Original

Telomerase activity in the occurrence and progression of oral squamous cell carcinoma

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Abstract: Chronic inflammation is considered to be one of the risk factors for carcinogenesis. It was recently reported that telomerase plays an important role in inducing such chronic inflammation. Although high telomerase activity is detected in cancer tissues, the activator of telomerase is still unknown. In this study, we used an immunohistochemical method to examine the expression of human telomerase reverse transcriptase (hTERT) in the dysplasia-carcinoma sequence in the oral cavity. Furthermore, the effects of inflammatory cytokines on the telomerase activity and migration of oral cancer cell lines (Ca9-22, HSC-3, and HSC-4) were examined. Immunoreactivity for hTERT was observed in squamous intraepithelial neoplasia 3 and squamous cell carcinoma. Telomerase activity in Ca9-22 cells was increased by treatment with TNF- α and INF- γ , while its activity in HSC-4 cells was decreased by IL-1 β . Although inflammatory cytokines did not affect the proliferative activity of any of the oral cancer cell lines, cytokines and hTERT siRNA promoted the migration of HSC-3 cells. These results suggest that the presence of long-term chronic inflammation may increase telomerase activity and therefore contribute to malignant transformation of the oral mucosal epithelium. Furthermore, inhibition of telomerase activity by inflammatory stimuli

increases the invasion of certain types of oral squamous cell carcinomas.

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Keywords: oral cancer; telomerase; progression; inflammation.

Introduction

The telomere is a structure composed of a specific six-base-pair repeat sequence (TTAGGG) located at both ends of a chromosome (1,2). Normal somatic cells have a finite cell division capacity because the telomere shortens at every round of cell replication (1,2). In contrast, cancer cells can divide indefinitely because telomerase is activated and extends the telomere. Telomerase is a telomere extension enzyme that was discovered by Greider and Blackburn in 1985 (3); it consists of a complementary RNA sequence (CAAUCCCAAUC), a telomerase RNA/telomerase RNA component, telomerase reverse transcriptase (TERT), and several proteins (4).

Most cancer cells, including oral squamous cell carcinoma cells, have telomerase activity (5-10), and that activity is related to differentiation and proliferation (6,10). It has also been reported that many factors including interleukin (IL), protein kinase C, Wnt, and epigenetic factors regulate telomerase activity (11-14). However, the activator of telomerase during the development of oral cancer is still unknown.

Since Virchow demonstrated that inflammatory cell infiltration occurs in tumor tissue in 1863 (15), it has been recognized that chronic inflammation is a major factor related to carcinogenesis. Gastric cancer due to

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chronic atrophic gastritis resulting from *Helicobacter pylori* infection, hepatocarcinoma due to chronic hepatitis/cirrhosis of the liver resulting from hepatitis B/C virus infection, and bowel carcinoma due to ulcerative colitis are well known forms of carcinogenesis caused by chronic inflammation/infection (16).

Periodontopathic bacteria such as *Porphyromonas gingivalis* and *Tannerella forsythensis* cause chronic inflammatory lesions that result in the destruction of the paradentium. Because of the high incidence of carcinogenesis in non-smokers and non-drinkers who have periodontal disease, periodontitis is thought to be an independent risk factor for oral cancer (17-19). In periodontitis, pathogens produce butyric acid and many inflammatory cytokines, and these factors are assumed to cause cancer. Butyric acid functions as a histone deacetylase (HDAC) inhibitor to maintain the loose structure of chromatin and induce gene expression (20).

In this study using an immunohistochemical method, we examined the expression of human telomerase reverse transcriptase (hTERT) in the dysplasia-carcinoma sequence in the oral cavity. We also investigated the effect of inflammatory cytokines and sodium butyrate treatment on the telomerase activity and migration of oral cancer cell lines.

