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Telomere lengths in the oral epithelia with and without carcinoma

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ABSTRACT

Aging appears to be intrinsically related to carcinogenesis. Genomic instability due to telomere shortening plays an important role in carcinoma development. In order to clarify telomere dysfunction in carcinoma development, we examined the uninvolved epithelium adjacent to carcinoma *in situ* (CIS), i.e. background of CIS, and CIS itself, compared to control without carcinoma, using an improved quantitative fluorescence *in situ* hybridization (Q-FISH) method. We also estimated anaphase bridge (AB), which is inferred to be related to chromosomal instability. In all cell types (basal, parabasal, and suprabasal), mean telomere lengths were significantly shorter in the background than in the control. We also demonstrated increased incidences of AB, not only in CIS, but also in the background and control epithelia with excessively shortened telomeres. Thus we have conclusively demonstrated that CIS arises from epithelium with short telomeres.

1. Introduction

Aging appears to be intrinsically related to carcinogenesis, as elderly individuals tend to have a much higher incidence of carcinoma and also multiple carcinomas. The progression of telomere shortening with age could lead to genomic instability during the initial stage of cancerization.[1] One of the cancers that have shown a rapidly increasing incidence worldwide is oral cancer.[2] This cancer is one of several head and neck cancers that are known to show field cancerization, where genetically altered cells can be detected over wide areas of the affected epithelium.[3] Therefore, we considered that telomere shortening might be present in histologically normal regions near oral carcinoma, and these regions may be representative of the earliest stage of cancerization.

We have confirmed that telomeres of all human tissues shorten with age, except for cerebral tissue and myocardium, using Southern blot analysis.[4–6] We have also analyzed telomere length distributions of different cells in the oesophagus, stomach, and breast by quantitative fluorescence *in situ*

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hybridization (Q-FISH) using the telomere to centromere ratio (TCR).[7–9] To estimate telomere lengths using our Q-FISH method, we employed a block of cultured cells of known telomere length as a control to obtain the normalized TCR (NTCR). By this method, we have demonstrated that basal cells have the longest telomeres in epithelia, suggesting the possible presence of tissue stem cells in the basal layer.[10] Concerning telomeres in the oral cavity, we have reported annual reduction rate (32 bp) in the lingual mucosa, analyzed by Southern blotting.[11] By Q-FISH method, using visual assessment, Meeker and colleagues have reported about telomeres in oral precancerous lesions.[12] However, they did not show differences among the component cell types. Therefore, in the present study, we have estimated telomere length of each cell type of the epithelium in the lingual mucosa with/without carcinomas.

For our present study, we postulated that there would be measurable differences of telomere length between oral epithelia with and without carcinoma, determined using our Q-FISH method on samples of both non-cancerous control and also cancerous tissue and its uninvolved adjacent epithelia (background). We did not examine advanced carcinoma in order to avoid any influential factors (such as severe inflammation) associated with large tumours, and confined our study to squamous cell carcinoma *in situ* (CIS).

We also carried out histologic estimation of the presence of anaphase bridges (ABs) as a morphologic indicator of chromosomal instability due to shortened telomeres; they resulted from the breakage–fusion–bridge cycle.[13–15]

2. Materials and methods

2.1. Subjects

Twenty-five cases of CIS of the tongue from 24 patients (12 men and 12 women, 35–99 years, mean: 63.3 years) were analyzed. These lesions had been diagnosed by five pathologists (J.A., T.I., M.S., T.A., K.T.). Twenty-five representative paraffin sections that included CIS with uninvolved epithelium were selected. Uninvolved epithelium located 1–10 mm (mean 3.8 mm) distant from the CIS was defined as background. CISs were resected along with a 5-mm-wide margin of non-cancerous tissue. Therefore, we did not perform estimation of telomere length and anaphase bridge analysis for tissue located at different distances from the CIS. It was not possible to obtain fresh tissue of cancer and background from cases of CIS for the h-TERT study, because it would have been ethically necessary to examine histologically the entire surgical margin, including the specimen edges and bases of CIS. One patient had two different CISs, and therefore we studied 25 CISs and 24 backgrounds.

Control specimens of normal lingual mucosa were obtained from 27 autopsied individuals without malignancy in the head and neck, oesophagus, stomach, or lung (within 3 h postmortem; 16 men and 11 women, age 0–96 years, mean 41.8 years). The specimens were stored at –80 °C until use. We used these autopsy specimens as normal controls because of the difficulty in obtaining specimens of normal mucosa unassociated with carcinoma from surgically resected materials. Although we were concerned that postmortem changes might affect telomere length, we had previously studied telomere lengths in the samples of cerebrum and heart obtained at different postmortem times using Southern blotting, but found no significant changes.[4] In the lingual mucosa, we had previously studied telomere lengths in autopsy cases.[11] Using Southern blotting, we also newly analyzed the correlation between telomere length and postmortem time, but our data revealed no significant decrease of telomere length (data not shown).

Control cases were categorized into two subgroups by age: a young subgroup (6 men and 4 women, age 0–18 years, mean 1.8 years) and an old subgroup (10 men and 6 women, age 45–96 years, mean 65.4 years), age-matched to the CIS patients. As our previous studies using Southern blotting[11] and Q-FISH[10] had indicated that telomeres in human tissue from young individuals are longer than those from aged individuals, we used samples from the young individuals for technical reconfirmation of our method.

It had been shown previously in a pilot study that differences in tissue processing, and storage of the tissue at –80 °C, did not affect the NTCR (data not shown). Approval for this study was obtained from the ethics committee of Tokyo Metropolitan Institute of Gerontology.

