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Telomere Length of Human Adult Bronchial Epithelium and Bronchogenic Squamous Cell Carcinoma Measured Using Tissue Quantitative Fluorescence in situ Hybridization

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Key Words
Telomere · Bronchial epithelium · Smoking · Lung squamous cell carcinoma · Quantitative fluorescence in situ hybridization

Abstract
Background: Telomeres are repetitive DNA sequences located at the ends of chromosomes. Chromosomal and genomic instability due to telomere dysfunction has been known to play an important role in the carcinogenesis of some organs. ***Objectives:*** The aim of this study was to examine the correlation between smoking and the telomere length of human bronchial epithelial cells in individuals with and without lung cancer. ***Patients and Methods:*** We examined 68 non-lung cancer adult autopsy cases and 24 surgically resected cases of lung squamous cell carcinoma. Telomere lengths of the basal cells of bronchial epithelium were measured using the tissue quantitative fluorescence in situ hybridization method and were expressed in normalized telomere-to-centromere ratios (NTCRs). ***Results:*** The autopsied individuals included 27 current smokers (CuS), 33 never-smokers (NeS), and 8 ex-smokers (ExS). The NTCRs in the central bronchi of CuS, NeS, and ExS were 1.515, 1.372, and 1.204, respectively. The bronchial epithelial telomeres of CuS were significantly longer than those of non-CuS (NeS + ExS). When the analysis was conducted separately for females and males, a significant difference between CuS and NeS + ExS was recognized only for males. The NTCRs of the bronchial epithelium of lung cancer cases and lung cancer tissue are 1.514 and 1.385, respectively. ***Conclusions:*** Our findings suggest that smoking causes telomeric elongation in the bronchial epithelium. Therefore, it appears that the mechanism of carcinogenesis in smoking-related carcinomas may differ from that of many other carcinomas in which genetic instability due to aging-related telomeric shortening is assumed to play a role.

Introduction

Telomeres are nucleoprotein complexes located at the ends of eukaryotic chromosomes, where they play an important role in the protection against end-to-end fusion, degradation, and recombination. Telomeric DNA se-

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quences consist of tandem TTAGGG repeats, which generally shorten by 50 to 200 base pairs with each normal somatic cell division as a result of incomplete replication of telomere repeats during DNA synthesis – the so-called end-replication problem [1]. Telomere shortening occurs with human aging in many organs and tissues, promoting genetic instability and increasing the risk of malignancy [2, 3].

Using quantitative fluorescence in situ hybridization (Q-FISH) and our originally developed software, Tissue Telo, employing the telomere-to-centromere ratio (TCR) or normalized TCR (NTCR), we have confirmed the telomere length distributions of different cell types in the tongue [2], stomach [3], breast [4], and esophagus [5]. We have also demonstrated that alcoholics show reduced telomere length in the esophageal epithelium [6].

In this study, we estimated the telomere lengths of human adult bronchial epithelial cells using the Q-FISH method and examined their correlation with aging and smoking. We also examined the differences between non-cancer cases and central-type bronchial squamous cell carcinoma cases, which are considered to have a close relationship with smoking.

Patients and Methods

Patients and Tissue Processing

We examined 24 surgically resected lung cancer cases (1 female and 23 males, patients' age range 45–76 years, average 62.6 years) and 68 non-lung cancer adult autopsy cases (25 females and 43 males, age range 51–100 years, average 83.7 years). There was a significant difference between the age of lung cancer cases and non-lung cancer cases ($p < 0.001$). The lung cancer cases were limited to squamous cell carcinomas with a maximum tumor diameter of <3 cm and located in the central side bronchi rather than in the segmental bronchi, because such central squamous cell carcinomas are considered to be closely related to smoking. In the non-lung cancer group, cases which showed no significant lung diseases such as pneumonia or pulmonary fibrosis were selected from adult autopsy cases, because any effects besides those of smoking should be avoided. The smoking histories of all individuals were confirmed from the hospital medical records. Samples of tissue were obtained from the tumor and the background bronchial mucosa. In non-lung cancer cases, samples taken from the segmental bronchi were considered representative of central bronchial samples, whereas samples taken from areas more peripheral than the sub-subsegmental bronchi were considered representative of peripheral bronchial samples. The tissues were fixed in 10% buffered formalin and then subjected to standard tissue processing and paraffin embedding. They were sliced serially into 3-μm-thick sections for hematoxylin and eosin staining and into 2-μm-thick sections for Q-FISH.

