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Telomere shortening in gastric carcinoma with aging despite telomerase activation

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Abstract In the present study, we analyzed both telomere length and telomerase activity in surgical and autopsy samples of non-neoplastic mucosa and carcinomas of the stomach. Telomere length, determined by Southern blot analysis, demonstrated progressive shortening with age in non-neoplastic gastric mucosal specimens from 38 human subjects aged between 0 and 99 years, with an average annual loss rate of 46 base pairs (bp). The mean (±SD) telomere length in 21 gastric carcinomas was $7.0 \pm 1.6 \times 10^3$ base pairs (1.6 kbp). In 20 (95%) of the 21 subjects, the values were smaller than those in the non-neoplastic gastric mucosa (mean shortening 1.8 kbp), although a strong correlation was observed for the paired data ($r = 0.69$, $P = 0.0004$). Similarly, telomere lengths in carcinomas were shorter than those for intestinal metaplasia (a mean difference of 1.1 kbp). Telomerase activity, estimated using the telomeric repeat amplification protocol assay, was positive in 18 (86%) of the 21 gastric carcinomas, without significant differences among the three histological types (well, moderately, and poorly differentiated adenocarcinomas) or with sex or age. The results suggest that telomere length and possibly shortening rates vary with the individual, and that examination of both non-neoplastic mucosa and tumors is necessary to improve our understanding of the significance of telomerase in neoplasia.

Key words Telomere · Telomerase · Gastric carcinoma · Aging

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Introduction

Telomeres, composed of many hundreds of tandem repeats of the sequence TTAGGG, play a role in chromosomal protection and replication (Blackburn 1991). With one normal somatic cell division, chromosomes lose 50–200 base pairs (bp) of the telomeric sequence (Harley et al. 1990; Vaziri et al. 1993), and thus telomeres shorten progressively with aging in somatic cells. In contrast, immortalized and some carcinoma cells show maintenance of telomere length despite repeated cell division due to the activity of telomerase (Counter et al. 1994).

We have conducted systemic studies to clarify the reduction rates each year from human tissue of all types and we have found values of 60 bp and 55 bp for esophageal mucosa and liver tissue respectively (Takubo et al. 1999; Takubo et al. 2000).

Although telomerase activity and telomere length have been investigated in gastric carcinomas (Hiyama et al. 1995; Ahn et al. 1997; Maruyama et al. 1997), information on aging-related changes with reference to neoplasia is limited. In the present report, we therefore document data for telomere length in samples of non-neoplastic gastric mucosa (0–99 years) and gastric carcinomas (39–99 years), comparing the findings with histopathological features and telomerase activity.

Materials and methods

Subjects

Surgical specimens of non-neoplastic and neoplastic gastric mucosa (including 5 of intestinal metaplasia) were obtained from 32 and 21

patients respectively (a total of 21 men and 11 women, aged between 39 and 99 years), with gastric carcinomas, along with autopsy specimens of gastric mucosa from 3 neonates (0–4 weeks), 2 infants (< 1 year), and 1 child (under 2 years). The patients with gastric carcinoma had not been previously treated and underwent operations at the Tokyo Metropolitan Geriatric Hospital or the Department of Surgery II, Saitama Medical School. All samples of gastric mucosa were stored at −80 °C until analyzed. The study protocol was approved by the Tokyo Metropolitan Institute of Gerontology Ethical Committee.

Southern blot analysis

Genomic DNA was prepared from each sample by treatment with proteinase K and sodium dodecyl sulfate (SDS) followed by repeated phenol/chloroform extraction. Aliquots of 5 μg DNA were digested with the restriction enzyme *Hin*fI (Boehringer Mannheim Biochemica, Germany) and complete cutting was confirmed by electrophoresis of the DNA digests on 0.8% agarose gels. Separated DNA fragments were transferred by an alkaline transfer technique using capillary blotting to nylon membranes (Hybond-N+, Amersham, UK), which were then incubated for 12 h at 50 °C in hybridization buffer [6× SSPE (1× 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4), 1% SDS] containing a $(TTAGGG)_4$ probe that had been labeled with [γ-^{32}P]ATP (Amersham) at the 5′ end by T4 polynucleotide kinase (Toyobo, Japan). The membranes were washed first in 2× SSC (17.55 g/l NaCl, 8.82 g/l sodium citrate) at room temperature, and then in 6× SSC, 0.1% SDS at 50 °C for 15 min with shaking, dried with filter papers and used to expose X-ray films and Fuji Imaging Plates (Fuji Photo Film Co. Ltd., Japan) for 3 h at room temperature. The Fuji Imaging Plates were then analyzed with a BAS-2500 Mac image analyzer (Fuji Photo Film Co. Ltd.) using the programs Image Reader (version 1.1, Fuji Photo Film Co. Ltd.) and Mac Bas (version 2.4, Fuji Photo Film Co. Ltd.). Terminal restriction fragment (TRF) lengths, determined by comparing the position of maximum radioactivity in each lane with these of molecular size markers, were regarded as the telomere lengths.

