Elevated Expression of hTERT Is Associated with Dysplastic Cell Transformation during Human Oral Carcinogenesis in Situ

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ABSTRACT

Purpose: Telomerase is a ribonucleoprotein complex composed of the catalytic protein subunit (human telomerase reverse transcriptase or hTERT) and the RNA template. This enzyme activity is a necessary and rate-limiting step of cellular immortalization and could provide a unique marker of aberrant cells, which may selectively be targeted. The current study was undertaken to quantitatively determine the degree of telomerase activation during multistage oral carcinogenesis using paraffin-embedded tissue samples.

Experimental Design: hTERT expression level was quantitatively compared between normal and cancerous oral tissues by real-time reverse-transcription-PCR (RT-PCR). Also, the presence of hTERT transcript in individual cells was surveyed in the biopsy specimens with varying degrees of histopathology by in situ RT-PCR.

Results: Low level of hTERT amplification was detected by real-time RT-PCR in most (11/13) normal human oral mucosa. hTERT expression was also detected in the majority (11/12) of squamous cell carcinoma tissues, and the level was significantly (P < 0.05) elevated, on the average, by a factor >6.9. By in situ RT-PCR, hTERT expression was noted in normal epithelium (0/10) nor in mild dysplasia (0/7) but was detected in moderate dysplasia (4/5) and in those tissues with a higher grade of histopathology: severe dysplasia (3/3) and invasive carcinoma (4/4).

Conclusions: These results indicate that enhanced expression of telomerase activity occurs early during human oral carcinogenesis and support the critical role of telomerase in the development of human oral cancer.

INTRODUCTION

Carcinogenesis proceeds in a step-wise, gradual fashion that is initiated when the checkpoint mechanisms that limit the replicative life span of normal cells is abrogated (1, 2). Such mechanisms involve a progressive shortening of telomeric DNA during each round of cell division (3). Telomerase sequences, flanking the two ends of eukaryotic chromosomes, constitute the critically important element, which preserves the chromosomal integrity and genetic stability (4). Continued shortening of telomere DNA beyond the critical length results in genetic catastrophe and loss of cell viability, most profoundly during the crisis stage (5–8). However, rarely some cells can escape from crisis by activating telomerase, which restores the minimal length of telomeres required to maintain their function (6–9).

Telomerase is a ribonucleoprotein complex consisting of the catalytic protein subunit, hTERT, and the RNA template, hTR (13). The enzyme activity correlates very closely with the level of hTERT expression and is detected in the vast majority of immortalized and cancer cell lines, whereas it is absent in most normal human somatic cells (14–16). Similarly, telomerase activation and elevated hTERT expression have been well correlated to malignant cell transformation in human tumor tissues (14, 15, 17). Functional telomerase is also necessary to sustain cellular immortality, because exposure of cells to telomerase inhibitors leads to apoptotic cell death (18–20). Therefore, understanding the role of telomerase in carcinogenesis could provide a unique opportunity to specifically mark cancer cells and to target these aberrant cells with remarkable selectivity.

The survey of telomerase activity during human oral carcinogenesis has primarily been conducted previously using the PCR-based TRAP (21–26). While this technique bears high sensitivity and is relatively simple, it does not convey the architectural information of telomerase expression in tissue samples and is merely semiquantitative at its best. The TRAP assay is also limited in its usage only for freshly isolated protein extracts, making it impossible to conduct retrospective studies of telomerase with archived materials. Accordingly, recent studies used in situ hybridization to detect hTERT transcript in various normal human tissues and biopsy specimens including those of oral epithelium (26–29).

