Detection of Telomerase Activity in Oral Rinses from Head and Neck Squamous Cell Carcinoma Patients[1]

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ABSTRACT

Telomerase is a ribonucleoprotein that maintains telomere length and whose activity is associated with escape from cellular senescence. Telomerase activity has been found in germline, immortalized, and malignant tumor cells. Using a modified PCR-based assay for telomerase activity, 26 of 35 (80%) primary, fresh, head and neck squamous cell cancer specimens and 3 of 6 head and neck squamous dysplastic lesions possessed telomerase activity. In addition, 14 of 44 (32%) oral rinses from a separate group of head and neck squamous cell cancer patients contained detectable telomerase activity, whereas 1 of 22 (5%) oral rinses from normal control patients exhibited telomerase activity. Telomerase activity in oral rinses was compared with corresponding activity in paired primary tumor samples for 19 cases: 7 of 19 demonstrated activity in both tumor and oral rinse, 2 of 19 lacked activity in both tumor and oral rinse, 10 of 19 tumors demonstrated activity that could not be detected in corresponding oral rinses, and there were no examples of positive oral rinses with corresponding negative tumors. Although currently limited in its sensitivity, analysis of telomerase activity in oral rinses represents a novel method to detect the presence of cancer cells shed in the upper aerodigestive tract.

INTRODUCTION

Telomeres are specialized structures at the ends of eukaryotic chromosomes that consist of a few hundred bp to approximately 30 kb of a simple six-bp repeat (1). Reduction in average telomere length has been noted during normal aging in human differentiated cells. Lower organisms also exhibit a progressive shortening of telomere length during progression to cellular senescence, and attempts to artificially reduce telomere length have resulted in premature cellular senescence and death (2, 3). It has been noted in cell culture experiments that progressive telomere shortening is associated with a stereotypical cell crisis in which genetic instability and cell cycle arrest precede an inevitable death in the majority of cells. However, those cells that are selected to survive this crisis period express a telomerase activity capable of maintaining telomere length indefinitely during subsequent cell divisions. Investigation of this activity in humans has determined that telomerase is a ribonucleoprotein that uses an RNA template to add the requisite six-bp DNA repeat units to the 3′ end of telomeres in a processive manner (4).

Initial investigations demonstrated telomerase activity in human cancer cells but not in surrounding normal tissues (5). Advances in

detection methods have resulted in a PCR-based assay that can detect as few as 50 telomerase-expressing cells/sample (6). Using this method, investigators have been able to demonstrate telomerase activity in nearly all immortal cell lines, a high percentage of primary tumor specimens from malignancies of diverse tissue origin including HNSCC,[3] and less frequently in various premalignant, immortalized lymphoid, and benign proliferative tissues. The ubiquitous nature of telomerase expression in a malignant phenotype and the sensitivity of PCR-based techniques suggests that measurement of telomerase activity may be an attractive strategy for detection of malignant cells that are shed into body fluids.

We have previously demonstrated that cancer cells are shed into the urine of bladder cancer patients and into the saliva of head and neck cancer patients using an assay based on hybridization of mutation-specific oligonucleotide probes to amplified PCR products from DNA derived from exfoliated cells (7, 8). This method of detection is highly sensitive and specific, but requires characterization of a unique mutation in a patient's tumor prior to analysis, precluding its use as an initial screening technique for asymptomatic, high-risk patients. A PCR-based telomerase screening assay does not require any characterization of a malignant lesion to facilitate detection since telomerase is ubiquitously expressed in most neoplasms and is not present in nonimmortalized cells. We therefore performed an initial screening of representative malignant and preinvasive HNSCC lesions and oral rinses from both HNSCC patients and normal controls to determine whether telomerase activity could be detected in neoplastic cells shed from HNSCC patients.

