e-ISSN: 0975-1556, p-ISSN:2820-2643

Available online on www.ijpcr.com

International Journal of Pharmaceutical and Clinical Research 2024; 16(1); 1597-1601

Original Research Article

Evaluation of Immunochemical Expression of Human Telomerase Reverse Transcriptase in Oral Leukoplakia and Oral Squamous Cell Carcinoma: A Case Control Study

Mahesh Nirujogi[1], Ayyagari Kameswara Rao[2], G. D. Anand Swaroop[3], Parameswar Peela[4], Sravya Kodati[5], Kuna Rajani[6]

[1]Dental Practitioner, Laasya Dental Clinic, Visakhapatnam
[2]Assistant Professor, Department of Dentistry, Andhra Medical College, Visakhapatnam
[3]Assistant Professor, Department of Dentistry, Rangaraya Medical College, Kakinada
[4]Assistant Professor, Department of Oral Pathology and Microbiology, ANIDS, Tagarapuvalasa, Visakhapatnam
[5]Assistant Professor, Department of Oral Pathology and Microbiology, Government Dental College and Hospital, Vijayawada
[6]Associate Professor, Department of Pathology, Government Medical College, Vizianagaram

Received: 25-10-2023 / Revised: 23-11-2023 / Accepted: 26-12-2023
Corresponding Author: Dr. Kuna Rajani
Conflict of interest: Nil

Abstract:

Background: Telomerase in association with oncogenes or with inactivation of tumor suppressor genes induces tumorigenic activity by inhibiting cellular senescence, promoting survival.

Aims and Objectives: The study was carried out to evaluate the Immunochemical expression of human telomerase reverse transcriptase (hTERT) in normal mucosa, Oral Leukoplakia (OLP) and Oral Squamous cell carcinoma (OSCC) patients by using Immunohistochemistry (IHC).

Settings and Design: The study was conducted in 40 individuals, including 10 healthy individuals, 15 individuals with clinical, histopathologically confirmed OLP and OSCC patients each.

Materials and Methods: Incisional biopsy was performed from the lesion proper and haematoxylin and eosin (H&E) staining was performed for confirmatory diagnosis, IHC was performed by using mouse monoclonal hTERT antibody.

Statistical Analysis: All the data was analyzed using SPSS software version 22.0. Nuclear labelling score (LS) was compared across the groups using T-test. The mean percentages of cells stained for normal, OLP, OSCC were compared using ANOVA.

Results: The Labelling Index (LI) for normal mucosa, normal to OLP and OSCC were 1.00±0.82, 2.20±1.01 and 2.93±0.59 respectively. The mean nuclear LI increased from normal (1.00±0.82), through normal to OLP (2.20±1.01) to OSCC (2.93±0.59). The mean nuclear Labelling Score (LS) increased from normal (1.00±0.82), through OLP (5.20±3.93) to OSCC (7.80±2.88). The mean LS was significantly higher in OSCC when compared to OLP.

Conclusion: The strong expression of hTERT in OLP and OSCC suggests that telomerase activity is involved in the development of dysplastic epithelium leading to multistage oral carcinogenesis.

Keywords: hTERT, OLP, OSCC, IHC.

Introduction

Head and neck cancers belong to a diverse group of malignancies and are the seventh most common malignancies worldwide, of which Oral Squamous Cell Carcinoma (OSCC) is the commonest one [1] and is the 6th most common cancer in all cancers.[3]

According to Global Cancer Observatory 2020, the highest annual incidence of OSCC is in Asia, followed by Europe and North America.[2] In India, incidence rates are 7-17 per 100,000 persons per year. 77,000 new cases develop in India and 52,000 die every year.[3] In India it ranks first in males and third in females among all cancer cases in many regions with a male to female ratio of 2:1.[3] The progression to OSCC requires multiple step mutations in somatic cells altering cellular growth, proliferation and differentiation. It is expressed as a progression from normal epithelium to OSCC through pre-cancerous lesions

clinically and histologically as mild, moderate, severe dysplasia to conventional OSCC.[4] One of such mutations is maintenance of Telomeric length. Telomeres are the DNA–protein complexes at chromosome ends; protect genome from degradation and interchromosomal fusion. Their length shortens after each DNA replication, and ultimately chromosomal degradation occurs and cell dies.[5]

Telomerase is an RNA dependant DNA polymerase enzyme complex with RNA template (hTR), an associated protein (hTP-1) and a catalytic sub unit (hTERT). It synthesizes telomeric DNA fragments using RNA moiety as template and compensates for loss of telomere during cell division. hTP-1 and hTR are expressed in both cancer and normal cells but hTERT is overexpressed only in cancer cells.[6] Postulations from previous studies indicate that reactivation of telomerase is vital for cellular immortalization causing genomic instability on its way of carcinogenesis.[7-10] This caused us to evaluate the over expression of hTERT in OLP and OSCC cases by IHC.

