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 pathophysiological 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.

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Key words: Parkinson's disease, DNA methylation, subtelomere, telomere, gender.

Acknowledgments: this work was supported, in part, by a Grant-in-Aid from the Ministry of Education, Science, and Culture of Japan (#23590885) and the National Natural Science Fund (NSFC) (81170329/H2501). We thank Ms. Ueda and Ms. Taguchi for their valuable technical assistance.

Conflict of interests: the authors report no potential conflict of interests.

Received for publication: 27 September 2011.
Revision received: 19 January 2012.
Accepted for publication: 16 May 2012.

Ageing Research 2012; 4:e9
doi:10.4081/ar.2012.e9

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

tubes. We added over 20 times the volume of 10 mm Tris-HCl, 1 mm EDTA (pH 8.0) to the blood sample to remove erythrocytes by lowering osmotic pressure. Next, peripheral leukocytes were collected by centrifugation.

Telomere detection

Telomere detection was performed as previously described with a modification (3). Methylation-sensitive and -insensitive isoschizomers, *Hpa*II and *Msp*I, were used. Briefly, blood cell DNA was extracted from samples and the DNA (1 μg) were 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 *Msp*I-restriction fragment lengths (TRFs) were estimated using the formula Σ(ODi-background)/Σ(ODi-background/Li), where ODi is the densitometric intensity and Li is the length of the 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 standard as previously described.[3,9] The Southern blot smear intensity was quantified as follows: each telomeric sample was divided into grid squares as follows according to the molecular size ranges: >9.4, 9.4> >4.4 and 4.4 >Kb. The percent of the divided intensity in each molecular weight range was measured (intensity of a defined region-background×100/total lane intensity-background). Subtelomeric methylation was assessed by comparing *Msp*I-TRF and *Hpa*II-TRF (H-M TRF) and by comparing *Msp*I telomere length distribution and *Hpa*II telomere length distribution. Subtelomeric methylation status of size-fractioned telomeres was analyzed to know the extents of subtelomeric methylation with different telomere sizes. The difference between the percent of *Msp*I signal intensity (%*Msp*I-TRF) and the *Hpa*II signal intensity (%*Hpa*II-TRF) in each molecular weight range was calculated. The proportion of the calculated difference (%*Hpa*II-TRF %*Msp*I-TRF) in >9.4 Kb range to %*Hpa*II-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 (%*Msp*I-TRF %*Hpa*II-TRF) in <4.4 Kb range to %*Msp*I-TRF in >4.4 Kb range was used to evaluate the methylation status of telomeres shorter than 4.4 Kb.

Statistical analysis

The normality of the data was examined with the Kolmogorov-Smirnov test and the homogeneity of variance with the Levene Median test. If both the normal distribution and equal variance tests were passed, the differences in the telomeres length including the mean TRF length and the telomere percentage analysis with age and condition (AD patients or age-matched healthy controls) were studied using a two-way analysis of variance (ANOVA) test followed by all pairwise multiple comparison procedures using Tukey's post hoc test. The data are expressed as the mean+standard deviation. The criterion for the significance is P<0.05. All analyses were carried out using a Sigma Statistical Analysis software package (Sigma 2.03, 2001; St. Louis, MO).

Results

The PD patients showed no significant changes in the *Msp*I-TRF in comparison to con-

Table 1. The ages of the participants

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

Age: the mean value±standard deviation in years. n: the numbers of the participants.

Table 2. The telomeric parameters of the participants

					P-value			
	PM	PF	NM	NF	PM/NM	PF/NF	PM/PF	NM/NF
*Msp*I-TRF (Kb)	6.9±0.9	6.6±1.2	6.6±1.3	7.4±1.5	0.350	0.083	0.341	0.079
*Hpa*II-TRF (Kb)	8.2±1.0	7.8±1.2	7.9±1.3	8.8±1.5	0.349	0.029	0.288	0.031
H-M-TRF (Kb)	1.3±0.5	1.2±0.6	1.3±0.5	1.4±0.5	0.941	0.208	0.745	0.223
>9.4 Kb *Msp*I (%)	33.3±11.6	29.6±12.2	31.0±12.0	38.3±13.6	0.523	0.043	0.353	0.058
9.4-4.4 Kb *Msp*I (%)	56.3±8.3	56.4±7.1	55.4±8.5	51.6±6.7	0.736	0.043	0.978	0.088
<4.4 Kb *Msp*I (%)	10.4±5.6	14.1±7.6	13.5±8.0	10.1±8.8	0.133	0.140	0.113	0.172
>9.4 Kb *Hpa*II(%)	48.4±10.7	45.9±12.4	45.2±10.8	54.9±13.0	0.332	0.035	0.526	0.008
9.4-4.4 Kb *Hpa*II (%)	47.9±9.2	48.7±9.6	50.4±9.0	41.9±9.9	0.362	0.038	0.796	0.003
<4.4 Kb *Hpa*II (%)	3.8±3.4	5.4±3.5	4.4±3.3	3.2±3.9	0.521	0.066	0.157	0.243
>9.4 Kb H-M (%)	15.1±6.1	16.3±6.6	14.2±6.5	16.6±6.5	0.635	0.893	0.562	0.197
<4.4 Kb H-M (%)	-6.6±3.5	-8.6±4.9	-9.1±5.6	-6.9±5.5	0.078	0.31	0.176	0.182
H-M/H >9.4 Kb	0.32±0.13	0.37±0.15	0.32±0.15	0.31±0.13	0.100	0.232	0.318	0.841
M-H/M <4.4 Kb	0.67±0.18	0.58±0.20	0.70±0.17	0.75±0.14	0.594	0.006	0.181	0.202