Approval for this study was obtained from the ethics committees of Mita Hospital, International University of Health and Welfare, and Tokyo Metropolitan Institute of Gerontology.

FISH and Probes

The slides were processed by the FISH method, as reported previously [2–5, 7]. Tissue sections were hybridized with peptide nucleic acid (PNA) probes for the telomere (Telo C-Cy3 probe: 5′-CCCTAACCCTAACCCTAA-3′; catalogue No. F1002, Fasmac, Japan) and the centromere (Cenp1-FITC probe: 5′-CTTCGTTGGAAACGGGGT-3′; custom-made, Fasmac), and the nuclei were stained with DAPI (Molecular Probes, Eugene, Oreg., USA).

Image Analysis of Telomeres

FISH digital images were captured by a CCD camera (Retiga-2000DC, QImaging, Surrey, B.C., Canada) mounted on an epifluorescence microscope (80i, Nikon, Tokyo, Japan) equipped with a triple-bandpass filter set for DAPI/FITC/Cy3 (part No. 61 010, Chroma Technology Corp., Rockingham, Vt., USA) and a ×40 objective lens (Plan Fluor ×40/0.75, Nikon).

Microscope control and image acquisition were performed using the Image-Pro Plus software package (version 7.0, Media Cybernetics Co. Ltd., Silver Spring, Md., USA). The captured images were analyzed with our own tissue analysis software, Tissue Telo, version 3.0, which estimates the TCRs of individual nuclei, as reported previously [2–5, 7]. Nuclear regions were identified manually from the composite color image: DAPI (blue channel), FITC (green channel), and Cy3 (red channel) (fig. 1a). Telomere and centromere signals were then determined as pixels showing the brightest intensities (top 5%) within each selected nuclear region. The measured signal intensities (or optical densities) were corrected for background autofluorescence, as determined from the mean of the pixels showing the lowest intensities (bottom 20%). The top 5% and bottom 20% thresholds had previously been shown to give consistent results [7]. As there is no guarantee that the entire nucleus is captured within any given tissue section, the total corrected telomere signal (integrated optical density) for each nucleus was further normalized by the corresponding integrated optical density of the centromere [2, 3]. TCR values were determined from individual bronchial basal cells of both lung cancer and non-lung cancer cases, and from lung cancer cells. Between 165 and 356 cells (mean 308.9 cells) were analyzed for each case.

TCR Normalization by Cell Block

As a control for variations in sample preparation, we also performed Q-FISH on a cell block section from a cultured cell strain, TIG-1 [8], with a population doubling level of 34 (telomere length: 8.6 kbp by Southern blot analysis; kept and cultured at our institution), and placed it on the same slides as the bronchial and lung cancer sections (fig. 1b). The TCR measurement for each bronchial basal or cancer cell was divided by the median TCR for the control cell block on the same slide to give the NTCR of the cell [2, 3].

Statistical Analyses

The NTCRs for bronchial basal and cancer cells were compared by t test. The correlation of the NTCR with age was examined using a least square method. In all comparisons, differences at $p < 0.05$ were considered to be significant.

