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Telomerase activity and expression of human telomerase catalytic subunit gene in esophageal tissues

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Background. Telomerase, the ribonucleoprotein enzyme that synthesizes telomeric DNA, is thought to be necessary for cellular immortality and carcinogenesis. Telomerase activity is associated with the majority of malignant human cancers. The mRNA that encodes the telomerase catalytic subunit (human telomerase repeat transcriptase; hTERT) has recently been identified, and the expression of the *hTERT* gene is thought to regulate the activation of telomerase. However, the expression of *hTERT* mRNA in esophageal tissues has not been reported. We investigated *hTERT* gene expression in cancerous and noncancerous esophageal tissues, and determined the relationship between *hTERT* mRNA expression and telomerase activity. ***Methods.*** Tissues from esophageal carcinomas in 14 patients, reflux esophagitis in 12 patients, esophageal acanthosis in 2 patients, esophageal papilloma in 1 patient, radiation esophagitis in 1 patient, and normal esophageal epithelium in 11 patients (including 3 specimens of normal epithelium from patients with esophageal carcinoma) were examined. All specimens were taken endoscopically. *hTERT* gene expression was investigated using reverse transcription-polymerase chain reaction (RT-PCR). Quantitative analysis of telomerase activity was analyzed by fluorescence telomeric repeat amplification protocol (F-TRAP) assay. ***Results.*** Thirteen of the 14 (93%) esophageal carcinoma specimens expressed *hTERT* mRNA and revealed detectable telomerase activity. Noncancerous esophageal lesions had not only *hTERT* mRNA expression with a high frequency (14 of 16 cases; 88%) but also detectable telomerase activity (12 of 13 cases; 92%). Normal esophageal epithelium also highly expressed *hTERT* mRNA (10 of 11 cases; 91%) and revealed detectable telomerase activity (all 9 cases; 100%). In 32 of the 35 specimens analyzed for both *hTERT* mRNA and telomerase activity (91%), the expression of *hTERT* mRNA was consistent with detectable telomerase activity. ***Conclusions.*** The expression of *hTERT* mRNA was detected not only in cancerous but also in noncancerous esophageal tissues at a high frequency. This result was different from that reported for other gastrointestinal epithelium. Moreover, telomerase activity in esophageal carcinoma was significantly stronger than that in reflux esophagitis and normal epithelium. In addition, there was a strong relation ship between the detection of telomerase activity and the expression of *hTERT* mRNA in cancerous and noncancerous esophageal tissues. Thus, the qualitative analysis of *hTERT* mRNA expression may not be useful as a biomarker of carcinoma in esophageal tissues. Nevertheless, the quantitative analysis of telomerase activity may be somewhat useful.

Key words: telomerase activity, human telomerase reverse transcriptase (hTERT), esophageal carcinoma, normal esophageal epithelium

Introduction

Esophageal squamous cell carcinoma is a frequent malignancy in Japan and in certain areas of China. Several studies have shown that the pathogenesis of esophageal squamous carcinomas involves the functional loss of a tumor-suppressor gene, such as the *p53* gene mutation, and allele loss of chromosomal loci, such as 17p.[1,2] Recently, cell immortalization has been found to be a very important event in carcinogenesis. Cell immortalization requires the activation of telomerase, an enzyme essential for stabilizing telomere length.[3,4] Telomerase is

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a ribonucleoprotein enzyme that maintains telomere length by adding telomeric DNA to the ends of eukaryotic chromosomes. Telomerase consists of human telomerase RNA component (hTERC/hTR),[5] telomerase-associated protein (TEP1/TP1/TLP1),[6,7] and human telomerase reverse transcriptase (hTERT, previously referred to as hTRT[8] and hEST2[9]). In 1994, Kim et al.[10] developed a telomeric reverse amplification protocol (TRAP) assay, which was a highly sensitive assay for measuring telomerase activity. Telomerase has been reported to be activated in reproductive cells and cancer cells, and to be related to the immortalization of the cell. Telomerase activity has been detected at a high rate in human carcinomas, such as gastric cancer,[11] ovarian cancer,[12] and lung cancer.[13] Because telomerase is activated in most carcinomas, the detection of telomerase activity has gathered attention as a biomarker of carcinoma.