Study samples:

The study was conducted in 40 individuals, which included 10 healthy individuals, 15 individuals with clinical, histopathologically confirmed OLP and OSCC patients each.

Materials and Methods:

After taking approval by the Institutional Review Board regarding the study protocol, informed consent from all subjects enrolled in the study, incisional biopsy was performed from the lesion proper under local anesthesia, tissues were fixed in 10% neutral buffered formalin and wax embedded. Haematoxylin and eosin (H&E) staining was performed for confirmation of histopathological diagnosis. OLP and OSCC were histologically graded as mild, moderate, severe and well, moderately, poorly differentiated respectively according to WHO 2005 classification.

IHC protocol for detection hTERT:

IHC was performed using monoclonal hTERT antibody. Tissues were sectioned at 4μm thickness and mounted on polysilane coated slides. The sections were deparaffinized by heating on the slide warmer at 600c for 1 hour. The sections were dewaxed in 2 changes of xylene, each of 15 minutes, dehydrated in graded alcohol (100%, 90%, 80%, 70%) each for 10minutes and washed in running tap water for 2 minutes.

The slides were placed in EDTA antigen retrieval solution bath which is preheated in a Dako PT link. Preheating the antigen retrieval solution was done at 650 c for 20 minutes, followed by heating at 970c for 20 minutes and then cooled to 650c for 20 minutes. The slides were removed from PT tank and immediately dipped in jar containing diluted EnVision™ FLEX Wash Buffer (20X) at room temperature for 5 minutes.

Sections on slides were rounded, washed in wash buffer once and were covered with 3% Hydrogen peroxide for 10 minutes followed by washing with wash buffer for 3 times. The sections were incubated with mouse anti-hTERT monoclonal primary antibody (Novocastra Laboratories, New York, USA) at 1:40 dilution at room temperature for 1 hour.

The biotinylated goat anti-rabbit immunoglobulin was used as secondary antibody (Novocastra Peroxidase Detection system), slides were kept for 30 minutes at room temperature, followed by addition of streptavidin - peroxidase conjugate for 30 minutes at room temperature. The sections were then treated with diaminobenzidine hydrochloride for 5 minutes, counterstained with Harris hematoxylin and mounted with DPX. Tissues obtained from tonsils during tonsillectomy were used as positive controls and normal oral mucosa served as negative controls in the present study. Presence of brown colour in immunostained cells was considered as positive immunoreactivity.

hTERT expression:

The cellular localization, nuclear localization, nature, percentage of nuclei stained with hTERT antibody Labelling Index (LI), staining Intensity (SI) and nuclear labelling scores (LS) were studied using the criteria of Luzar etal.[7] The LI was calculated as percentage of hTERT stained cells per hundred cells counted. Counting was done using high 40x power in microscope. The inter-observer correlations were considered. The SI of the samples were graded and assigned numerical scores (0, 1, 2, 3 for no stain, mild, moderate and intense stain respectively). Three investigators performed the assessment independently and each investigator used the positive control as standard bench mark. The nuclear labelling scores (LS) of the samples were determined using the formula LI × SI.

Statistical analysis used: All the data was analyzed using SPSS software version 22.0. Along with the cellular, nuclear localization of hTERT or both, staining intensity (SI), percentage of nuclei stained with hTERT antibody (LI) and nuclear labelling score (LS) was compared across the groups using T-test. The mean percentage of cells stained for normal. OLP, OSCC were compared using Analysis of Variance (ANOVA). A $P<0.05$ was considered as statistically significant.

Results:

The demographic details of all the patients included in the study are shown in [Table 1]. In OLP- mild, moderate and severe epithelial dysplasia constituted about 27% (4/15), 40% (6/15) and 33%

(5/15) respectively. In OSCC- well, moderate and poor differentiated were about 33% (5/15), 53% (8/15) and 13% (2/15) respectively.

hTERT staining characteristics:

Nuclear staining was observed in 40.0% (6/15) and combined nuclear and cytoplasmic staining in 60.0% (9/15) OLP samples. Nuclear staining was observed in 66.7% (10/15) and combined nuclear and cytoplasmic staining in 33.3% (5/15) OSCC samples. The difference in the cellular localization of hTERT immunostain in OLP and OSCC was statistically significant (P<0.01). However the cellular localization and intensity of hTERT stain across the grades of dysplasia and OSCC were not statistically significant (Figure1).