Results

The 68 autopsy cases included 27 current smokers (CuS), 33 never-smokers (NeS), and 8 ex-smokers (ExS). The average smoking quantity of CuS was 47.1 pack-years with a range of 10–160 pack-years. The average smoking cessation period of ExS was 43 years with a range of 20–54 years. The smoking quantity of ExS was not available. The 24 surgical cases included 21 CuS and 3 unknown cases (table 1). The NTCRs of the central and peripheral bronchi showed no correlation with age (p = 0.394 and 0.745, respectively). The NTCRs of the central bronchus of CuS, NeS, and ExS in autopsy cases were 1.515, 1.372, and 1.204, respectively. There was no significant difference between the NTCRs of NeS and ExS (p = 0.198). The NTCRs of CuS were significantly higher than those of non-CuS (NeS + ExS) (p = 0.032). The NTCRs in the peripheral bronchi of CuS, NeS, and ExS in autopsy cases were 1.553, 1.360, and 1.277, respectively. There was no significant difference between the NTCRs of NeS and ExS (p = 0.610). The NTCRs of CuS were significantly higher than those of non-CuS (NeS + ExS) (p = 0.011). When a separate analysis of females and males was conducted, significant differences in NTCRs between CuS and NeS + ExS were found only in males (central: p = 0.016; peripheral: p = 0.029); there were too few female CuS for statistical analysis, and therefore no obvious conclusions could be drawn about female CuS. The principal results for non-cancer cases are shown in table 2. The NTCRs of the background bronchial epithelium in lung cancer cases were approximately similar to the values of CuS of autopsy cases. The NTCRs of lung cancer tissue were lower than those of the background bronchial epithelium, but no statistically significant difference was observed. The mean NTCRs and standard deviations are shown in table 3. The representative FISH images of bronchial epithelium and cancer tissue are shown in figure 2.

Discussion

The present study showed that telomeres in the bronchial basal cells of CuS were longer than those of non-CuS, both in all subjects and in males. The difference was small but statistically significant. Telomeres can increase their length in two ways: through telomerase and alternative lengthening of telomeres [9, 10], and telomere length in most human tissues and tumors is maintained by telomerase [11]. Increased telomerase activity by the telomeric repeat amplification protocol (TRAP) method and

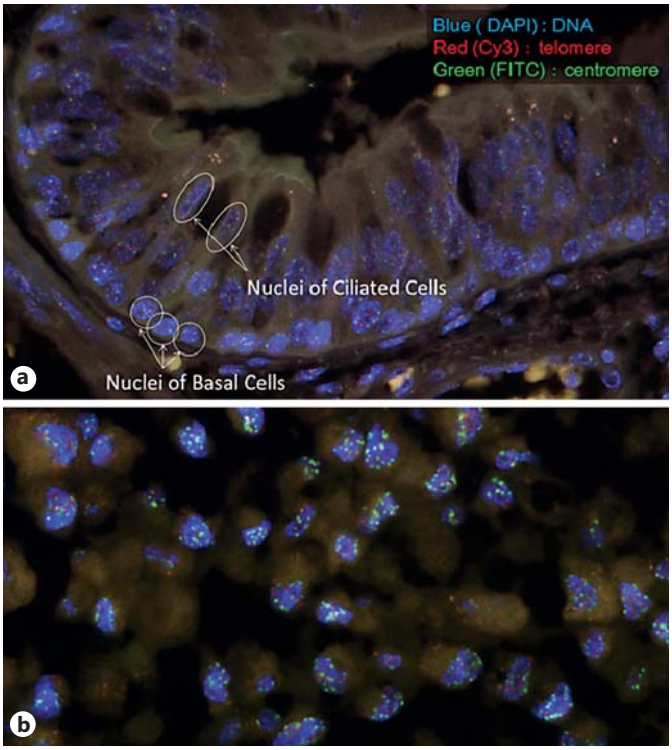

Fig. 1. FISH images showing DNA in blue (DAPI), telomeres in red (Cy3), and centromeres in green (FITC). **a** A tissue section of the bronchial epithelium. The nuclei of basal cells and ciliated cells can be easily distinguished. We manually selected the nuclei of basal cells, and their telomere lengths were measured as signal intensity TCR using the Tissue Telo original software which calculates the TCR automatically by circumscribing the nuclei. **b** A cell block section from a cultured TIG-1 cell strain which was placed on the same slides of the bronchial and lung cancer sections as a control for variations in sample preparation. The mean telomere length of TIG-1 is 8.6 kbp by Southern blot analysis.