In 1997, Nakamura et al.[8] identified the mRNA of human telomerase reverse transcriptase (hTERT/hTRT), a catalytic subunit of telomerase, and reported that the expression of *hTERT* mRNA correlated with telomerase activity in cell lines. Bodnar et al.[14] reported that significant telomerase activity was induced, and life span was extended when the *hTERT* gene was transfected in normal cells, and when the enforcement was manifested. Therefore, expression of the *hTERT* gene seems to be an important stage in the acquisition of telomerase activity. A significant correlation between *hTERT* gene expression and detectable telomerase activity was observed in hepatocellular carcinoma,[15] cervical cancer,[16] urothelial cancer,[17] and gastrointestinal stromal tumors.[18]

Esophageal squamous cell carcinoma has been reported to have a high frequency of detectable telomerase activity.[19–21] However, the expression of *hTERT* mRNA in esophageal carcinomas, esophagitis, and normal epithelium has not been reported. In this study, we investigated *hTERT* gene expression and telomerase activity in cancerous and noncancerous esophageal tissues, and determined the relationship between telomerase activity and the expression of *hTERT* mRNA.

Patients, materials, and methods

Tissue samples

The characteristics of the patients from whom the tissues were obtained are summarized in Table 1. All 41 tissue samples used were obtained during routine endoscopy at the Department of Gastroenterology, Tsukuba University Hospital, between 1998 and 1999 (30 men and 8 women; median age, 67 years; range, 26–87 years). All samples were obtained from biopsy specimens. Esophageal carcinoma specimens were obtained from 14 patients who had not received any treatment (13 men and 1 woman, median age, 73 years; range, 59–87 years). Twenty-seven specimens of noncancerous esophageal epithelium were obtained. They included 12 specimens of reflux esophagitis (from 7 men and 5 women; median age, 63 years; range, 52–80 years), 2 of esophageal acanthosis, 1 of radiation esophagitis, and 11 of normal esophageal epithelium (from 9 men and 2 women; median age, 70 years; range, 51–86 years). The normal esophageal epithelium samples included noncancerous esophageal epithelium from 3 of the patients with esophageal carcinoma (cases 3, 4, and 9). Glycogen produced in normal esophageal epithelium reacts with iodine, staining the normal epithelium brown. These samples were obtained from iodine-reactive epithelium at least 5 cm away from carcinoma. After tissue removal, samples for RNA analysis and TRAP assays were rapidly frozen in liquid nitrogen and stored at −80°C until use, and the samples for histopathological study were fixed in 10% buffered formalin. Informed consent, in writing, was obtained from all patients.

Table 1. Characteristics of patients

Diagnosis	No. of patients	Male/Female	Age (years; mean ± SD)
Esophageal carcinoma	14	13/1	73 ± 7
Squamous cell carcinoma	13	12/1	
Undifferentiated carcinoma	1	1/0	
Benign esophageal lesions	16	11/5	63 ± 12
Reflux esophagitis	12	7/5	
Esophageal acanthosis	2	2/0	
Papilloma	1	1/0	
Radiation esophagitis	1	1/0	
Normal esophageal epithelium	11	9/2	70 ± 11
With esophageal carcinoma	3	3/0	
Without esophageal carcinoma	8	8/2	

Cell lines

HeLa cells, derived from cervical cancers, were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; GIBCO BRL) and 45μg/ml gentamycin (GIBCO BRL) in the presence of 5% CO_2 at 37°C. These cells were used as a positive control.

RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from tissues with TRIzol (GIBCO BRL) according to the manufacturer's protocol. For *hTERT* mRNA detection, RT-PCR analysis was performed with 1.0μg of total RNA, using the mRNA selective PCR kit Ver.1.1 (Takara Shuzo, Kyoto, Japan). Primers used for the amplification of *hTERT* cDNA were MS113 (5′-AGAGTGTCTGGAG CAAGTTGC-3′) and MS114 (5′-CGTAGTCCATG TTCACAATCG-3′). These primers do not encompass any regions reported to be involved in alternative splicing of the *hTERT* gene.[22] The amplification conditions were 33 cycles, consisting of 85°C for 1min, 60°C for 1min, and 72°C for 1min, followed by 1 cycle of 50°C for 30min (reverse transcription). As an internal control, the detection of glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA was analyzed in each sample. Primers used for the amplification of *G3PDH* cDNA were G3PDH-F (5′-ACCACAGTCCATGCC ATCAC-3′) and G3PDH-R (5′-TCCACCACCCTGT TGCTGTA-3′). The amplification conditions were 25 cycles, consisting of 85°C for 1min, and 60°C for 1min, and 73°C for 1min, followed by 1 cycle of 50°C for 30min (reverse transcription). The *hTERT* PCR products were separated by electrophoresis on 3% agarose gels, and visualized by staining with SYBR Gold (Molecular Probes Eugere, OR, USA). The G3PDH PCR products, as an internal control, were separated on 2% agarose gels and stained with ethidium bromide.

