Telomerase Activity in Premalignant and Malignant Lesions of Human Oral Mucosa

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Abstract
Recent studies have demonstrated a strong association between carcinogenesis and re-activation of telomerase in various human tumors. In the present study, we have analyzed the telomerase activity in 105 oral mucosal samples, including normal mucosa and premalignant and malignant lesions, by using the telomeric repeat amplification protocol assay. The telomerase activity was detected in normal oral squamous epithelium and in 75% of the oral leukoplakias and oral carcinomas. Although the telomerase activity was observed in normal and hyperplastic squamous epithelium, it showed some relationship with certain clino-pathological factors in malignant lesions. Telomerase activity was found to have a relationship with the grade of tumor differentiation. Of 34 well-differentiated squamous cell carcinomas, only 10 (30%) exhibited high telomerase activity, whereas in moderately or poorly differentiated squamous cell carcinomas, all seven (100%) tumors displayed high activity. In addition, the level of telomerase activity had an inverse correlation with the treatment response in the early-stage tumors, and the activity differed significantly between the tumors in the following intraoral sites: nonkeratinizing mucosa (buccal mucosa, alveolus, and floor of mouth) and tongue. This preliminary result shows that telomerase activity is present in normal oral mucosal epithelium, as it is in normal hematopoietic cells and in carcinomas, and that telomerase activity has a relationship with degree of tumor differentiation and treatment response. Thus, assessing the telomerase activity may be a useful prognostic marker in oral squamous cell carcinomas.

Introduction
Cancer of the oral cavity is the most common neoplasm in India and in most southeast Asian countries (1). The high incidence of this disease has been attributed to the prevalence of tobacco chewing among the populations of these countries. Cancer data from India show that approximately 30% of all cancers occur in oropharyngeal regions and account for about 56,000 deaths annually (2). Recently, the incidence of oral cancer has increased in Western countries, also due to the wide use of smokeless tobacco (3). Oral cancer has a well-defined precancerous lesion, oral leukoplakia, which serves as a good model to study the processes of oral carcinogenesis (4). However, unlike the other common cancers, the molecular mechanism of oral carcinogenesis is not well understood. Only a few fragmentary reports are available regarding the molecular carcinogenic pathway of oral cancer (5–7). These studies showed a difference in genetic aberrations between the oral cancers from Western and southeast Asian countries, which may be due to the differences in the etiology. In Indian oral cancers, a higher incidence of ras mutations and a lower incidence of p53 mutations were reported, compared with Western cases (5).

In the process of transformation of a cell from normal state to malignant, one of the key steps is the immortalization of cells. Recent evidence indicates that telomerase plays an important role in cell immortalization (8, 9). Telomerase is a RNA-dependent DNA polymerase that synthesizes telomeric DNA fragments de novo, using its RNA moiety as a template, and compensates for the loss of telomere during the cell division (10–12). The current hypothesis is that, during immortalization of a somatic cell, the telomere length will be maintained by the reexpression of telomerase (13). Recently, a simple and highly sensitive PCR-based “single tube” method, called the TRAP assay, was developed for telomerase detection (14, 15). Thereafter, a number of studies were reported regarding the telomerase biology of normal, immortalized, and malignant cells. These studies have shown that somatic cells in general are devoid of telomerase, whereas germ-line, immortalized, and malignant cells express telomerase in varying degrees (8, 13, 14). Telomerase activity was found in the majority of human primary tumors, by us and others. It was detected in 85% of gastric cancers and 95% of colorectal cancers (16), 85% of liver cancers (17), 94% of neuroblastomas (18), 80% of lung cancers (19), 84% of prostate cancers (20), and 93% of breast cancers (21). Telomerase activity was not found in the corresponding normal tissues; however, its expression was observed in some preneoplastic lesions of gastric, colorectal, and liver cancers (16, 17).

In addition, the telomerase activity was well correlated with certain clinicopathological factors and proposed some

The abbreviations used are: TRAP, telomeric repeat amplification protocol; WDSCC, well-differentiated squamous cell carcinoma; HPV, human papilloma virus.