MATERIALS AND METHODS

Telomerase activity was detected using a modification of a previously published method (9). Informed consent was obtained from each patient. Briefly either (a) three 7-μm sections of fresh tumor, measuring approximately 0.5 × 0.5 cm, were snap frozen in liquid nitrogen and placed in 20 μl of CHAPS detergent buffer, mixed, and incubated at 4°C for one h or (b) 25 ml of sterile saline were rinsed and gargled briefly (15 s) and spit into a sterile 50-ml conical tube. Cells were pelleted at 2500 × g for 15 min at 4°C, the supernatant was discarded, and pelleted cells were resuspended in 1 ml of saline and snap frozen in liquid nitrogen for storage. Prior to testing, lysates were thawed and a 5-μl aliquot was placed in 20 μl of CHAPS detergent buffer (9), mixed, and incubated at 4°C for 1 h. Care was taken so that sample to sample contamination, PCR contamination, and RNase contamination did not occur, including handling all specimens with gloves, changing the cryostat knife, washing the cryostat apparatus with water, 100% ethanol, and xylene between sample cutting, and strict segregation of samples, oral rinses, and positive and negative controls. The digests were then centrifuged at 13,000 × g for 10 min, and the supernatant was divided so that a heat-treated (85°C for 15 min) negative control could be run with each sample. Four-μl aliquots of each supernatant were placed in 21 μl of TRAP master mix to yield a solution containing a final concentration of 20 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 63 mM KCl, 1 mM EGTA, 0.005% Tween 20, 0.5 mM each of deoxynucelotide triphosphate, and 0.25 μg of T4 gene 32 protein following incubation at 30°C for 1 h. Negative samples were then rerun in dilutions of 1:10 and 1:100 to

[3] The abbreviations used are: HNSCC, head and neck squamous cell cancer; TRAP, telomerase repeat amplification protocol; CHAPS, 3-[(3-cholamidopropyl)dimethylamino]-1-proanesulfonate.

attempt recovery of telomerase activity in the event that telomerase (or PCR) inhibitors prevented adequate extension. This was found to be true for a small minority of samples (<10%). DNA extension products were then phenol-chloroform extracted and ethanol precipitated in a standard fashion to eliminate any PCR inhibitors and resuspended in 25 μl of ddH$_2$O. Twenty-five μl of master mix, including 10 units of Taq polymerase and 4 μCi of [γ-^{32}P]dCTP, were added to the resuspended DNA extension product mixture to yield a final concentration of 20 mM Tris-HCl (pH 8.3), 1.5 mM MgCl$_2$, 63 mM KCl, 1 mM EGTA, 0.005% Tween 20, 0.25 mM each of deoxynucleotide triphosphate, and 1.5 $\times$ 10^{-17} g of an internal positive control for PCR. This internal control was made by amplifying microsatellite repeat $D4S243$ using modified primers such that each primer consists of a forward or reverse $D4S243$ primer on the 3' end and either CX or TS primer sequences on the 5' end of each primer, respectively. Primer sequences used to amplify this control amplicon are 5'-AATCCTTCGAGCAGAGTTTAGGAGCCTGTGGTCCTGTT-3' and 5'-CCCTTACCCTTACCCTTACCCTAATCAGTCTCTCTTTCTCCTTGCA-3'.

The resulting ~200-bp PCR amplified product was then gel purified and quantitated, and 1.5 $\times$ 10^{-17} g were added to each reaction mixture. This mixture was separated from 100 ng of CX primer by a wax barrier as described. Reactions were then incubated at 90°C for 1 min and then cycled at 95°C for 30 s and 50°C for 30 s and at 70°C for 1 min for 35 cycles with a final extension step of 70°C for 5 min. Five μl of 10× gel loading buffer were added and 25 μl of this mixture were run on a 10% polyacrylamide gel, dried, and exposed to autoradiographs as described for approximately 8–10 h. A CHAPS-only negative control and 293 renal carcinoma cell line extract positive control were included with each run. Frozen sections of tumor samples were checked to assure the presence of tumor cells and absence of necrosis.

RESULTS

An initial survey of randomly selected samples from 35 patients with HNSCC of varying stages and 6 patients with dysplastic lesions was performed using a modified TRAP assay. These tumors and preinvasive lesions originated from the oral cavity, oropharynx, pharynx, and larynx. This assay included an internal PCR control to identify PCR inhibition by sample components and to allow for semiquantitation of telomerase activity. The criteria for judging a sample telomerase positive included (*a*) >3 sequential bands of the 6-bp telomerase repeat on the sample lane with an appropriate band corresponding to the 200-bp internal control in the same lane; (*b*) a negative control lane with no detectable telomerase laddering but with a positive 200-bp internal control; and (*c*) repetition of the result with two or more repeat samplings of the primary material. This assay demonstrated that 26 of 35 (80%) HNSCC samples exhibited telomerase activity, whereas 3 of 6 (50%) premalignant lesions also exhibited telomerase activity (Table 1 and Fig. 1).

