Biomarkers of DNA damage in patients with end-stage renal disease: mitochondrial DNA mutation in hair follicles

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Abstract

Background. DNA damage was noted in patients with end-stage renal disease (ESRD). Mitochondrial DNA (mtDNA) mutations have been proposed as a genomic biomarker in the process of human ageing, degenerative diseases and carcinogenesis.

Methods. Polymerase chain reaction (PCR) techniques were applied to detect mtDNA deletions in hair follicles, an appendage of skin, from 162 patients with ESRD.

Results. The incidences of the 4977 bp deletion of mtDNA in hair follicles were found to increase with age in normal control and ESRD patients. As compared with normal subjects, ESRD patients had 3.5, 2.3, 2.7, 2.3 and 1.4 times higher incidences of the 4977 bp deletion of mtDNA in the age groups of 20–30, 31–40, 41–50, 51–60 and 61–70 years, respectively. Moreover, the difference in the proportion of mtDNA with the 4977 bp deletion was statistically significant between ESRD patients and normal subjects >50 years of age.

Conclusion. We suggest that the 4977 bp deletion of mtDNA in hair follicles may serve as one of the tissue biomarkers of genetic instability of the mitochondrial genome in ESRD patients.

Keywords: biomarker; deletion; end-stage renal disease; hair follicles; mitochondrial DNA

Introduction

In patients with end-stage renal disease (ESRD), a decrease in the capability of DNA repair in nuclear DNA (nDNA) has been demonstrated in the UV-irradiated lymphocytes of patients with ESRD with or without haemodialysis [1]. Increased incidences of structurally abnormal chromosomes and elevated rates of sister chromatid exchange were also found in the patients with ESRD [2]. Increase of DNA instability may be contributed to by an accumulation of mutagenic or carcinogenic heterocyclic amines in the dialysate or in the plasma of patients with ESRD [3]. These genotoxic substances may induce DNA mutation and promote high morbidity of cancer in ESRD patients [4].

As compared with nuclear DNA, human mitochondrial DNA (mtDNA) is much more susceptible to damage induced by mutagens or carcinogens as the result of a lack of proofreading and poor DNA repair during mtDNA replication [5]. In a previous study, we found that the incidence of the 4977 bp mtDNA deletion of hair follicles is not only a biomarker of ageing, but also an index of the genomic damage in cigarette smokers [6,7]. Moreover, the high proportion of the 4977 bp deletion of mtDNA in the hair follicles may be considered as one of the molecular events that are associated with the occurrence of smoking-associated cancers [7].

In this study, we used hair follicles of ESRD patients to investigate the frequency of occurrence and abundance of the 4977 bp deletion of mtDNA. Although environmental xenobiotics may be found in hair due to contamination by airborne pollutants, the components of hair are resistant to change by exposure to external chemicals including repeated shampooing, perming, relaxing and dyeing [8]. Since the hair follicles are deeply embedded in the dermis, the genotoxicity of hair follicular DNA may be considered as a suitable lifetime index of internal genomic damage in ESRD patients.

Subjects and methods

Patients

One hundred and sixty-two ESRD patients and 236 healthy controls were recruited for the present study. Informed
consent was obtained from each of the study subjects. All patients from a single dialysis centre were invited to participate in the present study. The baseline examination was conducted between May and June 1999. The causes of ESRD were chronic glomerulonephritis in 85 patients (53%), diabetic nephropathy in 30 (19%), chronic pyelonephritis in 18 (11%), polycystic kidney disease in three (2%) and other diseases in 16 (10%). The cause was unknown in 10 (6%) patients. One hundred and twenty-five patients received haemodialysis three times a week to maintain a Kt/V urea index of 1.2 per session. Thirty-seven patients under continuous ambulatory peritoneal dialysis received 4–51 bags to achieve a Kt/V urea index of 1.9 per week.

**Collection of human hair follicles**

Five to seven hair follicles were plucked from the occipital region of the scalp of the ESRD patients and normal subjects for the study of incidence of the mtDNA 4977 bp deletion, using the polymerase chain reaction (PCR). Thirty-six ESRD patients and 39 control subjects agreed to offer >15 hair follicles, from which there was sufficient DNA to perform the semi-quantitative PCR analysis of mtDNA with the 4977 bp deletion.