Materials and Methods

Tissue preparation

Specimens were collected from the archives of the Division of Pathology, Department of Diagnostic and Therapeutic Sciences, Meikai University, covering the period from 2006 through 2013. The samples comprised 46 cases of epithelial dysplasia (including carcinoma *in situ*) and 15 cases of oral squamous cell carcinoma (OSCC). Sections 5 μ m thick from paraffin-embedded tissue blocks were stained with hematoxylin-eosin for histologic diagnosis according to the World Health Organization (WHO) histologic classification (21). In this study, cases of epithelial dysplasia were classified as squamous intraepithelial neoplasia (SIN) 1 (mild dysplasia), SIN 2 (moderate dysplasia), and SIN 3 (severe dysplasia and carcinoma in situ).

The study protocol was reviewed and approved by the Research Ethics Committee of Meikai University Graduate School of Dentistry (A1321).

Immunohistochemistry

Serial sections were deparaffinized and immersed in methanol containing 0.3% (v/v) hydrogen peroxide for 15 min at room temperature to block endogenous peroxidase activity. After washing with running water and

phosphate-buffered saline (PBS, pH 7.4), the sections were immersed in 0.01 M citrate buffer (pH 6.0) and heated in a microwave oven for 15 min at low power for antigen retrieval. Anti-human TERT (hTERT) rabbit polyclonal antibody (1:100 dilution; Millipore, Temecula, CA, USA) was applied to each section overnight at 4°C. The sections were then incubated with peroxidase-labeled dextran polymer (Simple Stain MAX-PO; Nichirei Bio, Tokyo, Japan) for 60 min at room temperature, and the reaction products were visualized by immersing the sections in freshly prepared 2 mM DAB solution (0.05% 3,3'-diaminobenzidine tetrahydrochloride; Nacalai Tesque, Kyoto, Japan) in 0.05 M Tris-HCl (pH 7.6) and 0.01% H₂O₂. Nuclei were lightly stained using Mayer's hematoxylin (Muto Pure Chemicals, Tokyo, Japan). A case was defined as positive when at least one cell showed a positive reaction.

Cells

The human gingival squamous cell carcinoma-derived cell line, Ca9-22, and the human tongue squamous cell carcinoma-derived cell lines HSC-3 and HSC-4 were provided by RIKEN BRC through the National Bio-Resource Project of MEXT, Japan. Each cell line was grown routinely in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 100 U/mL penicillin-streptomycin (Gibco Invitrogen, Carlsbad, CA, USA), 10 U/mL fungizone (Gibco Invitrogen), and 10% fetal bovine serum (Gibco Invitrogen) in a humidified atmosphere of 5% CO₂ at 37°C. Prior to experiments, each cell line was incubated in serum-free medium containing 50 ng/mL tumor necrosis factor (TNF)- α (R&D Systems, Minneapolis, MN, USA), 50 ng/mL interleukin (IL)-1 β (R&D Systems), 50 ng/mL interferon (IFN)- γ (R&D Systems), 2 mM sodium butyrate (NaB; Wako, Osaka, Japan), 10 nM TERT siRNA (Santa Cruz Biotechnology, Dallas, TX, USA), or 10 nM control siRNA (Santa Cruz). To compare the hTERT activity between oral cancer cell lines and normal oral epithelial cells, human gingival epithelium progenitor (HGEP) cells were incubated in CnT-Prime medium (both from CELLnTEC, Bern, Switzerland).

PCR-ELISA

To assess telomerase activity, TeloTAGGG Telomerase PCR ELISA plus was performed in accordance with the manufacturer's instructions (Roche, Mannheim, Germany). A total of 2×10^5 cells were dissolved in lysis reagent, and polymerase chain reaction (PCR) was performed using a biotin-labeled P1-TS primer (to add TTAGGG repeats to the 3'-end) and an anchor-primer

