Telomerase Activity in Oral Squamous Cell Carcinoma

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Background: The riboprotein telomerase has been linked to cellular immortality and is believed to play a key role in tumorigenesis.

Objective: To determine if telomerase is expressed in patients with oral squamous cell carcinoma.

Design: Twenty patient samples of oral squamous cell carcinoma and 20 adjacent histologically normal mucosal samples were assayed using the telomeric repeat amplification protocol (TRAP) method for detection of telomerase activity. The leukemic cell line, K562, was used as a positive control and the human fibroblast line, Hs21Fs, as a negative control.

Patients: Consecutive series of patients with oral squamous cell carcinoma presenting to a tertiary referral center.

Main Outcome Measure: A sample was classified as telomerase positive when an RNase-sensitive hexameric repeat ladder was observed. Absence of laddering was considered a negative result.

Results: Eighteen (90%) of 20 tumor samples and 7 (35%) of 20 adjacent histologically normal samples were telomerase positive. A statistically significant difference was observed in telomerase activity for T1 and T2 cancers compared with T4 cancers (P<.05 by analysis of variance). No statistically significant difference was observed in activity for T1 and T2 cancers vs T3 cancers.

Conclusions: The finding of telomerase activity in 90% of tumor samples is consistent with the concept of telomerase playing a key role in tumorigenesis. Further study is needed to determine the usefulness of this enzyme as a molecular marker.


Esidual and recurrent disease following conventional treatment for squamous cell carcinoma of the oral cavity is a continuous problem despite improvements in surgical technique and radiotherapy during the past 2 decades. When all stages are included, the 5-year survival rate for common oral cavity subsites such as the tongue and floor of mouth is approximately 65%. Concurrent with recent advances has been a greater general understanding of the molecular events involved in the development of head and neck cancer. This has led to the development of a progression model to describe some of the genetic changes that may be involved. Identification of a factor that consistently distinguishes cancer cells from normal cells could have major implications for early diagnosis, analysis of surgical resection margins, and management of patients with head and neck cancer, including patients with cancer of the oral cavity. Recently, the telomere-telomerase hypothesis of cancer progression has gained considerable support. This hypothesis is based on the finding of the ribonucleoprotein enzyme telomerase in most human cancers. Normal somatic tissue, except germ-line cells, do not express enzyme activity. Telomerase allows tumor cells to escape cellular senescence by sustaining proliferation and preventing cell death. The enzyme has this capability because it maintains telomeres that are short segments of 6-nucleotide DNA sequences (TTAGGG), located at chromosomal ends. Telomeres are vital for chromosomal stability and gene expression. They protect vital coding sequences of the genome from being lost during each cell division. Telomeres shorten with replication of human somatic cells in culture and with cell aging in vivo, and their length is an indicator of the replicative capacity of cells. Progressive loss of telomeric DNA has been proposed to act as a mitotic clock signaling entry of the cell into senescence, or cell death. In this way, telomeres keep a biochemical tally of the number of divisions remaining in a cell's lifetime. Acting as a reverse transcriptase, telom-
MATERIALS AND METHODS

TISSUE PREPARATION

Tumor samples were obtained following surgical resections from 20 previously untreated oral cavity squamous cell carcinomas. Normal tissue was taken adjacent to cancer tissue and from histologically normal surgical margins. Care was taken not to contaminate normal tissue with tumor samples by changing gloves and by using different surgical blades when cutting tissue. Once obtained, specimens were divided into 2 parts. One part was stained with hematoxylin-eosin and examined histologically by an experienced head and neck pathologist (C.M.) to confirm the diagnosis of malignant or normal. The second part was flash frozen in liquid nitrogen and stored at −70°C for subsequent molecular analysis. Squamous cell carcinomas were classified according to the loss of differentiation that the lesions exhibited, using the UICC staging system (G1 for well differentiated, G2 for moderately well differentiated, and G3 for poorly differentiated squamous cell carcinoma) and the TNM classification system.