Patients and control subjects were instructed to avoid overexposure to the sun or chemicals. None of the patients or normal subjects recruited for this study had a history of any known mitochondrial diseases.

**Preparation of DNA from hair follicles**

Total DNA was prepared from hair follicles according to a procedure developed in this laboratory with some modification [9]. The hair follicles with a 2-cm hair shaft of ~0.5 cm in length were used for the extraction of total DNA. The lysis buffer contained 2% sodium dodecylsulphate, 10 mM dithiothreitol and 100 µg/ml proteinase K in 50 mM Tris–HCl buffer (pH 8.3). After digestion at 55°C for 1 h, the mixture was heated to 100°C for 5 min to inactivate proteinase K and then centrifuged at 14 000 g for 20 min to remove proteins and debris. The supernatant was extracted once each with phenol, phenol/chloroform (1:1 v/v), and chloroform, respectively. The aqueous layers were pooled and an equal volume of isopropanol was added, and the mixture was allowed to stand at 4°C for ~8 h. The DNA was then pelleted and dissolved in 10 mM Tris–HCl (pH 8.3) containing 1 mM EDTA (TE buffer).

**Synthesis of oligonucleotide primers**

The primers encompassing the target DNA sequences were chemically synthesized by Bio-Synthesis, Inc. (Lewisville, TX). The sequences of the synthesized oligonucleotides and the sizes of PCR products amplified from mtDNA molecules with a specific 4977 bp deletion or tandem duplication are shown in Table 1.

**Analysis of the incidence of mtDNA with a 4977 bp deletion in hair follicles**

Each desired fragment of mtDNA was amplified in a 50 µl reaction mixture containing 100 ng template DNA, 200 µM each of dNTPs, 1 U of Taq DNA polymerase (Perkin-Elmer/Cetus), 20 pmol of each primer, 50 mM KCl, 1.5 mM MgCl₂, and 10 mM Tris–HCl (pH 8.3). A total of 35 cycles of PCR was performed for each sample in a Perkin-Elmer/Cetus DNA thermal cycler. The first cycle was performed by 3 min denaturation at 94°C, 3 min annealing at 55°C and 1 min primer extension at 72°C. The other PCR cycles were conducted as follows: denaturation at 94°C for 40 s, annealing at 55°C for 40 s and extension at 72°C for 50 s.

**Primer-shift PCR for the confirmation of the 4977 bp deletion of mtDNA**

The authenticity of the 4977 bp deletion of mtDNA was ascertained by a primer-shift PCR method [10]. By using different primer sets listed in Table 1, we found that the length of the amplified DNA changed in parallel to the shift of the distance between each primer pair of Table 1. Since no DNA fragment could be amplified from the wild-type mtDNA with the PCR condition used (short extension time), the only DNA fragment was that amplified from the mtDNA molecules with the 4977 bp deletion. By using this method, we were able to conclude that the PCR-amplified DNA fragments were not generated by misannealing of the primers to DNA. The sizes of the PCR products amplified from the mtDNA molecules with the 4977 bp deletion by different primer pairs were 608 bp (L2–H4), 469 bp (L4–H2), 389 bp (L3–H3) and 275 bp (L2–H2), respectively. Figure 1 illustrates the primer-shift PCR for the detection of the 4977 bp deletion of mtDNA in human hair follicles.

**Analysis of the proportion of mtDNA with the 4977 bp deletion in hair follicles**

The total DNA extracted from each of the specimens was serially diluted 2-fold with distilled water. The range of dilution was usually between 2⁰ and 2¹⁷. The primers L1 (3304–3323) and H1 (3753–3734) were used for the amplification of a 450 bp DNA fragment from total mtDNA, and primers L4 (8150–8169) and H2 (13595–13576) were used for the amplification of a 469 bp product from mtDNA with the 4977 bp deletion. Amplified DNA fragments were then separated by electrophoresis on a 1.2% agarose gel at 100 V for 40 min and were visualized fluorographically after staining with 0.5 µg/ml of ethidium bromide. The gel patterns