The current study was undertaken to make a quantitative...
comparison of hTERT expression between human oral SCC tissues and normal counterparts and to define the clinicopathological stage of human oral carcinogenesis at which hTERT expression is activated in situ. We have determined the level of hTERT in total RNA extracted from paraffin-embedded tissues with histological diagnosis of SCC (n = 18) or NHOM (n = 20) by quantitative, real-time RT-PCR. Furthermore, hTERT expression was surveyed by in situ RT-PCR in oral epithelial tissue samples of varying degrees of histopathology that reflects the full spectrum of human oral carcinogenesis in situ. hTERT expression was detected in the majority of SCC tissues and was significantly increased in these samples by a factor >6.9 compared with normal tissues, which also exhibited low level of hTERT expression. Also, the elevated expression of hTERT was found at the single-cell level, primarily in tissues of moderate dysplasia and of higher grade of histopathology. Thus, our data indicate that telomerase activation occurs early during human oral carcinogenesis and support the critical role of telomerase in the development of human oral cancer.

MATERIALS AND METHODS

Tissue Samples. We obtained NHOM (n = 20) and tissues samples with varying degrees of dysplasia: mild (n = 7), moderate (n = 5), and severe (n = 3) dysplasia, and SCC (n = 18). These samples were paraffin-embedded biopsy specimens archived at the Section of Oral Pathology, University of California Los Angeles School of Dentistry. The SCC tissues used in this study contained 80% of epithelial cells with atypical nuclei. Thin (6 μm) paraffin sections of each tissue were stained with H&E, and the grade of epithelial dysplasia was determined according to the standard criteria (30). These tissue samples were used either to extract total RNA for quantitative determination of hTERT expression or to identify hTERT expression in situ.

cDNA Synthesis. Total RNA was isolated from two 20-μm thick sections of NHOM and SCC tissues using Paraffin Block RNA Isolation kit (Ambion Inc., Austin, TX) according to the manufacturer’s guidelines. The isolated RNA was dissolved in 7.5 μl of H2O, and reverse-transcription reaction was performed in the first strand buffer (Life Technologies, Inc., Rockville, MD), containing 200 units of Superscript II (Life Technologies, Inc.), 40 units of RNase inhibitor (Perkin-Elmer, Foster City, CA), 10 μM of dithiotreitol, 250 ng of random hexamer (Perkin-Elmer), and 2.5 μM deoxynucleotide triphosphate. The annealing reaction occurred for 10 min at 25°C, and cDNA synthesis was performed for 50 min at 42°C, followed by incubation for 10 min at 70°C for enzyme inactivation.

Real-Time PCR. The principle of real-time PCR was first described by Heid et al. (31). Briefly, amplification of the target sequence is monitored per PCR cycle by detecting the fluorescence signal emitted by the internal probe that is degraded by the 5’ nuclease activity of the Taq polymerase. The emission signal accumulates in each sample, and the Ct value required to reach a given fluorescence threshold is determined. Thus, the Ct value of a sample inversely correlates to the quantity of the starting cDNA. Using the cDNA of known quantity, a standard curve can be generated and used to determine the starting amount of cDNA based on the Ct value of each sample.

In our study, we constructed the standard curves for hTERT and 18S rRNA using serially diluted (0.008–5 ng) cDNA of HOK-16B, an immortalized HOK, which highly expresses telomerase activity (16). The PCR reaction for the standard curves and individual samples (18 SCC and 20 NHOM tissues) was carried with 5 μl of each RT samples and 20 μl of the master mix prepared with 1× TaqMan Universal Master Mix (Perkin-Elmer) and 200 mM of primers and the internal probe. We have used the identical primer and probe sequences as described by Bieche et al. (32) for amplification of hTERT. Amplification of 18S rRNA was performed with the primers and the internal probe provided by Perkin-Elmer. The obtained data for 18S rRNA was used to normalize the sample-to-sample variation in the amount of input cDNA and also to evaluate the quality of the isolated RNA and RT efficiency. The thermal cycling and collection of the fluorescence emission spectra were performed with an ABI Prism 7700 Sequence Detection System (Perkin-Elmer) under the parameters described elsewhere (31–33).