NM, normal males; NF normal females; PM Parkinson's males; PF Parkinson's females.

trols in both genders (Table 2, Figure 1). On the other hand, the *Hpa*II-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 *Hpa*II-*Msp*I 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), *Msp*I-TRF and *Hpa*II-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 *Msp*I-TRF (>9.4 Kb) was a more sensitive measure to detect the telomeric length change than the mean *Msp*I-TRF. The subtracted distribution, the *Hpa*II-TRF distribution minus the *Msp*I-TRF distribution, was not statistically different between controls and PD patients (Table 2, Figures 3, 4). Moreover, we tried to analyze the *Msp*I- and *Hpa*II-TRF distribution by comparing the proportions between the %*Msp*I-TRF and %*Hpa*II-TRF in the size ranges in order to detect relative subtelomeric methyaltion status without the bias associated with the absolute percentages of *Msp*I-TRF or *Hpa*II-TRF. For this purpose, we used (%*Msp*I-TRF - %*Hpa*II-TRF)/%*Msp*I-TRF for the TRF shorter than 4.4 Kb and (%*Hpa*II-TRF - %*Msp*I-TRF)/%*Hpa*II-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.

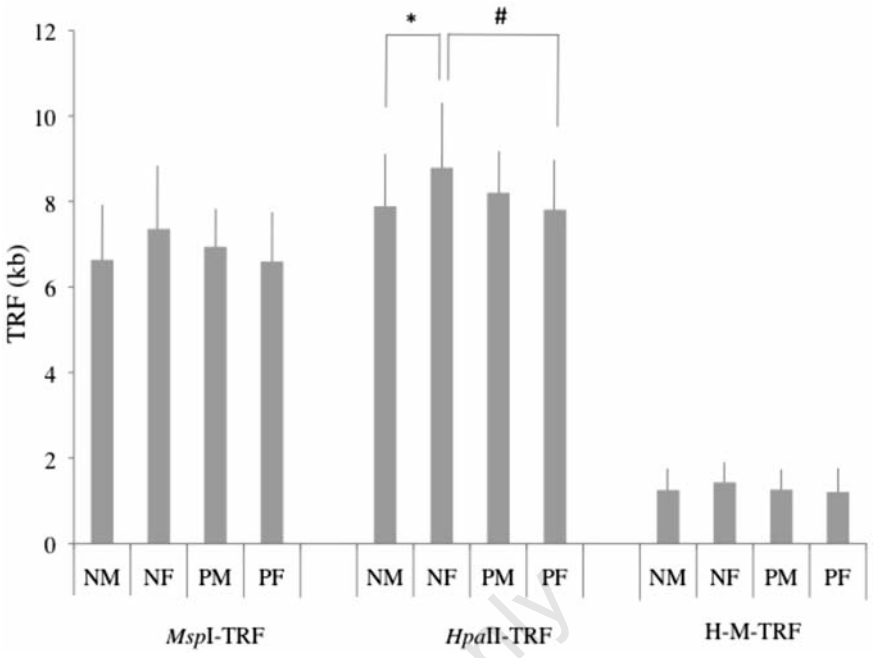

Figure 1. The mean *Msp*I-TRF (telomere length), *Hpa*II-TRF (methylated telomere length) and *Hpa*II-*Msp*I-subtracted length (H-M; subtelomeric methylated range) of the healthy control participants and the Parkinson's patients. Vertical bars depict the standard deviations. Abbreviations: NM, normal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