Table 1 Base sequences of the primers

Product name	Sequences (5'→3')	Accession number
E-cadherin	Forward: ggattgcaaattcctgccattc	NM_004360
	Reverse: aacgtgtgccgggtgtca	
Vimentin	Forward: tgagtaccggagacaggtgcag	NM_003380
	Reverse: tagcagcttcaacggcaaagttc	
GAPDH	Forward: gcaccgtcaaggctgagaac	NM_002046
	Reverse: tggagaagacgccagtga	

P2. The PCR products were denatured and hybridized separately with digoxigenin-(DIG)-labeled detection probes specific for the telomeric repeats (P3-T) and for the Internal Standard (IS) (P3-Std), respectively. The products were then immobilized on a streptavidin-coated microplate via the biotin label, and detected using a horse-radish peroxidase-conjugated anti-digoxigenin antibody and the sensitive peroxidase substrate 3,3',5,5'-tetramethylbenzidine. Finally, the absorbance was measured using an autokinetic enzyme scaling meter at a wavelength of 450/690 nm and the relative telomerase activities for IS and the control template were measured.

Cell growth inhibition test (MTT assay)

Each cell line (1×10^4 cells/well) was incubated in serum-free medium containing inflammatory cytokines or NaB in 96-well plates overnight at 37°C. The culture media were discarded after 24 h, and 200 μ L/well thiazolyl blue tetrazolium bromide (Sigma-Aldrich) solution (0.2 mg/mL) was added. After 4 h of incubation at 37°C, 200 μ L/well dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added, and the absorbance was measured at 540 nm using an autokinetic enzyme scaling meter.

Cell migration assay (scratch assay)

Cells were seeded in 12-well tissue culture slides at a density of 1×10^6 cells/well. A scratch was made gently through the central axis of the plate using a pipette tip. The cells were then incubated with serum-free medium containing inflammatory cytokines, NaB, or siRNAs. The migration of cells into the scratch was observed under a microscope and the distance migrated was measured after 8 h using Image J (National Institutes of Health [NIH], Bethesda, MD, USA).

Western blotting

Cellular proteins were extracted using PAREx (Takara Bio, Shiga, Japan). The resulting cell lysates were centrifuged at 12,000 rpm for 5 min at 4°C, and the supernatants were recovered to determine the protein concentration. A total of 10 μ g protein per lane was separated on mini-protein TGX precast mini gels (Bio-

Table 2 Positive number of hTERT in oral epithelial dysplasia and oral cancer tissues

	Positive number	Total number	%
SIN1	0	10	0
SIN2	0	11	0
SIN3	5	15	33.3
OSCC	9	15	60

Rad, Hercules, CA, USA) using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto pure nitrocellulose membranes (Bio-Rad). Specific proteins on the membranes were detected by incubation with specific primary antibodies against β -actin (1:200 dilution; Santa Cruz) or hTERT (1:100 dilution; Millipore) overnight at 4°C, followed by species-specific secondary antibodies conjugated to peroxidase and DAB/H₂O₂ solution. The intensity of the bands was measured using Image J, and a normalized value was calculated as a control.

Real-time RT-PCR

Real-time RT-PCR was performed using a Thermal Cycler Dice Real-Time System (Takara Bio) in accordance with the manufacturer's instructions. A One-Step SYBR PrimeScript RT-PCR Kit II (Takara Bio) was used for the RT-PCR reaction. The primers, based on sequences for E-cadherin, vimentin and GAPDH, are shown in Table 1. Each PCR mixture (final reaction volume, 20 μ L) contained 10 μ L One Step SYBR RT-PCR buffer 4, 0.8 μ L PrimeScript 1-step Enzyme Mix 2, 0.8 μ L forward primer (0.4 μ M), 0.8 μ L reverse primer (0.4 μ M), and 100 ng/mL total RNA. The PCR conditions were 95°C for 10 s, followed by 45 cycles of 95°C for 5 s, and 60°C for 30 s. Dissociation was performed using a melting program.

Statistical analysis

Statistical significance was determined using the Mann-Whitney *U* test.