2.2. Tissue processing and histologic assessment

CIS specimens immediately after resection and control samples were fixed for 5 h in 10% buffered formalin v/v. Both types of samples were then subjected to standard tissue processing and paraffin embedding. Tissues were sliced into sections 3 μm thick for haematoxylin and eosin (HE) staining and immunohistochemical examination, and into sections 5 μm thick for Q-FISH.

In histologic examination, the slides were checked for autolysis, inflammatory cell accumulation, and fibrosis. Cells in non-cancerous epithelium and CIS were classified into three types[10] and evaluated by immunohistochemistry and Q-FISH: (a) basal: cells in a single layer on the basement membrane, (b) parabasal: cells in the second to third layers on the basal membrane, and (c) suprabasal: cells in the fourth or upper layer above the parabasal (Fig. 1A).

2.3. Immunohistochemical staining for Ki-67 and its evaluation

Tissue sections were stained with an antibody against Ki-67 (monoclonal antibody: MIB-1; Dako Cytomation, Glostrup, Denmark, 100×) by the Polymer-Immuno Complex method (ENVISION+/HRP(DAB); Dako Japan). Antibody-stained sections were observed quantitatively by three pathologists (J.A., T.A., K.T.), who estimated the numbers of each cell type showing positivity in the lingual mucosa and divided the positivity rates into four grades: >50% of cells positive ++, 5–<50% of cells positive +, <5% of cells positive +, and negative –.

2.4. Q-FISH

2.4.1. FISH and probes

The slides were processed by the FISH method, as reported previously.[7] Tissue sections were hybridized peptide nucleic

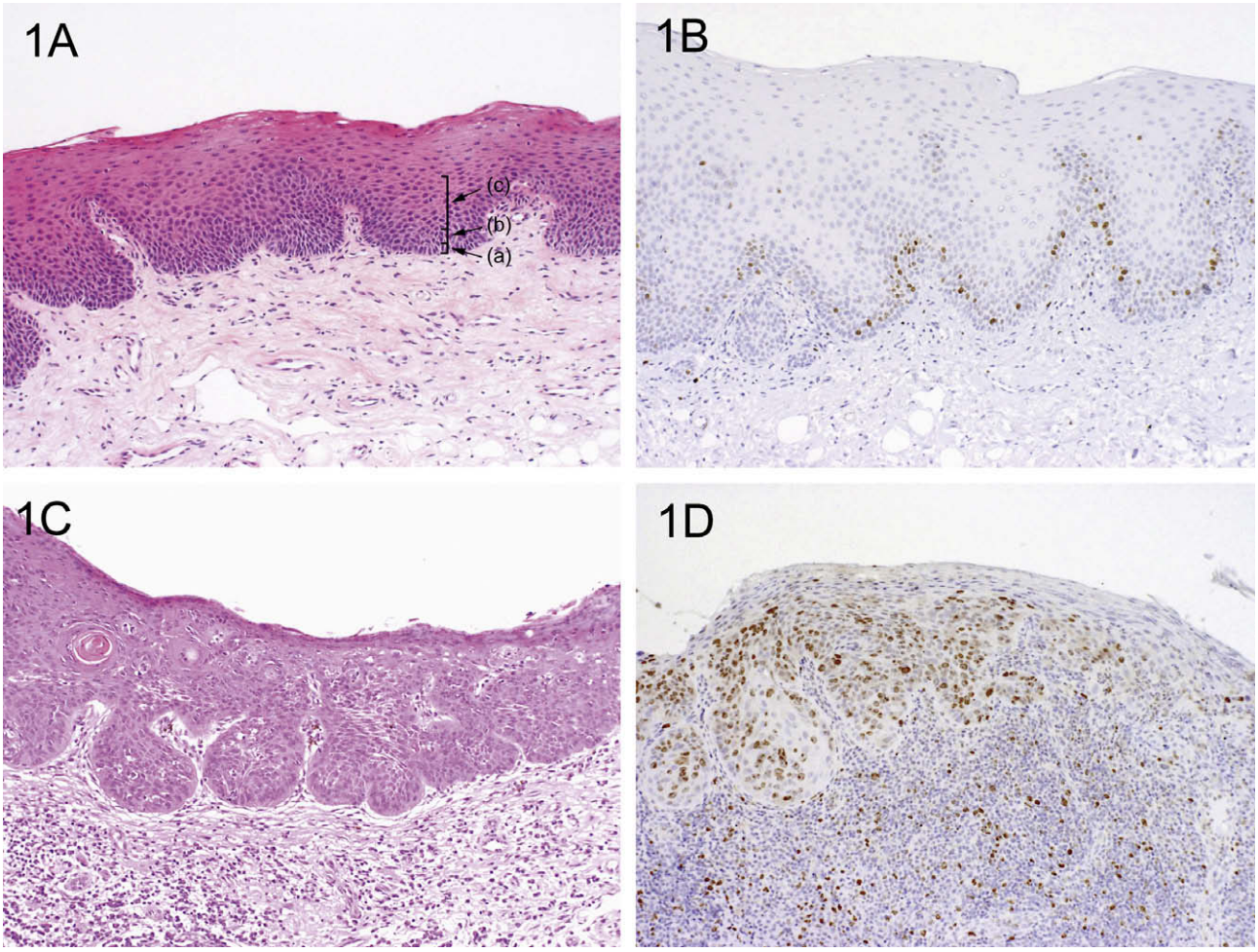

Fig. 1 – Control epithelium and carcinoma *in situ* (CIS) of the tongue. Original magnification: ×100. (A) Normal lingual mucosa without malignancy (73-year-old man). (a) Basal, (b) parabasal, and (c) suprabasal cells. (B) Immunohistochemical staining for Ki-67, same cases as A. Scattered positive cells are evident only in the parabasal layer. (C) CIS in lingual mucosa (82-year-old woman). Basal cell-like atypical cells are proliferating and have replaced almost the whole layer, with parakeratosis. (D) Immunohistochemical staining for Ki-67, same case as C. Multiple positive cells are evident throughout almost the whole layer.