We then prospectively collected and analyzed 44 oral rinses obtained from a separate group of HNSCC patients and 22 oral rinses from normal, nonsmoking control patients for telomerase activity. During the course of analysis of these oral rinses, several observations became noteworthy. A majority of the oral rinses possessed a PCR inhibitory activity that could be abrogated by inclusion of a phenol-chloroform extraction and ethanol precipitation step after elongation of the telomerase template. Apparent nonspecific amplification yielding occasional isolated longer length (>80 bp) bands was noted in several oral rinse samples from both HNSCC and normal control patients despite satisfactory negative controls, but there was no evi-
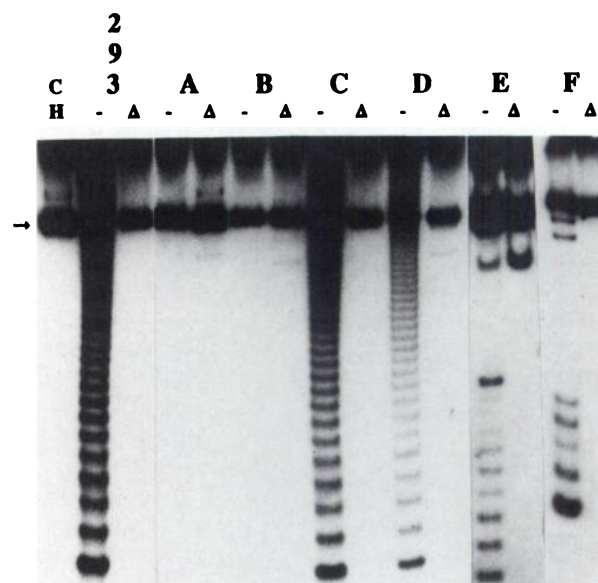

Fig. 1. Telomerase activity in primary tumors and oral rinses. Representative samples of telomerase-positive tumors, telomerase-positive oral rinses, and telomerase-negative oral rinses. −, nonheat-treated sample; Δ, heat-treated negative control; *arrow*, position of internal PCR control band. *CH*, CHAPS buffer alone without heat treatment; *293*, 293 cell extract-positive control; *A* and *B*, telomerase-negative oral rinses; *C* and *D*, telomerase-positive tumors; *E* and *F*, telomerase-positive oral rinses. This figure is a composite of four different autoradiographs displaying variations of some banding patterns due to a slight variation in electrophoresis conditions.

Table 2 *Telomerase in HNSCC oral rinses and matched tumors*

	Tumor positive	Tumor negative
Oral rinse positive	7	0
Oral rinse negative	10	2

dence of any ladder pattern characteristic of telomerase-mediated primer extension. This nonspecific amplification may represent nonspecific binding of PCR primers to telomeres or telomere-like sequences present in residual DNA present in crude protein extract that is normally overwhelmed by competition from telomerase-extended template products. These samples were interpreted as negative according to the criteria outlined above.

In all, 14 of 44 (32%) oral rinses from patients with HNSCC were found to possess telomerase activity (Table 1 and Fig. 1). Adequate corresponding snap-frozen primary tumor samples were available for 19 of the 44 HNSCC patients (Table 2). Seven of these tested positive for telomerase activity in both the oral rinse and primary tumor. Ten oral rinses did not possess telomerase activity despite the presence of telomerase activity in the matching tumor. Only two of these tumors did not possess telomerase activity, and the oral rinses from both patients did not possess telomerase activity. Of the 22 normal, nonsmoking controls, only 1 had an oral rinse positive for telomerase activity. This control subject did not have any risk factors for HNSCC, did not have any signs of respiratory tract infection during sampling, and tested positive with repeat testing.

The possibility that false-negative oral rinse results were due to inadequate cellular content or inadequate protein concentration in the sample was entertained, but the protein concentration of the cell extract was measured using the bicinchoninic acid assay (10) and was normalized for most specimens. Some oral rinses (approximately one third) yielded protein concentrations below the normalized value, but when compared to those with normal protein concentrations, there was no difference in the rate of telomerase-positive samples or quan-

Table 1 *Telomerase activity in HNSCC patients and oral rinses*

	Telomerase positive (%)	Telomerase negative	Total
Invasive HNSCC	26 (80)	9	35
Premalignant lesions	3 (50)	3	6
HNSCC oral rinses	14 (32)	30	44
Normal control oral rinses	1 (5)	21	22

tity of telomerase activity. Moreover, the internal control PCR template was equally amplified in the telomerase-negative oral rinse samples. False-negative oral rinses may be due to difficulties in obtaining high-quality oral rinse specimens: telomerase activity is sensitive to RNase and heat, and false-negative oral rinses may have resulted from endogenous RNase degradation of telomerase template or from prolonged exposure to room temperature conditions due to logistical factors in the collection process. This also holds true for primary tumor specimens and may have resulted in underestimation of the frequency of telomerase activity in primary tumors. Conversely, strict segregation of samples from collection to processing and final analysis was observed to prevent contamination or degradation. In addition, it is conceivable that telomerase inhibitors could be present in saliva. To test this hypothesis, we added an aliquot of cell digestion extract equivalent to the activity derived from $\sim 10^3$ cells from a telomerase-positive cell line (293 renal carcinoma) to telomerase-negative saliva specimens and observed no change in telomerase activity, indicating that inhibition of telomerase activity by factors in saliva is probably not a major cause of false-negative tests (data not shown).