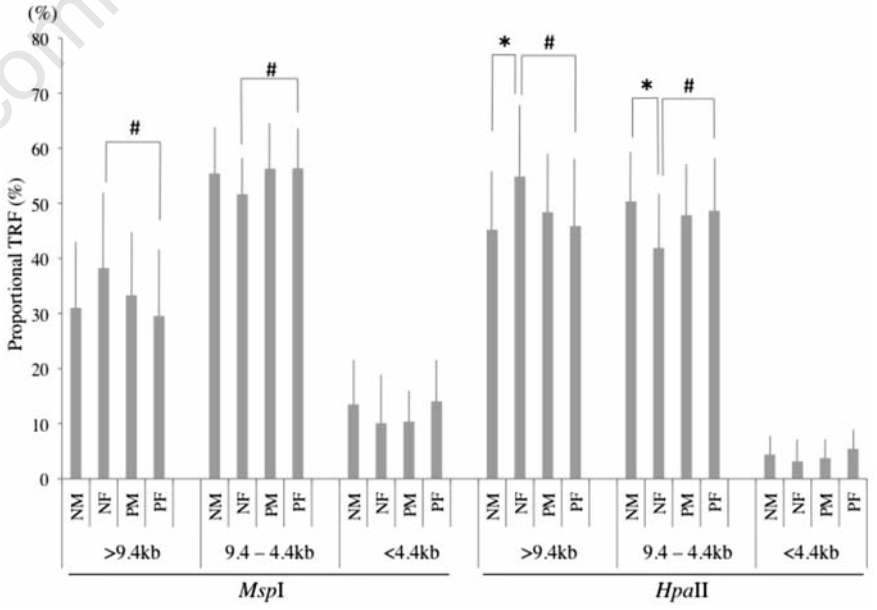

Figure 2. The distribution of methylation-insensitive (*Msp*I)- and -sensitive (*Hpa*II)-TRF lengths in the healthy controls and the Parkinson's patients. Telomere fragment length percentage profiles of long (>9.4 Kb), intermediate (9.4-4.4 Kb) and short (<4.4 Kb) length ranges are shown. The densitometry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

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

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.[21] 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.[22] For example, some recent reports have shown that 8-oxo-deoxyguanosine, an oxidized product of deoxyguanosine which is a constituent of the genomic DNA sequence, increases in patients with PD.[23-25] This mutation can lead to mutations of genomic sequences containing guanosine, like the CCGG sequence, the recognition site of *Msp*I or *Hpa*II. 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.[26] 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.[27] 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,[28] thereby, increasing the activity of manganese superoxide dismutase and suppressing NADPH-oxidase activity.[29-31] 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.[23] These results indicate that disease conditions can affect the telomere structure in a gender-associated manner. The correlation between the

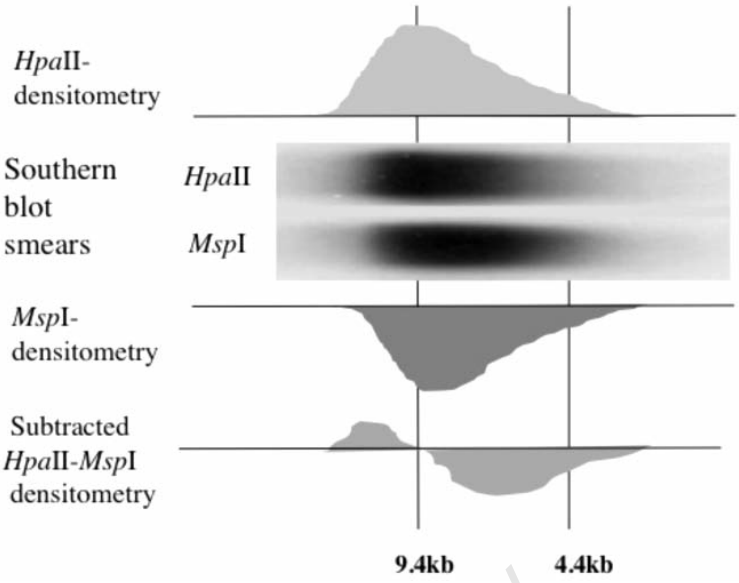

Figure 3. A schema for the proportional densitometric analysis of the isoschizomeric TRFs of *Msp*I- and *Hpa*II-digest. The densitometry data were divided into three parts, >9.4 Kb, 9.4-4.4 Kb, and <4.4 Kb. A representative genomic Southern blot of leukocyte DNA with telomere probe using *Msp*I and *Hpa*II and the analysis of the *Hpa*II-*Msp*I-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.

