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Demonstration and semi-quantification of mtDNA from human dentine and its relation to age

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Abstract In order to study if mitochondrial DNA (mtDNA) could be retrieved from isolated human dentine, small pieces of dentine were cut out from the central part of the apical half of wisdom teeth from 21 individuals aged 15 to 85 years. The dental pulp was used as a control. After extraction, amplification and agarose gel electrophoresis the amount of mtDNA was semi-quantified from the intensity of the stained bands in the gel. Mitochondrial DNA was retrieved from all samples and the sequences were identical in pulp and dentine from each individual. There was a clear age-dependent decrease in the amount of amplified mtDNA. Since the odontoblastic processes in the apical dentine undergo degeneration with age and the dentinal tubules subsequently become occluded with calcium phosphate crystals, the conclusion was drawn that even after dissolution of the odontoblastic processes, at least remains of the mtDNA are trapped in the dentine. This well protected mtDNA could thus be regarded a good source of DNA in identification cases with severe degradation.

Key words Mitochondrial DNA · Quantification · Dentine · Age

Introduction

The forensic application of mtDNA has gained increasing interest over the past few years. Because of the stable circular structure of the molecule and the occurrence of many copies per cell [1], mtDNA typing appears to be more effective than nuclear DNA in old remains with advanced decomposition or skeletonisation [2–4].

In several studies [5–9], teeth have been shown to be a good source of both nuclear and mitochondrial DNA. In most of these studies as much soft tissue as possible was usually collected, including the cell-rich pulp, the dentine (odontoblastic processes), the cementum (cementoblasts and cementocytes) and the periodontal tissue. No study has so far been dedicated to study the various tissues, or tissue components, separately.

The odontoblastic processes (also called Tomes' dentinal fibres) project from the odontoblasts of the pulp into the dentine [10]. In a cross section there are 20,000–45,000 processes per mm^2. These processes contain numerous mitochondria which can be expected to be extremely well protected in the dentinal canaliculae. The odontoblastic processes undergo regression in an age dependent manner with the subsequent occlusion of the canaliculae with calcium phosphate crystals.

It is not known whether the odontoblastic processes are completely disintegrated, or if there are remains of mitochondria with mtDNA trapped in the canaliculae. If there is remaining mtDNA, this would be a good source of mtDNA for identification of decomposed bodies because of its protected localisation.

This experiment was performed to study if mtDNA can be retrieved from dental canaliculae and if a correlation to the age of the person exists.

Material and methods

Samples and sample preparation

Wisdom teeth (n = 21) from unrelated Koreans aged 15 to 85 years were obtained from dentists immediately after extraction. The teeth were divided into seven 10-year-groups with three teeth in each group and stored at –20 °C until used. After mechanical cleaning, the teeth were washed in sterile distilled water followed by a 15 s wash in 10% sodium hypochlorite commercial bleach, a 15 s wash in 95% ethanol and air dried (following the instructions

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of the AFDIL – Armed Forces DNA Identification Laboratory, Rockville, MD, USA). Using a diamond cutting disc the crowns were separated from the roots. The roots were then cut along the midline, the pulp was removed using a spoon excavator and from the central part of the apical half of the root a small block of dentine was separated using a diamond disc. The isolated dentine was briefly washed in 10% commercial bleach in order to destroy possible remains of soft tissue DNA. Finally the dentine pieces were dried, mechanically ground and divided into aliquots of 10 mg each.

DNA extraction

To each dentine and pulp sample and to the reagent blank 700 µl extraction buffer (10 mM Tris, pH 8.0; 100 mM NaCl; 50 mM EDTA, pH 8.0; 0.5% SDS) and 20 µl proteinase K (20 mg/ml) were added, vortexed and incubated at 56 °C overnight. To the extract volume 720 µl phenol/chloroform/isoamyl alcohol (25:24:1) was added, vortexed and centrifuged for 2 min at 10,000–15,000 rpm in a microcentrifuge. The upper aqueous layer was transferred to a sterile labelled microcentrifuge tube and extraction was repeated twice, 720 µl 1-butanol was added and the tubes were vortexed and centrifuged for 2 min at 10,000–15,000 rpm. The lower aqueous layer was transferred to the sample reservoir of a Centricon-100 concentrator (Amicon), 1 ml TE buffer was added and the columns were centrifuged at 1000 × g for 20 min or until the sample had spun through. The washing step with TE buffer was repeated twice. The retentate was transferred into a microcentrifuge tube and stored at –20 °C.

mtDNA amplification and sequencing

The amplification for hypervariable region 2 (HV2) of the mitochondrial D-loop was performed in a Perkin Elmer 9600 thermal cycler for all samples in one amplification run.

Primers (AFDIL instructions):

F15/34 (5′ CAC CCT ATT AAC CAC TCA CG)

R448/465 (5′ TGA GAT TAG TAG TAT GGG AG).