In Situ RT-PCR. The paraffin-embedded tissue samples were sectioned at 6-μm thickness. Three consecutive sections of each sample were placed per one In Situ PCR Glass Slide (Perkin-Elmer). The slides were baked at 60°C for 3 h to immobilize the sections, deparaffinized in xylene, and air-dried after dehydrating them in graded alcohol. To allow access to nucleic acids, the sections were digested with 2 mg/ml pepsin (Sigma Chemical Co., St. Louis, MO) in 0.1 n HCl for 30 min at 37°C in a humid chamber, and two of the three sections were treated with 20 units of DNase (Stratagene, La Jolla, CA) overnight. One remaining section was maintained untreated, and, thus, contained intact genomic DNA to control the efficiency of PCR amplification in situ. Subsequently, each section was submerged in 50 μl of one-step RT-PCR reaction solution containing 0.3 mM deoxynucleotide triphosphates, 0.06% BSA, 40 units of RNase inhibitor, 1.2 μM primers, 12 nm digoxigenin-dUTP (Roche Molecular Biochemicals, Indianapolis, IN), 2.5 mM Mn(OAc)2, and 5 units of rTth DNA polymerase. The slides were assembled with AmpliCover Discs (Perkin-Elmer) and reverse-transcribed at 65°C for 30 min. The thermal cycling was then allowed in a GeneAmp In situ PCR System 1000 (Perkin-Elmer) at 94°C/3 min once and for 20 cycles of 94°C/45 s and 60°C/1.5 min. To control genomic DNA contamination, thermal cycling was performed in the two DNase I-treated sections in the absence of RT reaction. hTERT sequence was amplified with the primers 5’-ACTTT-TGTTCAAGGTGGATGTCGACG-3’ (forward) and 5’-AAGAAATCATCCACC AAACCGCA GG-3’ (reverse), yielding 493-bp amplicons (34). The forward primer is located on exon 6 and the reverse on exon 10 (35). To visualize hTERT amplification in situ, the slides were blocked in 0.1 × SSC/0.2% BSA at 45°C for 15 min. The incorporated digoxigenin-dUTP was probed with antidigoxigenin antibody using the DIG Nucleic Acid Detection kit (Roche Molecular Biochemicals).
RESULTS

hTERT Expression Was Elevated in Human Oral SCC Tissues. We have quantitatively analyzed the level of hTERT expression in paraffin-embedded biopsy specimens, 18 of which were histologically diagnosed with oral SCC and 20 with NHOM. The validity of the real-time RT-PCR for quantitative amplification of hTERT has been established previously (31–33). In our study, hTERT amplification was first assessed with serially diluted cDNA (0.008–5 ng) of cultured HOK-16B, an immortalized HOK cell line (36), which strongly expresses telomerase activity (16). During the amplification reaction, the emission spectra were collected at every cycle, and the Ct value, i.e., the minimal cycle number at which the fluorescence threshold was reached for individual amplification reaction, was determined for each sample with an increasing amount of input cDNA using the standard curve shown in Fig. 1. A similar result (r = 0.993; P < 0.001) was also obtained for amplification of 18 s rRNA that served as an internal control with which we normalized the variation in the starting amount of cDNA in different samples. This abundantly expressed rRNA was also used to evaluate the quality of the total RNA extracted from each tissue sample.

The Ct values obtained from amplification of hTERT in each SCC and normal samples were converted to nanograms of cDNA of HOK-16B cell line and respective Ct values (A) for hTERT amplification. The input cDNA amount and the Ct values were negatively correlated with remarkable consistency (r = -0.995; P < 0.001). This standard curve was found to be inclusive of all Ct values of individual samples (○) and was used to convert the Ct numbers to the input cDNA amount equivalent to the known cDNA quantity of HOK-16B. B, fluorescence emission spectra were collected from each sample once per PCR cycle, and the kinetics of hTERT amplification was monitored over the entire thermal cycling reaction. The Ct values of individual samples were determined at the minimal number of cycles needed to reach the fluorescence threshold.

hTERT Activation Occurred in Moderate Dysplasia during Human Oral Carcinogenesis in Situ. The above data clearly indicated a quantitative difference in the level of hTERT expression between NHOM and SCC tissues. It was additionally necessary to delineate the hTERT expression level in individual cells of the tissues and to identify the clinical stage at which hTERT activation occurred. For this purpose, we determined hTERT expression by in situ RT-PCR in NHOM and in resected tissues with varying degrees of histopathology.