Cells of the HeLa cell line were used as a positive control; 0.5μg of total RNA isolated from HeLa cells was used for both RT-PCR analyses.

Telomerase repeat amplification protocol (TRAP) assay

Telomerase activity was measured by a quantitative fluorescence-telomeric repeat amplification protocol (F-TRAP) assay, using the TRAP-EZE telomerase detection kit (Intergen, Purchase, NY, USA), reported by Hisatomi et al.[23] Tissue extracts were isolated from samples, using homogenization in 200μl ice-cold 1× CHAPS lysis buffer (10mM Tris-hydrochloric acid, pH 7.5); 1mM magnesium chloride; 1mM ethylene glycol tetraacetic acid [EGTA]; 0.1mM phenylmethylsulfonyl fluoride; 5mM β-mercaptoethanol; 0.5% 3-[3-(cholamidopropyl)-dimethylamino]-propane sulfate [CHAPS], and 10% glycerol), as described. After 30min of incubation on ice, the tissue extracts were centrifuged at 15000*g* for 20min at 4°C and the supernatant was rapidly frozen and stored at −80°C. Protein concentrations of the extracts were determined using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). 2.0μg of protein concentrations were used for the TRAP assay. Telomerase quantification control template (TSR8), including the TRAP-EZE telomerase detection kit, was added for each set of assays. After 30-min incubation at 30°C, PCR was done for 30 cycles, consisting of 94°C for 30s, followed by 60°C for 30s for extension. The PCR products were separated by electrophoresis on 12.5% nondenatured polyacrylamide gels, and visualized by staining with SYBR Gold. We also analyzed the extracts of HeLa cells as a positive control, and inactivated samples by heat degeneration (85°C for 10min prior to TRAP assay) and lysis buffer as a negative control.

For the quantification of telomerase activity, we used the STORM chemofluorescence imager (Amersham Biosciences, Buckinghamshire, UK), and data from the imager were analyzed, using Image Quant ver.1.1 (Amersham Biosciences) for the quantitative analysis of telomerase. After electrophoresis, we measured the signal in the region of the gel lane corresponding to the TRAP product ladder bands from all samples, including non-heat-treated (x) and heat-treated sample extracts (x_0), 1× CHAPS lysis buffer control (negative control) (r_0), and TSR8 quantification control (r). Then, we measured the signal from the internal standard in non-heat-treated samples (c) and the TSR8 quantification control (c_R). Telomerase activity was calculated by the following formula:

$$\text{TPG (units)} = [\{(x - x_0)/c\}/\{(r - r_0)/c_R\}] \times 100$$

The unit for quantitative telomerase activity used was TPG (total product generated). Each TPG unit corresponds to the number of substrate oligonucleotide (TS) primers (in 1×10^{-3} mole or 600 molecules) extended with at least four telomeric repeats by telomerase in the extract in a 10-min incubation at 30°C.

Macroscopic and histological classification

All specimens were fixed in 10% buffered formalin, embedded in paraffin, stained with hematoxylin and eosin (H & E), and assessed by a pathologist who was blind to the identity of the subjects and their clinical conditions. Esophageal carcinoma specimens were classified macroscopically and histopathologically accord-

ing to the criteria of the Japanese Society of Esophageal Diseases.[24]

Statistical analysis

The χ^2 test was used to test differences between proportions. Analysis of variance (ANOVA) was used for further analysis, and if there were significant differences, Fisher's protected least significant difference (PLSD) test was used for post-hoc analysis. P values less than 0.05 were considered to be significant. All analyses were performed using StatView 4.5 for Macintosh (Abacus Concepts Berkeley, CA, USA).

Results

All specimens expressed *G3PDH* mRNA (Table 2; Fig. 1 shows representative results).

The expression of *hTERT* mRNA was detected in 13 of the 14 specimens of esophageal carcinoma (93%). These samples of esophageal carcinoma were derived from 13 cases of squamous cell carcinoma and 1 case of undifferentiated carcinoma, small-cell type. Telomerase activity was detected in all 13 cases (100%). Although 1 squamous cell carcinoma (case 1) did not express *hTERT* mRNA, it had detectable telomerase activity (Table 2).