acid (PNA) probes for the telomere (telo C-Cy3 probe: 5′-CCCTAACCCTAACCCTAA-3′; Fasmac, Atsugi, Japan, 0.32 μmol/L in final concentration) and the centromere (Cenp1-Fluoresceinisothiocyanate isomer-1; FITC probe: 5′-CTTCGTTGGAAACGGGGT-3′; Fasmac, 0.12 μmol/L in final concentration), and the nuclei were counterstained with 4′6-diamidino-2-phenylindole (DAPI, Molecular Probes, Eugene, OR).

2.4.2. *Image analysis of telomeres*

Q-FISH digital images were captured by a Charge Coupled Device (CCD) camera (ORCA-ER-1394, Hamamatsu Photonics KK, Hamamatsu, Japan) mounted on an epifluorescence microscope (80i, Nikon, Tokyo, Japan) equipped with a triple band-pass filter set for DAPI/FITC/Cy3 (Part #61010, 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 5.0, Media Cybernetics Co. Ltd., Silver Spring, MD, USA). The captured images were analyzed with our own telomere analysis software, 'TissueTelo Ver. 2′, where TCRs of individual nuclei are estimated automatically, as reported previously.[7] TCR measurements were extracted for each of the three cell types in the tissue section based on histologic assessment. At least 703 cells (range: 703–2021 cells, mean: 1353.9 cells) were analyzed for each case. The mean value was defined as the representative TCR value for all analyzed cells of each type in each case.

2.4.3. *Preliminary comparison of TCR with terminal restriction fragment (TRF) value*

Cultured TIG-1 cells[16] were obtained at four different PDLs (20, 34, 41, and 60), and subjected to telomere analysis using two different methods: (a) fixation with 10% buffered formalin, preparation of a paraffin-embedded cell block, and estimation of TCR by Q-FISH, and (b) DNA extraction followed by digestion with *Hinf*-1 (Boehringer Mannheim Biochemica, Germany), and estimation of the terminal restriction fragments

(TRF) value by Southern blotting.[4,11] Each regression line was calculated, as was the correlation between the TCR and TRF values.

2.4.4. *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 line, TIG-1,[16] with a population doubling level (PDL) of 34 (telomere length: 8.57 kbp by Southern blot analysis, see Section 3.2) and placed it on the same slides as the lingual sections. The TCR measurement for each lingual cell was divided by the mean TCR for the control cell block on the same slide to give the NTCR of the cell.

2.4.5. *Anaphase bridges*

The presence of ABs as a morphological indicator of chromosomal instability[13–15] was examined in representative HE slides by two pathologists (J.A., K.T.). We counted the numbers of total mitoses and ABs in control, background, and CIS, and calculated the total mitotic and AB counts per epithelial length (per millimetre, AB index) of tissue preparation.

2.4.6. *Statistical analyses*

The NTCRs for basal, parabasal, and suprabasal cells were compared within individual cases using paired *t*-test. The NTCRs and AB index of background mucosa and CIS were compared by paired *t*-test, and those of normal control and background or CIS, were compared by *t*-test. Correlations were analyzed using the Pearson correlation coefficient test and single regression analysis. In all comparisons, differences at $p < 0.05$ were considered to be significant.

3. Results

3.1. *Histology and Ki-67 evaluation*

Autolysis, inflammatory cell accumulation, or fibrosis was not observed in the controls or backgrounds. However, chronic and acute inflammatory cell accumulation was often observed beneath CIS (Fig. 1A and C).

Results of Ki-67 staining evaluation are summarized in Table 1. In the controls and background, positive cells were mostly located in the parabasal layer, and scattered in basal in adults (Fig. 1B). In the background, positive cells were slightly increased. In CIS, basal cells were mostly positive, and distributed in several layers from the basement membrane (Fig. 1D).

Table 1 – Ki-67 index of control, background of CIS[a], and CIS by immunohistochemical staining.

Cell layer	Age-matched control[b] ($n = 17$)	Background of CIS[c] ($n = 24$)	CIS ($n = 25$)
Basal cell layer	(±)	(+)	(++)
Parabasal layer	(+)	(+)	(+)–(++)
Suprabasal layer	(–)	(–)	(±)

(++): >50% cells positive.
(+): 5–<50% cells positive.
(±): <5% cells positive.
(–): Negative.
[a] CIS: carcinoma *in situ*.
[b] Age-matched control: older subgroup without malignancy in the head and neck, oesophagus, or lung.
[c] Background of CIS: uninvolved epithelium of CIS.

