Journal of Advanced Medical and Dental Sciences Research

@Society of Scientific Research and Studies

Journal home page: www.jamdsr.com doi: 10.21276/jamdsr

(e) ISSN Online: 2321-9599; (p) ISSN Print: 2348-6805

Original Article

Telomerase Activity in Oral Leukoplakia and Oral Squamous Cell Carcinoma

Fahad M. Samadi[1], Shaista Suhail[1], Manjari Sonam[1], Shruti Singh[1], Diksha Singh[1]

[1]Department of Oral Pathology and Microbiology, King George's Medical University, Lucknow, U.P., India.

ABSTRACT:
Background: Telomerase is a ribonucleoprotein complex responsible for *de novo* telomere synthesis and addition of telomeric repeats to existing telomeres. Telomerase activity can be measured *in vitro* by using the telomeric repeat amplification protocol (TRAP). Method: The study was done to investigate the presence of telomerase activity in oral leukoplakia (OL) and oral squamous cell carcinoma (OSCC) by TRAP assay. Result: Telomerase activity was detectable in 18 of 20 human OSCC and 7 of 20 OL tissues. The expression of telomerase in the premalignant lesions was associated with phenotypic progression, the degree of dysplasia. Conclusion: These results indicate that telomerase is activated frequently during the late stage of oral premalignancy and may play a crucial role in OSCC.
Key words: Biomarker, squamous cell carcinoma, telomerase, telomere.

Received: 2 May 2018 Revised: 20 June 2018 Accepted: 20 June 2018

Corresponding Author: Dr. Fahad M. Samadi, Assistant professor, Department of Oral Pathology and Microbiology, King Georges Medical University, Lucknow, U.P., India

This article may be cited as: Samadi FM, Suhail S, Sonam M, Singh S, Singh D. Telomerase Activity in Oral Leukoplakia and Oral Squamous Cell Carcinoma. J Adv Med Dent Scie Res 2018;6(7):120-124.

INTRODUCTION:
Oral cancer is among the leading cancer type in South Central Asian men[1], including India**.** Oral precancerous lesions (PCLs), a benign morphologically altered tissue that has a greater than normal risk of malignant transformation, such as oral leukoplakia etc., is also very common. Precancerous condition is a condition that does not necessarily alter the clinical appearance of local tissue but is associated with a greater than normal risk of precancerous lesion or cancer development in that tissue such as sub mucous fibrosis etc. (Neville *et al.* 2005). Oral leukoplakia and oral sub mucous fibrosis (OSMF) are early indicators of damage to the oral mucosa with a transformation rate of 2–12% to frank malignancies.[2]
Oral cancer affects approximately 0.5% (5 million people) of the population in the Indian subcontinent and is now a public health issue in many parts of the world, including the United Kingdom[3], South Africa, and many Southeastern Asian countries.[4] Reichart suggested that as a result of transmigration of populations, an increasing number of Oral Submucous Fibrosis (OSMF) cases are being found in other countries. It constitutes one of the major oral health problems in countries like India[5]. Oral lesions and cancers are now affecting even young individuals.
OL is a common precancerous lesion, characterized by morphologically altered tissue, in which cancer is more likely to occur than its apparently normal counterpart**.** OL is defined as a predominantly white lesion of the oral mucosa that cannot be removed by scraping and cannot be characterized clinically or microscopically as any other definable lesion. There is a wide range in the malignant transformation rates (0.3-17.5) of OL. The frequency of malignant transformation for OL varies between 0-20 % in an observation period of 1-30 years. Generally it is more or less accepted that approximately 3% of OL will be transformed into oral cancer in an average period of 5 years**.** OL occurs commonly in the age group of 35-45 years[6]. Clinically various forms of OL have been reported such as homogenous, ulcerated, nodular (speckled), verrucous and candidal**.** Males are more affected than females. Interestingly it has also been reported that the OL on the oral floor or ventral tongue shows malignant transformation of 16% to 19% but those occurring in females are high (47%)[7]**.**

OSMF is a pre-cancerous condition characterized by the accumulation of collagen in the lamina propria of oral mucosa. According to Tilakratne it is characterized by a juxtra-epithelial inflammatory reaction followed by fibro elastic changes in the lamina propria and associated epithelial atrophy[8]. The disease affects most part of the oral cavity as well as the upper third of the esophagus. The incidence of OSMF has increased manifold in various parts of the Indian subcontinent[9].