1 Received 10/2/96; accepted 2/24/97.
2 The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. The present work was done as part of the Research Training Fellowship Programme of Dr. S. Kannan awarded by the IARC, at the First Department of Pathology, Hiroshima University School of Medicine, Hiroshima, Japan. The present study was also supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare, Japan, for a comprehensive 10-year strategy for cancer control.
prognostic significance. Although the incidence of squamous cell carcinomas of head and neck is high, telomerase activity in these tumors has been reported by only two groups (14, 22). A recent study has demonstrated telomerase activity in 88% of head and neck squamous cell carcinomas and 39% of oral leukoplakia samples examined (22). In the present study, we have examined the telomerase activity in normal oral mucosa, oral leukoplakia, and oral squamous cell carcinoma tissues obtained from Indian patients by using TRAP assay. We have also studied the relation between the telomerase activity and clinico-pathological factors of the malignant lesions.

Materials and Methods

Cell Lines. Six cell lines established from the human oral squamous cell carcinoma, namely, HO-1-u-1 (floor of the mouth), HO-1-N-1 (buccal mucosa), Ca9-22 (gingiva), HSC-2 (buccal mucosa), HSC-3 (tongue), and HSC-4 (tongue), were examined for telomerase activity. All of these cell lines were obtained from Japanese Cancer Research Resources Bank, Tokyo, Japan, and were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate at 37°C with 5% CO2. Confluent cultures were trypsinized and washed with sterile PBS, and the cell pellets were snap frozen in liquid nitrogen and stored at −80°C until use. Before pelleting, small aliquots of culture suspension were taken from each dish, and cell numbers were counted.

Tissue Sample Collection. A total of 105 oral mucosal samples, including 52 incision biopsies of oral cancer, 5 wide excised (surgical cases) oral squamous cell carcinoma tissues, 36 punch biopsies of oral leukoplakia, and 2 normal oral mucosa, were examined for the telomerase activity. All of these tissue samples were collected and recorded pro forma.

TRAP Assay. TRAP assay in cell lines and tissues was done essentially the same way as we have described previously (16). Briefly, cell pellets were suspended in 400 μl of TRAP lysis buffer and incubated on ice for 30 min. Similarly, small bits of tissues were homogenized with TRAP lysis buffer (50–250 μl, depending upon the size of the tissue bit) in Kontes tubes with matching pestles rotated with a Micromix at 450 rpm. After incubation on the ice, both the extracts were centrifuged at 16,000 × g for 20 min at 4°C. The supernatants (cell extracts) were aliquoted and immediately stored at −80°C. Protein concentrations of tissue extracts were determined using Coomassie brilliant blue assay. TRAP analyses were done in three concentrations of tissue extract, 6 μg (1 X), 0.6 μg (10X), and 0.06 μg (100X) protein/assay, in all of the samples except cell lines. In cell lines, extracts equivalent to 1 × 10^6 cells were used for TRAP assay. The TRAP assay was done according to the method described earlier (15, 16), and it consisted of two steps. In the first step, the telomerase in the extract was allowed to synthesize telomeric oligonucleotides on TS primer (5′-AACCTGCGAGCGAGGTT-3′). In the second step, the telomerase-synthesized new oligonucleotides were amplified using PCR by including reverse CX primer (5′-CCTTACCTTACCTAAGGTT-3′) in the presence of [32P]dCTP. Then, the PCR-amplified products were resolved on 12% nondenaturing polyacrylamide gel, and the reaction products were finally visualized by autoradiography. All of the gels were autoradiographed for short (14 h) and long (48 h) exposure times. The telomerase activity was graded as strong (dark and prominent ladders in both 14- and 48-h exposure autoradiograms), moderate (weak ladder in 14-h exposure and dark ladder in 48-h exposure), weak (detectable only in 48-h exposure), and negative (not detectable in either 14- or 48-h exposure). We have also confirmed the specificity of the telomerase activity by RNase treatment of the tissue extract prior to TRAP assay and have run a negative control (instead of tissue extract, lysis buffer alone was added) with all sets of TRAP assay to avoid false positivity.

We have analyzed the relationship between the level of telomerase activity and clinico-pathological factors such as histopathology, stage of disease, treatment response, and introral site of the lesions in malignant cases. For this, samples with strong and moderate activity of telomerase were taken together as high-activity group, and samples with negative or weak telomerase activity were taken as a negative group because we observed a weak telomerase activity in samples from normal oral mucosa. χ² test was applied to validate the significance of the difference between groups.