EXTRACT PREPARATION FROM CRYOPRESERVED TISSUE SAMPLES

Tissue was first weighed and then transferred to an RNase-free mortar and pestle. Once crushed to a fine powder by continuous addition of liquid nitrogen, 200 µL of lysis buffer (10 mmol/L Tris-hydrochloride [pH 7.5], 1 mmol/L magnesium chloride, 1 mmol/L egtaciz acid, 5 mmol/L β-mercaptoethanol, 0.5% 3-(3-cholamidopropyl)dimethylammonio propane sulfonate, 10% glycerol) was added per 40 to 100 mg of tissue. The buffer and tissue were then removed to a microfuge tube. Remaining tissue was washed with an additional 200 µL of lysis buffer and added to the microfuge tube. Following incubation on ice for 30 minutes, the sample was centrifuged at 1600 RCF (relative centrifugal force) for 20 minutes at 4°C. The supernatant was carefully removed and the additional 200 µL of lysis buffer was added to the microfuge tube. Following incubation on ice for 30 minutes, the sample was centrifuged at 1600 RCF (relative centrifugal force) for 20 minutes at 4°C. The supernatant was carefully removed and the protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, Calif). A protein concentration of 4 µg/µL was used for all reactions. The remaining extract was flash frozen in liquid nitrogen and stored at −70°C.

TELOMERASE ASSAY (TRAP MIX)

Initially, a TRAP mix was prepared as described by Kim et al.9 The cell or tissue extract (4 µg) made up to a volume of 4 µL was incubated with 36-µL reaction mixture containing TRAP buffer (20 mmol/L Tris-hydrochloride [pH 8.3], 1.5 mmol/L magnesium chloride, 63 mmol/L potassium chloride, 0.05% polysorbate 20 [Tween 20, Sigma Chemical Co, St Louis, Mo], 1 mmol/L egtaciz acid, DEPC [diethylpyrocarbonate] distilled deionized H2O, 0.1 µg/µL of TS [substrate oligonucleotide] primer sequence (5'-AATCCGTGGCAGCAGAGTTT-3'), 5 µg/µL of T4 gene 32 protein [Boehringer Mannheim, Mannheim, Germany], 10 µg/µL of bovine serum albumin, 2.5 mmol/L of each deoxynucleotide triphosphate, and DEPC-treated [RNase free] distilled deionized H2O.

For every sample, an RNase control was included. The cell or tissue sample to be tested was treated with RNase (1 U/µL) for 10 minutes before adding the 36 µL of the TRAP reaction mixture. Tubes were then incubated at 23°C for 30 minutes and overlaid with 50 µL of oil. During this time, a separate polymerase chain reaction (1) PCR mix was made containing TRAP buffer (see above), 2.5 mmol/L of each deoxynucleotide triphosphate, 0.1 µg/µL TS oligonucleotide, 5 µg/µL of T4 gene 32 protein, 10 µg/µL bovine serum albumin, DEPC distilled deionized H2O, 5 µL Taq polymerase, α32P dATP (10-mCi/µL; Amersham International, Oakville, Ontario), and 1 µL of CX primer (3’-TCCATTTGATCCATTCCC-5’). Ten microliters of this reaction mix was added to each TRAP reaction tube, below the oil layer.

Polymerase chain reaction was then performed for 30 cycles (Biometra Inc, Tampa, Fla) with a 30-second denaturation at 94°C, a 30-second annealing step at 50°C, and a 90-second extension at 72°C. A final extension at 72°C for 3 minutes completes unfinished ends. The products of the reaction were resolved by electrophoresis in a 10% non-denaturing polyacrylamide gel in 10 × TBE (Tris borate EDTA) buffer after 8 µL of dye was added to each sample. The gel was run for 2 hours at 200 V, then dried, exposed to x-ray film (Kodak, Rochester, NY), and processed.

The method used in the telomerase assay (TRAP) is a highly sensitive means of detecting telomerase activity. In the first part of the reaction, telomerase adds hexameric telomere repeats (TTAGGG) onto the 3’ end of the substrate oligonucleotide (TS). During the second step, the extended products are amplified by the PCR using the TS and reverse primers to generate a ladder of products with 6 base increments starting at 50 nucleotides, ie, 50, 56, 62, 68, etc (Figure 1). The detection of this ladder indicates the presence of enzymatically active cell or tissue extract and therefore a positive result is recorded. The absence of a ladder indicates absence of telomerase activity and a negative result is recorded. The signal from the ladder was semiquantitatively analyzed by densitometry (ImageQuant IBM, Sunnyvale, Calif) and subjected to statistical analysis (Graphpad Software, San Diego, Calif). The intensity of the telomerase ladder was measured, after background subtraction, for each sample. The area measured included all telomerase bands and was then expressed relative to the positive control that was run with each experiment.