Results

Localization of hTERT in oral epithelial dysplasia and oral cancer tissues

To identify the timing and localization of hTERT expression, immunohistochemistry was used to analyze oral epithelial dysplasia and OSCC tissue with anti-hTERT antibodies. hTERT expression was observed in SIN3 (5/15, 33.3%) and OSCC (9/15, 60.0%), but was not detected in SIN1 and SIN2 (Fig. 1; Table 2). In HSC-3 cells, a few positive reactions for hTERT were recognized

Fig. 1 Localization of hTERT in oral epithelial dysplasia and cancer. To study the localization of hTERT in SIN1 (A-C), SIN2 (D-F), SIN3 (G-I), and oral cancer tissues (J-L), immunohistochemistry was performed using rabbit anti-human TERT antibodies. Positive reactions are indicated using arrows (I, L). Original magnification: $\times 40$ (A, B, D, E, G, H, J, K); original magnification: $\times 100$ (C, F); original magnification: $\times 200$ (I, L).

in the cytoplasm of prickle cells, whereas many positive reactions were recognized in the cytoplasm of prickle cells in OSCC tissues.

Comparison of telomerase activity in normal oral epithelium and OSCC cell lines

The activation of hTERT in HGEP, Ca9-22, HSC-3, and HSC-4 cells was measured using a TeloTAGGG Telomerase PCR ELISA plus. hTERT activity was significantly higher in OSCC cell lines than in HGEP cells ($P < 0.001$; Fig. 2A). In OSCC cell lines, the telomerase activity in HSC-3 and HSC-4 cells was more than 2 times higher than that in Ca9-22 cells.

Effects of inflammatory cytokines and NaB on telomerase activity and cell proliferation in OSCC cell lines

Because the telomerase activator in human oral lesions

remains unknown, hTERT activation was studied after OSCC cell lines had been incubated with $\text{TNF-}\alpha$, $\text{IL-1}\beta$, $\text{IFN-}\gamma$, or NaB. Telomerase activation was increased by $\text{TNF-}\alpha$ and $\text{INF-}\gamma$ in Ca9-22 cells ($P < 0.001$), but was decreased by $\text{IL-1}\beta$ in HSC-4 cells ($P < 0.001$). The treatments had no significant influence on HSC-3 cells (Fig. 2B). MTT assays were performed to investigate the effects of inflammatory cytokines and NaB on cell proliferation using media containing $\text{TNF-}\alpha$, $\text{IL-1}\beta$, $\text{IFN-}\gamma$, or NaB. Cell proliferation was not markedly affected by inflammatory cytokines or NaB (Fig. 2C).

Correlation between hTERT activity and OSCC cell migration

Scratch assays were performed to investigate the effects of inflammatory cytokines and NaB on cell migration using media containing $\text{TNF-}\alpha$, $\text{IL-1}\beta$, $\text{IFN-}\gamma$, or NaB.

Fig. 2 Influence of cytokines and sodium butyrate on telomerase activity and the proliferation of oral cancer cell lines. The telomerase activity in HGEp, Ca9-22, HSC-3, and HSC-4 cell lines (A), and in Ca9-22, HSC-3, and HSC-4 cells treated with inflammatory cytokines or NaB (B). Cell proliferation was assessed using MTT assays after treatment with inflammatory cytokines or NaB (C). Mann-Whitney *U* tests were performed to compare hTERT activity in cancer cell lines to that in HGEp cells (A), and data obtained from cells in normal medium (RPMI-1640) (B, C); *, $P < 0.001$. Each bar represents the mean $\pm$ SD of three independent experiments each using duplicate samples.

Fig. 3 Effects of cytokines and sodium butyrate on the migration of oral cancer cell lines. Scratch assays were performed after incubation of Ca9-22, HSC-3 and HSC-4 cells with inflammatory cytokines or NaB (A). The width of the scratch in each image was measured using Image J, and Mann-Whitney *U* tests were performed to compare data with those obtained from cells in normal medium (RPMI-1640) (B); *, $P < 0.05$; **, $P < 0.01$. Each bar represents the mean $\pm$ SD of three independent experiments.