In OLP 26.7% (4/15) of the cases had mild staining, 40% (6/15) moderate staining, while 33.3% (5/15) exhibited intense staining. In OSCC cases 46.7% (7.15) showed moderate staining, while 53.4% (8/15) exhibited intense staining. The statistical difference of staining intensity from normal to OLP, normal to OSCC is highly significant. (Table 2) The Labelling Index (LI) for normal mucosa, normal to OLP and OSCC were 1.00±0.82, 2.20±1.01 and 2.93±0.59 respectively. The mean LI was significantly higher in OSCC when compared to OLP. The mean nuclear Labelling Score (LS) increased from normal (1.00±0.82), through OLP (5.20±3.93) to OSCC (7.80±2.88) and is statistically significant (Table 3).

Table 1: Demographic details of study participants

Groups	Histological grading	Frequency	Percentage
OLP	Mild	4	26.7
	Moderate	6	40
	Severe	5	33.3
	Total	15	100
OSCC	Moderately differentiated OSCC	5	33.4
	Well differentiated OSCC	8	53.3
	Poorly differentiated OSCC	2	13.3
	Total	15	100

Table 2: Staining Intensity (SI) between three groups

SI	Mean	SD	P value	Inference
Normal	0.7	0.48	<0.01	Highly significant
OLP	2.07	0.80		
OSCC	2.60	0.63		

Table 3: Labelling Index (LI) and labelling score (LS) between three groups

LI	Mean	SD	P value	Inference
Normal	1.00	0.82	<0.01	Highly significant
OLP	2.20	1.01		
OSCC	2.93	0.59		
LS	**Mean**	**SD**	**P value**	**Inference**
Normal	1.00	0.82	<0.01	Highly significant
OLP	5.20	3.93		
OSCC	7.80	2.88		

Discussion:

Escape from death crisis or evasion of apoptosis is one of the hallmarks of a cancer cell. Maintenance of telomeric length is the prime reason for immortality of a cell. The cancer cell does that by various mechanisms including activation of telomerase or by genetic homologous recombination.[5] Telomerase is an RNA dependant DNA polymerase enzyme complex with RNA template (hTR), an associated protein (hTP-1) and a catalytic sub unit (hTERT). It synthesizes TTAGGG telomeric DNA sequences using RNA moiety as template and compensates for loss of telomere during cell division.[9,11]

The most widely used strategy for detecting telomerase activity is the Telomerase Repeat Amplification Protocol (TRAP) assay and RT-PCR. By these methods, though telomerase activity can be detected even in few immortal cancer cells, cellular and nuclear localization cannot be done, which is a possibility with hTERT and IHC [9-12] and so in the present study, IHC expression of hTERT protein was analysed and compared among normal, OLP and OSCC samples.

The present study was conducted on 40 individuals, which included 10 healthy individuals, 15 individuals with clinical, histopathologically confirmed OLP and OSCC patients each whereas Palani J et al[6] conducted study on 10 normal, 15 OLP, 30 OSCC cases, while Raghunandan BN et al[9] performed the same study in 10 normal, 21 OLP and 20 OSCC cases and Mutirangura et

al.[14] performed in 26 OLP cases and 14 OSCC cases.

In the present study, nuclear staining was observed in 40.0% and combined nuclear and cytoplasmic staining in 60.0% OLP samples. Nuclear staining was observed in 66.7% and combined nuclear and cytoplasmic staining in 33.3% OSCC samples. Whereas study conducted by palani et al [6] only one sample of normal oral mucosa had cytoplasmic staining, while in OSCC, OLP and oral sub mucous fibrosis tissues, hTERT cytoplasmic expression ranged from 13-33%. However Chen et al [10] showed a correlation between cytoplasmic and nuclear hTERT expression in histologically different grades of OSCC, suggesting that hTERT expression was a biomarker for this type of lesion. The authors reported that cytoplasmic hTERT was increased in OLP and OSCC compared to normal epithelium, whereas nuclear hTERT was decreased in OSCC. The roles of hTERT in the cytoplasm have not as yet been clarified. Some reports, however, implicate cytoplasmic h TERT in the inhibition of apoptosis. Many studies have shown that intracellular translocation of hTERT from the cytoplasm to the nucleus constitutes one of the mechanisms involved in telomerase activation and that nuclear localization is associated with phosphorylated hTERT protein.[11]

In the present study the normal cells (5/10) showed nuclear positivity that too in the basal layer only and remaining samples didn't take positive hTERT staining whereas in the previous studies of Kumar et al.[12] reported that more than 50% of the cells showed hTERT expression in the basal and parabasal layers which are the normal proliferative progenitor compartment and contain stem cells which have hTERT activity. Abrahao et al.[13] reported 100% (5/5) in oral epithelial hyperplasia without dysplasia.

In the present study all cases of OLP have taken positive staining as similar results observed in the study conducted by kumar et al.,[12] and Abrahao et al.,[13] whereas it is not similar to the studies like Mutirangura et al.[14] where they reported telomerase expression in 43% (10/26) of OLP. In the current investigation, all OLP cases were hTERT positive, and no correlation was found between hTERT and dysplasia grade. We suggest that the intense hTERT staining in the OLP cases may reflect a high proliferative cell capacity in oral lesions with epithelium presenting as dysplasia.