3.2. *Preliminary confirmation of our method validity*

Regression analysis of TRF values for TIG-1 cells at the four different PDLs is shown in Fig. 2A. The regression line also

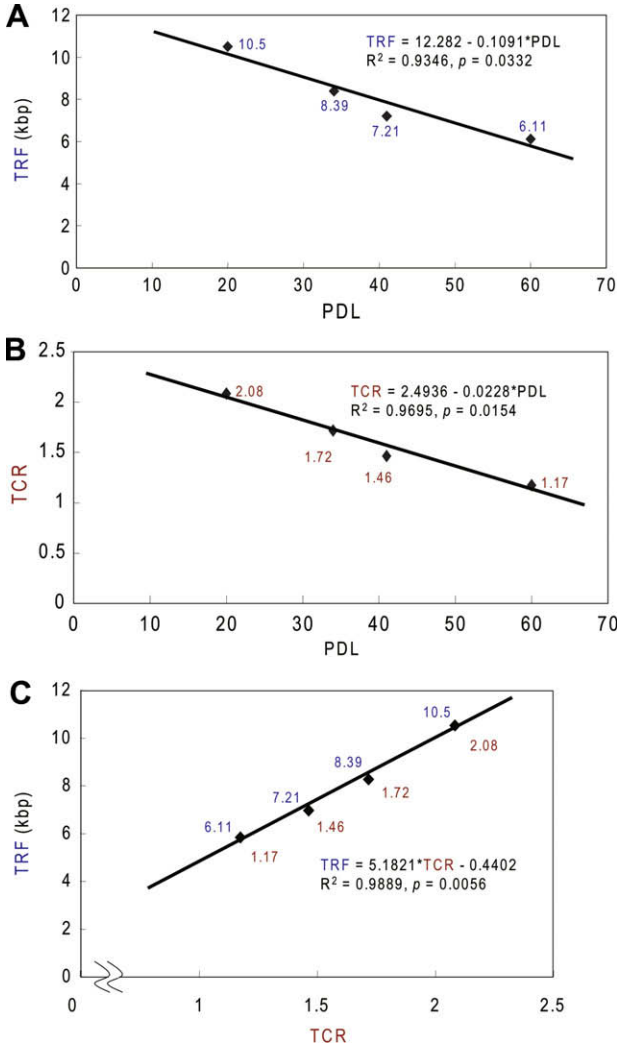

Fig. 2 – Regression analysis of TCR value using Q-FISH, and telomere restriction fragment (TRF) values by Southern blot analysis. (A) Regression analysis of PDLs of TIG-1 and TRF values by Southern blotting. TIG-1 cells were obtained at four different PDLs (20, 34, 41, and 60). TRF values with standardized incidence ratio (SIR) in each PDL were 10.52 ± 3.39, 8.27 ± 2.58, 6.96 ± 2.16, and 5.84 ± 1.91, respectively. (B) Regression analysis of population doubling levels (PDLs) of TIG-1 and TCR values obtained by Q-FISH. Cultured TIG-1 cells for the cell blocks were obtained at the same PDL for Southern blotting. TCR values with standard deviation (SD) were 2.08 ± 6.48, 1.72 ± 3.01, 1.46 ± 3.49, and 1.17 ± 2.03, respectively. (C) Correlation between TCR values obtained by Q-FISH and TRF values obtained by Southern blotting in TIG-1 cells at four different PDLs.

indicated significant telomere shortening ($p = 0.0332$). Regression analysis of TCRs for TIG-1 cells at the four different PDLs is shown in Fig. 2B. The regression line also indicated significant telomere shortening ($p = 0.0154$). The TCR and TRF values were significantly correlated ($p = 0.0056$) (Fig. 2C). These findings indicated that our methodology was valid.

3.3. NTCRs of the four groups

Representative Q-FISH images of a control epithelium without malignancy and CIS are shown in Fig. 3A(a and b). NTCRs for each cell type in four groups, (1) young subgroup, (2) old subgroup, (3) background, and (4) CIS, are shown in Fig. 3B. Mean NTCRs and numbers of cells analyzed in the background and CIS are summarized in Table 2.

3.3.1. *Pattern of telomere length within epithelium and CIS*

NTCRs of basal cells were significantly larger than those of parabasal and suprabasal cells in the two control subgroups. NTCR of basal cells was significantly larger than those of parabasal ($p = 0.0052$) in the background. On the other hand, in CIS, there was no significant difference between NTCRs of basal and parabasal cells by paired *t*-test ($p = 0.064$). There was also no significant difference between NTCRs of basal and suprabasal cells by paired *t*-test ($p = 0.058$).

3.3.2. *Comparisons of NTCRs among the four groups*

In the control (including young and old subgroups, $n = 27$), NTCRs of basal, parabasal, and suprabasal cells were 2.75, 2.03, and 1.82, respectively. In the young subgroup, NTCRs of basal, parabasal, and suprabasal cells were 6.15, 5.22, and 3.98, respectively. These NTCRs were larger than those of the old subgroup for all cell types (basal, parabasal, and suprabasal NTCRs were 1.70, 1.48, and 1.52, respectively). NTCRs of the old subgroup were significantly larger than those of the background (basal, parabasal, and suprabasal NTCRs were 1.33, 1.18, and 1.26), and CIS (basal, parabasal, suprabasal NTCRs were 1.69, 1.33, and 1.28, respectively), for all cell types.