Cell migration was increased by IL-1 β (in HSC-4 cells; $P < 0.05$), IFN- γ (in HSC-4 cells; $P < 0.05$), and NaB (in HSC-3 and HSC-4 cells; $P < 0.05$ and $P < 0.01$, respectively) (Fig. 3).

To assess the correlation between hTERT activation and cell migration, OSCC cell lines were incubated with siRNA for hTERT, and scratch assays were performed. Cell migration was increased by hTERT siRNA in HSC-3 cells ($P < 0.05$) (Fig. 4A, B). In contrast, hTERT siRNA had no effect on Ca9-22 cells (data not shown). The downregulation of hTERT expression and activation by siRNA was confirmed using Western blotting (Fig. 4C) and TeloTAGGG Telomerase PCR ELISA plus (Fig. 4D).

Effects of inflammatory cytokines on OSCC cell invasion

To assess why cytokines increased telomerase activity

in Ca9-22 cells, real-time RT-PCR was performed using specific primers for E-cadherin and vimentin. The results revealed that vimentin expression tended to be increased by TNF- α and INF- γ in comparison with E-cadherin expression, whereas vimentin expression was decreased by TNF- α and INF- γ in comparison with E-cadherin expression in HSC-4 cells (Fig. 5).

Discussion

Epidemiological studies have shown that tobacco, alcohol, viruses, and chronic inflammation are risk factors for carcinogenesis (22-24). A recent report has suggested that the telomere extension enzyme, telomerase, promotes the expression of inflammatory cytokines such as TNF- α and IL-6 by activating NF- κ B to induce chronic inflammation (25). Telomerase is a large enzyme complex consisting of a complementary RNA sequence,

Fig. 4 Effects of hTERT siRNA on cell migration and hTERT expression and activation. After incubating HSC-3 and HSC-4 cells with hTERT or control siRNA for 24 h, cell migration was investigated using scratch assays (A). The width of the scratch in each image was measured using Image J (B). hTERT expression was assessed using Western blotting (C), and hTERT activation was examined (D). Mann-Whitney *U* tests were performed to compare data with those obtained from cells in normal medium (RPMI-1640); *, *P* < 0.05. Each bar represents the mean ± SD of three independent experiments.

Fig. 5 Effects of cytokines on the epithelial-mesenchymal transition in OSCC cells. Expression of E-cadherin and vimentin was assessed using real-time RT-PCR after Ca9-22, HSC-3, and HSC-4 cells had been incubated with TNF-α and IFN-γ. Mann-Whitney *U* tests were performed to compare data with those obtained at 0 h; *, *P* < 0.05; **, *P* < 0.001. Each bar represents the mean ± SD of three independent experiments.

a telomerase RNA/telomerase RNA component, TERT, and several proteins (4). Telomerase is always activated in germ cell lines and maintains the telomere at a certain length. However, in somatic cell lines, telomerase is

inactivated and the telomere and chromatin are cleaved at every cell division. Telomerase is re-activated during carcinogenesis, resulting in cell immortalization (26-28). In bowel cancer, telomerase is activated gradually in parallel with the progression of the adenoma-carcinoma sequence (29). Similarly, the present findings suggest that telomerase plays a role in carcinogenesis and the malignant transformation of oral lesions; however, it is not related to the development of epithelial dysplasia.

TNF-α, IL-1β, and IFN-γ were selected as typical inflammatory cytokines. TNF-α and IL-1β are important cytokines during the early stages of inflammation. They are produced by macrophages and promote the expression of chemokines and cell adhesion molecules (30). High concentrations and/or the long-term presence of TNF-α induce cachexia and sapraemia (31,32). IL-1β promotes the expression of additional ILs, including IL-2, IL-3, and IL-6 (33). IFN-γ is one of the main activators of macrophages and it has anti-viral, anti-tumor, and cell differentiation-inducing functions (34). The present study revealed that the effects of cytokines on telomerase activity differed between OSCC cell lines. It is thought

that each cell line may have a different kind of receptor for cytokines, and that therefore confirmation of the presence of receptors on these cell lines is necessary.