Table 2. *hTERT* mRNA expression and telomerase activity in esophageal tissues

Case no.	Age (years)	Sex	Diagnosis	*G3PDH* mRNA	*hTERT* mRNA	TPG
1	75	M	SCC (IIb)	+	−	212.39
2	87	F	SCC (2)	+	+	91.46
3	63	M	SCC (2)	+	+	61.69
4	70	M	SCC (2)	+	+	197.31
5	70	M	SCC (2)	+	+	51.38
6	76	M	Small (1pl)	+	+	122.42
7	71	M	SCC (2)	+	+	12.49
8	75	M	SCC (2)	+	+	36.11
9	77	M	SCC (2)	+	+	103.54
10	70	M	SCC (IIc)	+	+	151.43
11	76	M	SCC (IIc)	+	+	175.44
12	79	M	SCC (IIa)	+	+	236.21
13	62	M	SCC (2)	+	+	107.68
14	59	M	SCC (IIc)	+	+	NE
15	56	M	Reflux esophagitis	+	+	55.70
16	51	M	Reflux esophagitis	+	+	44.63
17	74	F	Reflux esophagitis	+	+	74.19
18	55	F	Reflux esophagitis	+	+	NE
19	70	M	Reflux esophagitis	+	+	3.54
20	80	M	Reflux esophagitis	+	+	15.70
21	63	F	Reflux esophagitis	+	+	8.86
22	64	M	Reflux esophagitis	+	+	187.40
23	68	M	Reflux esophagitis	+	+	NE
24	52	M	Reflux esophagitis	+	−	130.62
25	57	F	Reflux esophagitis	+	+	NE
26	64	F	Reflux esophagitis	+	−	80.61
27	26	M	Esophageal acanthosis	+	+	92.91
28	62	M	Esophageal acanthosis	+	+	125.19
29	63	M	Esophageal papilloma	+	+	45.75
30	72	M	Radiation esophagitis	+	+	32.54
31[a]	63	M	Normal epithelium	+	+	16.00
32[a]	70	M	Normal epithelium	+	+	23.98
33	86	M	Normal epithelium	+	+	NE
34	63	M	Normal epithelium	+	−	NE
35	60	F	Normal epithelium	+	+	49.77
36	51	M	Normal epithelium	+	+	69.33
37	78	F	Normal epithelium	+	+	36.78
38	62	M	Normal epithelium	+	+	19.82
39	82	M	Normal epithelium	+	+	33.55
40	78	M	Normal epithelium	+	+	113.08
41[a]	77	M	Normal epithelium	+	+	60.54

TPG, Total product generated; SCC, squamous cell carcinoma; Small (1pl), undifferentiated carcinoma small-cell type; 2, IIa, IIb, IIc, macroscopic classification of esophageal cancer according to the criteria of the Japanese Society of Esophageal Diseases; NE, not examined

[a] Noncancerous epithelium from patients with esophageal carcinoma (case 31 from case 3, case 32 from case 4 and case 41 from case 9)

