The gender-related alterations in the telomere length and subtelomeric methylation status in patients with Parkinson’s disease

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Abstract

The aim of this study was to determine whether Parkinson’s disease affects somatic telomeric features. Some recent reports have shown that telomere length is not changed in patients with Parkinson’s disease (PD). In this study, we more closely evaluated possible Parkinson’s disease-associated telomeric alterations than has been done previously. We analyzed the telomere length distribution, the subtelomeric methylation status, and their gender-related differences, as well as the mean telomere length in PD patients in comparison to age-matched controls. The telomeric parameters of the peripheral leukocytes of Parkinson’s disease outpatients and normal healthy volunteers, including family members of the participating outpatients were determined by analyzing the densitometries of the Southern blot results obtained with methylation-sensitive and insensitive isoschizomers. The Parkinson’s patients had gender-related differences in the alterations of their telomere length and subtelomeric status. Only female patients had significant Parkinson’s disease-associated telomeric and subtelomeric changes. The female Parkinson’s patients bore proportionally decreased long telomeres (>9.4 Kb) and less methylation of short telomeres (<4.4 Kb) in comparison with healthy controls, both of which have been regarded to be a part of aging-associated telomeric and subtelomeric changes. These results suggested that the aging-related telomeric and subtelomeric changes are accelerated specifically in female Parkinson’s patients, and that genomic DNA is more strongly affected by Parkinson’s disease in females than in males.

Introduction

A telomere is a structure consisting of thousands of hexamer (TTAGGG/AATCCC) repeats and accessory peptide factors located at the termini of human chromosomes.1,2 Telomeres become shortened little by little because of the inability of complete DNA duplication at the chromosome ends. This process is known as the end-replication problem. Such telomere shortening has been observed in peripheral blood nuclear cells with aging in a gender-related manner.3 In addition, telomere shortening is accelerated by various pathological conditions including physical and mental stress, which yield systemic or local oxidative stress. In fact, telomere shortening is accelerated by disease conditions such as mental stress, obesity, smoking, type 2 diabetes mellitus, ischemic heart diseases, Alzheimer’s disease, and sarcoidosis.4,9 In all of these reported diseases, increased oxidative stress has been suggested to potentially relate to the enhanced shortening of telomere. Such pathological conditions can be hypothesized to affect not only the telomeric structure itself but also the surrounding genomic structures including the epigenetic status, such as DNA methylation. Shortened telomeres have been reported to tend to accompany subtelomeric hypomethylation in mice of fifth generation the telomerase activity-deficient tert/tert-mutant mouse.10 Therefore, aging-associated telomere length shortening may also be affected by less methylation in the subtelomeric region. The subtelomeric methylation status can be associated with aging-related telomere attrition, which is enhanced in various kinds of disease conditions. Parkinson’s disease (PD) is a neurodegenerative disorder characterized by a progressive degeneration of dopaminergic neurons. Localized chronic inflammation and mitochondrial dysfunction are the causative factors of pathogenetical oxidative stress for neurodegeneration in PD.11-15 Restricted motor behavior enhances mental stress of PD patients. The causative and the secondary stress for PD may cause fragility in the telomeric and subtelomeric structure of circulating leukocytes. It has been hypothesized that the elevated oxygen stress associated with PD may lead to telomere shortening. Some reports, however, have shown that the somatic telomere length is not shorter in PD patients.16-18 We herein tried to confirm whether or not the telomere structure is altered in PD patients. In order to detect the detailed aging-related changes in the telomeric structure in PD patients, the subtelomeric methylation changes associated with aging as well as the telomere length changes were analyzed in healthy Japanese subjects and Japanese PD patients.