DISCUSSION

The role of telomerase expression in the process of malignant transformation is currently undergoing intense scrutiny. Initial investigations have focused on the association of cellular immortalization with the ability of cells to maintain telomere length during cell division. In lower organisms, and more recently in cell culture systems, manipulation of telomere length has resulted in altered proliferative capacity, in agreement with the hypothesis that maintenance of telomere length permits continued cellular proliferation. Concurrent investigations in primary human tumor specimens have demonstrated a unique association between telomerase activity and neoplastic transformation as well as cellular immortalization. In this study, we found that telomerase activity is present in all stages of tumor progression in patients with HNSCC, ranging from early preinvasive dysplasias to fully malignant invasive tumors. Our results are consistent with a recent report describing telomerase activity in oral leukoplakia and HNSCC in patients from Thailand (11).

The ability to detect malignant cells in saliva using this assay is dependent on (a) the ability to detect small amounts of telomerase activity using a PCR-based assay; (b) the presence of telomerase activity in premalignant and invasive head and neck lesions; and (c) the constant shedding of squamous epithelia into saliva. Theoretically, telomerase activity should be detected in more than 75% of the oral rinses from HNSCC patients based on our initial analysis of telomerase positivity in invasive HNSCC and other reports in the literature for telomerase expression in primary tumors (6, 11). The inability of the assay to detect telomerase activity in the majority of oral rinses may be due to a number of factors. Telomerase is sensitive to both heat and RNase; the presence of RNase in saliva is well documented and could significantly degrade enzyme activity, and some oral rinses may have been stored at room temperature for a prolonged period of time before processing. An additional explanation for false-negative results may be that a very small percentage of cells obtained by oral rinses are actually tumor cells [as defined by our previous investigation using a sensitive and quantitative plaque hybridization technique (8)] and may be below the threshold required for detection of telomerase activity.

Appropriate controls for false-positive results were performed, including a RNase- or heat-treated negative control for each sample, as well as appropriate digestion buffer and control positive cell line samples for each run. Lymphocytic infiltration of tumor may also occasionally contribute to a false-positive result for primary lesions. The possibility of false-positive oral rinse results from lymphoid elements (activated B and T cells) also exists due to the presence of large amounts of tonsillar and adenoidal lymphoid tissue in the upper aerodigestive tract. Only 1 of the 22 oral rinses from control subjects showed any telomerase activity, despite the fact that at least one third of these were sampled while the subject had symptoms of viral upper respiratory tract infection and optimal collection conditions were used. We chose not to include oral rinses from smokers as controls in the event that these patients harbored asymptomatic premalignant or malignant lesions.

The false-negative rate for detection of telomerase activity from malignant cells shed into oral rinses appears to be a major limiting factor for cancer detection. An increase in the sensitivity of detection of telomerase activity may yield additional positive oral rinses. Prospective clinical trials are needed to provide an accurate sensitivity, specificity, and positive predictive value for this test using a clinically appropriate control group (patients at risk for HNSCC) rather than healthy, nonsmoking subjects. Although telomerase detection is not yet an ideal diagnostic test, analysis of telomerase activity in oral rinses may be useful in the design of a preliminary screening strategy for HNSCC. We were not able to obtain oral rinses from patients with premalignant lesions of the upper aerodigestive tract, but it is encouraging that these premalignant lesions do express telomerase. It is possible that detection of telomerase activity in oral rinses may allow for detection of lesions before an invasive phenotype has evolved, allowing for early therapeutic intervention. These detection strategies may also be applicable to other tumor types, including lung cancer, in which expectorated sputum might contain telomerase-expressing neoplastic cells. A combination of detection techniques, including microsatellite-based assays (12), may also be used in a complementary fashion after initial telomerase screening. Improvements in detection of telomerase or a telomerase protein complex based on an ELISA may become available when cloning efforts for the telomerase gene(s) succeed.

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