Results

Telomerase Activity in Oral Cancer Biopsies and Cell Lines. Telomerase activity in oral cancer tissues is summarized in Table 1. Of 52 oral cancers, 39 (75%) exhibited telomerase activity, whereas the remaining 13 (25%) tumors were negative. Strong telomerase activity was seen in 8 (15%), moderate activity was seen in 14 (27%), and weak activity was seen in 17 (33%) cases. All of the positive tumors were sensitive to RNase treatment (Fig. 1). Initially, 16 samples were negative for TRAP assay with 6-μg tissue extracts. But on

<table>
<thead>
<tr>
<th>Histopathology</th>
<th>No. of cases</th>
<th>Level of telomerase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Strong (3%)</td>
</tr>
<tr>
<td>WDSCC</td>
<td>34</td>
<td>Moderate (27%)</td>
</tr>
<tr>
<td>Moderately differentiated squamous cell carcinoma</td>
<td>6</td>
<td>Weak (38%)</td>
</tr>
<tr>
<td>Poorly differentiated squamous cell carcinoma</td>
<td>1</td>
<td>Negative (32%)</td>
</tr>
<tr>
<td>Verrucous carcinoma</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Atypical squamous epithelium</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>52</td>
<td></td>
</tr>
</tbody>
</table>

Table 1 Relationship between telomerase activity and histopathology in oral cancers
Telomerase activity in various histological types of oral cancers. Numbers, case numbers. Case 2, verrucous carcinoma; case 3, moderately differentiated squamous cell carcinoma; case 47, poorly differentiated squamous cell carcinoma. All other cases were WDSCCs. Tissue extracts without (−) or with (+) RNase treatment were assayed for telomerase activity.

Telomerase activity in oral cancer tissue extracts with different protein concentrations. Numbers, case numbers.

dilution analysis, of 16 negative cases, 3 expressed weak telomerase activity with 0.6 μg of tissue extract. However, we have not found much processivity or ladder extension in diluted oral cancer tissue extracts (Fig. 2), as we observed in colorectal and gastric cancers (16).

Histopathological analysis of the tumor samples showed that majority of them (34 samples, 65%) were WDSCCs, 6 were moderately differentiated squamous cell carcinomas, and 1 was poorly differentiated squamous cell carcinomas. Of the remaining tumors, seven were verrucous carcinomas, and four specimens had no frank malignant cells, only atypical squamous epithelium. The grade of differentiation of tumors exhibited a relation with the level of telomerase activity. Of 34 cases of WDSCCs, 11 (33%) were negative and 13 (38%) had only weak telomerase activity. However, all moderately and poorly differentiated squamous cell carcinomas displayed high telomerase activity. Verrucous carcinomas showed a pattern of telomerase activity similar to that of WDSCCs (Table 1). The statistical analysis also showed a significant difference between telomerase activity and grade of differentiation in oral squamous cell carcinomas ($\chi^2 = 9.19; P < 0.005$).

Lesions were classified as either early (including stage I and II cancers) and late (including stage III and IV cancers), on the basis of their composite clinical stage of disease, as described by the Union Internationale contre le Cancer (23). Of 52 cases, 34 (65%) were late-stage tumors and 13 were early-stage tumors. With respect to telomerase positivity, no significant difference was observed between the early and late stage of the disease ($\chi^2 = 0.00; P > 0.9$). All these cases were treated with radiotherapy, except for three early-stage verrucous carcinomas, which were surgically excised. We have grouped the patients into three categories: good (no clinical evidence of disease after treatment), bad (partial or no response to treatment and persistence of disease clinically), and no follow-up, on the basis of the responsiveness of the tumor to treatment, judged in the first follow-up visit (1–2 months) after the completion of the therapy. On assessing the responsiveness of tumors to radiotherapy, it was found that 26 cases (50%) had only poor response and 11 cases (21%) had responded well to the treatment. No follow-up details were available in the remaining 15 cases. By correlating the telomerase activity with treatment response of tumors, it was found that 50% (13 of 26) of the cases with poor response to radiotherapy had a high expression of telomerase. In the group that responded well, only 28% (3 of 11) showed high levels of the telomerase activity (Table 2), but it was not statistically significant ($\chi^2 = 0.83; P > 0.3$). Regarding the total telomerase positivity (strong, moderate, and weak), no significant difference was found between good (73%) and bad (81%) groups for treatment response.