Materials and Methods

Study population

Parkinson’s disease patients (19 men and 17 women) visiting the outpatient clinic of the Kyushu University Beppu Hospital from November 2008 through December 2010 were enrolled, and some of their family members and some of our hospital’s healthy workers, who passed a regular medical check-up within a year before the enrollment, were also enrolled as healthy controls (27 men and 22 women). The present research was performed, following the approval by the Conjoint Health Research Ethics Board of Kyushu University, and written consent was obtained from all the participants. DNA samples were obtained from peripheral leukocytes of de novo female PD patients diagnosed according to the Japan Parkinson’s Disease Society Brain Bank criteria. Blood samples were collected from the patients before the administration of anti-Parkinson agents started. The numbers and the ages of the participants are described in Table 1. There were no statistical differences in the ages between males and females, and between controls and PD patients. Blood samples were drawn, using heparinized syringes and 10 mL vacutainer
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Telomere detection

Telomere detection was performed as previously described with a modification (3). Methylation-sensitive and -insensitive isoschizomers, HpaII and MspI, were used. Briefly, blood cell DNA was extracted from samples and the DNA (1 μg) was digested. The digests (10 μL) were Southern hybridized to a probe of 500 bp long (TTAGGG)n labeled with digoxigenin (dig). The smears of the autoradiogram were captured on an Image Master, and the telomere length was then assessed quantitatively. Every sample was measured in triplicate.

Terminal restriction fragment length analysis

The mean terminal MspI restriction fragment length analysis (TRFs) were estimated using the formula \( \frac{OD_i - \text{background}}{\Sigma(OD_i - \text{background})/\Sigma(OD_i - \text{background})L_i} \), where \( ODi \) is the densitometric intensity of a TRF fragment at position \( i \). Telomere length distribution was analyzed by comparing the telomere length using a telomere percentage analysis with three intervals of length as defined by a molecular weight range was calculated. The percent of telomeric methylation with different telomere sizes. The difference between the percent of MspI signal intensity (%MspI-TRF) and the HpaII signal intensity (%HpaII-TRF) in each molecular weight range was calculated. The proportion of the calculated difference (%MspI-TRF %HpaII-TRF) in >9.4 Kb range to %HpaII-TRF in >9.4 Kb range was used to evaluate the methylation status of telomeres with telomeric and subtelomeric methylated region longer than 9.4 Kb. Similarly, the proportion of the calculated difference (%MspI-TRF %HpaII-TRF) in <4.4 Kb range to %MspI-TRF in >4.4 Kb range was used to evaluate the methylation status of telomeres shorter than 4.4 Kb.

Results

The PD patients showed no significant changes in the MspI-TRF in comparison to con-

Table 1. The ages of the participants

<table>
<thead>
<tr>
<th></th>
<th>Normal (n)</th>
<th>Parkinson (n)</th>
<th>P-value (disease-related)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>54.3±4.6 (27)</td>
<td>56.0±4.4 (19)</td>
<td>0.21</td>
</tr>
<tr>
<td>Age range</td>
<td>49-64</td>
<td>49-62</td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>55.0±4.6 (22)</td>
<td>57.4±4.4 (17)</td>
<td>0.12</td>
</tr>
<tr>
<td>Age range</td>
<td>48-62</td>
<td>47-61</td>
<td></td>
</tr>
<tr>
<td>P-value (gender-related)</td>
<td>0.57</td>
<td>0.36</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2. The telomeric parameters of the participants