Telomeric repeat amplification protocol (TRAP) assay

Lysates were prepared by powdering tissues frozen under liquid nitrogen, followed by homogenization in 200 μl ice-cold lysis buffer (10 mM TRIS/HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM 2-mercaptoethanol, 0.5% CHAPS, 10% glycerol) and incubation for 30 min on ice. After the incubation, the lysates were centrifuged at 10 000*g* for 20 min at 4 °C and the supernatant and precipitate were rapidly frozen separately and stored at −80 °C. Protein concentrations were determined by the Bradford assay (Bio-Rad, Hercules, Calif.). For the TRAP assay, tubes were prepared by sequestering 0.1 μg CX primer (5′-CCCTTACCCTTACCCTTACCTAA-3′) under a wax barrier (Ampliwax; Perkin-Elmer Cetus, Foster City, Calif.). Extracts, equivalent to 6 μg protein, were assayed in 50 μl reaction mixture containing 20 mM TRIS/HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.005% Tween 20, 1 mM EGTA, 50 μM dNTP, 150 kBq [α-^{32}P]dCTP (Amersham, UK), 0.1 μg TS primer (5′-AATCCGTCGAGCAGAGTT-3′) 1 μg T4 gene 32 protein (Boehringer Mannheim, Germany) and 2 units of Taq DNA polymerase (Gibco-BRL, Gaithersburg, Md.). After a 30-min incubation at room temperature for telomerase-mediated extension of the TS primer, the reaction mixture was heated at 90 °C for 90 s and then subjected to 31 polymerase chain reaction cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 45 s. The products were then electrophoresed on 10% polyacrylamide gels.

Clinicopathological assessment

All gastric specimens from surgical and autopsy cases were examined histopathologically. The tumors were classified into histological types and staged according to the TNM classification of the UICC (Hermanek and Sobin 1987).

Statistics

Differences in mean values were analyzed by Student's *t*-test and correlations with Fisher's test.

Results

As determined from histopathological features, the 21 carcinomas comprised 3 well, 13 moderately and 5 poorly differentiated adenocarcinomas (Table 1). Typical

Table 1 Telomere length and telomerase activity in non-neoplastic gastric mucosa and gastric carcinomas, in relation to clinicopathological data for all subjects. *M* male, *F* female, *Well* well-differentiated adenocarcinoma, *Mod* moderately differentiated adenocarcinoma, *Poor* poorly differentiated adenocarcinoma, *N-TRF* terminal restriction fragments of non-neoplastic gastric mucosa, *T-TRF* terminal restriction fragments of gastric carcinoma, *kbp* 10^3 base pairs, *N-telomerase activity* telomerase activity in non-neoplastic gastric mucosa, *T-telomerase activity* telomerase activity in gastric carcinoma; + positive, − negative

