Sensitive Detection of the 4977-bp Deletion in Human Mitochondrial DNA of Young Individuals

Christoph Meissner and Nicole von Wurmb
Medical University of Luebeck, Luebeck, Germany

Specific deletions in mitochondrial DNA (mtDNA) are present at high levels in skeletal muscle and other tissues (7). It has been suggested that an accumulation of these somatic deletions could be caused by oxidative damage that increases with age (2). In recent years, more than 20 different types of deletions have been identified using polymerase chain reaction (PCR) techniques (16). The most frequently observed deletion is 4977-bp long and occurs at a presumed deletion “hot spot” involving two 13-base direct repeats beginning at positions 8469 and 13447 in the mitochondrial genome sequence (4). This deletion shows a marked correlation to age (6,11) and has also been found in human brain tissue of patients with Parkinson’s disease (8), in heart-muscle tissue of patients with cardiomyopathy (12), in skeletal muscle of patients with Kearns-Sayre syndrome (17) and in several other degenerative diseases (13).

The undeleted mtDNA and the basal level of the 4977-bp deletion can be detected under specific PCR conditions (4,9). In the past few years, the amplification products were detected on agarose gels followed by ethidium bromide staining (9). Unfortunately, this technique does not detect very small quantities of DNA, and the signal is not permanent (15). Highly sensitive detection of nucleic acids in the picogram range has been achieved by the specific chemical reduction of silver ions (5).

Our former study showed that the smaller the deletion-specific fragments, the better the detection threshold (unpublished observations), because smaller fragments have a higher amplification efficiency (14). For this reason, we chose new primer sequences that were located nearer to the deletion breakpoints, so that smaller fragments could be amplified from the deleted mitochondrial genome (Figure 1). The use of these new primers and the detection on ultrathin polyacrylamide gels (PAG) led to an improved detection threshold and to the detection of the 4977-bp deletion in subjects younger than 20 years without any mitochondrial diseases, which has not yet been described by others.

Autopsy tissues were obtained from individuals representing a wide range of ages. Total DNA was isolated from 100 mg of skeletal muscle (musculus iliopsoas) using the Super QUIK-GENE SQG1 Kit (Immucor, Rödermark, Germany) following the manufacturer’s instructions. The amount of DNA was quantified by the Human DNA Quantitation System (Life Technologies, Gaithersburg, MD, USA). This slot-blot method is specific for human nuclear DNA and is very sensitive, with a detection limit of 20 pg DNA. In contrast to photometric quantitation, it makes possible the determination of the concentration of highly degraded DNA. Unfortunately this method does not detect mtDNA. Currently, to our knowledge, there is no mtDNA-specific slot blot for a routine analysis available. Assuming that nearly 1% of total extracted DNA is mtDNA, we used the nuclear-specific slot blot to get an ap-

Figure 1. Demonstration of the strategy for a specific amplification of deleted mtDNA. Schematic presentation. The primers flank the part of the 4977-bp deletion and should produce an approximately 5-kb PCR product from normal genomes and the described products (109, 238 and 260 bp) from 4977-bp deleted mitochondrial genomes under specific PCR conditions.
proximate clue as to how much mtDNA is in the sample to be analyzed. The primers were chosen according to the Anderson sequence (3) and used together with modified primers that had already been established (9,11). They were synthesized by MWG Biotech (Ebersberg, Germany).

First, the amplification of the undeleted mitochondrial DNA was performed using the primer pair L15/H16 (nucleotide positions [np] 3304–3328 and 3563–3539) to standardize the total mtDNA content in the sample. In a second PCR, the deletion-specific products were amplified. The primer pair L45/H25 (np 8412–8436 and np 13650–13626) revealed a 260-bp, deletion-specific fragment; the primer pair L35/H45 (np 8285–8309 and np 13499–13475) yielded a 238-bp PCR product. The new primers L45/H45 led to the smallest fragment (109 bp) as shown in Figure 1. PCR was carried out using standard buffer (PE Applied Biosystems, Weiterstadt, Germany) and an AmpliTaq Gold™ Polymerase (PE Applied Biosystems) concentration of 1 U in a 25-µL reaction mixture. The use of AmpliTaq Gold Polymerase enabled a hot-start PCR since activation of the enzyme for 7 min at 94°C initiated simultaneous amplification in all specimens.