<table>
<thead>
<tr>
<th></th>
<th>PM</th>
<th>PF</th>
<th>NM</th>
<th>NF</th>
<th>PM/NM</th>
<th>PF/NF</th>
<th>PM/PM</th>
<th>P-value (gender-related)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MspI-TRF (Kb)</td>
<td>6.9±0.9</td>
<td>6.6±1.2</td>
<td>6.6±1.3</td>
<td>7.4±1.5</td>
<td>0.350</td>
<td>0.083</td>
<td>0.341</td>
<td>0.079</td>
</tr>
<tr>
<td>HpaII-TRF (Kb)</td>
<td>8.2±1.0</td>
<td>7.8±1.2</td>
<td>7.9±1.3</td>
<td>8.8±1.5</td>
<td>0.349</td>
<td>0.029</td>
<td>0.288</td>
<td>0.031</td>
</tr>
<tr>
<td>H-M-TRF (Kb)</td>
<td>1.3±0.5</td>
<td>1.2±0.6</td>
<td>1.3±0.5</td>
<td>1.4±0.5</td>
<td>0.941</td>
<td>0.208</td>
<td>0.745</td>
<td>0.223</td>
</tr>
<tr>
<td>&gt;9.4 Kb MspI (%)</td>
<td>33.3±11.6</td>
<td>29.6±12.2</td>
<td>31.0±12.0</td>
<td>38.3±13.6</td>
<td>0.523</td>
<td>0.043</td>
<td>0.353</td>
<td>0.058</td>
</tr>
<tr>
<td>9.4-4.4 Kb MspI (%)</td>
<td>56.3±8.3</td>
<td>56.4±7.1</td>
<td>55.4±8.5</td>
<td>51.6±6.7</td>
<td>0.736</td>
<td>0.043</td>
<td>0.978</td>
<td>0.088</td>
</tr>
<tr>
<td>&lt;4.4 Kb MspI (%)</td>
<td>10.4±5.6</td>
<td>14.1±7.6</td>
<td>13.5±8.0</td>
<td>10.1±8.8</td>
<td>0.133</td>
<td>0.140</td>
<td>0.113</td>
<td>0.172</td>
</tr>
<tr>
<td>&gt;9.4 Kb HpaII (%)</td>
<td>48.4±10.7</td>
<td>45.9±12.4</td>
<td>45.2±10.8</td>
<td>54.9±13.0</td>
<td>0.332</td>
<td>0.035</td>
<td>0.526</td>
<td>0.008</td>
</tr>
<tr>
<td>9.4-4.4 Kb HpaII (%)</td>
<td>47.9±9.2</td>
<td>48.7±9.6</td>
<td>50.4±9.0</td>
<td>41.9±9.9</td>
<td>0.362</td>
<td>0.038</td>
<td>0.796</td>
<td>0.003</td>
</tr>
<tr>
<td>&lt;4.4 Kb HpaII (%)</td>
<td>3.8±3.4</td>
<td>5.4±3.5</td>
<td>4.4±3.3</td>
<td>3.2±3.9</td>
<td>0.521</td>
<td>0.066</td>
<td>0.157</td>
<td>0.243</td>
</tr>
<tr>
<td>&gt;9.4 Kb H-M (%)</td>
<td>15.1±6.1</td>
<td>16.3±6.6</td>
<td>14.2±6.5</td>
<td>16.6±6.5</td>
<td>0.635</td>
<td>0.083</td>
<td>0.562</td>
<td>0.197</td>
</tr>
<tr>
<td>&lt;4.4 Kb H-M (%)</td>
<td>6.6±3.5</td>
<td>8.6±4.9</td>
<td>9.1±5.6</td>
<td>6.9±5.5</td>
<td>0.078</td>
<td>0.31</td>
<td>0.176</td>
<td>0.182</td>
</tr>
<tr>
<td>H-MH &gt;9.4 Kb</td>
<td>0.32±0.13</td>
<td>0.37±0.15</td>
<td>0.32±0.15</td>
<td>0.31±0.13</td>
<td>0.100</td>
<td>0.232</td>
<td>0.318</td>
<td>0.841</td>
</tr>
<tr>
<td>H-MH &lt;4.4 Kb</td>
<td>0.67±0.18</td>
<td>0.58±0.20</td>
<td>0.70±0.17</td>
<td>0.75±0.14</td>
<td>0.594</td>
<td>0.006</td>
<td>0.181</td>
<td>0.202</td>
</tr>
</tbody>
</table>

