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Simplified qPCR method for detecting excessive mtDNA damage induced by exogenous factors

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ABSTRACT

Damage to mitochondrial DNA (mtDNA) is a meaningful biomarker for evaluating genotoxicity of drugs and environmental toxins. Existing PCR methods utilize long mtDNA fragments (~8–10 kb), which complicates detecting exact sites of mtDNA damage. To identify the mtDNA regions most susceptible to damage, we have developed and validated a set of primers to amplify ~2 kb long fragments, while covering over 95% of mouse mtDNA. We have modified the detection method by greatly increasing the enrichment of mtDNA, which allows us solving the problem of non-specific primer annealing to nuclear DNA. To validate our approach, we have determined the most damage-susceptible mtDNA regions in mice treated *in vivo* and *in vitro* with rotenone and H_2O_2. The GTGR-sequence-enriched mtDNA segments located in the D-loop region were found to be especially susceptible to damage. Further, we demonstrate that H_2O_2-induced mtDNA damage facilitates the relaxation of mtDNA supercoiled conformation, making the sequences with minimal damage more accessible to DNA polymerase, which, in turn, results in a decrease in threshold cycle value. Overall, our modified PCR method is simpler and more selective to the specific sites of damage in mtDNA.

1. Introduction

Accumulation of lesions in mitochondrial DNA (mtDNA) due to oxidative and other types of damage is believed to be one of the causes of energy crisis in aging tissues (Harman, 2009). Lesion formation is promoted by the prokaryotic organization of mitochondrial genome, lack of histones, and less efficient than the nuclear one DNA repair system (Bogenhagen, 2012). Since mitochondrial reticulum contains multiple copies of mitochondrial genome, mtDNA lesions can be viewed not only as causes of metabolic dysfunctions, but as biomarkers of the development of these dysfunctions and can be used to estimate the level of oxidative stress in mitochondria in various tissues.

The classical methods for evaluating the extent of DNA damage, such as Southern blot and high performance liquid chromatography, have a number of limitations. In particular, they require considerable amounts of DNA for analysis (10–50 μg) (Furda et al., 2012). Because of these limitations, many researchers have used long-range PCR (Van Houten et al., 2000; Ayala-Torres et al., 2000; Santos et al., 2006; Chan et al., 2011; Maslov et al., 2013; Czarny et al., 2013; Lehle et al., 2014) to access the levels of DNA damage, based on the assumption that DNA lesions (single-strand breaks, modified bases or their adducts) inhibit DNA polymerase and slow down accumulation of the PCR product. Therefore, the rate of PCR product accumulation would be inversely proportional to the number of damaged DNA molecules (Furda et al., 2012).

Many types of DNA lesions are induced by oxidative stress (Wallace, 2002). Mitochondria could be severely damaged not only by superoxide radical, singlet oxygen, and hydroxyl radical, but also by hydrogen peroxide. H_2O_2-induced DNA impairments are mediated by iron ions that catalyze formation of hydroxyl radicals ($^{\bullet}OH$) and cause single- and double-strand DNA breaks (Panayiotidis et al., 1999; Barbouti et al., 2001; Hegde et al., 2012). Another type of DNA lesions is thymine modification that leads to the formation of thymine dimers (TT) and thymine glycol (Basu et al., 1989). The eighth position of the purine imidazole ring is most susceptible to oxidative damage – its oxidation causes formation of 8-oxo-7,8-dihydro-2′-deoxyadenosine (8-oxoA) and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxoG) (Wallace, 2002).

Most of these lesions inhibit DNA polymerase activity (Sikorsky et al., 2007) and therefore, can be detected by qPCR. The only

Abbreviations: mtDNA, mitochondrial DNA; 8-oxoG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; FPG, formamidopyrimidine DNA glycosylase; HSP, H-strand promoter; OR, origin of replication; TAS, termination-associated sequence.

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exception is 8-oxoG, since amplification efficiencies and Cq values for templates containing a single 8-oxoG were not significantly perturbed, although the presence of two tandem 8-oxoGs substantially hindered amplification (Sikorsky et al., 2004). Numerous studies have shown that 8-oxoG is the most common type of DNA lesions in the nuclear genome (Cooke et al., 2003). A number of works demonstrated that aging is accompanied by accumulation of primarily 8-oxoG in DNA (Sohal et al., 1994; Rattan et al., 1995). In genomic DNA, these lesions are repaired mostly by DNA glycosylases that cleave oxidized purine bases. Thus, formamidopyrimidine DNA glycosylase (FPG), an enzyme specific to 8-oxoG, (Nelson et al., 2014) create breaks in DNA stands at the sites of 8-oxoG and prevents DNA synthesis by DNA polymerase (Maslov et al., 2013).

It is known that mitochondrial antioxidant systems differ in different organs (*e.g.*, liver and brain), mostly in the activity of catalase and its involvement in the protection of mitochondria from H_2O_2 (Andreyev et al., 2015). For this reason, we used isolated brain and liver mitochondria to exclude the effects of cytosolic antioxidant systems, as well as to identify regions of mtDNA that are more vulnerable to damage induced by exogenous H_2O_2 and genotoxic agents, such as rotenone.