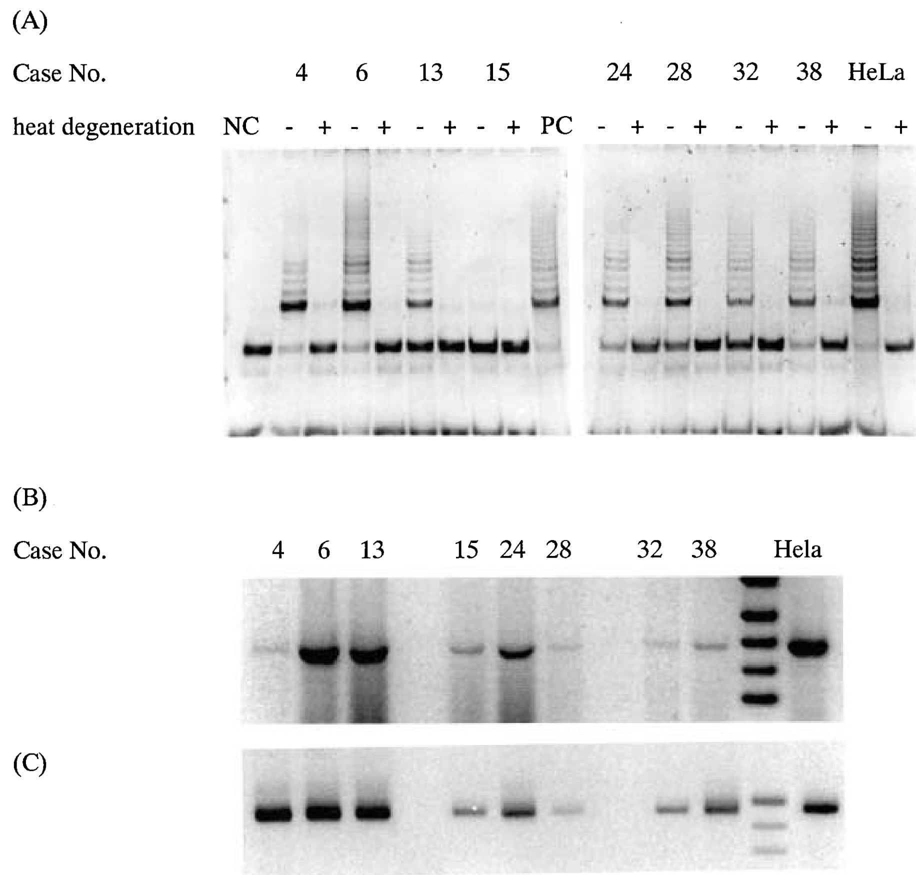

Fig. 1. A Telomerase activity, **B** *hTERT* mRNA expression, and **C** *G3PDH* mRNA expression in esophageal tissues. *NC*, Negative control; *PC*, positive control

We analyzed a case of small-cell type of undifferentiated carcinoma in the esophagus (case 6), and detected both *hTERT* mRNA and telomerase activity (Table 2).

hTERT mRNA was detected in 10 of the 12 cases (83%) of reflux esophagitis. In addition, telomerase activity was detected in 8 of 9 cases (89%). One case of reflux esophagitis (case 15) expressed *hTERT* mRNA, but had very weak telomerase activity. Two cases of reflux esophagitis (cases 24 and 26) did not express the *hTERT* gene, but had relatively high telomerase activity. The 2 cases of esophageal acanthosis (cases 27 and 28), the case of esophageal papilloma (case 29), and the case of radiation esophagitis (case 30) not only had expression of the *hTERT* gene but also had detectable telomerase activity. The 16 benign esophageal lesions not only had *hTERT* mRNA expression at a high frequency (14 of 16 cases; 88%) but also had detectable telomerase activity (12 of 13 cases; 92%) (Table 2).

Of the 11 samples of normal esophageal epithelium, 3 (cases 31, 32, and 41) were derived from patients with esophageal carcinomas (cases 3, 4, and 9). No samples of normal mucosa contained carcinoma cells histopathologically. Furthermore, normal esophageal epithelium also highly expressed *hTERT* mRNA (10 of the 11 cases; 91%) and revealed detectable telomerase activity (all 9 cases; 100%). With or without carcinoma, normal esophageal epithelium showed *hTERT* gene expression and detectable telomerase activity (Table 2).

We also investigated the quantification of telomerase activity. Telomerase activity in the esophageal carcinoma, reflux esophagitis, and normal epithelium was 120.0 ± 57.8, 66.8 ± 45.9, and 47.6 ± 21.6 TPG (mean ± SD). Moreover, telomerase activity in the carcinoma tissue was greater than that in the normal epithelium or that in the reflux esophagitis with significant difference ($P < 0.05$). On the other hand, there was no significant difference in quantitative telomerase activity between reflux esophagitis and normal epithelium (Fig. 2).

The relationship between *hTERT* gene expression and telomerase activity was investigated. We analyzed both *hTERT* mRNA and telomerase activity in 35 samples; 13 samples of esophageal carcinoma, 13 of benign esophageal lesions, and 9 of normal epithelium. Thirty-one of the 32 *hTERT*-positive esophageal mucosae were positive for detectable telomerase activity (Table 3). We considered that there was a strong relationship between *hTERT* gene expression and telomerase activity; 32 of the 35 (91%) samples were concordant for *hTERT* gene expression and detectable telomerase activity. In contrast, 3 of the 35 samples