Table 1. Clinical characteristics of the subjects

	Non-cancer cases	Lung cancer cases
Subjects (f/m)	68 (25/43)	24 (1/23)
Mean age (range), years		
Female	86.5 (63–100)	61
Male	82.1 (51–94)[a]	62.7 (45–76)[a]
Total	83.7 (51–100)[b]	62.6 (45–76)[b]
Smoking history		
CuS (f/m)	27 (1/26)	21 (1/20)
NeS (f/m)	33 (23/10)	0
ExS (f/m)	8 (1/7)	0
Unknown (f/m)	0	3 (0/3)

[a]p < 0.001 and [b]p < 0.001, significant difference between non-cancer and cancer cases.

Table 2. Telomere length in bronchial epithelium of non-cancer cases

	n	Central bronchus, mean NTCR (SD)	Peripheral bronchus, mean NTCR (SD)
CuS			
Female	1	1.365 (–)	1.591 (–)
Male	26	1.520 (0.323)[a]	1.551 (0.294)[b]
Subtotal	27	1.515 (0.325)[c]	1.553 (0.294)[d]
Non-CuS (NeS + ExS)			
Female	24	1.381 (0.323)	1.358 (0.374)
Male	17	1.280 (0.291)[a]	1.323 (0.335)[b]
Subtotal	41	1.339 (0.317)[c]	1.344 (0.363)[d]

SD = Standard deviation. [a]p = 0.016, [b]p = 0.029, [c]p = 0.032, and [d]p = 0.011, significant difference between CuS and non-CuS.

Table 3. Telomere length in bronchial epithelium and tumor tissue of lung cancer cases

	n	Background bronchus, mean NTCR (SD)	Tumor tissue, mean NTCR (SD)
CuS			
Female	1	1.245 (–)	1.402 (–)
Male	19	1.539 (0.775)	1.417 (0.630)
Subtotal	20	1.524 (0.757)	1.417 (0.613)
Unknown smoking history			
Female	0	–	–
Male	4	1.464 (0.433)	1.227 (0.348)
Subtotal	4	1.464 (0.433)	1.227 (0.348)
Total			
Female	1	1.245 (–)	1.402 (–)
Male	23	1.526 (0.725)	1.384 (0.589)
Subtotal	24	1.514 (0.712)	1.385 (0.576)

SD = Standard deviation.