2. Materials and methods

2.1. Laboratory animals

Six months-old male C57BL6 mice were used in experiments. The animals were obtained from the Stolbovaya Nursery (Scientific Center for Biomedical Technology, Russia) and housed in plastic cages under standard conditions (25 °C; 12-h light/dark cycle; relative humidity, >40%) with *ad libitum* access to food (type ssniff Spezialdiäten GmbH, Germany) and water. Animal maintenance, injections and sacrifice were performed strictly in accordance with the rules set by Institutional Animal Care and Use Committee of Voronezh State University

2.2. mtDNA isolation from frozen tissue

mtDNA was isolated from frozen brain and liver tissues. Fifty mg of tissue was homogenized in 2 ml of PBS buffer (Invitrogen, USA). The homogenate was centrifuged at 13,000*g* for 1 min, and mtDNA was isolated with a Plasmid Miniprep Kit (Evrogen, Russia) as recommended by the manufacturer.

2.3. mtDNA isolation from isolated mitochondria

Three hundred mg of tissue was homogenized with a Dounce tissue grinder in 25 ml of the mitochondria isolation buffer containing 220 mM mannitol, 100 mM sucrose, 1 mM EGTA, 20 mM HEPES, 2 mg/ml BSA, pH 7.4. The homogenate was centrifuged at 900*g* for 5 min. The pellet was discarded, and the supernatant was centrifuged at 10,000*g* for 10 min. The resulting pellet was resuspended in the mitochondria isolation buffer without BSA and centrifuged at 10,000*g* for 10 min. The pellet was then resuspended in 1 ml of PBS buffer and divided between two 1.7-ml microtubes. H_2O_2 (500 μM) was added to one of the tubes, and the tubes were incubated for 30 min. mtDNA was them isolated from the treated and control mitochondria with a Plasmid Miniprep Kit.

mtDNA was additionally purified with Agencourt AMPure XP magnetic beads (Beckman Coulter, USA). The beads were added to mtDNA solution at a 0.4x (v/v) ratio; the beads with the bound mtDNA were washed twice with 70% ethanol. mtDNA was eluted with 25 μl of 0.1X TE buffer.

The extent of mtDNA enrichment was determined by qPCR as described by Quispe-Tintaya et al. (2013) and calculated using the standard equation RQ = 2(-ΔΔCq), were 1 was the ΔCq value for total DNA isolated from tissue homogenate using Quick-gDNA MiniPrep kit (Zymo Research, USA).

2.4. Quantitative PCR

To validate qPCR as a method for assessing mtDNA damage, mtDNA fragments of different lengths (295, 1326, 2069, 4546, and 9158 bp) were amplified using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA) (Table 1) (). The reaction conditions were: 5 min at 95 °C, followed by 35 cycles of 95 °C for 10 s, 61 °C for 30 s, 72 °C for 2–16 min (depending on the fragment length). The reaction mixture contained 0.4 μl of Encyclo-polymerase, 2 μl of 10X Encyclo buffer, 0.2 mM of each dNTP (all reagents from Evrogen, Russia), 1X SYBR Green Master Mix (BioDye, Russia), and 0.5 μM forward and reverse primers in a total volume of 20 μl. The linear character of PCR was confirmed using serial mtDNA dilutions (from 1 ng to 1 pg of template DNA); the reaction efficiency was calculated from the Eq. (1) (Yuan et al., 2006):

$$E = 10^{(-1/\text{slope})} \quad (1)$$

After qPCR optimization mentioned above, primers for the detection of lesions were designed. Primers for amplification of nine mtDNA fragments (Table 2) () were designed using the primer3 software (Untergasser et al., 2012). The extent of excessive mtDNA damage which was induced by H_2O_2 or rotenone was estimated using the ΔΔCq method: ΔCq for the control and experimental (damaged) long fragments was compared to ΔCq for the control and experimental short fragments. Since the amplified fragments differed in length, the number of mtDNA lesions was calculated per 10 kb of mtDNA using the Eq. (2) (Rothfuss et al., 2010)

$$\textit{Lesions per } 10\text{ kb} = \left(1 - 2^{-(\Delta long - \Delta short)}\right) * {}^{10000}\,\text{bp}/_{f}\textit{ragment length, bp} \quad (2)$$

To detect 8-oxoG, 50 ng of mtDNA was incubated in 50 μl of reaction mixture containing 4 U of FPG (NEB, USA) at 37 °C for 1 h followed by 20 min incubation at 60 °C to inactivate the enzyme. mtDNA fragments were then amplified, and the number of lesions was calculated.

The size of the PCR products was determined by electrophoresis in 2% agarose gel.

Table 1
Optimized mtDNA qPCR primer set.

		PCR product	Length fragment	PCR efficiency, %	Linearity, R^2
ChrM: For.	ACGAGGGTCCAACTGTCTCTTA				
ChrM: Rev. 1	TAGGGTAACTTGGTCCGTTGAT	2078–2372	295 bp	99.7	0.9956
ChrM: Rev. 2	CCGGCTGCGTATTCTACGTT	2078–3403	1326 bp	92.2	0.9974
ChrM: Rev. 3	TAGTTGAGTACGATGGCCAGGA	2078–4146	2069 bp	90.1	0.9919
ChrM: Rev. 4	GCCCAGGAAATGTTGAGGGA	2078–6623	4546 bp	64.1	0.9498
ChrM: Rev. 5	TGGCTATAAGTGGGAAGACCATT	2078–11235	9158 bp	33.6	0.9186