Dissection of the relationship between the stage of the disease and treatment response revealed that 86% (19 of 22) of the late-stage cases responded poorly, whereas 55% (6 of 11) of the early-stage cases showed good response to the treatment (Table 2). This difference in the treatment response was also statistically significant ($\chi^2 = 6.19; P < 0.025$). With regard to the telomerase activity, we found that the majority of the early-stage cases (5 of 6) revealed weak or negative activity. Of seven early cases with weak or negative telomerase activity, five responded well to the treatment, whereas of four early lesions with moderate or strong telomerase activity, only one responded well to the treatment (Table 2). However, this difference was statistically not a valid one ($\chi^2 = 2.21; P > 0.05$). It is well known that intraoral localization of the cancer has a significant effect on the response of radiotherapy or patient prognosis. To examine whether any such relation existed with respect to the telomerase activity, the tumors were classified into two groups, tongue cancer and buccal cancer (inclusive of tumors located in the nonkeratinizing oral epithelium, such as buccal mucosa, alveolus, and others). Surprisingly, the majority (13 of 16, 81%) of the tongue cancers exhibited weak or negative telomerase activity, irrespective of stage. Conversely, in buccal tumors, the majority (27 of 31) of tumors expressed various levels of telomerase activity ($\chi^2 = 4.24; P < 0.05$). We have also found some conspicuous differences in treatment response between these two groups. Although most of the tongue cancers were negative or weak in telomerase activity, one negative case has responded well to the treatment. However, 28% (10 of 36) of buccal cancers responded well to the treatment, of which 7 (70%) had only negative or weak telomerase activity, but 71% (10 of 14) of the poorly responded buccal tumors exhibited high telomerase activity. This difference in the telomerase activity and treatment response in buccal cancers was also statistically significant ($\chi^2 = 4.03; P < 0.05$). By analyzing all of the variables, such as stage of the disease, treatment response, and intraoral site of the lesion, together with telomerase activity, we demonstrated that, in early-stage buccal cancers, all (four of four) having negative or weak telomerase activity responded well to the treatment, whereas two of the three cases with high activity of telomerase...
responded poorly to the treatment (Table 3). Statistical analysis also showed a significant \( \chi^2 = 3.73; P = 0.05 \) relationship between the level of telomerase activity and the treatment response in early buccal cancers. In the late stage, irrespective of the level of telomerase activity, the treatment responses were poor in majority of the lesions (88% in high telomerase activity cases and 67% in weak or negative cases). We have also examined whether this difference in treatment response between buccal and tongue cancers has any relationship with the histological differentiation in tumors. But we could not find any significant difference, and the majority of them were the well-differentiated type in both the sites.

Because we found a conspicuous difference in telomerase activity between tongue and buccal cancers, we further searched for telomerase activity in cell lines established from squamous cell carcinoma of the various sites of oral cavity, as detailed in “Materials and Methods.” But we could not find any difference in telomerase activity in any of the cell lines, because all of them displayed identically a very high activity (Fig. 3). However, these cell lines were not established from the oral cancers of Indian origin.

**Telomerase Activity in Oral Leukoplakias.** Of 36 oral leukoplakias examined, 27 (75%) showed telomerase activity. Among them, 63% (17 of 27) expressed only weak telomerase activity. All of the positive cases were sensitive to RNase treatment, and negative cases did not exhibit any telomerase activity, even in diluted extracts (Fig. 4). Histopathological examination of these leukoplakia revealed that none of the lesions contained any notable dysplastic features. With regard to the relationship between telomerase activity and the clinical type of the leukoplakia, only 3 of 20 (15%) showed high telomerase activity in homogenous group, whereas in the non-

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**Table 2** Relationship between telomerase activity, stage of disease, and treatment response in oral cancers

<table>
<thead>
<tr>
<th>Telomerase activity</th>
<th>Early stage</th>
<th>Late stage</th>
<th>Total</th>
<th>No follow-up</th>
<th>Early stage</th>
<th>Late stage</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Good</td>
<td>Bad</td>
<td>Total</td>
<td>Good</td>
<td>Bad</td>
<td>Total</td>
<td>Early stage</td>
</tr>
<tr>
<td>Strong</td>
<td>1</td>
<td>1</td>
<td>2 (18%)</td>
<td>0</td>
<td>4</td>
<td>4 (18%)</td>
<td>1</td>
</tr>
<tr>
<td>Moderate</td>
<td>0</td>
<td>2</td>
<td>2 (18%)</td>
<td>1</td>
<td>5</td>
<td>6 (28%)</td>
<td>0</td>
</tr>
<tr>
<td>Weak</td>
<td>2</td>
<td>1</td>
<td>3 (27%)</td>
<td>2</td>
<td>6</td>
<td>8 (36%)</td>
<td>1</td>
</tr>
<tr>
<td>Negative</td>
<td>3</td>
<td>1</td>
<td>4 (37%)</td>
<td>0</td>
<td>4</td>
<td>4 (18%)</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>6 (55%)</td>
<td>5 (45%)</td>
<td>11</td>
<td>3 (14%)</td>
<td>19 (86%)</td>
<td>22</td>
<td>2 (14%)</td>
</tr>
</tbody>
</table>