We obtained paraffin-embedded biopsy specimens with mild (n = 7), moderate (n = 5), and severe (n = 3) dysplasia, and invasive carcinoma (n = 4). The grades of epithelial dysplasia and histological diagnosis were made according to the standard criteria (30). In particular, we evaluated cytological atypia, atypical mitoses, or nuclear atypia, and other cellular alterations along the basal and parabasal layers. Using these tissue specimens, the sections were first deparaffinized and digested with DNase I to degrade genomic DNA. Subsequently, hTERT mRNA was reverse transcribed and amplified in a one-step reaction containing digoxigenin-dUTP. hTERT amplification was then detected in situ by direct immunohistochemical staining using antidigoxigenin antibody coupled with alkaline phosphatase. The use of this technique was validated by in situ staining of the sample without DNase I digestion, which showed intranuclear staining where amplification of genomic DNA occurred (Fig. 2). However, after the enzyme digestion, the PCR reaction without reverse transcription yielded no visible amplification, indicating that DNase I digestion in situ was sufficient to prevent amplification of cognate genomic DNA sequence. The above two controlled reactions were carried out in parallel with individual samples of interest for each in situ RT-PCR reaction on the same slide.

None of the 10 tested NHOM showed hTERT amplification (Table 2), albeit some normal tissues with focal epithelial hyperplasia contained positively stained cells primarily along the
basal cell layer (Fig. 3A). The signals detected in these cells, in contrast with the genomic DNA amplification (Fig. 2C), were localized to the cytoplasmic region resembling a “donut-shaped” staining pattern. hTERT amplification remained undetectable in tissues of mild dysplasia (Fig. 3B) until moderate dysplasia in which discrete amplification signal was visible in the cytoplasmic region (Fig. 3C). Similar pattern and intensity of staining was detected in tissues of severe dysplasia and invasive carcinoma (Fig. 3, D and E). Thus, hTERT expression and, hence, telomerase activation appears to occur during moderate dysplasia.

DISCUSSION

We report for the first time the quantitative difference between the level of hTERT expression in oral SCC tissues and NHOM by real-time RT-PCR. The great majority of SCC tissues contained detectable amount of hTERT transcript, which was also found in multiple NHOM specimens, albeit the transcript level in the latter group was at least 6.9-fold less than that of the former. These findings are in agreement with the previous studies, which showed high hTERT expression or telomerase activity primarily in malignant oral lesions (21-26, 28, 29). However, in some studies low levels of hTERT transcript and weak enzyme activity were also detected in normal oral tissues by RT-PCR and TRAP assay, respectively (26, 29). Likewise, our results reproducibly indicated a weak but readily detectable level of hTERT transcript in 85% of the tested NHOM tissues.