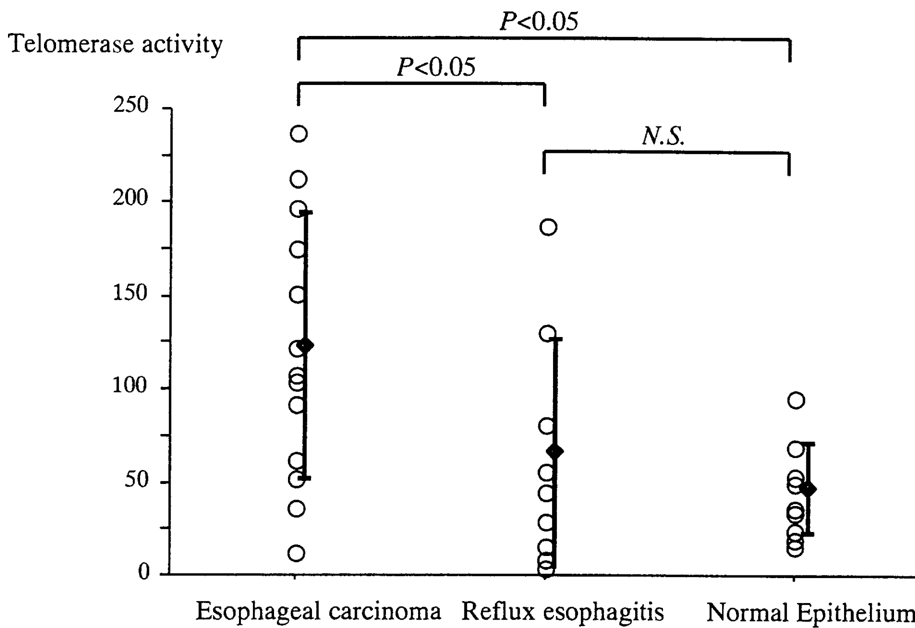

Fig. 2. Statistical analysis of quantitative telomerase activity. The *vertical axis* indicates the mean ± SD value for telomerase activity in the samples (total product generated; TPG). Telomerase activity in esophageal carcinoma, reflux esophagitis, and normal epithelium was 120.0 ± 57.8, 66.8 ± 45.9, and 47.6 ± 21.6 TPG, respectively. *N.S.*, No significant difference

Table 3. Relationship between *hTERT* mRNA expression and telomerase activity

Telomerase activity	*hTERT* +	*hTERT* −
Esophageal carcinoma		
+ (n = 13)	12	1
− (n = 0)	0	0
Reflux esophagitis		
+ (n = 8)	6	2
− (n = 1)	1	0
Esophageal acanthosis		
+ (n = 2)	2	0
− (n = 0)	0	0
Esophageal papilloma		
+ (n = 1)	1	0
− (n = 0)	0	0
Radiation esophagitis		
+ (n = 1)	1	0
− (n = 0)	0	0
Normal esophageal epithelium		
+ (n = 9)	9	0
− (n = 0)	0	0

were discordant for *hTERT* mRNA expression and telomerase activity. These samples did not express the *hTERT* gene, but were positive for telomerase activity (cases 1, 24, and 26).

Discussion

Nakamura et al.[8] reported that *hTERT* mRNA expression was correlated with telomerase activity. In the present study, *hTERT* mRNA expression was detected in 30 of the 35 cases with detectable telomerase activity (86%). This result was similar to the results obtained in cervical cancer and normal cervical tissues (82%),[16] urothelial cancer and normal urothelial tissues (80.8%),[17] and in normal skin and skin tumor (82.8%).[25] Because we did not have samples that had neither *hTERT* gene expression nor detectable telomerase activity, there was no significant difference between *hTERT* gene expression and detectable telomerase activity in the statistical analysis using the χ^2 test ($P > 0.1$). However, our results showed that *hTERT* gene expression was consistent with detectable telomerase activity in 91% of esophageal tissues. This suggests a strong relationship between the expression of *hTERT* mRNA and telomerase activity in esophageal tissues.

It is accepted that over 85% of squamous cell carcinomas of the esophagus had high levels of detectable telomerase activity. Takubo et al.[19] reported that 87% of esophageal carcinomas had telomerase activity. Koyanagi et al.[20] reported that telomerase activity was detected in all examined esophageal squamous cell carcinoma tissues. Ikeguchi et al.[21] reported that 40 of 52 (77%) esophageal squamous cell carcinomas had detectable telomerase activity. In our study, telomerase activity in all examined esophageal squamous cell carcinomas was greater than that in the other esophageal tissues. Furthermore, the *hTERT* gene was expressed in all but one of the samples of squamous cell carcinomas. Our results also revealed that esophageal squamous cell carcinomas expressed the *hTERT* gene as well as showing detectable telomerase activity. In addition, we detected *hTERT* gene expression and telomerase activity

in the small-cell type of undifferentiated carcinoma of the esophagus. To our knowledge, this is the first report of the detection of telomerase activity and the *hTERT* gene expression in undifferentiated carcinoma of the esophagus. Telomerase activity in undifferentiated carcinoma has been reported in lung cancers.[26] Hiyama et al.[26] reported that all 11 cases of small-cell lung cancers had strong telomerase activity. Our results suggest that the small-cell type of undifferentiated carcinoma of the esophagus, as well as small-cell carcinoma in the lung, has strong telomerase activity and the expression of *hTERT* mRNA.