A highly sensitive method of extracting telomerase from tissue using a telomeric repeat amplification protocol (TRAP) method has recently become available, making possible the study of telomerase activity in a variety of human tumors and normal tissues.3 Telomerase activity has been detected in a wide variety of tumors and tumor cell lines whereas it has largely been found absent from normal tissue. Because of the paucity of information on telomerase in the head and neck literature, we investigated a consecutive group of patients with oral cavity squamous cell carcinoma for the presence of telomerase and correlated the level of activity with...
tumor stage and grade according to the Union Internationale Contre le Cancer (UICC) staging system.

## RESULTS

Twenty oral cavity squamous cell carcinomas and 20 adjacent histologically normal samples were assayed using the TRAP method. Tumors were representative of the different subsites within the oral cavity (Table) and all T stages were studied. Sixteen tumors were graded as moderately differentiated, 2 as well differentiated, and 2 as poorly differentiated. Eleven patients were men and 9 were women, aged between 38 and 76 years (mean age, 59 years). Most samples were taken from tongue (n = 9), with floor of mouth (n = 4), alveolus (n = 3), retromolar trigone (n = 2), and buccal (n = 2) making up the remainder of sites.

Telomerase activity was identified in 18 (90%) of 20 tumors and 7 (35%) of 20 histologically normal samples. As predicted, enzymatic activity was destroyed by pretreatment with RNase (Figure 1). A semiquantitative assessment of telomerase activity was made by densitometry for each assay (Figure 2). A statistically significant difference was observed when levels for T1 and T2 cancers were compared with densitometry recordings for T4 cancers and when T3 cancers were compared with T4 cancers (P < .05; nonparametric analysis of variance). No statistically significant difference was observed between activity in T1 and T2 tumors vs T3 tumors.

Fifteen patients remain disease free, and 4 are alive with locoregional disease. Three of the 7 patients with telomerase activity in histologically normal mucosa have developed recurrence. Recurrences in these 3 patients developed at 5, 6, and 10 months following completion of treatment. Of the remaining 4 patients with telomerase activity in histological normal tissue, only 1 had pathological nodal disease and the remainder have been treated in the past 7 months. One patient developed recurrence at 8 months in the group who had no telomerase activity in the adjacent mucosa. One patient in the series died, due to renal failure in the immediate postoperative period.

The telomerase densometric results for tumor and adjacent normal tissue were analyzed independently with regard to recurrence and time to recurrence. There was no statistical difference between densometric readings in patients with recurrence compared with those who remain disease free. No correlation could be identified between densomet-

