA. The TaqMan genotyping method has proved to be highly robust, and requires less manual handling and time than PCR–RFLP procedures. Sample turnaround time in the clinical pathology laboratory has also been improved using the TaqMan method. Individuals with specific NQO1 genotypes were accurately identified by the TaqMan procedure and verified by RFLP and direct sequencing of target DNA containing the polymorphic site. The standard TaqMan allelic discrimination protocol developed by Perkin-Elmer/Applied Biosystem requires both the homozygous wild-type and mutant control DNA to provide automatic identification by means of the sequence detection software. A great improvement in the current method for detecting NQO1 polymorphism is that we can manually assign genotypes under the "dye component" command found in the sequence detection software (fig 1). This manual genotype calling was based on the ratio of the two probes FAM to TET. Therefore, TaqMan genotyping can be initiated without the homozygous wild-type and mutant control DNA. This method will be particularly useful for screening homozygous or heterozygous mutant alleles even when the allele frequency is very low. Once identified, the rare homozygous variants could be validated by sequencing and other existing methods. The homozygous mutants identified could then be used in the standard TaqMan genotyping test as control alleles. Although direct TaqMan genotyping is very useful for rapid identification of rare polymorphisms, it is recommended that the TaqMan genotyping procedure described in fig 3 should be followed by including all proper control alleles.

NQO1 mutant allele frequencies in the white population have been reported by several groups, and have ranged from 0.13 to 0.25.[7 8 12 13] NQO1 polymorphism is highly variable among different ethnic groups,[8 13] with the mutant allele frequency being significantly higher in Chinese (0.49) and native North American populations (0.46) than in whites (0.16).[8] The genotype distribution in our study population was in Hardy-Weinberg equilibrium. With the ethnic background of our study population not specified, the allele frequency for the NQO1 mutants in our study was 0.17. This is in agreement with reports on white populations.[8] The homozygous variant frequency in our study was ~ 4.2% of the population, which is also consistent with the white population genotype distribution.[8 13]

In summary, we have developed and validated a high throughput genotyping method to detect NQO1 polymorphism using TaqMan probes. This method is very useful for screening and identifying SNPs in large population groups, and possibly for the early identification of individuals at risk in molecular epidemiology studies. The development of this important genotyping method brings genetic analysis closer to the goal of fully automated, accurate, high throughput determination of single nucleotide markers.

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