No.	Age (years)	Sex	Histology	N-TRF (kbp)	T-TRF (kbp)	N-Telomersae activity	T-Telomerase activity
1	2 W[a]	F		10.7		(−)	
2	2 W[a]	F		10.7		(−)	
3	3 W[a]	M		13.7		(−)	
4	1	M		12.3		(−)	
5	1	M		12.7		(−)	
6	2	M		10.9		(−)	
7	39	F	Mod	12.1	7.1	(−)	(+)
8	44	M	Mod	12.4	11.5	(+)	(+)
9	47	M		9.5		(−)	
10	47	F		11.0		(−)	
11	50	M		7.1		(−)	
12	55	M		12.6		(−)	
13	55	M		10.3		(−)	
14	56	M		8.5		(+)	
15	57	M	Mod	7.7	5.1	(+)	(+)
16	57	M		8.1		(−)	
17	58	M	Poor	7.9	5.6	(+)	(+)
18	62	F		8.8		(+)	
19	64	M	Well	6.4	5.4	(−)	(+)
20	64	M		6.7		(+)	
21	64	M		9.2		(+)	
22	66	M		10.0		(−)	(+)
23	67	M	Mod	11.9	9.6	(−)	(+)
24	72	M	Poor	11.7	5.7	(+)	(+)
25	74	M	Mod	9.9	8.8	(−)	(+)
26	76	F	Mod	10.2	8.1	(+)	(+)
27	77	M	Well	8.0	7.5	(+)	(+)
28	78	F	Mod	9.1	8.1	(−)	(+)
29	78	M	Mod	10.0	8.5	(−)	(+)
30	80	F	Mod	8.0	6.8	(−)	(−)
31	80	M	Mod	8.5	6.7	(−)	(+)
32	81	F	Poor	6.4	6.4	(+)	(−)
33	81	M	Mod	8.2	6.4	(−)	(−)
34	81	F	Poor	9.0	5.9	(+)	(+)
35	81	F	Well	7.6	6.4	(−)	(+)
36	81	M	Poor	5.8	4.7	(+)	(+)
37	87	F	Mod	7.7	6.3	(−)	(+)
38	99	F	Mod	6.5	6.3	(−)	(+)

[a] *W* weeks

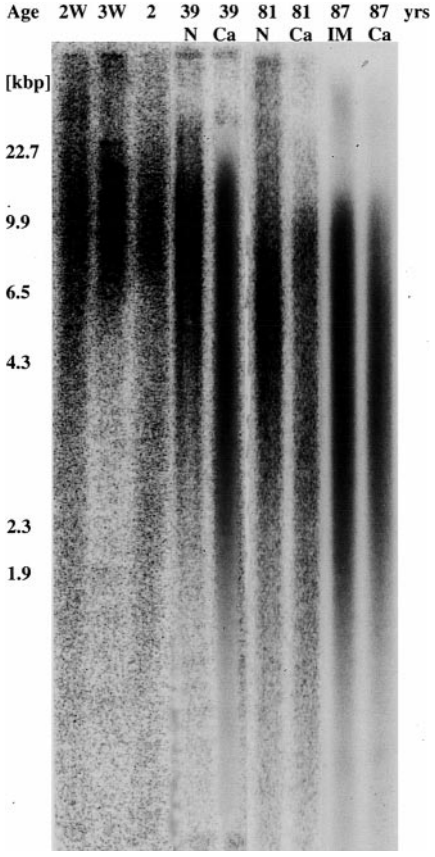

Fig. 1 Representative autoradiogram showing results of Southern blot analysis of telomeric DNA from gastric tissues of subjects aged 0–87 years. Molecular mass markers are indicated on the *left*. Radiographic results for six subjects aged 2 weeks, 3 weeks, and 2, 39, 81 and 87 years. *N* normal, non-neoplastic gastric mucosa, *IM* intestinal metaplasia, *Ca* carcinomas. The telomere lengths are 10.7, 13.7, 10.9, 12.1 (N), 7.1 (Ca), 8.2 (N), 6.9 (Ca), 7.7 (IM) and 6.3 (Ca) kbp respectively. Note that the smears of telomeric DNA of the gastric mucosa on the Southern blotting radiographs are short for the two neonates and the infant and long for the three older subjects. *W* weeks, *yrs* years, *kbp* 10^3 base pairs