On the other hand, controversial results have been reported for telomerase activity in normal esophageal epithelium. Takubo et al.[19] reported that the normal esophageal mucosa from 21 of 92 (23%) autopsied patients had detectable telomerase activity. Koyanagi et al.[20] reported that only 5 of 50 (10%) normal esophageal tissues showed telomerase activity. However, Ikeguchi et al.[21] showed that telomerase activity was detected in 45 of 52 (87%) normal tissue samples adjacent to carcinomas and in 8 of 11 (73%) normal esophageal epithelium samples from non-cancerous patients. Moreover, Bachor et al.[27] reported that all tissues of normal esophageal epithelium showed telomerase activity. We investigated 11 samples from normal esophageal epithelium; *hTERT* mRNA was expressed in all but one sample (case 34), and telomerase activity was detected in all examined cases. We examined why this discrepancy occurs. Therefore, we compared our materials and methods with those in other reports.

First, we carefully compared our source of tissue samples and our tissue treatment with those of other researchers. We obtained the samples from normal esophageal epithelium using endoscopic biopsy, and the samples were frozen immediately using liquid nitrogen. Ikeguchi et al.[21] obtained their samples from surgical resection or endoscopic biopsy, and they also immediately froze the samples after tissue removal. Bachor et al.[27] also used snap-frozen samples from endoscopic biopsy. The similarity of their tissue treatment may have brought about a similar result for telomerase activity in normal epithelium. Regarding the sampling, Takubo et al.[19] obtained samples of normal esophageal tissues only from autopsy. Because more time was needed to obtain their samples than to obtain our samples, there is a possibility that telomerase may have been inactivated in the samples from autopsy. Therefore, the telomerase activity they detected in normal epithelium may be lower than the level that we detected. Secondly, regarding the isolation of tissue samples, Koyanagi et al.[20] only treated the samples with washing buffers (10mM HEPES-potassium hydroxide, pH 7.5, 1.5mM magnesium chloride, 10mM potassium chloride, 1mM dithiothreitol) before they the homogenized samples. They also used this treatment in another study[28] and reported that no tumor tissue was observed in telomerase-negative samples. Although it is not known whether or not the treatment using this washing solution affects telomerase activity, there is some possibility that this solution hinders the detection of telomerase activity when the activity is relatively low. Therefore, this treatment using the washing solution may also lead to a difference in detectable telomerase activity in normal epithelium. Thirdly, for the TRAP assay, Ikeguchi et al.[21] and Bachor et al.[27] used a kit by Oncor (Gaithersburg, MD, USA) and we used a TRAP-EZE telomerase detection kit (Intergen). These two kits have the same contents and are based on the protocol established by Kim et al.[10] Takubo et al.[19] and Koyanagi et al.[20] also analyzed their samples using the TRAP assay established by Kim et al.[10] Slight differences were seen between the conditions in our experiments and those of the others, such as incubation temperature and amplification conditions. However, we could not explain whether the sensitivity of the TRAP assays was changed under the influence of the different experimental details for the TRAP assays. From this point of view, it might be hard to explain the different results for telomerase activity in normal esophageal epithelium obtained by the various reporters.

In previous studies on the stomach and intestine, telomerase activity and *hTERT* mRNA expression were either absent in normal epithelium or were detected at a lower rate than in carcinomas.[11,29–31] However, we now demonstrate that almost all normal esophageal epithelium has *hTERT* gene expression and detectable telomerase activity. Thus, it is necessary to explain why normal esophageal epithelium has telomerase activity and *hTERT* mRNA expression. First, the architectural characteristics of esophageal epithelium may have affected our result. In our results, all telomerase-positive normal epithelium expressed *hTERT* mRNA. In addition, we investigated all samples histopathologically, and we verified that all normal esophageal epithelium samples contained the basal layer of the epithelium. The esophageal epithelium which has squamous stratified epithelium, has different architectural characteristics compared with other gastrointestinal epithelium. This tissue architecture is similar to the epidermis. In the epidermis, telomerase activity is detected in the basal layer, the proliferative compartment.[32] Moreover, Kolquist et al., using in situ hybridization,[33] reported that cells of the basal layer in normal skin expressed *hTERT* mRNA. Notably, they also reported that telomerase-positive esophageal epithelium had *hTERT* expression in the basal layer of the esophageal epithelium. Frost et al.[34] reported that telomerase activity and *hTERT* mRNA expression was localized predominantly to the lower suprabasal levels