In the present study the normal tissues the percentage of hTERT positive expressing cells was less as compared to study conducted by Raghunandan, et al.,[9] the mean percentages of hTERT positive cells in normal oral mucosa samples were 62.91%. Kumar et al.[12] reported that 100% (10/10) cases of OSCC expressed hTERT.

Abrahao et al.[13] reported 85.5% of hTERT positive cells in their study of well to poorly differentiated OSCCs. In the study of Raghunandan et al.,[9] 61.90% (13/21) of oral epithelial dysplasia, 65% (13/20) of OSCC showed significant nuclear staining and some cases showed combined nuclear and cytoplasmic staining which is not similar to present study in which we have observed only 40% and 33.7% cases were nuclear staining alone in OLP and OSCC respectively.

In the study conducted by Palani et al,[6] the mean LI across the grades of dysplasia did not reveal any statistical significance, they observed that the increase in mean nuclear LS from mild (60.2±20.9), to moderate (93.8±17.2) to severe (108.6±3.21) dysplasia was statistically significant (P=0.004). But in this present study the mean LI and LS across the grades of dysplasia did not reveal any statistical significance.

In this study the mean nuclear LI increased from normal, through OLP to OSCC. The present study reveals the increased LI and LS from the normal to OSCC through OLP like in the study of Palani et al., implying that hTERT expression increases during malignant transformation.

Conclusion

The intense expression of hTERT in OLP and OSCC suggests that telomerase activity is involved in the development of dysplastic epithelium leading to multistage oral carcinogenesis. The biological significance of hTERT expression in differentiation of committed and proliferating cells needs further studies with increased sample size to determine if hTERT may be a useful diagnostic or prognostic marker in OLP and OSCC.

References

1. Mody MD, Rocco JW, Yom SS, Haddad RI, Saba NF. Head and neck cancer. Lancet. 2021 Dec 18; 398(10318):2289-2299.
2. Oral cancer - the fight must go on against all odds. Evid Based Dent. 2022 Mar; 23(1):4-5.
3. Borse V, Konwar AN, Buragohain P. Oral cancer diagnosis and perspectives in India. Sens Int. 2020; 1:100046.
4. Shafer, Hine, Levy. Text book of Oral Pathology. 7th Ed. New Delhi: Elsevier; 2012. Chapter 2: Benign and malignant tumors of the oral cavity: p.89-121.
5. Shammas MA. Telomeres, lifestyle, cancer and aging. Curr Opin Clin Nutr Metab Care. 2011; 14(1):28–34.
6. Palani J, Lakshminarayanan V, Kannan R. Immunohistochemical detection of human telomerase reverse transcriptase in oral cancer

and pre-cancer. Indian J Dent Res 2011; 22:362.
7. Luzar B, Poljak M, Gale N. Telomerase catalytic subunit in laryngeal carcinogenesis-an immunohistochemical study. Mod Pathol 2005; 18:406-1.
8. Downey MG, Going JJ, Stuart RC, Keith WN. Expression of telomerase RNA in oesophageal and oral cancer. J Oral Pathol Med 2001; 30:577-81.
9. Raghunandan BN, Sanjai K, Kumaraswamy J, Papaiah L, Pandey B, Jyothi BM, et al. Expression of human telomerase reverse transcriptase protein in oral epithelial dysplasia and oral squamous cell carcinoma: An immunohistochemical study. J Oral Maxillofac Pathol. 2016; 20(1):96-101.
10. Chen HH, Yu CH, Wang JT, Liu BY, Wang YP, Sun A, et al. Expression of humantelomerase reverse transcriptase (hTERT) protein is significantly associated with the progression, recurrence and prognosis of oral squamous cell carcinoma in Taiwan. Oral Oncol 2007; 43:122-9.
11. Cong YS, Wright WE, Shay JW. Human telomerase and its regulation. Microbiol Mol Biol Rev 2002; 66(3):407-25.
12. Kumar SK, Zain RB, Ismail SM, Cheong SC. Human telomerase reverse transcriptase expression in oral carcinogenesis – A preliminary report. J Exp Clin Cancer Res 2005; 24:639-46.
13. Abrahao AC, Bonelli BV, Nunes FD, Dias EP, Cabral MG. Immunohistochemical expression of p53, p16 and hTERT in oral squamous cell carcinoma and potentially malignant disorders. Braz Oral Res 2011; 25:34-41.
14. Mutirangura A, Supiyaphun P, Trirekapan S, Sriuranpong V, Sakuntabhai A, Yenrudi S, et al. Telomerase activity in oral leukoplakia and head and neck squamous cell carcinoma. Cancer Res 1996; 56:3530-3.