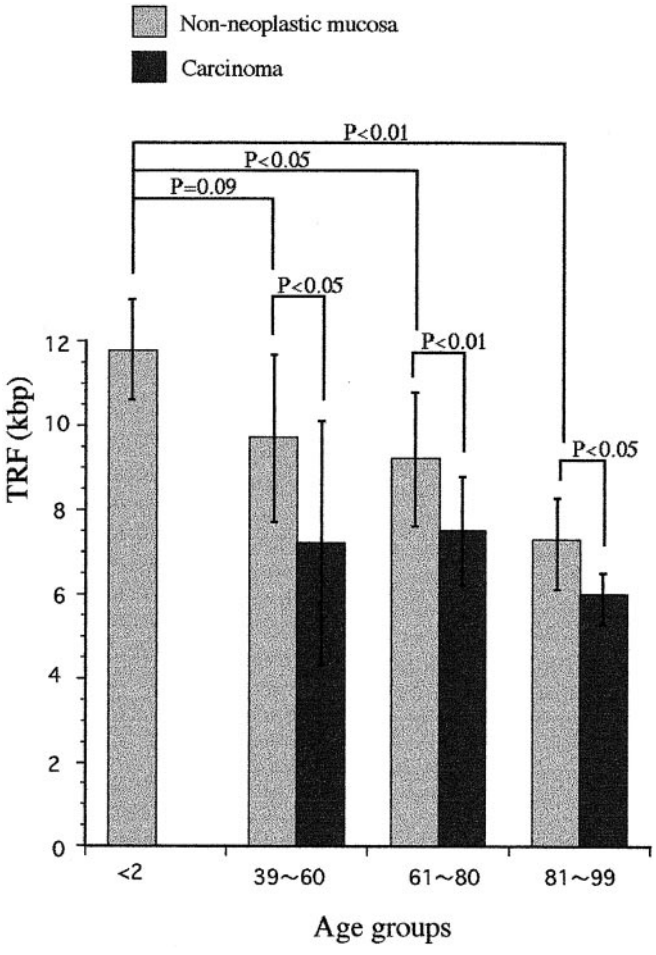

Fig. 2 Mean telomere lengths in non-neoplastic gastric mucosa and carcinomas. The mean (±SD) telomere length of the six subjects less than 2 years old was 11.8 ± 1.2 kbp. Mean (±SD) telomere lengths for non-neoplastic mucosa were 9.7 ± 2.0, 9.2 ± 1.6 and 7.3 ± 1.1 kbp for the three age groups, 39–60 years (n = 11), 61–80 years (n = 14), and 81–99 years (n = 7) respectively. Mean (±SD) values in carcinomas were 7.2 ± 2.9 (n = 4), 7.5 ± 1.3 (n = 10), and 6.0 ± 0.6 kbp (n = 7) for the age groups 39–60 years, 61–80 years and 81–99 years respectively. Note the decrease with aging and that telomere lengths for gastric carcinomas are significantly shorter than those for non-neoplastic mucosa. *TRF* Terminal restriction fragment

band widths on Southern blot analysis of telomeres of gastric mucosa are shown in Fig. 1, these being wider in adults than in children.

The mean (±SD) telomere length of the 6 subjects under 2 years old was ($11.8 \pm 1.2 \times 10^3$) base pairs (kbp) (3 neonates, mean 11.7 kbp, 2 infants, mean 12.5 kbp and 1 child, 10.4 kbp). Mean (±SD) telomere lengths for non-neoplastic mucosa were 9.7 ± 2.0, 9.2 ± 1.6 and 7.3 ± 1.1 kbp for the three age groups, 39–60 years (n = 11), 61–80 years (n = 14), and 81–99 years (n = 7) respectively. Data for telomere lengths for the carcinomas are summarized in Table 1. Mean (±SD) values were 7.2 ± 2.9 (n = 4), 7.5 ± 1.3 (n = 10), and 6.0 ± 0.6 kbp (n = 7) for the age groups 39–60 years, 61–80 years and 81–99 years respectively (Fig. 2). Although a tendency was noted for the mean telomere length in the carcinoma tissues to shorten with age, there were no significant differences among the three older age groups.