Detection of hTERT expression in normal human tissues appears to depend on the type of tissue origin; normal human skin and intestinal epithelial tissues contained hTERT transcript (17), whereas urothelial cells and the tissues of terminally differentiated, nonreplicative cells, e.g., adipose tissue, skeletal muscle, and peripheral nerve, did not (17, 33). Furthermore, moderate level of telomerase activity was reported in cultured normal HOKs, which were explanted and expanded from NHOM (16, 26, 37). Thus, hTERT expression in normal human tissues, like telomerase enzyme activity, may be limited to those that undergo constant cycles of cellular replication, differentiation, and regeneration. The level of hTERT expression in NHOM appears also to depend on the replicative activity of the constituent cells because the tissues with basal cell hyperplasia contained visible hTERT amplification that was not evident in NHOM specimens by in situ RT-PCR. This finding is in keeping with the previous report showing that exponentially replicating normal HOKs in culture demonstrated telomerase activity that was rapidly lost in nonreplicating cells during senescence (16). Therefore, hTERT expression in histologically normal epithelium appears also to depend on the replicative activity of the constituent cells because the tissues with basal cell hyperplasia contained visible hTERT amplification that was not evident in NHOM specimens by in situ RT-PCR. This finding is in keeping with the previous report showing that exponentially replicating normal HOKs in culture demonstrated telomerase activity that was rapidly lost in nonreplicating cells during senescence (16).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sex/age</th>
<th>Location</th>
<th>hTERT</th>
<th>18s rRNA</th>
<th>Normalized hTERT (10⁻³)</th>
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</thead>
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<tr>
<td>NHOM</td>
<td></td>
<td></td>
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</tr>
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<td>0.20</td>
<td>88.0</td>
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<td>2</td>
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<tr>
<td>3</td>
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</tr>
<tr>
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<td>26.2</td>
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<td>22.7</td>
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<tr>
<td>8</td>
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<td>0.54</td>
<td>202.0</td>
<td>2.7</td>
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<td>9</td>
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<td>0.27</td>
<td>11.0</td>
<td>24.5</td>
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<tr>
<td>10</td>
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<td>17.5</td>
<td>8.6</td>
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<tr>
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<td>0</td>
<td>52.0</td>
<td>0</td>
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<tr>
<td>12</td>
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<td>306.0</td>
<td>0.60</td>
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<td>13</td>
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<td>lower lip</td>
<td>0.35</td>
<td>76.0</td>
<td>4.6</td>
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<tr>
<td>Mean ± SE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7.0 ± 2.3</td>
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<tr>
<td>SCC</td>
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<tr>
<td>1</td>
<td>F/81</td>
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<td>0.07</td>
<td>1.0</td>
<td>70.0</td>
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<td>2</td>
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<td>floor of mouth</td>
<td>0.24</td>
<td>4.4</td>
<td>54.5</td>
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<tr>
<td>3</td>
<td>F/75</td>
<td>mandible</td>
<td>0.66</td>
<td>4.5</td>
<td>146.7</td>
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<td>0.36</td>
<td>24.4</td>
<td>14.8</td>
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<tr>
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<td>maxilla</td>
<td>0.37</td>
<td>26.6</td>
<td>13.9</td>
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<tr>
<td>6</td>
<td>F/78</td>
<td>mandible</td>
<td>0.81</td>
<td>303.0</td>
<td>2.7</td>
</tr>
<tr>
<td>7</td>
<td>F/81</td>
<td>lateral tongue</td>
<td>0</td>
<td>54.6</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>F/55</td>
<td>floor of mouth</td>
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<td>23.3</td>
<td>18.0</td>
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<td>ventral tongue</td>
<td>0.27</td>
<td>16.7</td>
<td>16.2</td>
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<td>1.91</td>
<td>10.0</td>
<td>191.0</td>
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<td>maxilla</td>
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<td>17.9</td>
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<td>F/79</td>
<td>buccal mucosa</td>
<td>1.06</td>
<td>27.0</td>
<td>39.3</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>48.6 ± 17.4</td>
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</tbody>
</table>

* Expression of hTERT and 18s rRNA was from Cₜ number, which was converted to the relative amount of HOK-16B cDNA using standard curve.
* Ratio of hTERT: 18s rRNA expression.
* P < 0.05.
lium may be a marker of cell proliferation rather than of malignant cell transformation. Alternatively, the quantity of hTERT transcript detected in NHOM specimens may have also been contributed in part by infiltrating hematopoietic cells, which also demonstrate moderate level of telomerase activity (38). For the reasons described above, some (3/20) NHOM specimens exhibited relatively abundant hTERT transcript by real-time RT-PCR as shown in Table 1.

The quantitative difference between the hTERT expression in NHOM and SCC tissues was rather small compared with the apparent difference of telomerase activity between normal oral mucosa and neoplastic oral lesions (21–26). It should also be noted that telomerase activity was determined by means of enzyme assay, i.e., TRAP, which could amplify the actual difference in the absolute quantity of the hTERT transcript by real-time RT-PCR as shown in Table 1.

The detection sensitivity of this method is apparently less than that of real-time RT-PCR, because the in situ assay is based on the visual inspection of colorimetric signals, whereas the real-time RT-PCR yields emission signal from the fluorogenic probe that is captured by the charged-coupled device (31–33). The reduced sensitivity was in fact advantageous for our purposes, because it allowed us to visually differentiate between the positively stained cells with high hTERT expression and those with low or no hTERT expression, which did not stain at all. Accordingly, hTERT expression was not noted in NHOM and mild dysplasia samples by in situ RT-PCR.