The concentrations of the primers, the magnesium chloride and the nucleotide triphosphates were 0.0004, 1.2 and 0.2 mM per dNTP, respectively. The samples were placed in a GeneAmp® PCR System 2400 (Perkin-Elmer, Norwalk, CT, USA) with cycling conditions set as follows: 1 step of 7 min at 94°C for enzyme activation, 30 cycles at 94°C for 40 s, 65°C for 40 s and 72°C for 50 s, with a final extension for 7 min. PCR products were separated on ultrathin polyacrylamide gels at 1000 V, 50 mA, 20 W on a horizontal electrophoresis chamber (Biophoresis; Bio-Rad, Munich, Germany) for 45 min and silver-stained according to standard methods (1).

To confirm that the amplified DNA fragments were not due to mis-annealing of primers, we further analyzed the deletion of mtDNA by the primer-shift PCR method. Four different fragments of deleted mtDNA were amplified with the following primer pairs: L35/H45 (389 bp), L45/H25 (260 bp), L35/H45 (238 bp) and L45/H45 (109 bp). In 20 examined DNA samples from skeletal muscle of aged subjects, each of the PCR products had the expected fragment length of the Anderson sequence (3). In addition, the PCR products of the deleted and the undeleted mtDNA were sequenced on a ABI PRISM™ 310 Genetic Analyzer (PE Applied Biosystems). The sequences obtained were consistent with the mtDNA sequence published by Anderson et al. (3). Thus, we could verify that the signals were authentic and not the result of nonspecific artifacts of the PCR. It was also observed that the PCR products amplified from the 4977-bp deleted mtDNA had exactly the predicted length. Sequencing these products of 50 different individuals showed that the deletion breakpoints were always between the same nucleotide positions, either np 8469 and 13447 or np 8482 and 13460, as previously described (17).

Under the PCR conditions described above, we were able to obtain signals up to a detection limit of 10 fg template DNA for the undeleted and 10 pg for the deleted mtDNA, depending on the age of the individual (Figure 2). This detection threshold is better than the lowest values shown in the literature by Mansouri and coworkers (10), with 100 fg for the undeleted and 2.5 ng for the deleted mtDNA. Additionally, we were able to detect the 4977-bp mtDNA deletion in skeletal muscle from...
subjects younger than 20 years old without any mitochondrial diseases (Figure 3).

We think that this improvement is the result of our new primers, which yield smaller fragments with a higher amplification efficiency and of our detection on silver-stained PAG. Improvement of the amplification conditions enables easy amplification of the specific signals of mtDNA from total human DNA and eliminates the need for difficult and time-consuming isolation of mitochondria or preparation of mtDNA. It suffices to prepare high-molecular-weight DNA from various tissues using commercially available kits, which are quick and usually free of toxic contents such as phenol.

Moreover, the lower threshold makes it possible to reduce the cycle number from the previously needed 40 to 30 cycles. This reduces the contamination risk and may be crucial for forensic applications and for quantitative analysis to determine the age at death in soft tissues of unidentified persons as previously described (11).

It is now possible to detect the 4977-bp deletion of mtDNA even in younger subjects and from less amount of materials. This could be important for the investigation of biopsy material. Up to now, most results indicate that the deletion occurs in relation to age or degenerative diseases. We could demonstrate that even in younger individuals without mitochondrial diseases, very low levels of the 4977-bp deletion are detectable in muscle. Thus use of our new protocol can probably provide more information towards understanding the origin and the genesis of the 4977-bp deletion of human mtDNA.

REFERENCES