It was suggested that tobacco chewing is the most important risk factor for multiple oral premalignant lesions and may be a major etiological factor for cancers on the oral epithelium in the Indian population[7]. Similarly, oral cancer is associated with tobacco chewing in various forms in several other countries of the world.

Although the available epidemiological evidence indicates that the chewing of the tobacco/pan masala/areca nut is an important risk factor for OSMF, not all chewers develop the disease indicating the importance of genetic predisposition. Genetic predisposition might explain such an individual variability. Metabolic enzymes that are potentially involved in either the activation (Phase I) or detoxification (Phase II) of chemical carcinogens in tobacco smoke/pan masala have received a great deal of attention recently as possible genetic susceptibility factors for a variety of cancers.

Telomeres are repetitive sequences at the ends of chromosomes that protect chromosomes from incomplete replication, nuclease degradation, and end-to-end fusion during replication. Telomeres are required for chromosome segregation during meiosis and mitosis. Telomerase is a ribonucleoprotein complex responsible for *de novo* telomere synthesis and addition of telomeric repeats to existing telomeres. Telomerase activity can be measured *in vitro* by using the telomeric repeat amplification protocol (TRAP)[10].

Telomerase activity has been demonstrated in a high percent of extracts from most tumor types. For example, telomerase has been demonstrated in 75% of oral carcinomas, 80% of lung cancers[11], 94% of neuroblastomas[12], 95% of colorectal cancers[13], and 98% of bladder cancers.[14]

Telomerase activity is readily detected in most cancer biopsies, but not in premalignant lesions or in normal tissue samples with a few exceptions that include germ cells and hemopoietic stem cells. Telomerase activity may, therefore, be a useful biomarker for diagnosis of malignancies and a target for inactivation in chemotherapy or gene therapy. These observations have led to the hypothesis that activation of telomerase may be an important step in tumorgenesis. To test this hypothesis, we studied telomerase activity in tissue samples of normal human oral cells and in biopsies of superficial and transitional squamous cell carcinoma (TCC) of oral cavity.

The expression of telomerase, a ribonucleoprotein complex, is necessary to overcome cellular senescence, and it is associated with immortal cells and cancer. However, its role in precancerous lesions such as OL is less known.[15] The purpose of this study is to investigate the presence of telomerase activity in OSCC, OL and the relationship between the enzyme and multistep tumorigenesis.

Telomerase is a ribonucleoprotein enzyme that synthesizes telomeres, the specialized structures containing unique simple repetitive sequences (TTAGGG in vertebrate) at the end of chromosomes[16,17]. The enzyme compensates for the end replication problem and allows cells to proliferate indefinitely[18]. Recent studies, using the TRAP, have shown that telomerase is activated in most human cancer tissues but not in most normal tissues and tissues adjacent to malignant or benign tumors[19]. In addition, previous studies have shown that the lack of telomerase activity correlates with critically shortened telomeres and frequent spontaneous cancer remission[20]. Thus, the expression of telomerase is important and may be a rate-limiting step for tumor progression[19].

MATERIALS AND METHODS

Methodology

Study was conducted in the Department Oral Pathology and Department of Biochemistry of King George's Medical University Lucknow. Cancer patients undergoing, radiotherapy, chemotherapy and or surgery were excluded in this study. Patients were examined for signs or symptoms of oral cancer.

Detailed information of each patient was noted in a pre-tested proforma. Information regarding the patient's name, age, sex, occupation, background, dietary habits, dental hygiene, personal habits and present complaints were gathered. Emphasis was given to addictions like areca nut, tobacco (smoke and smokeless) and alcohol. Detailed clinical examination of each patient was done to assess the site, size and type of lesion. Epidemiological information was collected as per preset proforma.

Study population

In the study we have collected a total of 20 samples of tissue from oral lesion of oral leukoplakia (OL), 20 samples of Oral Squamous Cell Carcinoma(OSCC) and 20 samples of oral normal mucosa (control) patients visiting to the Department of Oral Pathology and Microbiology, K.G.M.U, Lucknow. A detailed history of all participants was taken. Individuals were excluded if they had received antibiotic, steroid, or antifungal therapy during the previous three months, if they had a history of underlying systemic disease, or if they were HIV-seropositive or had any other condition that could potentially decrease their immunity. We also excluded oral cancer patients who were undergoing or had undergone radiation therapy or surgical treatment for an oral lesion.