The present study demonstrated that TNF- α and IFN- γ induced telomerase activation in Ca9-22 cells, and tended to promote vimentin expression. However, the use of hTERT siRNA inhibited vimentin expression. This phenomenon, whereby epithelial cells acquire mesenchymal features, was proposed by Hay in 1995 (35), and is now called the epithelial-mesenchymal transition (EMT). One indication of the EMT is downregulation of E-cadherin (an epithelial molecule) or upregulation of vimentin (a mesenchymal molecule). It has been reported that telomerase activation promotes the EMT in bowel cancer or bone sarcoma cell lines (36,37). These observations suggest that inflammatory cytokines induce EMT in certain oral cancer cells, such as Ca9-22, via the same mechanism that occurs in bowel cancer and/or bone sarcoma cells.

Telomerase activity was detected in normal oral epithelial cells (HGEP cells) in spite of its negative activity in normal somatic cells. This could be because these normal cells have similar properties to basal and/or stem cells because they are gingival progenitor cells. The present study revealed that telomerase activity was higher in oral cancer cell lines relative to normal oral epithelial cells, and that the degree of activity in cancer cells differed considerably according to tissue origin. With 2-3-fold higher telomerase activity, the degree of malignancy may be higher in tongue cancer than in gingival cancer. It will be necessary to examine the relationship between telomerase activity and malignancy in cancers derived from various oral regions.

NaB functions as an HDAC inhibitor *in vivo* and regulates chromatin structure and gene expression (20). Research has shown that telomerase activity is regulated by epigenetic factors such as methylation at promoter regions and chromatin acetylation (38-40), and that HDAC inhibitors suppress hTERT activation in prostate cancer and brain cancer (41,42). This suggests that NaB regulates hTERT activation. The present study demonstrated that NaB tended to promote hTERT slightly in Ca9-22 and HSC-3 cells, but inhibited activation slightly in HSC-4 cells. The mechanisms that regulate telomerase activity in HSC-4 cells resemble those in prostate cancer or brain cancer, although the exact details are unclear. As such, additional studies are needed to reveal the regulatory mechanism operating in these cell lines or tissues.

As immortalization via telomerase activation is an important process in cancer cells, inhibitors of telomerase activity have received attention as novel anti-cancer agents. To

date, many such molecules have been identified, including 3'-azido-3'-deoxythymidine (AZT), which inhibits human immunodeficiency virus (HIV)-1 by interfering with DNA synthesis (43), PIPER (*N,N'*-bis[2-(1-piperidino)ethyl]-3,4,9,10-perylenetetra-carboxylic diimide), which binds to G-quadruplexes (44), and epigallocatechin-3-gallate (EGCG), which is derived from polyphenols in green tea (45). Of these, EGCG has been reported to inhibit the invasion and proliferation of oral cancer cells (46,47). In addition, there is growing support for the use of NaB as an anti-cancer agent. However, as shown in the present study, NaB tends to promote the migration of certain types of oral cancer cells by inhibiting telomerase expression or activation. Therefore, care must be taken when considering its use.

By analogy with the adenoma-carcinoma sequence in bowel cancer (29), it has been suggested that carcinogenesis in the oral cavity results from a dysplasia-carcinoma sequence whereby epithelial dysplasia arises from normal mucosa (48). Our present findings suggest that chronic inflammation, progressive epithelial dysplasia and long-term exposure to inflammatory cytokines lead to telomerase expression. This in turn leads to malignant transformation and regulates the invasion of certain types of oral cancer cells.

Conflicts of interest

The authors have no potential conflicts of interest to declare with respect to the authorship and/or publication of this article.

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