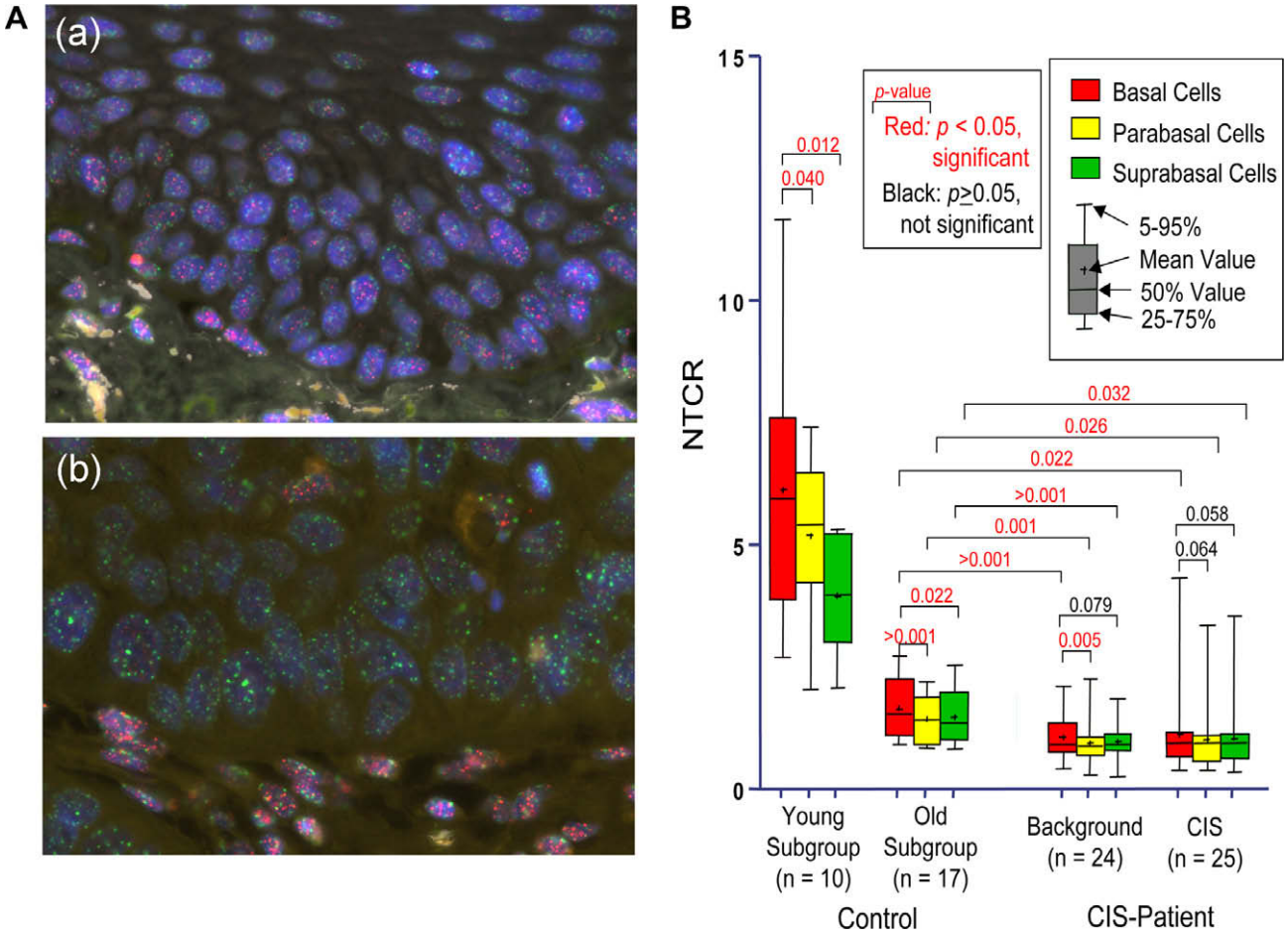

Fig. 3 – (A) Representative Q-FISH images, original magnification; ×40. Red signal: telomere-Cy3 signal, green signal: centromere-FITC signal, blue: DAPI counterstaining for DNA. (a) Q-FISH image of the normal control from a 73-year-old man. NTCRs of basal, parabasal, and suprabasal cells were 2.75, 2.03, and 1.82, respectively. Scattered cells with prominent red (Cy3) signals reside mainly in the basal layer. (b) Q-FISH image of CIS (61-year-old woman). NTCRs of basal, parabasal, and suprabasal cells were 0.48, 0.50, and 0.71, respectively. Red signals are considerably weaker than in the control case shown in Fig. 3A(a). (B) All analyzed NTCR data for controls ($n = 27$) and CISs ($n = 25$) are summarized as a box and whisker plot. The control group was categorized into young ($n = 10$) and old ($n = 17$) subgroups.

Table 2 – Normalised TCR[a] (NTCR) of the three cell types in background of CIS[b], and CIS.

Case number	Age/sex	Total count of cell No.	Background of CIS[c]			CIS		
			Basal[d]	Parabasal	Suprabasal	Basal	Parabasal	Suprabasal
1	35/W	1058	1.24	0.96	1.13	2.04	1.50	1.48
2	41/W	1001	1.03	0.98	1.01	1.14	1.17	1.09
3	43/W	1771	0.93	0.91	0.90	4.28	3.02	3.15
4	46/W	1770	0.93	0.68	0.86	0.52	0.43	0.43
5	52/M	1406	0.94	0.75	0.86	0.83	0.74	0.76
6	53/W	703	0.42	0.42	0.34	0.62	0.51	0.47
7	53/W	1615	0.90	0.82	1.21	0.68	0.66	0.80
8-1[e]	58/W	962	2.23	1.58	1.80	0.95	1.51	1.28
8-2[e]	58/W	742	2.23	1.58	1.80	2.29	1.76	2.07
9	61/W	1063	0.83	0.55	0.68	0.48	0.50	0.71
10	62/M	971	1.26	1.22	1.47	1.12	1.30	0.97
11	62/M	1126	0.97	1.02	0.96	1.19	1.06	1.00
12	64/M	1876	1.08	0.88	0.88	0.90	0.93	1.08
13	65/M	947	1.30	0.94	1.13	12.39	6.77	6.26
14	66/M	1432	1.43	1.08	1.11	1.07	0.85	0.93
15	67/M	1238	1.94	1.80	2.00	0.71	0.52	0.58
16	67/M	1062	0.59	0.39	0.35	0.55	0.49	0.43
17	68/M	1470	1.05	0.97	0.94	1.05	1.07	0.95
18	70/M	1568	1.46	1.16	1.30	1.17	1.08	1.22
19	70/M	1796	0.83	0.74	0.73	0.61	0.63	0.54
20	72/M	1180	4.83	5.67	4.66	1.59	1.52	1.14
21	77/W	1126	1.20	1.05	1.17	1.25	1.17	1.09
22	82/W	1154	0.64	0.63	0.62	1.19	0.89	0.78
23	85/W	1674	0.94	0.85	0.90	1.04	1.02	1.00
24	99/W	914	2.06	1.82	2.75	2.47	2.05	1.91