Sample Collection

Samples were obtained by punch biopsy or surgical resection from 20 samples of OL, 20 samples of OSCC, and 20 samples of normal oral mucosa adjacent to OL or

oral cancer. These samples were each divided into two pieces. The first part was sent for routine histological examination. The second part was immediately stored at -80^{o} C until used. All H&E stained slides were reviewed by pathologist to determine histological differentiation of tissues according to the criteria described previously. The hyperplastic lesions (were classified according to the increased number of cells in the epithelium. Fresh tissue specimens obtained from oral cavity were immediately placed in RNA stabilizing reagent and frozen for cell extract preparation. The study protocol was divided into three major steps – RNA extraction, telomeric repeat amplification protocol (TRAP) assay, and gel electrophoresis.

RNA extraction

The tissue specimens placed in RNA stabilizing reagent and frozen were washed twice in cold phosphate‑buffered saline (PBS) by adding 500 μl PBS to the sample placed in microfuge tube and centrifuging at 5000 ×*g* for 1 min.

The tissue specimens were then treated with 1 ml Lysis Buffer and incubated on ice for 30 min. Cell debris was pelleted (12,000 ×*g* for 30 min at 4°C), and the supernatant was collected and frozen at −80°C for future use. Protein concentration of the tissue was determined using biophotometer (Eppendorf, USA). Aliquots of extracts containing 20 μg proteins were used for each TRAP assay.

Telomeric repeat amplification protocol assay

TRAP assay for test reactions (OSCC and normal oral mucosa samples) consisted of two steps. The aliquoted supernatant or cell extract was thawed to room temperature. A volume of 2.5 μl of cell extract was mixed with 1 μl of CX reverse primer and 6.5 μl of diethylpyrocarbonate (DEPC) to make the volume of the RNA primer mix to 10 μl. The above mix was incubated at 65°C for 10 min and immediately chilled on ice. This step allowed telomerase in the extract to synthesize telomeric oligonucleotides on TS primer.

Master mix was prepared by adding the components in the following order: 200 μl of reaction mix provided in the kit was added, followed by 16 μl each of TS forward primer and enzyme mix, respectively. Finally, 16 μl of DEPC water was added to complete the master mix composition.

15.5 μl of this master mix was added in each polymerase chain reaction (PCR) tube having 10 μl of RNA primer mix. In the second step, the telomerase‑synthesized new oligonucleotides were amplified using PCR by including reverse CX primer in the presence of deoxynucleotide triphosphates.

The above‑prepared final mix was placed in a thermocycler in which three‑step PCR was performed. The thermal cycler was preheated to 50°C before placing samples in it. The cycle conditions were 42°C for 30 min followed by 94°C for 15 min for denaturation process. The annealing step comprised 36 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec. Following the annealing step, extension was done at 72°C for 5 min and 4°C for 1 min. This final extension completed the unfinished ends. The products of reaction were removed from thermocycler and stored at 4°C for gel electrophoresis. Control reaction which was later used as internal standard for comparison of TA was repeated at several dilutions (1:2, 1:5, 1:10, 1:25, and 1:50) of control RNA template.

Gel electrophoresis

After 7 μl of bromophenol blue dye was added to each tube containing the PCR product, the products of the reaction (10 μl of each) were resolved by electrophoresis in a 12% non denaturing polyacrylamide gel containing ethidium bromide, in 10X TAE (Tris acetate ethylene diaminetetraacetic acid). 10 μl of control reaction PCR products in the above mentioned several dilutions were also resolved on the gel. The minimum dilution of control reaction at which detectable activity could be appreciated was 1 μg. Therefore, for each run of test reactions, a control reaction PCR product (1 μg) was included for later comparison. The gel was run for 2 hrs at 25 V and 16 mA. After separation of products in ladder pattern, the gel was removed and wet gel was scanned directly using 254 nm ultraviolet transilluminator.

Ethidium bromide that had bound specifically to nucleic acids depending on the molecular weight and concentration of the nucleic acid was excited by ultraviolet irradiation, and on excitation, it emitted fluorescent light which was captured by builtin camera.

Data analysis

Total Lab Phoretic Software was used for gel analysis. The sum of total integrated fluorescence intensities of telomerase ladders in each gel lane from test reaction was divided by the signal from the co-amplified internal standard (control RNA reaction) to get quantified telomerase levels. Since the TA of control RNA template was 1 μg, this was considered as the cut off value. The test reactions showing TA levels of >1 were considered positive and those showing TA levels of <1 were considered negative.