![Fig. 1. Electrophoretogram of PCR products amplified from mtDNA with the 4977 bp deletion from scalp hair follicles. Lanes 1 to 4 indicate 275, 389, 469 and 608 bp PCR products amplified by primer pairs L2–H2, L3–H3, L4–H2 and L2–H4, respectively, from mtDNA with the 4977 bp deletion. Lane M represents the 100 bp ladder DNA size marker.](image-url)
Table 1. Primers used for PCR amplification of mtDNA with the 4977 bp deletion

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Amplified position and size 5'→3' (bp)</th>
<th>Size of the PCR product (bp) amplified from mtDNA with the 4977 bp deletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1–H1*</td>
<td>3304–3753 (450)</td>
<td>450</td>
</tr>
<tr>
<td>L2–H2</td>
<td>8344–13595 (5252)</td>
<td>275</td>
</tr>
<tr>
<td>L3–H3</td>
<td>8285–13650 (5366)</td>
<td>389</td>
</tr>
<tr>
<td>L4–H2</td>
<td>8150–13595 (5446)</td>
<td>469</td>
</tr>
<tr>
<td>L2–H4</td>
<td>8344–13928 (5585)</td>
<td>608</td>
</tr>
</tbody>
</table>

*The primer set L1–H1 was used for the determination of the total mitochondrial DNA. L1 (3304–3323) 5'-AACATACCCATGCTCAGCTG-3', L2 (8344–8363) 5'-ACCAACACCTCTTTCAGTG-3', L3 (8285–8305) 5'-CTCTGACGCCGTAAAGC-3', L4 (8150–8169) 5'-CCGGGATTATCAGCGTGGA-3'.

Table 2. The frequency of occurrence of mtDNA with the 4977 bp deletion in the hair follicles of normal subjects and patients with ESRD

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Normal subjects</th>
<th>ESRD patients</th>
<th>OR</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ b</td>
<td>– b</td>
<td>+ (%)</td>
<td></td>
</tr>
<tr>
<td>20–30</td>
<td>3</td>
<td>32</td>
<td>8.6</td>
<td>3</td>
</tr>
<tr>
<td>31–40</td>
<td>7</td>
<td>43</td>
<td>14.0</td>
<td>15</td>
</tr>
<tr>
<td>41–50</td>
<td>6</td>
<td>36</td>
<td>14.3</td>
<td>14</td>
</tr>
<tr>
<td>51–60</td>
<td>19</td>
<td>39</td>
<td>20.4</td>
<td>18</td>
</tr>
<tr>
<td>61–70</td>
<td>19</td>
<td>41</td>
<td>31.6</td>
<td>13</td>
</tr>
<tr>
<td>All</td>
<td>45</td>
<td>191</td>
<td>19.1</td>
<td>63</td>
</tr>
</tbody>
</table>

*Significant level determined by χ² (Fisher’s exact test was applied if any one of the expected frequency was <5).

+ /– denotes the presence/absence of mtDNA with the 4977 bp deletion.

Incidence of the mtDNA mutation in the hair follicles.

of PCR products were photographed under UV-light transillumination. The proportion of mtDNA with the 4977 bp deletion was determined by the ratio between the highest fold of dilution that allowed the 469 bp PCR product amplified from mtDNA with the 4977 bp deletion to be detectable in the gel, and the fold of dilution that allowed the 450 bp PCR product to be visibly amplified from total mtDNA under identical conditions [10].

Statistical analysis

The χ² test was applied (Fisher’s exact test was used if any one of the expected frequencies was <5) to compare the results from patients with those of age-matched control groups. The overall odds ratio (OR), 95% confidence interval (CI) and the P value were calculated using the SAS statistical package (SAS Institute, Cary, NC).

Results

Incidence of the 4977 bp deletion of mtDNA in hair follicles

Using the PCR techniques with the primer pairs of L4–H2, the 4977 bp mtDNA deletion in the total DNA of human hair follicles from all the subjects recruited in this study was screened first. The incidence of the 4977 bp deletion was found to increase with age in the normal control subjects and ESRD patients. In the patients with ESRD, the incidences of the 4977 bp deletion of mtDNA in hair follicles were found to be 30.0%, 31.9%, 40.0%, 43.9% and 44.8% in the 20–30, 31–40, 41–50, 51–60 and 61–70 years age groups, respectively (Table 2). In contrast, the incidences of the mtDNA deletion in the hair follicles of the healthy subjects of the five age-matched groups were 8.6%, 14.0%, 14.3%, 20.4% and 31.6%, respectively (Table 2). As compared with the normal subjects, the ESRD patients had 3.5-, 2.3-, 2.7-, 2.3- and 1.4-fold higher incidences of the 4977 bp deletion of mtDNA in each of the five age groups, respectively. However, we noted that there were only significant differences in the incidence of the 4977 bp deletion of mtDNA between the ESRD patients and normal subjects in the 31–40, 41–50 and 51–60 years age groups (Table 2).