With this technique, moderate dysplasia samples and the tissues with higher grade of histopathology, i.e., severe dysplasia and invasive carcinoma, reproducibly exhibited visible hTERT expression, most profoundly along the basal and mid-spinous layer of the epithelial squame. The tissue samples frequently contained regions of moderate dysplasia and juxtaposed mild dysplasia in the same biopsy specimen, which also demonstrated the presence and the absence of hTERT expression, respectively. Thus, our results support the involvement of telomerase in human oral cancer. The induction of hTERT expression has been reported to be progressive and to occur throughout the entire length of oral carcinogenesis (22, 25). However, in our study hTERT expression was visible in moderately dysplastic tissues, and the intracellular pattern and the intensity of hTERT signal were not altered in tissues with higher grade of histopathology. Our results are in agreement with a previous report, which showed telomerase activity by TRAP assay in human oral tissues with moderate or higher grade of dysplasia but not in those with mild dysplasia (39). Therefore, detection of hTERT expression by in situ RT-PCR or of telomerase activity by TRAP assay appears to indicate the presence of moderate or higher grade dysplasia in human oral biopsy specimens. Whether telomerase activation occurs in individual cells in moderately dysplastic tissues or whether cells with high telomerase activity are selected to constitute such lesion remains to be elucidated.

### Table 2 hTERT mRNA expression in oral cancer, dysplasia, and in normal mucosa by in situ RT-PCR

<table>
<thead>
<tr>
<th>Histology</th>
<th>No. of cases</th>
<th>hTERT expression</th>
</tr>
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<tbody>
<tr>
<td>Normal mucosa</td>
<td>10</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Mild dysplasia</td>
<td>7</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Moderate dysplasia</td>
<td>5</td>
<td>4 (75%)</td>
</tr>
<tr>
<td>Severe dysplasia</td>
<td>3</td>
<td>3 (100%)</td>
</tr>
<tr>
<td>Invasive carcinoma</td>
<td>4</td>
<td>4 (100%)</td>
</tr>
</tbody>
</table>

*a% positive for hTERT among the specimens tested.*

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Fig. 2 In situ RT-PCR of hTERT in NHOM. A, thin sections (6 μm) of NHOM were stained for H&E. B, a consecutive section of the same tissue was used for in situ RT-PCR using hTERT primers (see “Materials and Methods”) after DNase I digestion of genomic DNA. C, in the absence of DNase I digestion, the cognate genomic sequence was amplified with hTERT primers and was shown as intranuclear staining. The area within the box was magnified ×4 (inset). D, without reverse-transcription and with DNase I digestion, no amplification of hTERT occurred. Bar, 50 μm.

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![Image](https://clincancerres.aacrjournals.org/content/3/7/3083/F2.large.jpg)
Fig. 3 Expression of hTERT during multistep human oral carcinogenesis in situ. hTERT transcripts were detected in situ by RT-PCR in paraffin-embedded human oral tissues with varying degree of histopathology: basilar hyperplasia (A), mild dysplasia (B), moderate dysplasia (C), severe dysplasia (D), and squamous carcinoma (E). The left half of each panel shows the histopathology by H&E staining, and the right half the in situ RT-PCR of the same tissue for hTERT expression. Arrows, examples of positive hTERT staining; arrowhead, an example of mitotic figure at the basal cell layer. Bar, 50 μm.
It is noteworthy that both moderate dysplasia and cellular crisis mark the pivot of oral carcinogenesis at which hTERT or telomerase is activated (6). Also, after-crisis HOKs in “raft” culture resembled the cellular constituents of moderately dysplastic tissues (40). These observations strongly suggest the analogy between the two events that moderate dysplasia is an in situ counterpart of cellular crisis in vitro. Although this notion cannot entirely be validated at present, it provides a means by which we can resonate our understanding from the in vitro studies onto oral carcinogenesis in situ.

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