M: man, W: woman.
a TCR: telomere to centromere ratio.
b CIS: Carcinoma *in situ*.
c Background of CIS: uninvolved epithelium of CIS.
d Larger than parabasal cells in each case, $p = 0.0052$ by paired *t*-test.
e Case 8 has two CISs in the same patient.

3.4. ABs in lingual epithelia and CIS

Representative ABs are shown in Fig. 4A. Results of mitosis and AB analyses are summarized in Table 3. The total number of mitoses in CIS was significantly larger than in the old subgroup ($p = 0.0315$). AB index in CIS were higher than in the old subgroup, but not to a significant degree ($p = 0.0782$).

3.4.1. Correlations between age and AB index

Correlations between age and AB index in each epithelium are shown in Fig. 4B. In the controls, no AB was observed in individuals under 55 years of age ($n = 15$) and had a significant positive correlation (AB index = 0.0008 * Age – 0.0051, $p = 0.002$ (Fig. 4B(a)). However, in the background, even a 35-year-old individual had ABs (Fig. 4B(b and c)).

3.4.2. Correlations between NTCR and AB index

In order to demonstrate the correlation between telomere length and the AB-index, the NTCRs of basal cells and the AB index for each case were plotted, as shown in Fig. 4B(d–f). The control group ($n = 27$) showed only 1 or 2 ABs in each case, with a significant correlation between NTCR and the AB index (Fig. 4B(d)), whereas more individuals with CIS had a larger AB index in the background and CIS. In all types of epithelium, 31 of 32 cases (96.8%) with ABs, except for the background of a case, had a NTCR of less than 3. The mean NTCR of basal cells in all AB-positive cases ($n = 32$) was 1.27, whereas that in AB-negative cases ($n = 44$) was 2.77 (AB-positive < AB-negative cases, $p = 0.0022$).

4. Discussion

The present study produced three main findings: (A) non-cancerous epithelia showed basal that had longer telomeres than parabasal and suprabasal, whereas CIS had no evidence of the normal telomeric pattern. (B) Telomeres in the background were significantly shorter than in the control. (C) ABs were observed in individuals with excessively short telomeres, aged 55 years old or more in the control group, and having a NTCR of more than 3, except for one case of CIS.

Immunohistochemistry for Ki-67 gave results for the controls similar to those in our previous study.[10] In the old subgroup and background, Ki-67-positive cells were present in the parabasal layer, but in CIS they were present in the basal, parabasal, and suprabasal layers. This suggests that the amplifying proliferative zone of the normal lingual epithelium is almost limited within the parabasal layer, whereas that of CIS extends to a wider area. Therefore, in normal epithelium, telomere shortening occurs mainly in the parabasal layer.

In the present study, we analyzed TCRs in TIG-1 cells at four different PDLs and compared them with the TRF values.