Statistical analysis

TA levels were compared between OSCC, OL and normal oral mucosa using Student's *t*‑test. Student's *t*‑test and ANOVA test were applied to validate the significance of the difference between groups.

Histological examination

The tissues were fixed in 4% paraformaldehyde solution, serially sectioned at 5 μm thickness and then stained routinely with hematoxylin and eosin (H&E) following standard procedures. The staining procedure for tissue sections with Haematoxylin and Eosin stain was as follows

The sections were:

- Deparaffinized on slide warming table
- Cleared in xylene.
- Hydrated through descending grades of alcohol :100%, 90%, 70%, 50% (5 minutes each)
- Stained with Harris Haematoxylin for 5 minutes
- Differentiated in 1% acid alcohol (one dip)
- Brought to running water for about 5 minutes
- Washed in water (blueing)
- Stained in Eosin Y for 2 minutes
- Dehydrated in ascending grades of alcohol (50%, 60%, 70%, 80%, 96%, 100%)
- Cleared in xylene
- Mounted with DPX

RESULTS

The results of the present study showed TA in 90% of OSCC, 35% of Oral Leukoplakia and 5% of normal oral mucosa. The TA levels ranged from 0.19 to 6.91 (mean 2.05, standard deviation [SD] 1.37) in OSCC, from 0.17 to 4.5 (mean 0.28, standard deviation 4.25) in OL and 0.21 to 1.09 (mean 0.54, SD 0.27) in normal oral mucosa. TA level differed significantly between OSCC, and OL (t = 3.9691, P = 0.0000).

The Student's t-test did not show statistically significant difference in the mean TA levels among males and females, among patients with or without adverse habits, and also did not differ among groups with different types of tobacco habits.

A statistically significant difference in TA according to overall clinical stage was not observed in the present study (P = 0.2703). The TA levels show pattern of increase with progression of clinical stage.

No obvious correlation was demonstrated with age, sex, history of risk factor exposure

Table:1 ANOVA test for comparison of telomerase activity in clinical stages of oral squamous cell carcinoma

OSCC stages	No. of patients	TA level (mean±SD)
I+II	2(10)	1.96±0.19
III	7(35)	1.63±1.2
IVA	9(45)	2.37±1.4
IVB	4(20)	2.19±1.9

Table: 2 ANOVA test for comparison of telomerase activity in clinical stages of oral leukoplaia

OLP Stages	No. of patients	TA level (mean±SD)
L0	7	1.47±0.29
L1	5	1.19±1.31
L2	13	2.47±0.17
L3	5	1.28±1.63

DISCUSSION:

Telomerase activity has been readily found in most cancer biopsies, in premalignant lesions or in normal tissue samples with a few exceptions in include germ cells and hemopoietic stem cells in which its activity is absent. It is concluded that activity of telomerase can be used as a biomarker for diagnosis of malignant oral cancer and a target for inactivation in chemotherapy or gene therapy. Its expression will also prove to be an important diagnostic tool as well as a novel target for cancer therapy. The activation of telomerase may be an important step in tumorgenesis which can be controlled by inactivating its activity during chemotherapy therapy. The expression and activity of telomerase are indispensable for cancer formation. In light of the role of hTERT in carcinogenesis, targeting hTERT can be a promising tool to inhibit cancer initiation and progression.

CONCLUSION

It was concluded that telomerase, a specialized reverse transcriptase enzyme, had a salient role in the process of tumorgenesis. Telomerase activity has been readily found in most cancer biopsies, in premalignant lesions or in germ cells. Activity of telomerase, is generally absent in normal tissues. It is known to be induced upon immortalization or malignant transformation of human cells such as in oral cancer cells. Maintenance of telomeres plays an essential role during transformation of precancer to malignant stage. The roles of telomeres in regulating both stability of genome and replicative immortality seems to contribute in essential ways in cancer initiation and progression. Its expression will also prove to be an important diagnostic tool as well as a novel target for cancer therapy. The activation of telomerase may be an important step in tumorgenesis which can be controlled by inactivating its activity during chemotherapy.