Progressive telomere shortening with aging was studied using non-neoplastic gastric mucosal specimens from the 32 patients and 6 autopsy subjects (less than 2 years old) aged between 0 and 99 years to determine the annual rate of telomere loss. The telomere length for non-neoplastic gastric mucosae ranged from 5.8 kbp to 13.7 kbp. We observed age-related shortening of telomeres in the non-neoplastic gastric mucosa, at an average rate of 46 bp/year. The slope (−46 bp/year) of the linear regression line of non-neoplastic gastric mucosae proved to be significantly different from zero (r = −0.649, P = 0.0001) (Fig. 3). Similarly, in carcinoma specimens from the 21 patients aged between 39 and 99 years, we observed age-related shortening at a rate of 33 bp/year. However, the linear regression line of

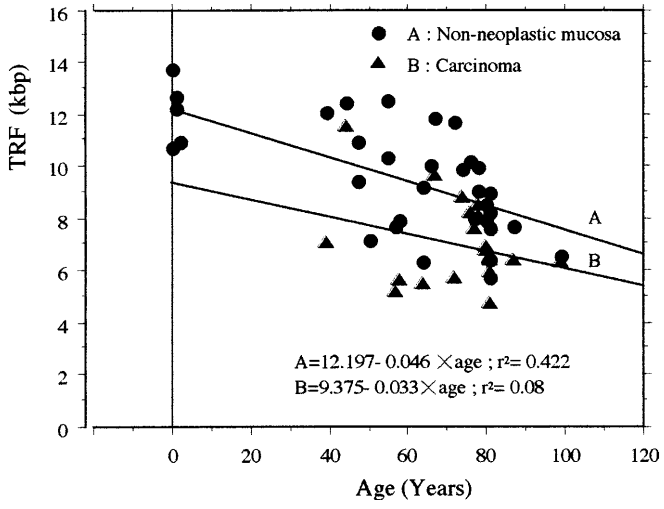

Fig. 3 Telomere lengths for non-neoplastic gastric mucosa, and carcinomas. The mean telomere length for each individual, determined by quantitative analysis, is plotted against age. The slope (−46 bp/year) of the linear regression line of non-neoplastic mucosae (*A*) is significantly different from zero ($r = -0.649$, $P = 0.0001$). However, this is not the case for carcinomas (*B*) ($r = -0.283$, $P = 0.217$)

gastric carcinomas was not significantly different from zero ($r = -0.283$, $P = 0.217$) (Fig. 3).

Furthermore, comparison of telomere lengths revealed lower values for carcinomas than for non-neoplastic gastric mucosa in 20 (95%) of 21 subjects (the mean difference was 1.8 kbp). The telomere lengths for non-neoplastic gastric mucosa and gastric carcinomas in the same patients strongly correlated ($r = 0.69$, $P = 0.0004$) (Fig. 4). Telomeres in carcinomas were shorter than in counterpart intestinal metaplasia (mean difference of 1.1 kbp).

The gastric carcinoma telomere lengths in cases with telomerase activity ranged from 4.7 kbp to 11.5 kbp, whereas without the enzyme they ranged from 6.4 kbp to 6.8 kbp (mean values 7.7 kbp and 6.5 kbp respectively, without significant difference). Of the 21 carcinomas, 18 (86%) had detectable telomerase activity (Table 1), and 3 (100%) of 3 well-differentiated adenocarcinomas, 11 (85%) of 13 moderately differentiated adenocarcinomas, 4 (80%) of 5 poorly differentiated adenocarcinomas, and 3 (60%) of 5 samples of intestinal metaplasia were positive. Moreover, 13 (41%) of the 32 non-neoplastic gastric mucosae (from patients aged between 39 and 99 years) also had detectable telomerase activity. No telomerase activity was evident in the 6 samples from individuals less than 2 years of age.

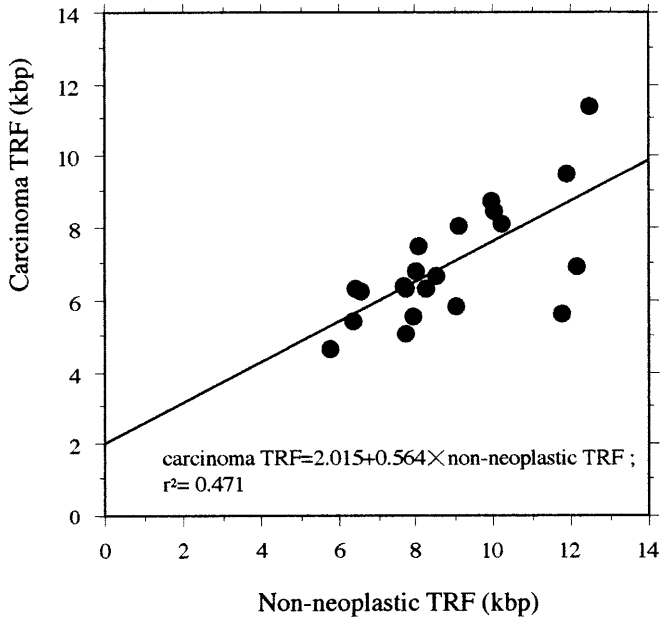

Fig. 4 Relationship between non-neoplastic gastric mucosa and gastric carcinoma telomere lengths. Note the strong correlation ($r = 0.69$, $P = 0.0004$)