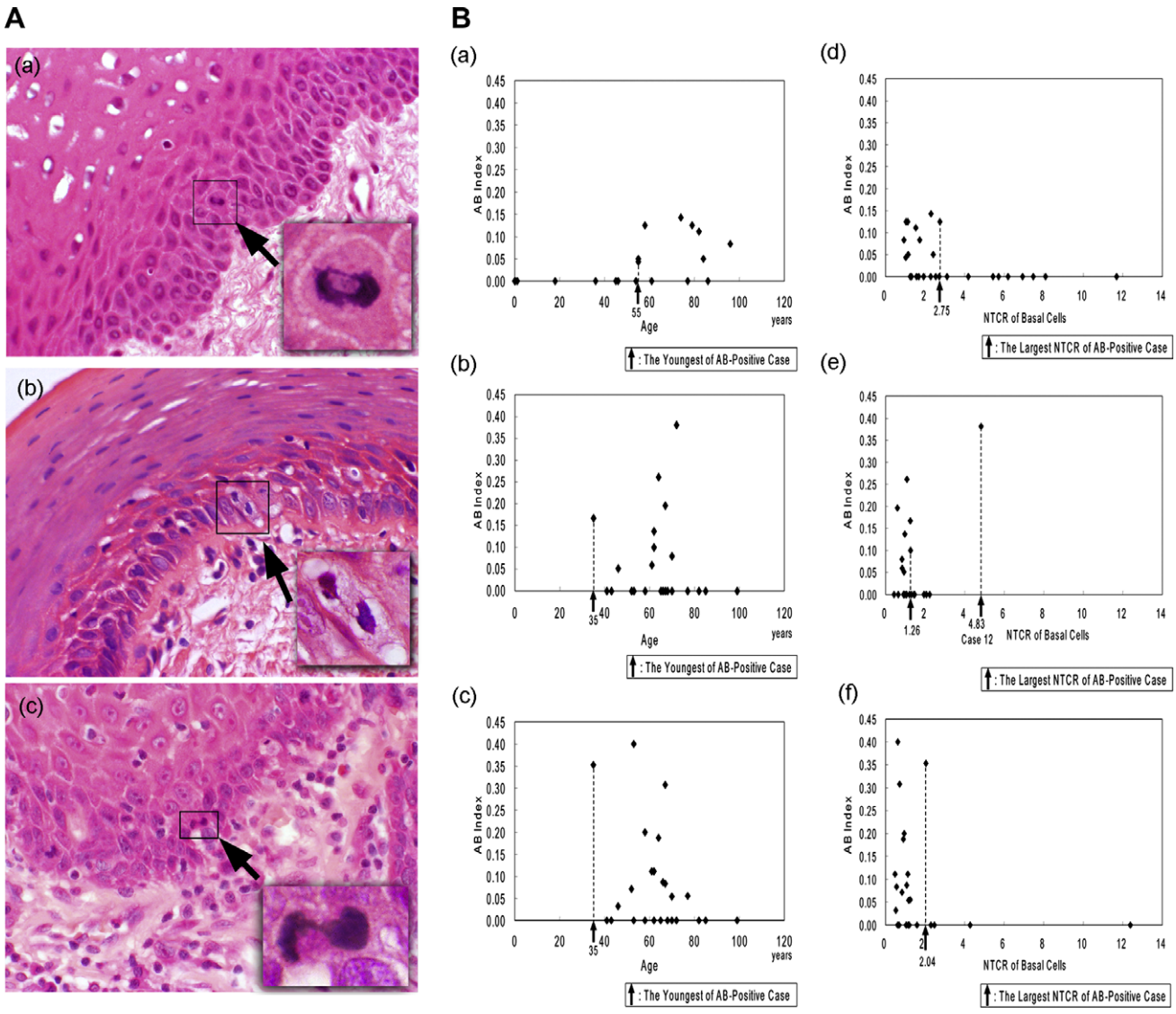

Fig. 4 – (A) Representative images of anaphase bridges (ABs) observed in (a) control epithelium from a 96-year-old woman, (b) background (62-year-old man), and (c) CIS (64-year-old man). Original magnification, ×40, inset; ×100 (oil immersion). (B) Correlations between AB index (anaphase bridge count/mm) and age (a–c), and between AB index and NTCR of basal cells (d–f). Arrows: The youngest case (a–c) and the largest NTCR (d–f) among AB-positive cases are indicated. (a) AB index and age in controls (n = 27) with significant correlation (AB index = 0.0008 * Age – 0.0051, p = 0.002). ABs were observed in individuals 55 years old or older (n = 12). (b) AB index and age in background (n = 24). ABs were observed even in individuals 35 years old or older. (c) AB index and age in CIS (n = 25). ABs were observed even in individuals 35 years old or older. (d) AB index and NTCR in controls with significant correlation (NTCR 4.1453 – 23.289 * AB index; p = 0.0233). AB-positive cases had a NTCR of less than 2.75. (e) AB index and NTCR in background. AB-positive cases had a NTCR of less than1.26 except for one case (NTCR 4.83). (f) AB index and NTCR in CIS. AB-positive cases had a NTCR of less than 2.04.

The results appear to confirm the accuracy and reproducibility of this method for comparing telomere lengths among different cell types or tissues.

In non-cancerous epithelia including the control and background, basal cells had the longest telomeres among the three cell types, suggesting that stem cells may reside in the basal layer.[10] In the present study, we reconfirmed our previous results.

In the pancreas[17] and liver,[18] it has been reported that carcinomas have shorter telomeres than the surrounding background tissues. In the present study, comparison among the NTCRs (three cell types) of the old subgroup, background, and CIS showed that those of the background and CIS were significantly smaller than those of the old subgroup. This finding is the first evidence to corroborate previous studies showing that cancerous and/or precancerous lesions have shorter telomeres than those of non-cancerous tissues in the colon[14] and liver.[19] In those studies, telomere lengths were compared mainly between background and carcinoma or between background with chronic inflammation, e.g. ulcer-

Table 3 – Frequency of anaphase bridges and mitoses per mm in lingual epithelia.

	Control		Background of CIS	CIS
	Young subgroup (n = 10)	Old subgroup (n = 17)	(n = 24)	(n = 25)
AB index[a]	0.00	0.04	0.06[c]	0.08[d]
Mitotic index[b]	1.04	0.99	1.68[e]	2.50[f]

Background of CIS: uninvolved epithelium of CIS.
CIS: carcinoma *in situ*.
a AB index: frequency of anaphase bridges per epithelial length (millimetre).
b Mitotic index: total mitotic count per epithelial length (millimetre).
c AB index of background is larger than in the old subgroup, but not significantly ($p = 0.296$).
d AB index of CIS is larger than in the old subgroup or background, but not significantly ($p = 0.078$, 0.204, respectively).
e Mitotic index of background is larger than in the old subgroup, but not significantly ($p = 0.054$).
f Mitotic index of CIS is larger than in the old subgroup and background ($p < 0.001$, 0.029, respectively).

ative colitis or chronic hepatitis. However, our present data for the lingual epithelium revealed that the background, lacking any histologic change, also had short telomeres like those in ulcerative colitis or chronic hepatitis. Oral CIS occurred in telomere-shortened epithelium without histologic changes or inflammation.