Proportion of mtDNA with the 4977 bp deletion in hair follicles

The proportions of mtDNA with the 4977 bp deletion in hair follicles increased with age in both ESRD patients and normal controls. The proportions of
mtDNA with the 4977 bp deletion in hair follicles were estimated as 0.068 ± 0.019, 0.147 ± 0.021 and 0.594 ± 0.102% for ESRD patients in the 21–30, 31–50 and 51–70 years age groups, respectively. In contrast, the proportions of mtDNA with the 4977 bp deletion were determined to be 0.039 ± 0.023, 0.096 ± 0.056 and 0.291 ± 0.047%, respectively, for the normal controls of the corresponding age-matched groups. A statistically significant difference in the proportion of mtDNA with the 4977 bp deletion in hair follicles was found between ESRD patients and normal subjects in the 51–70 years age group (P < 0.05) (Figure 2).

Discussion

Human mtDNA is a 16 569 bp double-stranded closed circular DNA molecule, which codes for 13 polypeptides that are essential for oxidative phosphorylation, 22 tRNAs and two rRNAs that constitute the protein synthesis machinery in mitochondria [11]. As compared with nDNA, mtDNA is much more susceptible to damage induced by mutagens or carcinogens as the result of a lack of protective histones, poor proofreading during DNA replication and lower efficiency of DNA repair [5]. These particular characteristics of mtDNA have rendered it vulnerable to both intrinsic and extrinsic oxidative damages. For example, carcinogenic polycyclic aromatic hydrocarbons (PAH) are accumulated preferentially in the mitochondria of animal cells, and the PAHs of differing carcinogenicity were found to bind to the mtDNA of cultured mouse embryo cells 50 to >500 times more readily than to nDNA [12,13]. Several ESRD-associated carcinogens or mutagens have also been shown to promote the formation of not only nDNA adducts, but also mtDNA adducts [14,15]. The incidence of mtDNA with the 4977 bp deletion in hair follicles was recently disclosed to be greatly increased in subjects who were long-term smokers [7]. Thus, mtDNA with the 4977 bp deletion may also be a valuable biomarker in the study of genotoxicity of the uroemic milieu in ESRD patients. However, in the groups of haemodialysis-treated patients and continuous ambulatory peritoneal dialysis-treated subjects, there was no significant difference in the incidence of mtDNA with the 4977 bp deletion between these two groups (data not shown).

The 4977 bp deletion of mtDNA, which removes a DNA segment from the nucleotide position (np) 8483–13459, has been established as the most common and abundant large-scale deletion of mtDNA in various human tissues [16]. Three major mechanisms, including homologous recombination, topoisomerase cleavage and slip-replication, have been proposed to do this [17]. Slipped mispairing during mtDNA replication is one of the most widely accepted mechanisms to explain the occurrence of this common mtDNA deletion, whose breakpoints are flanked by a 13 bp direct repeat (5'–ACCTCCCTACCA-3'). The direct repeats may be mis-recognized during annealing, when the open circular or linear forms of mtDNA are generated by the single-strand breaks via the attack of electrophilic mutagens or carcinogens [18,19]. Thus, researchers can predict that the 4977 bp deletion of mtDNA can be easily induced in tissue cells that are exposed to ESRD-associated toxic metabolites [20].

In this study, we have demonstrated for the first time that ESRD is an add-on factor to promote mtDNA mutation in human hair follicles. The predominant 4977 bp deletion of mtDNA may imply the existence of genetic instability in the mitochondrial genome of ESRD patients, and this common deletion may serve as one of the biomarkers for the study of genotoxicity of the uroemic milieu in patients with ESRD.

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