\( \text{NM, normal males; NF, normal females; PM, Parkinson’s males; PF, Parkinson’s females.} \)
controls in both genders (Table 2, Figure 1). On the other hand, the HpaII-TRF was shorter in female PD patients, indicating that the methylated terminal fragment length can be affected in a gender-specific manner. However, the subtracted HpaII-Msp1 TRFs were not significantly different between controls and PD patients. Therefore, the mean length range of the methylated subtelomeres did not seem to be changed in PD patients. In the proportional telomere length distribution, the long TRFs (>9.4 Kb), Msp1-TRF and HpaII-TRF, decreased, and the intermediate TRFs (9.4-4.4 Kb) increased specifically in female PD patients (Table 2, Figure 2). This indicated that the proportional amount of long Msp1-TRF (>9.4 Kb) was a more sensitive measure to detect the telomeric length change than the mean Msp1-TRF. The subtracted distribution, the HpaII-TRF distribution minus the Msp1-TRF distribution, was not statistically different between controls and PD patients (Table 2, Figures 3, 4). Moreover, we tried to analyze the Msp1- and HpaII-TRF distribution by comparing the proportions between the %Msp1-TRF and %HpaII-TRF in the size ranges in order to detect relative subtelomeric methylation status without the bias associated with the absolute percentages of Msp1-TRF or HpaII-TRF. For this purpose, we used (%Msp1-TRF - %HpaII-TRF)/%Msp1-TRF for the TRF shorter than 4.4 Kb and (%HpaII-TRF - %Msp1-TRF)/%HpaII-TRF for the TRF longer than 9.4 Kb (Table 2, Figure 5). The subtelomeres of short telomeres turned out to be proportionally hypomethylated in female PD patients. In summary, these data showed that long telomeres decreased and the subtelomeric regions of short telomeres were hypomethylated in PD in female PD patients but not in male patients.

**Discussion**

Telomere length shortening with aging can be enhanced by various human pathophysiological conditions. The analysis of a telomerase-deficient mouse mutants have indicated that telomere shortening affects the neighboring subtelomeric hypomethylation and that shortened telomeres are associated with subtelomeric hypomethylation.10 In addition, mutations in DNA methyltransferase (DNMT3B) in humans lead to autosomal recessive ICF (immunodeficiency, centromeric region instability, facial anomalies) syndrome.19 The telomeres of the somatic cells in patients with the syndrome are abnormally short.20 Therefore, the hypomethylated state of subtelomeric regions is thought to result in telomere shortening. The present study showed that the long telomeres decreased and the short telomeres with methylated subtelomeres decreased in PD patients, but these PD-associated telomeric

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**Figure 1.** The mean Msp1-TRF (telomere length), HpaII-TRF (methylated telomere length) and HpaII-Msp1-subtracted length (H-M; subtelomeric methylated range) of the healthy control participants and the Parkinson’s patients. Vertical bars depict the standard deviations. Abbreviations: NM, normal males; NF normal females; PM Parkinson’s males; PF Parkinson’s females. *# P<0.05; *Gender-related difference; #Parkinson’s disease-related difference.

**Figure 2.** The distribution of methylation-insensitive (Msp1)- and -sensitive (HpaII)-TRF lengths in the healthy controls and the Parkinson’s patients. Telomere fragment length percentage profiles of long (>9.4 Kb), intermediate (9.4-4.4 Kb) and short (<4.4 Kb) length ranges are shown. The densitometry was examined for both cases of Msp1 and HpaII digestion. The abbreviations are similar to those in Figure 1. *# P<0.05; *Gender-related difference; #Parkinson’s disease-related difference.