The PCR master mix for a 25 µl reaction consisted of 2.5 µl 10 × Perkin-Elmer PCR buffer (1 × 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$), 2 µl 2.5 mM dNTP mix, 1 µl 10 µM forward amplification primer, 1 µl 10 µM reverse amplification primer, 0.25 µl of AmpliTaq DNA Polymerase and 2.5 µl of the DNA extract. The PCR conditions were 94 °C for 30 s followed by 32 cycles at 94 °C for 20 s, 56 °C for 10 s, 72 °C for 30 s, followed by 5 °C soak. Of the PCR product 5 µl was added to 1 µl 6 × agarose gel loading buffer, loaded on a 2% agarose gel, electrophoresis was carried out at constant voltage (90 V) for approximately 30 min and visualized on a trans-illuminator. A sizing ladder was included on the gel (1 µl of 123 base pair ladder, 4 µl dH_2O and 1 µl of 6 × loading buffer). The gel was photographed with a Polaroid camera fitted with an orange filter. Prior to sequencing the PCR product was purified using Centricon 100 spin dialysis columns (Amicon). Direct sequencing was performed as described before [11]. HV2 was analysed between the positions 73 and 340 according to the Anderson sequence [12].

Semi-quantification of amplified mtDNA

The amount of mtDNA obtained after amplification was semi-quanitatively assayed on the photograph of the agarose gel.

Results

Mitochondrial DNA was successfully extracted, amplified and sequenced in HV2 from all 21 pulp and dentin samples. In all cases the nucleotide sequences of the pulp and the dentine samples were identical. The result of the sequencing and the discussion of the obtained data base have been reported elsewhere [11]. There was a clear decrease in the amount of mtDNA in the dentine with age (Fig. 1). The highest amounts were found in the lowest age groups and the lowest amounts in the highest age groups. Four samples did not contain enough mtDNA to show bands on the gel after amplification from 2.5 µl extract, but gave unambiguous sequencing results when the concentrations of the DNA extract and of Taq polymerase were increased in later amplification reactions.

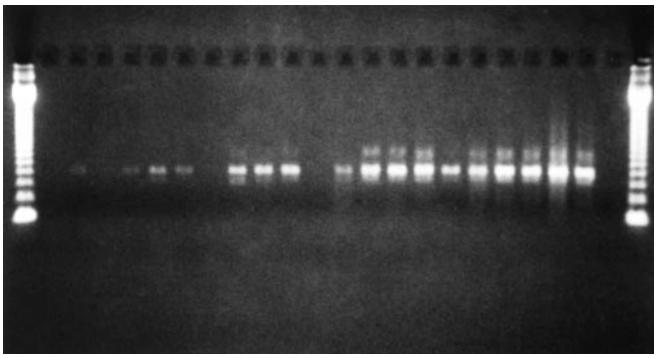

Fig. 1 Amplification products in HV2 of the mtDNA D-loop region (2.5 µl of the DNA extract in a 25 µl PCR reaction mix). DNA was extracted from isolated dentine from individuals of different ages. From left to right lane 1: 123 bp ladder, lanes 2–4: 71–80 years, lanes 5–7: 61–70 years, lanes 8–10: 51–60 years, lanes 11–13: 41–50 years, lanes 14–16: 31–40 years, lanes 17–19: 21–30 years, lanes 20–22: ≤ 20 years, lane 23: reagent blank, lane 24: 123 bp ladder

Discussion

The main purpose of this study was to clarify if mtDNA could be recovered from mitochondria of the odontoblastic processes. These processes take part in the formation of the dentine, but subsequently retract and/or disintegrate starting from the apical end of the dental root. The formation of sclerotic, also called translucent, root dentine is an age-dependent process starting a few years after the tooth is fully formed and then processing in an occlusal direction until the complete root is translucent at the age of about 80 years [13, 14]. Since mtDNA could be found in samples from old individuals we would suggest that the odontoblastic processes do not retract, but rather disintegrate leaving mitochondria, with mtDNA, or at least fragments of mtDNA, intermingled between the calcium phosphate crystals. The possibility that there is an age-dependent accumulation of inhibitors of the PCR reaction is unlikely, since no such age-dependent decrease has been noted in nuclear DNA from the pulp.

At present there do not exist exact methods to quantify mtDNA directly. Available slot-blot-hybridisation techniques are non-specific and do not separate human mtDNA from RNA, and human from non-human mtDNA. Therefore an indirect, semi-quantitative measurement of the rel-

ative intensities of the stained bands of the gel after electrophoreses of amplified DNA was used in this study. Even if single samples after PCR amplification were too small to show up on the gel after electrophoresis, there was a clear age-dependent decrease in the amount of mtDNA in the samples.

The hypervariability within the human mitochondrial D-loop region is high enough to be used for identification case work since the rate of mutations in mtDNA is 10 times faster than in nuclear DNA [15]. However, questions about its use have been raised since heteroplasmy seems to be frequent in the noncoding mitochondrial regions of bradytrophic tissues like the brain [16] and individual hair roots [17].

Mitochondrial DNA in the odontoblastic processes could thus be expected to show variable levels of heteroplasmy. In this study the sequences from the odontoblastic processes and from the dental pulp were identical in all cases, indicating that heteroplasmy is not a major problem using nuclei-free dentine as source for mtDNA. However, the present sequencing technique is not optimal for determination of minor amounts of heteroplasmic mtDNA, because of the background noise in the electropherograms. Further studies are underway to explore the degree of heteroplasmy in dentine with the DGGE (denaturing gradient gel electrophoresis) technique.

In conclusion, we have shown that isolated human dentine from extracted healthy teeth contains mtDNA which can be amplified and used for personal identification and that the amount of mtDNA decreases with age. Further systematic studies are underway to confirm and extend our pilot study on decomposed bodies, skeletons and teeth.

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