*The disease stages of five cases were not available. The treatment responses in these cases were: two bad (telomerase activity was strong in one case and weak in the other case), two good (telomerase activity was moderate in one case and weak in other case), and one no follow-up (telomerase activity was negative).*

**Table 3** Relationship between telomerase activity and treatment response, stage of disease, and intraoral site of the cancers

<table>
<thead>
<tr>
<th>Telomerase</th>
<th>Treatment response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tongue cancer</td>
</tr>
<tr>
<td></td>
<td>Early stage</td>
</tr>
<tr>
<td></td>
<td>Good</td>
</tr>
<tr>
<td>Strong</td>
<td>0</td>
</tr>
<tr>
<td>Moderate</td>
<td>0</td>
</tr>
<tr>
<td>Weak</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>1 (6%)</td>
</tr>
</tbody>
</table>

*FU, 5-fluorouracil.

*The disease stages of five buccal cancers were not available. The treatment response in these cases were: two bad (telomerase activity was strong in one case and weak in other case), two good (telomerase activity was moderate in one case and weak in other case), and one no follow-up (telomerase activity was negative).*

**Fig. 3.** Telomerase activity in cell lines established from the oral squamous cell carcinomas. Details are given in “Materials and Methods.” Cell extracts without (-) or with (+) RNase treatment were assayed for telomerase activity.

**Fig. 4.** Homogenous group. 7 of 16 (44%) showed strong or moderate telomerase activity (\( \chi^2 = 3.66; P < 0.06; \) Table 4). **Telomerase Activity in Normal Oral Mucosa.** As we examined telomerase activity in oral leukoplakia, we also examined telomerase activity in normal oral mucosa adjacent to the ma-
malignant lesions. For this, we collected samples from tumor area, the adjacent mucosa, and the distal uninvolved mucosa of the same patient from five surgically excised tissues. Surprisingly, telomerase activity was detected in adjacent and distal mucosa, as well as in cancers of all samples except one, which was negative in all areas, even in diluted extracts (Fig. 5). The histopathological examination of these tissues did not reveal any malignant cells in adjacent or distal mucosae. However, the distal mucosa of one case (patient 36) had dysplastic changes showing strong telomerase activity. Because adjacent normal mucosa exhibited telomerase activity, we also examined the telomerase activity in two normal mucosal samples obtained during nononcological maxillofacial reconstructive surgery. Weak telomerase activity was found in both of the normal mucosae, which were also sensitive to RNase treatment (Fig. 4A). On dilution analysis, one of them showed more activity in the 10-fold diluted tissue extract (Fig. 4B).

In addition to that observation, another interesting observation was found in one case of the carcinoma of tongue. A WDSCC located in the right side of the tongue was telomerase negative, and the second biopsy from the left side of the tongue, which was a homogenous leukoplakia, exhibited high telomerase activity. On dilution of the tumor extract, a very weak telomerase activity was found in the 10-fold diluted sample (Fig. 6).

**Discussion**

In the present study, we made a distinctive observation that differs from other available reports on telomerase activity in various solid tumors. Normal oral mucosa, hyperplastic epithelium, and oral leukoplasias showed a weak telomerase activity. One important reason for this difference may be that, with the exception of a few reports (14, 22, 23), all telomerase studies thus far performed have focused on telomerase biology in human cancers that originated in nonsquamous epithelium. Squamous epithelium is unique in its stratification and complex differentiation pattern, and patients with squamous epithelium carcinomas also greatly vary in their treatment response (3, 4). Another peculiar feature of squamous epithelium, especially of the oral mucosa, is that a patient with oral cancer has a 100-fold probability of developing a second primary tumor in oral epithelium: the “field cancerization” concept (24).