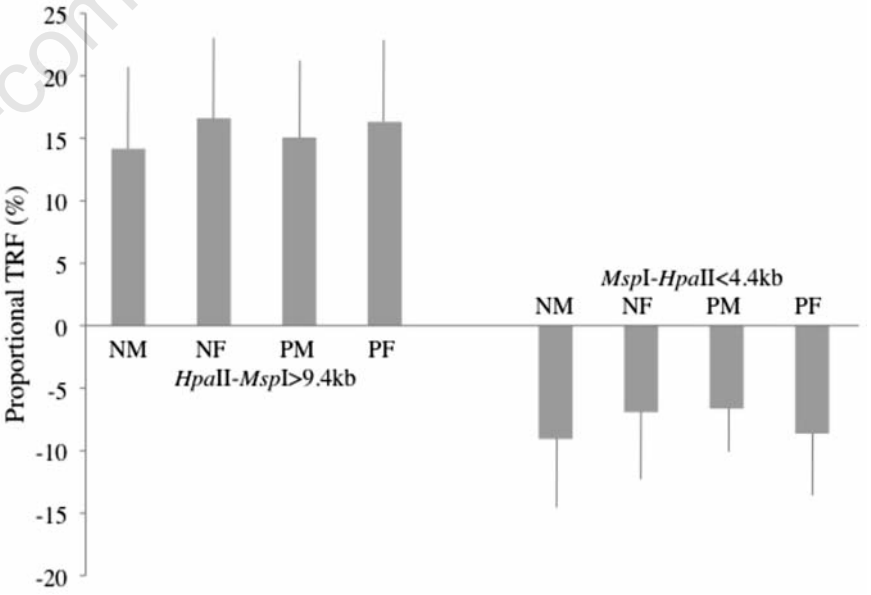

Figure 4. The subtracted distribution of methylation-insensitive (*Msp*I)- and methylation–sensitive (*Hpa*II)-TRF lengths in the healthy controls and in the Parkinson's patients. The subtracted value of the *Msp*I-TRF densitometry from the *Hpa*II-TRF densitometry in the subdivided parts (>9.4kb, <4.4kb) 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.

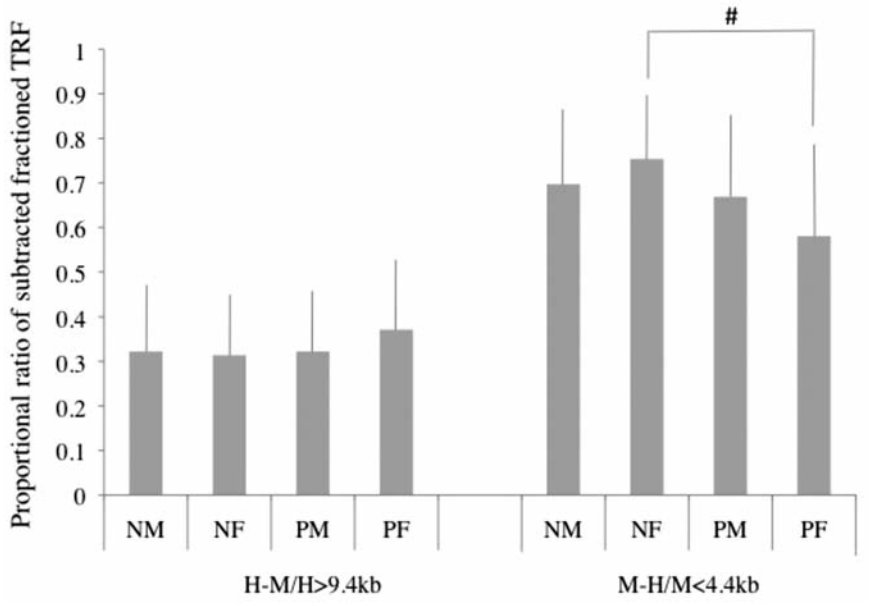

Figure. 5 The relative methylation status of subtelomeres of long (>9.4 Kb) and short (<4.4 Kb) telomere length ranges in controls and PD patients. Ratio of the subtracted percentage of *Hpa*II-*Msp*I *vs* percentage of *Hpa*II >9.4 Kb ((*Hpa*II-*Msp*I)/*Hpa*II (>9.4 Kb)) and that of *Msp*I-*Hpa*II *vs* *Msp*I <4.4 Kb ((*Msp*I-*Hpa*II)/*Msp*I (<4.4 Kb)) are used as indices indicating the subtelomeric methylation of longer (than 9.4 Kb) and shorter (than 4.4 Kb) telomeres, respectively. Vertical bars depict the standard deviations. #P<0.05.

attrition rate of a telomere and its subtelomeric hypomethylation range in individuals should be elucidated 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.

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