The oral cavity often generates multiple carcinomas distributed in the oropharyngeal, laryngeal, or oesophagogastric mucosae, a tendency referred to as field cancerization or Slaughter's concept, which has been explained by widely occurring genetic alterations.[3] Meeker and colleagues reported that telomere length abnormalities (excessive shortening) appear to be one of the earliest and most prevalent genetic alterations.[12] Because shortened telomeres induce the breakage–fusion–bridge cycle, which is a major source of genetic divergence, cells with short telomeres easily become malignant.[1] The presence of telomeres that are excessively shorter in the background epithelia than in control samples appears to support this concept. If telomere shortening occurs over wider areas that are usually not differentiated by routine histologic diagnostic techniques, multiple tumours may arise within such telomere-shortened fields.

In the present study, ABs were observed in all types of epithelium with telomere shortening. In the background, telomere length was similar to that of CIS. We have demonstrated an increased incidence of ABs, which are inferred to cause chromosomal instability, in both control specimens with telomere shortening and in the background. It has also been reported that telomere shortening is the initial phenomenon in cancerization, and that genomic instability is accelerated by chromosomal instability due to telomere shortening.[1]

In conclusion, we have estimated telomere lengths of cells in control, background, and CIS, and found that the pattern of telomere length in CIS differs from that in the control and background epithelia. Excessive telomere shortening and a high incidence of ABs with possible chromosomal instability were evident in the background, suggesting that lingual CIS arises in a telomere-shortened field without histologic inflammation. These findings also suggest that telomere shortening may be one of the important causes of field cancerization in the oral mucosa, and that close clinical follow-up is warranted for patients with oral carcinoma in whom a second carcinoma may arise.

Conflict of interest statement

None declared.

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REFERENCES

1. Londono-Vallejo JA. Telomere instability and cancer. *Biochimie* 2008;**90**(1):73–82.
2. Sciubba JJ. Oral cancer. The importance of early diagnosis and treatment. *Am J Clin Dermatol* 2001;**2**(4):239–51.
3. Braakhuis BJ, Tabor MP, Kummer JA, Leemans CR, Brakenhoff RH. A genetic explanation of Slaughter's concept of field cancerization: evidence and clinical implications. *Cancer Res* 2003;**63**(8):1727–30.
4. Takubo K, Izumiyama-Shimomura N, Honma N, et al. Telomere lengths are characteristic in each human individual. *Exp Gerontol* 2002;**37**(4):523–31.
5. Nakamura K, Takubo K, Izumiyama-Shimomura N, et al. Telomeric DNA length in cerebral gray and white matter is associated with longevity in individuals aged 70 years or older. *Exp Gerontol* 2007;**42**(10):944–50.
6. Ishii A, Nakamura K, Kishimoto H, et al. Telomere shortening with aging in the human pancreas. *Exp Gerontol* 2006;**41**(9):882–6.
7. Aida J, Izumiyama-Shimomura N, Nakamura K, et al. Telomere length variations in 6 mucosal cell types of gastric tissue observed using a novel quantitative fluorescence in situ hybridization method. *Hum Pathol* 2007;**38**(8):1192–200.

8. Kurabayashi R, Takubo K, Aida J, et al. Luminal and cancer cells in the breast show more rapid telomere shortening than myoepithelial cells and fibroblasts. *Hum Pathol* 2008;**39**:1647–55.
9. Shiraishi H, Mikami T, Aida J, et al. Telomere shortening in Barrett's mucosa and esophageal adenocarcinoma and its association with loss of heterozygosity. *Scand J Gastroenterol* 2009;**44**(5):538–44.
10. Aida J, Izumiyama-Shimomura N, Nakamura K, et al. Basal cells have longest telomeres measured by tissue Q-FISH method in lingual epithelium. *Exp Gerontol* 2008;**43**(9):833–9.
11. Nakamura K, Izumiyama-Shimomura N, Sawabe M, et al. Comparative analysis of telomere lengths and erosion with age in human epidermis and lingual epithelium. *J Invest Dermatol* 2002;**119**(5):1014–9.
12. Meeker AK, Hicks JL, Iacobuzio-Donahue CA, et al. Telomere length abnormalities occur early in the initiation of epithelial carcinogenesis. *Clin Cancer Res* 2004;**10**(10):3317–26.
13. Gisselsson D, Jonson T, Petersen A, et al. Telomere dysfunction triggers extensive DNA fragmentation and evolution of complex chromosome abnormalities in human malignant tumors. *Proc Natl Acad Sci USA* 2001;**98**(22):12683–8.
14. O'Sullivan JN, Bronner MP, Brentnall TA, et al. Chromosomal instability in ulcerative colitis is related to telomere shortening. *Nat Genet* 2002;**32**(2):280–4.
15. Montgomery E, Wilentz RE, Argani P, et al. Analysis of anaphase figures in routine histologic sections distinguishes chromosomally unstable from chromosomally stable malignancies. *Cancer Biol Ther* 2003;**2**(3):248–52.
16. Ohashi M, Aizawa S, Ooka H, et al. A new human diploid cell strain, TIG-1, for the research on cellular aging. *Exp Gerontol* 1980;**15**(2):121–33.
17. van Heek NT, Meeker AK, Kern SE, et al. Telomere shortening is nearly universal in pancreatic intraepithelial neoplasia. *Am J Pathol* 2002;**161**(5):1541–7.
18. Satyanarayana A, Manns MP, Rudolph KL. Telomeres and telomerase: a dual role in hepatocarcinogenesis. *Hepatology* 2004;**40**(2):276–83.
19. Nakajima T, Moriguchi M, Katagishi T, et al. Premature telomere shortening and impaired regenerative response in hepatocytes of individuals with NAFLD. *Liver Int* 2006;**26**(1):23–31.