REFERENCES

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005;55:74-108.
2. Sankaranarayanan,R. et al. Chemoprevention of oral leukoplakia with vitamin A and beta carotene: an assessment. Oral Oncol. 1997;33, 231– 236.
3. Canniff JP, Harvey W, Harris M (1986). Oral sub mucous fibrosis: its pathogenesis and management. Br Dent J 160:429-434.
4. Maher R, Lee AJ, Warnakulasuriya KA, Lewis JA, Johnson NW 1994. Role of areca nut in the causation of oral submucous fibrosis: a case-control study in Pakistan. J Oral Pathol Med 23 :65-69.
5. Reichart PA, Philipsen HP: Oral submucous fibrosis in a 31-yearold Indian women: first case report from Germany. Mund Kiefer Gesichtschir. 2006, 10(3):192-196.
6. Thompson 3rd G.R, Patel P.K, Kirkpatrick W.R, Westbrook S.D, Berg D, Erlandsen J, ReddingS.W and Patterson T.F (2010). Oropharyngeal candidiasis in the era of antiretroviral therapy. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 109: 488–492.

7. Shafer, Hine, Levy. Text Book of Oral Pathology (1993). 4th edition. Philadelphia: W.B. Saunders Company.
8. Tilakratne WM, Klinikowski MF, Saku T, Peters TJ, Warnakulasuriya S: Oral sub mucous fibrosis: Review on aetiology and pathogenesis. Oral Oncol 2005, 30:30-32.
9. Mehrotra R, Pandya S, Chaudhary AK, Kumar M, Singh M (2008). Prevalence of oral pre-malignant and malignant lesions at a tertiary level hospital in Allahabad, India. Asian Pacific J Cancer Prev. 9: 263-266
10. Blackburn, E. H. Structure and function of telomeres. Nature (Land.), 350: 569-573, 1991
11. Fujiwara, M., Okayasu, I., Takemura, T., Tanaka, I., Masuda, R., Furuhata, Y., Noji, M., Oritsu, M., Kato, M., and Oshimura, M. Telomerase activity significantly correlates with chromosome alterations, cell differentiation, and proliferation in lung adenocarcinomas. Mod. Pathol., *13:* 723–729, 2000.
12. Hiyama, E., Hiyama, K., Yokoyama, T., Matsuura, Y., Piatyszek, M. A., and Shay, J. w. Correlating telomerase activity levels with human neuroblastoma outcomes. Nat. Med., 1: 249-257,1995.
13. Kono, S., Ikeda, M., Tokudome, S., and Kutratsune, M. A case-control study of gastric cancer and diet in northern Kyushu. Jpn. J. Cancer Res., *79:* 1067–1074, 1988
14. Ji, B. T., Chow, W. H., Hsing, A. W., McLaughlin, J. K., Dai, Q., Gao, Y. T., Blot, W. J., and Fraumeni, J. F. Jr. Green tea consumption and the risk of pancreatic and colorectal cancers. Int. J. Cancer, *70:* 255–258, 1997
15. Van der Waal I, Schepman KP, Van der Meij EH, Smeele LE, Oral leukoplakia: A clinicopathological review. Oral Oncol. 1997 Sep; 33(5):291-301
16. Blackburn, E. H. Structure and function of telomeres. Nature (Land.), 350: 569-573, 1991.
17. Feng, J., Funk, W. D., Wang, S., Weinrich, S. L., Avilion, A. A., Chiu, C., Adams, R. R., Chang, E., Allsopp, R. C., Yu, J., Le, S., West, M. D., Harley, C. B., Andrews, W. H., Greider, C. W., and Villeponteau, B. The RNA component of human telomerase. Science (Willhington DC), 269: 1236-1241, 1995.
18. Counter, C. M., Avilion, A. A., LeFeuvre, C. E., Stewart, N. G., Greider, C. W., Harley, C. B., and Bacchetti, S. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. EMBO J., 11: 1921- 1992.
19. Kim, N. M., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L C., Coviello, G. M., Wright, W. E., Weinrich, S. L, and Shay, J. W. Specific association of human telomerase activity with immortal cells and cancer. Science (Willhington DC), 226: 2011-2015, 1994.
20. Hiyama, E., Hiyama, K., Yokoyama, T., Matsuura, Y., Piatyszek, M. A., and Shay, J. w. Correlating telomerase activity levels with human neuroblastoma outcomes. Nat. Med., 1: 249, 257,1995.

Source of support: Nil **Conflict of interest:** None declared