Unlike other human tissues, the squamous epithelium has a very high cell turnover rate, and the constant loss of superficial cells (desquamation) is replaced by continuous division in the basal cells (25, 26). Hence, comparatively more stem cells are likely to be present in the squamous epithelium for the active self-renewal process (25). As in the case of immortalized cells, the stem cells of human hematopoietic tissue have been shown to possess telomerase activity (27, 28). Cellular kinetic studies have also demonstrated a similarity in cell proliferation pattern between the stem cells of squamous epithelium and hematopoietic tissues (25). Thus, the telomerase activity in normal oral mucosa may be due to the presence of stem cells. Cell kinetic studies have shown that proliferation rate is comparatively higher in the hyperplastic epithelia of the oral leukoplakia and carcinoma (29, 30). Thus, these lesions may have more stem cells, and the telomerase activity may also be higher. The degree of telomerase activity in oral squamous cell carcinoma was related to the histological differentiation. It has been reported that WDSCCs are slow-growing, compared with moderately and poorly differentiated squamous cell carcinomas, which are also more aggressive in nature (29, 31). It is likely that less differentiated oral squamous cell carcinomas might contain more immortal cells than the former histological type; hence, telomerase activity would also be high. Studies on mouse squamous cell carcinoma have found a similar pattern of

**Table 4** Relationship between telomerase activity and clinical type in oral leukoplakia

<table>
<thead>
<tr>
<th>Clinical type of leukoplakia</th>
<th>Telomerase activity</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Strong</td>
</tr>
<tr>
<td>Homogenous</td>
<td>0</td>
</tr>
<tr>
<td>Speckled</td>
<td>1 (17%)</td>
</tr>
<tr>
<td>Erosive</td>
<td>1 (13%)</td>
</tr>
<tr>
<td>Verrucous</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>2 (5%)</td>
</tr>
</tbody>
</table>

Fig. 4. Telomerase activity in oral leukoplakia tissues and in normal oral mucosa. Numbers: case numbers. Cases 57–62: leukoplasias; cases 90 and 91: normal oral mucosa. A, RNase sensitivity analysis in the tissue extracts. –, without RNase treatment; +, with RNase treatment. B, dilution analysis of tissue extracts. Lane C, negative control.
Telomerase activity in relation to the grade of tumor differentiation (32).

An earlier study on the squamous cell carcinomas of the head and neck have demonstrated telomerase activity in 88% of the cancer tissues and in 38% (6 of 16) of the adjacent mucosa of these tumors (14). Recently, Taylor et al. (33) have demonstrated the telomerase activity in sun-protected and exposed normal human skin and precancerous and cancerous skin lesions. Studies on mouse skin tumors also showed a high telomerase activity in premalignant papillomas and demonstrated the correlation between tumor progression and telomerase activity (34). All these results thus indicate that telomerase activity is present in normal squamous epithelium, as observed in normal hematopoietic progenitor cells, and its activity shows a correlation with tumor progression in some instances. Also, the presence of high telomerase activity in adjacent or distal normal mucosa of the malignant lesions may be because of “condemned mucosa,” which are due to the field cancerization concept, and sometimes the activity may be related to the incidence of second primary tumors or recurrences. Further study is needed to establish this relationship. However, a recent study on oral lesions only showed telomerase activity in 1 (5%) of the 18 adjacent normal mucosa samples examined (22), but activity was detected in 88% of carcinomas and 39% of leukoplakia biopsies. In leukoplakia, a correlation between telomerase activity and grade of dysplasia was found. It is difficult to explain the difference in telomerase activity in normal adjacent oral mucosa between that study and the present study, but the variation may be due to racial differences.

With our present results, we have failed to detect telomerase activity in 25% of the both leukoplakias and malignant lesions. The reason for this cannot be explained at present. However, one of the reasons that may be considered is the inactivation of telomerase enzyme due to improper tissue collection and storage or the presence of Taq polymerase inhibitors in tissue. We took the utmost care during the collection of tissues, and all of the samples were collected in a uniform manner. Also, we have analyzed tissue extracts in 10- and 100-fold dilutions to nullify the effect of tissue inhibitors. Hence, inactivation or the presence of tissue inhibitors could not be a reason for telomerase negativity. Moreover, the presence of strong telomerase activity in leukoplakia and its absence in malignant tissue of the same patient (samples were collected in an identical manner) also do not support this reason. Hence, some biological differences may exist for this reason, or there may have more than one pathway in oral carcinogenesis, a telomerase-involved and an uninvolved mechanism. Further detailed analyses are required to elucidate the reason for this difference. A recent study on cellular immortality in human oral squamous cell carcinomas showed that cultures established from some of the late-stage carcinomas were senescent ones (35). Because telomerase is closely associated with immortality, the low or absent telomerase activity in some of the tumor samples might account for many mortal cancer cells. A similar observation was made in lung cancers (19).