### Telomerase Activity and Patient Details in 20 Patients With Squamous Cell Carcinoma of the Oral Cavity *

<table>
<thead>
<tr>
<th>Patient No./Sex/Age, y</th>
<th>Tumor Site</th>
<th>TNM Status</th>
<th>Telomerase Activity (Tumor Sample)</th>
<th>Status (Posttreatment Surgery, mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/F/60</td>
<td>Retromolar trigone</td>
<td>T2 N0 M0</td>
<td>+</td>
<td>DOD (17)</td>
</tr>
<tr>
<td>2/M/58</td>
<td>Buccal</td>
<td>T4 N0 M0</td>
<td>+</td>
<td>NED (4)</td>
</tr>
<tr>
<td>3/F/38</td>
<td>Lateral tongue</td>
<td>T3 N1 M0</td>
<td>−</td>
<td>NED (4)</td>
</tr>
<tr>
<td>4/M/69</td>
<td>Lateral tongue</td>
<td>T4 N2a M0</td>
<td>+</td>
<td>AWD (14)</td>
</tr>
<tr>
<td>5/F/55</td>
<td>Lateral tongue</td>
<td>T3 N0 M0</td>
<td>+</td>
<td>NED (13)</td>
</tr>
<tr>
<td>6/F/72</td>
<td>Floor of mouth</td>
<td>T2 N2a M0</td>
<td>+</td>
<td>AWD (9)</td>
</tr>
<tr>
<td>7/M/48</td>
<td>Floor of mouth</td>
<td>T3 N2a M0</td>
<td>+</td>
<td>NED (16)</td>
</tr>
<tr>
<td>8/M/57</td>
<td>Lateral tongue</td>
<td>T3 N1 M0</td>
<td>+</td>
<td>NED (12)</td>
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<tr>
<td>9/F/66</td>
<td>Lateral tongue</td>
<td>T2 N0 M0</td>
<td>+</td>
<td>NED (10)</td>
</tr>
<tr>
<td>10/M/54</td>
<td>Lateral tongue</td>
<td>T3 N2a M0</td>
<td>+</td>
<td>AWD (11)</td>
</tr>
<tr>
<td>11/M/48</td>
<td>Floor of mouth</td>
<td>T4 N0 M0</td>
<td>+</td>
<td>NED (7)†</td>
</tr>
<tr>
<td>12/F/65</td>
<td>Lateral tongue</td>
<td>T1 N0 M0</td>
<td>+</td>
<td>NED (6)</td>
</tr>
<tr>
<td>13/M/59</td>
<td>Buccal</td>
<td>T3 N0 M0</td>
<td>+</td>
<td>AWD (6)†</td>
</tr>
<tr>
<td>14/M/57</td>
<td>Anterior tongue</td>
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<td>+</td>
<td>NED (6)</td>
</tr>
<tr>
<td>15/M/76</td>
<td>Lateral tongue</td>
<td>T2 N0 M0</td>
<td>+</td>
<td>NED (10)†</td>
</tr>
<tr>
<td>16/M/50</td>
<td>Floor of mouth</td>
<td>T2 N0 M0</td>
<td>+</td>
<td>NED (5)</td>
</tr>
<tr>
<td>17/F/63</td>
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<td>T4 N0 M0</td>
<td>+</td>
<td>NED (5)</td>
</tr>
<tr>
<td>18/F/73</td>
<td>Lateral tongue</td>
<td>T3 N0 M0</td>
<td>+</td>
<td>NED (3)</td>
</tr>
<tr>
<td>19/M/55</td>
<td>Buccal</td>
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<td>+</td>
<td>NED (4)</td>
</tr>
<tr>
<td>20/F/66</td>
<td>Upper alveolus</td>
<td>T2 N0 M0</td>
<td>−</td>
<td>NED (4)†</td>
</tr>
</tbody>
</table>

* Plus sign indicates positive result (typical ladder pattern seen with polymerase chain reaction); minus sign, negative result (absence of typical ladder pattern seen with polymerase chain reaction); DOD, dead of other disease; NED, no evidence of disease; and AWD, alive with disease.

†Combination of surgery and radiotherapy.
ric values and early recurrence, either in the tumor itself or in adjacent normal mucosa. The overall mean (SD) result for adjacent normal tissue was 0.08 (0.17), which was significantly lower than tumor samples, 0.19 (0.23) ($P < .05$).

**COMMENT**

Our findings demonstrate that telomerase is present in 18 (90%) of 20 patients with oral cavity squamous cell carcinoma and in 7 (35%) of 20 samples of adjacent histologically normal tissue samples. Our data indicate that telomerase is activated in oral cavity carcinoma and this is in keeping with the concept that the enzyme plays a key role in tumorigenesis.9,15,16 Telomerase activity increases with late-stage carcinoma and is present at lower levels in all T stages. The finding of telomerase activity in histologically normal tissue suggests the enzyme may be useful as a molecular marker of disease and may have a role in the molecular assessment of tumor margins.

A previous report17 suggested that p53 analysis of surgical margins may augment standard histological assessment and may improve the prediction of local tumor recurrence. Although potentially informative, limitations of this method include a turnaround time of several days, and the innumerable permutations and possible mutations detectable. Also, p53 mutation has only been described in approximately 50% of head and neck cancers. The presence of telomerase in a high percentage of oral squamous cell carcinomas suggests it may be a useful method of detecting tissue abnormality at a molecular level. A telomerase assay can be performed within 24 hours, using a small amount of tissue material.

To our knowledge, this study is the first description of telomerase activity in histologically normal tissue surrounding squamous cell carcinoma of the oral cavity. It is possible that the highly sensitive TRAP assay detects genetically primed mucosa in keeping with the concept of "field cancerization."18 The identification of p53 mutations in separate tumors from the same individual supports this concept.19 Alternatively, telomerase may be present in dysplastic cells or activated lymphocytes that could account for the levels of activity seen in these samples. Conflicting reports have appeared concerning the activation of telomerase in lymphoid tissue. Shay20 found no activity in normal lymph nodes or splenic tissue, while activity has been found in some early hemopoietic precursor stem cells.21 We found a variable inflammatory response when a number of patient slides were carefully examined. **Figure 3** represents a patient with a T2 N0 M0 floor-of-mouth carcinoma who shows telomerase activity in the adjacent histologically normal tissue of this patient.