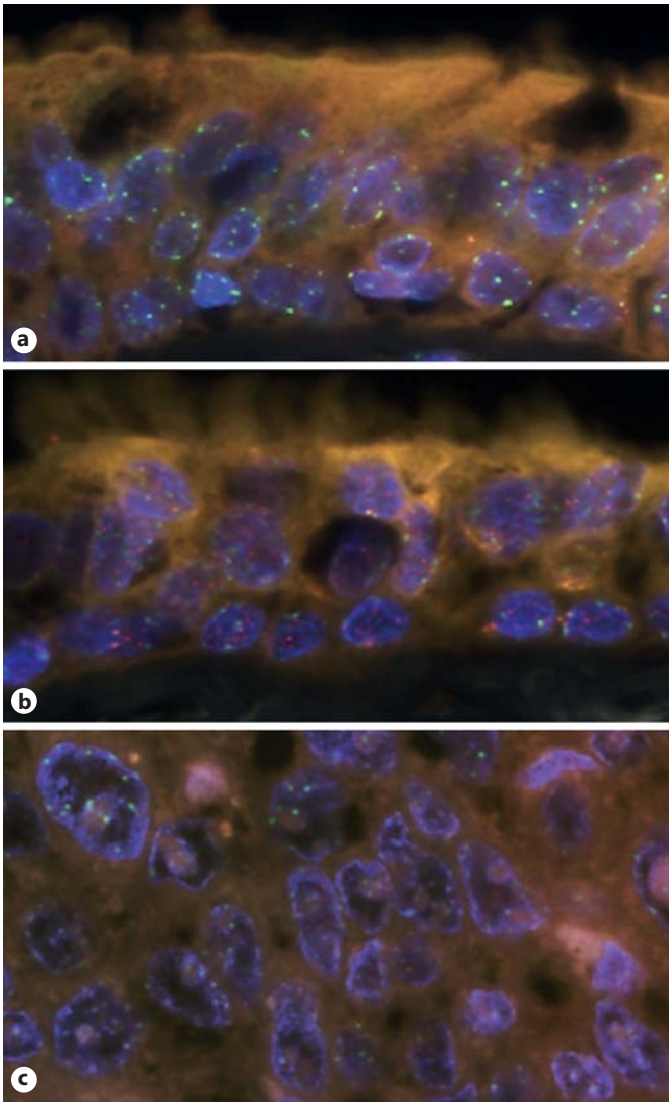

Fig. 2. FISH images of a tissue section showing DNA in blue (DAPI), telomeres in red (Cy3), and centromeres in green (FITC). **a** Bronchial epithelium from a 91-year-old male NeS. Telomere signals appear weaker than those of centromeres. The mean signal intensity NTCR of the basal cells is 0.880. **b** Bronchial epithelium from a 94-year-old male CuS. Telomere signals appear relatively stronger than those of centromeres. The mean NTCR of the basal cells is 1.450. **c** Squamous cell carcinoma tissue from a 71-year-old male CuS. The intensity of telomere and centromere signals varies with each nucleus. The mean NTCR of the cancer cells is 1.772.

expression of human telomerase reverse transcriptase (hTERT) mRNA have been described in the bronchial epithelium of smokers and patients with lung squamous cell carcinoma [12–14]. Both our results and those of previous reports suggest that smoking induces telomerase in the bronchial epithelium, causing extension of telomeres.

Only one previous investigation of telomere length in the bronchial epithelium has described that telomeres become shortened in bronchial metaplasia and gradually lengthen during the transition from dysplasia to invasive squamous cell carcinoma [15]. However, the association between telomere length and smoking was not mentioned. In the present study, CuS had no histologically obvious bronchial lesions such as dysplasia, but the telomeres were significantly elongated, whereas invasive squamous cell carcinoma was associated with shorter telomeres than those in the bronchial epithelium of smokers. Considering the results of this previous paper and

those of the present study, it can be suggested that telomerase is induced in some smoking-related bronchial conditions which can be either morphological dysplasia or merely molecular changes without morphological alterations. However, the discrepancy of telomere lengths in carcinomas remains difficult to explain. It is questionable whether the data of the previous study are directly comparable with ours, because the Q-FISH method employed for estimation of telomere length differed from ours. Unlike our method, it did not use computerized image analysis or control cell blocks, and the measured values were calculated as 4 grades that were visually assessed from the intensity and frequency of telomere staining. As we consider that our method using semi-automatic computerized image analysis and control cell blocks is more accurate, telomere length in bronchial dysplasia and metaplasia should be re-estimated using our method.

We have reported that telomere length is reduced in the background mucosal epithelium of oral and esophageal cancer [16, 17], which is considered to promote genetic instability and to be related to carcinogenesis. The present study revealed that smoking-related lung cancer appears to differ from oral or esophageal cancer in terms of telomere dynamics.

Some genetic differences between lung carcinomas of smokers and those of nonsmokers have been reported. The p53 mutation is significantly more frequent in lung carcinomas of smokers than in those of nonsmokers [18], and some papers have described widely dispersed p53 point mutations in the precancerous dysplastic bronchial epithelium of smokers [19, 20]. On the other hand, EGFR mutations and ALK rearrangements are less common in smoking-related than in non-smoking-related lung cancer [21, 22]. These reports and the results of the present study suggest that the mechanism of lung carcinogenesis in smokers differs from that in nonsmokers. Telomeres in the bronchial epithelium may lengthen in some smoking-related conditions, which are possibly precancerous, and smoking-related lung cancer is considered to be caused by gene mutations that frequently occur under these conditions, such as p53 mutations, in the absence of genetic instability resulting from telomere shortening.

In conclusion, our study has demonstrated changes in telomere dynamics in bronchial epithelium as a result of smoking, especially in males. Our data suggest that the mechanism of smoking-related carcinogenesis differs from that in other carcinomas and, therefore, that strategies of gene-targeting therapy and prevention of lung cancer might need to take smoking status into account.

Financial Disclosure and Conflicts of Interest

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