Discussion

Southern blot analysis is a general method to measure telomere length. Terminal restriction fragment lengths, determined by comparing the position of the maximum radioactivity in each lane with the molecular size markers, were regarded as the telomere lengths in these experiments. The clear difference in smear widths evident for telomeres, indicative of more variation from cell to cell in older individuals, was also found in esophageal mucosae and liver in our previous studies (Takubo et al. 1999; Takubo et al. 2000).

Human telomere shortening with aging has been reported in peripheral lymphocytes (41 bp/year) (Vaziri et al. 1993), epidermal cells (19.8 bp/year) (Lindsey et al. 1991), peripheral blood cells (33 bp/year) (Hastie et al. 1990), human intestinal mucosa of the large and small intestines (42 bp/year) (Hiyama et al. 1996), esophageal mucosa (60 bp/year) (Takubo et al. 1999), and liver (55 bp/year) (Takubo et al. 2000). The annual telomere loss in gastric mucosa was 46 bp from a mean telomere length for neonatal gastric mucosa of 11.8 kbp. In one normal somatic cell division, chromosomes would be expected to lose 50–200 bp of the telomeric sequence (Harley et al. 1990; Vaziri et al. 1993) and the turnover of gastric epithelia is very rapid, occurring about every 2–6 days (Eastwood 1977), with germ cells demonstrating frequent mitoses. Our data for telomere length in non-neoplastic mucosa, point to a variation across tissues, if one takes into account the finding that the telomeres of human esophageal mucosa and liver are 12–15 kbp long at the time of birth (Takubo et al. 1999; Takubo et al. 2000). Given the fact that the mucous membrane of the stomach is thought to have a renewal time of 5 days or less, 50 or more cell divisions would be required per year for maintenance (Eastwood 1977). Therefore, if telomeres shorten by 50 bp at each cell division, the expected rate of telomeric shortening would be 2.5 kbp/year. However, the oldest subject of this study was 99 years old and this subject had a

telomere length of 6.5 kbp in the non-neoplastic mucosa. Thus germ cells may indeed have some telomerase activity that cannot be detected by the telomere repeat amplification protocol assay using whole mucosal tissue. Alternatively there must be another mechanism to extend telomeres.

Telomerase activity has been detected in a wide variety of human tumors and tumor-derived cell lines. Ahn et al. reported telomerase activity to be positive in 85 of 95 gastric carcinomas (89%) and that expression was independent of age, sex, tumor location, tumor stage, histological grade or presence of lymph node metastasis (Ahn et al. 1997). Maruyama et al. demonstrated that telomerase activity may become evident from very early stages of gastric carcinogenesis, finding positive rates of 15%, 45% and 89% for cases of intestinal metaplasia, adenoma, and gastric carcinoma respectively. However, in normal gastric mucosae, telomerase activity could not be detected (Maruyama et al. 1997). The findings of the present study are thus in line with the literature on tumors, additionally demonstrating a relatively high frequency in non-neoplastic gastric mucosa (41%).

In the present study, the telomeres of carcinomas were about 1.8 kbp shorter than in the corresponding non-neoplastic mucosa in several age groups. Telomere length in gastric carcinomas may be influenced by the stromal and inflammatory cells, including lymphocytes, having shortened telomeres as they age. Annual reduction rates of peripheral blood cells (Hastie et al. 1990) and lymphocytes (Vaziri et al. 1993) have been reported to be 33 bp and 41 bp respectively. The most striking result was the highly significant correlation between non-neoplastic and carcinoma values in individual patients. Although, as mentioned above, we must consider heterogeneity within tissues, the present data point to the necessity for comparison with background levels before drawing conclusions about the significance of telomerase and telomere shortening for neoplasia.

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