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[Ageing Research 2012; 4:e9]
changes were observed only in females. Our previous studies demonstrated the aging-associated changes in the telomere of peripheral leukocytes of healthy Japanese subjects, that is, long telomeres with hypomethylated subtelomeres decrease and short telomeres with hypomethylated subtelomere increase with age. In the present study, some of these aging-associated telomeric and subtelomeric changes were detected in PD females, and these aging-associated telomeric changes seemed to be accelerated in female PD patients. Oxidative stress can induce genomic mutations including base modifications, deletions and rearrangements. For example, some recent reports have shown that 8-oxodeoxyguanosine, an oxidized product of deoxyguanosine which is a constituent of the genomic DNA sequence, increases in patients with PD. This mutation can lead to mutations of genomic sequences containing guanosine, like the CCAG sequence, the recognition site of Msp1 or HpaII. This change is detectable by the method introduced in the present study. Such a mutation inhibits genomic methylation, because the DNA methyltransferases cannot react with the mutated DNA, so the mutated region remains unmethylated. Such interference with the methyltransferase activity may result in telomeric and subtelomeric hypomethylation. An enhancement of the oxidative stress may therefore yield not only an acceleration of telomeric erosion but also telomeric and subtelomeric hypomethylation. For this reason, the enhanced oxidative stress may be followed possibly by accelerated telomere attrition and genomic hypomethylation in the telomeric and subtelomeric regions in PD patients. The age-related telomere shortening can be ameliorated by estrogen, because estrogen augments the telomerase activity by promoting TERT gene expression. Furthermore, estrogen has been reported to prevent superoxide production via activation of the respiratory function, which leads to a reduction of reactive oxygen leaks in the mitochondria, thereby, increasing the activity of manganese superoxide dismutase and suppressing NADPH-oxidase activity. Such estrogen-derived effects are expected to slow the attrition of telomeres with hypomethylated subtelomeres in females, compared with that in males. After menopause, such estrogen-associated telomere protecting mechanisms will decline with aging. This may explain why the PD-associated telomeric changes are more prominent in females than in males. In addition, Kikuchi et al. have shown that the level of an oxidized DNA product, 8-hydroxy-2′-deoxyguanosine, is higher in female PD patients, but not in male PD patients, in comparison to controls. These results indicate that disease conditions can affect the telomere structure in a gender-associated manner. The correlation between the

![Figure 3](image-url)  
**Figure 3.** A schema for the proportional densitometric analysis of the isoschizomeric TRFs of MspI- and HpaII-digest. The densitometry data were divided into three parts, >9.4 Kb, 9.4-4.4 Kb, and <4.4 Kb. A representative genomic Southern blot of leukocyte DNA with telomere probe using MspI and HpaII and the analysis of the HpaII-MspI-subtracted TRF distribution. Densitometric curves are shown above or below the Southern blot smear results. A subtracted distribution pattern calculated from subtraction of the two densitometries is shown below.

![Figure 4](image-url)  
**Figure 4.** The subtracted distribution of methylation-insensitive (MspI)- and methylation-sensitive (HpaII)-TRF lengths in the healthy controls and in the Parkinson’s patients. The subtracted value of the MspI-TRF densitometry from the HpaII-TRF densitometry in the subdivided parts (>9.4kb, <4.4kb) are shown as columns. The percentage of the respective area was presented as the fraction of the whole densitometric area (set as 100%). The abbreviations are similar to those in Figure 1.
methylomstate in the telomeric shortening of the disease-related subtelomericies are also required to confirm the involvement of intermediate, and long telomeres. Further studies could allow the determination of the fate of cells bearing short, this finding. Further investigation is necessary to elucidate in a cohort study. The present study also showed that the analysis of the telomere length distribution and subtelomeric methylation status is more useful to detect disease-associated telomeric changes than an analysis of the mean TRF measurement. However, we could not clarify why the PD-associated telomeric changes could be observed only in the telomere length distribution and in the subtelomeric methylation status. The simple influence of oxidative stress on the telomere structure described above cannot explain this finding. Further investigation is necessary to determine the fate of cells bearing short, intermediate, and long telomeres. Further studies are also required to confirm the involvement of the disease-related subtelomeric methylation state in the telomeric shortening process in a cell.

Reference

10. Benetti R, García-Cao M, Blasco MA. Telomere length regulates the epigenetic status of mammalian telomeres and sub-