of normal epithelium of the cervix. According to this architectural resemblance, the stem cells of the basal layer in the esophageal epithelium may have strong telomerase activity and *hTERT* gene expression. Therefore, almost all of our specimens of normal esophageal epithelium may have *hTERT* gene expression and detectable telomerase activity. Rohde et al.[35] reported that 75% of normal renal tissue had *hTERT* mRNA expression. They reported that normal tissue that expressed *hTERT* did not always have detectable telomerase activity. However, our result showed that there was a strong relationship between *hTERT* mRNA expression and detectable telomerase activity in normal esophageal epithelium. Second, our analyzed samples may have been contaminated with carcinoma cells or lymphocytes. It has been reported that mature lymphocytes have detectable telomerase activity.[36,37] In our histopathological analysis, no specimens of normal mucosa contained carcinoma cells, and lymphocytes were rarely seen in samples from normal esophageal epithelium. There is little chance that the telomerase-activated cells were contaminated, causing the detection of telomerase activity and expression of *hTERT* mRNA in samples from normal esophageal epithelium.

Our results for the noncancerous esophageal lesions, such as reflux esophagitis, esophageal acanthosis, and esophageal papilloma, also showed telomerase activity in over 80% of these samples, and the *hTERT* gene was expressed 78% to 100%. Bachor et al.[27] described telomerase activity in esophageal inflamed tissues. Inflamed tissues, such as reflux esophagitis, often include small numbers of activated lymphocytes. It is accepted that activated lymphocytes have detectable telomerase activity. We cannot deny that telomerase activity in the inflamed tissues is affected by the presence of lymphocytes. In addition, most of our samples from inflamed tissues also contained cells of the basal layer. Furthermore, cell proliferation is often activated in inflamed tissues. However, we compared quantitative telomerase activity in samples of reflux esophagitis and those of normal epithelium (Fig. 2), and there was no significant difference between reflux esophagitis and normal epithelium. This result suggests that telomerase activity in inflamed lymphocytes cannot be neglected, but that their telomerase activity may be not very strong. On the other hand, Taylor et al.[38] found telomerase activity in inflammatory lesions of the skin, and they showed that telomerase activity was localized to the epidermis in newborn foreskin. Therefore, a similar phenomenon may occur in inflammatory lesions of the esophagus. Consequently, *hTERT* gene expression and detectable telomerase activity in inflamed esophageal tissues may be due to the presence of the cells in the basal layer.

On the other hand, we confirmed, histopathologically, that the samples from esophageal carcinomas did not contain cells of the basal layer. Therefore, we think that the expression of *hTERT* mRNA in esophageal carcinoma may be derived from carcinoma cells, and that *hTERT* gene expression in normal epithelium or inflamed tissues may be derived from cells of the basal layer.

In our study, a few samples were discordant for telomerase activity and *hTERT* mRNA; three samples (cases 1, 24, and 26) did not express *hTERT* mRNA, but had detectable telomerase activity. This discordance may be caused by various factors. First, we considered the possibility of methylation of the *hTERT* gene, especially the CpG island of *hTERT*. Horikawa et al.[39] reported that the highly GC-rich content of the 5′ end of the *hTERT* cDNA spans to the 5′-flanking region and intron 1, making a CpG island. They also reported that these contents might regulate the *hTERT* gene.[39] In our study, three of the *hTERT* mRNA-negative samples had detectable telomerase activity (one sample, of case 34, was not analyzed). Dessain et al.[40] reported that the *hTERT* CpG island was methylated in many telomerase-negative and telomerase-positive cultured cells and tumors, and that the extent of methylation did not correlate with the expression of *hTERT*. Therefore, the methylation of *hTERT* might not contribute to the discordance in a few cases. Next, we considered that another factor may cause this discordance. A similar discordance has also been reported in various other tissues.[17,25,41] However, the previous reports could not explain this discordance. Moreover, it has not been reported the molecular mechanism of acquisition of telomerase activity independent of the *hTERT* gene. Therefore, at present, we cannot explain the reason for this discordance, and further study will be necessary.

In conclusion, our results revealed that *hTERT* mRNA was expressed not only in cancerous tissues but also in noncancerous tissues, including normal epithelium, in the esophagus at a high frequency. Furthermore, telomerase activity in esophageal carcinoma was relatively stronger than that in normal epithelium and benign esophageal lesions. In addition, there was a strong relationship between the expression of *hTERT* mRNA and the detection of telomerase activity in esophageal tissues. Consequently, the qualitative analysis of *hTERT* mRNA expression may not be useful as a biomarker of carcinoma in esophageal tissues. Nevertheless, the quantitative analysis of telomerase activity may be somewhat useful. Further in situ investigations of *hTERT* gene expression, and telomerase activity, may be necessary.

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