Because telomerase activity was reported in normal hematopoietic cells, we also looked for any differences in inflammatory cell infiltration in these telomerase-negative and positive lesions with routine histopathological examination. We could not find any significant differences between these groups with regard to inflammatory cell infiltration, because the telomerase-negative lesions also have marked inflammatory cell infiltration, as do telomerase-positive lesions (data not shown). In vitro studies have demonstrated a pivotal role for viral proteins in the activation of telomerase and immortalization of various cell types (8, 13). Recently, E6 proteins of HPV

Fig. 5. Telomerase activity in oral cancer (T), adjacent normal mucosa (A), and distal normal mucosa (D) of the same patients. Numbers, case numbers.

Fig. 6. Differences in telomerase activity between WDSCC on the right side of the tongue (left panel) and oral leukoplakia on the left side of the tongue (right panel) of the same patient. Lanes a, 0.10 μg of tissue extract; Lanes b, 0.6 μg of tissue extract with RNase treatment; Lanes c, 0.6 μg of tissue extract without RNase treatment; Lanes d, 0.06 μg of tissue extract; Lanes e, 0.06 μg of tissue extract.
type 16 were shown to activate telomerase in early passage human keratinocytes and mammary epithelial cells of precrisis state (36). As in cervical neoplasia, a role for HPV has also been implicated in oral carcinogenesis, due to the more frequent occurrence of HPV infection in oral precancerous and cancerous tissues relative to normal oral tissues in some studies (6). A recent study on the HPV prevalence in oral premalignant and malignant tissues from the patients of same demographic areas of the present study showed a high prevalence, 74% HPV infection (37). Thus, there is a possibility that the HPV infection status could bring about the telomerase activity of the present samples. However, detailed studies are required to attribute an *in vivo* role for HPV proteins in the activation of telomerase.

Studies on telomerase activity in cancers such as gastric, liver, breast, and prostate indicated the usefulness of telomerase activity as a prognostic marker and also proposed that the presence of telomerase activity could be used as an early detection marker to distinguish malignant and benign tissues (16, 17, 20, 21). Although telomerase activity was observed in normal and hyperplastic oral mucosa, the telomerase activity in malignant lesions showed some significant associations with certain clinico-pathological factors. The present results showed a significant relationship between the level of telomerase activity and treatment response in oral squamous cell carcinomas, especially in early lesions. However, the present results need to be reconfirmed in a study with larger sample size because the sample size is limited in the present study, and the results might therefore have occurred by chance. We also demonstrated a marked difference in telomerase activity between tongue and buccal cancers. One of the reasons for the poor response of radiotherapy in the majority of tongue cancers may be the superior blood and lymphatic supply, and the response may not be related to telomerase status. These variations also indicate a possible difference in carcinogenic and telomerase-involved pathways in these tissues. The consistent high telomerase activity in oral cancer cell lines obtained from different intraoral sites might be due to the selection of cells in cell lines with strong telomerase activity, due to their growth advantage under *in vitro* conditions. We have also performed an analysis to see whether any relationship was existed between personal details, including sex, age, and oral habits like smoking and betel quid chewing, and the telomerase activity in their tumor tissues. We could not find any significant relationship between these factors and telomerase activity (*data not shown*).

In conclusion, the results of the present study showed that telomerase activity was present in normal oral squamous epithelium and maintained its expression in premalignant and malignant lesions of the oral mucosa in varying degrees. In oral malignant lesions, the degree of telomerase activity varied, depending upon the grade of tumor differentiation. The response of radiotherapy also correlated well with the level of telomerase activity, especially in the early stages of disease that develops in nonkeratinizing sites of oral mucosa. Thus, telomerase activity can be an additional tool as a biomarker for assessing the prognosis or biological grade of the oral carcinomas. Because telomerase activity is present in normal and hyperplastic mucosa, it cannot be used as a marker for the early detection of oral cancer, as proposed in other solid tumors. However, further study on large number of samples is necessary to confirm the prognostic significance of telomerase activity in oral squamous cell carcinomas, including the rigorous follow-up of the patients.

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*Cancer Epidemiol Biomarkers Prev* 1997;6:413-420.

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