**Figure 2.** Telomerase activity of tumor samples expressed relative to 4 µg of K562 (positive control). The signal intensity from each tumor sample is measured and normalized to K562. Asterisk indicates a statistically significant difference between T4 carcinomas and other T stages. Mean (SD) values for each T stage are as follows: T1, 0.08 (1 patient); T2, 0.1 (0.11); T3, 0.09 (0.07); and T4, 0.6 (0.23).

**Figure 3.** Specimen stained with hematoxylin-eosin (original magnification × 250) processed routinely in formalin and paraffin embedded, demonstrating a mild to minimal chronic lymphocytic inflammation along the advancing edge of tumor. Telomerase activity was present in the adjacent histologically normal tissue of this patient.

**Figure 4.** Specimen stained with hematoxylin-eosin (original magnification × 250) showing an intense, moderate to severe chronic inflammatory response of lymphocytes and plasma cells at the advancing edge and surrounding nests of tumor. Telomerase was absent from the adjacent normal tissue in this case.

Telomerase has been detected at different stages of cancer progression depending on the type of malignancy.
investigated. In non–small cell lung cancer and gastric cancer, the enzyme is activated at the late stages of malignancy. In comparison, telomerase has been detected in premalignant stages of hepatocellular carcinoma. The enzyme may be an important molecular marker in brain tumor malignancy as activity correlates with prognosis in patients with malignant astrocytic tumors. A previous report found activation of the enzyme in 10 of 26 patients with oral leukoplakia, suggesting it may predict lesions that have a premalignant tendency or a propensity for recurrence. Since squamous cell carcinomas of the oral cavity progress through multiple steps from initial dysplasia to carcinoma in situ to overt invasive carcinoma, these lesions are ideal models to determine the timing of telomerase activation and further study is needed to determine this. Before the development of invasive carcinoma, these steps can be identified clinically as leukoplakia and erythroplakia. Identifying a biomarker that would predict patients likely to develop invasive carcinoma would be a useful molecular tool to assess cancer risk.

A recent report of laryngeal squamous cell carcinoma identified telomerase activity in 32 (89%) of 36 patients, indicating that other subsites in the head and neck region are likely to express this enzyme in addition to the oral cavity. Activation of telomerase was proportional to T stage and in some patients activity was present in histologically normal tissue, concurring with our findings. Telomerase activity was studied in a number of sites distant from the primary tumor and a gradient of activity was suggestive of a spread of telomerase-positive cells. The detection of telomerase was found to be independent of the presence of inflammatory cells or dysplasia. Alternatively, activation of telomerase may be due to an independent up-regulation of the enzyme in previously damaged mucosa from mutagenic carcinogens in cigarette smoke and alcohol.

The finding of telomerase activity in such a variety of human tumors studied to date has led to attempts at inhibiting the enzyme activity in vitro and this has met with some success. Blocking telomerase in cancer cells should allow their telomeres to shorten with subsequent cell divisions. These cells should then enter the stage of cell senescence and eventually die. Peptide nucleic acids with a sequence complementary to the RNA component of human telomerase have been found to be specific inhibitors of the enzyme in vitro. Developing oligonucleotides as gene-specific agents that bind to double-stranded DNA or that mimic protein binding ligands may have a future role in cancer therapy. Peptide nucleic acid is neutralized charged in comparison to DNA's negative charge and has a 10- to 50-fold greater inhibitory effect than phosphorothioate oligomers that were previously used as gene-specific inhibitors. The in vivo use of this type of agent is a potential means of suppressing tumor growth.

In conclusion, telomerase is activated in oral squamous cell carcinoma, supporting the concept that it is an important factor in tumorigenesis. The finding of telomerase activity in some histologically normal mucosa was unexpected. Further study to correlate surgical margins with patient outcome is clearly needed. In situ methods may resolve the role of dysplastic tissue and activated lymphocytes in the expression of telomerase activity. Telomerase inhibition may have an important adjunctive role in cancer therapeutics especially if combined with more conventional therapy.

Accepted for publication January 23, 1998.

This work was sponsored in part by The Charlie Conacher Cancer Research Fund, The Toronto Hospital, Toronto, Ontario; Synthes Maxillofacial Corporation, Paoli, Pa; and the Temmy Latner Dynecare Foundation, Toronto.

We thank Richard Leung of The Toronto Hospital, Division of Electron Microscopy, Department of Pathology, for his contribution to the photography.

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