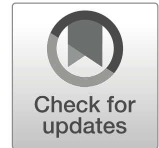
Pathology & Oncology Research (2020) 26:1573–1582
https://doi.org/10.1007/s12253-019-00700-6

ORIGINAL ARTICLE



Expression of hTERT in Oral Submucous Fibrosis and Oral Squamous Cell Carcinoma – an Immunohistochemical Analysis

Lizbeth Raju K[1] · Vanishri C. Haragannavar[1] · Shankargouda Patil[2] · Roopa S. Rao[1] · Tejavathi Nagaraj[3] · Dominic Augustine[1] · Sowmya S. Venkatesiah[1] · Shwetha Nambiar[1]

Received: 28 March 2019 / Accepted: 17 July 2019 / Published online: 5 September 2019
© Arányi Lajos Foundation 2019

Abstract

Human telomerase reverse transcriptase enzyme, the catalytic subunit of telomerase are seen to be frequently reactivated in cancers including Oral squamous cell carcinoma (OSCC). Increased hTERT expression have been seen in potentially malignant conditions including Oral submucous fibrosis (OSMF). The aim of the study was to evaluate the expression levels in OSMF, OSCC in the background of OSMF and OSCC using immunohistochemistry and also to correlate hTERT expression with clinicopathologic parameters. A total of 50 histopathologically diagnosed cases of 20 OSMF, 20 OSCC wherein 5 were OSCC in the background of OSMF and 10 Normal oral mucosae were retrieved from the departmental archives and subjected to immunohistochemical analysis of hTERT. The expression of hTERT increased from normal, OSMF, to OSCC with statistically significant differences in mean labelling score (LS). We also found a shift in cellular localization of stain where, normal mucosal tissues showed a nuclear stain unlike OSMF, where combined nuclear and cytoplasmic staining as noted. The tumor cells in OSCC showed predominant cytoplasmic staining. There was no correlation between hTERT expression and clinicopathological parameters of OSMF. However, a significant increase of hTERT expression was seen with increasing histological grading of OSCC. These results suggest the role of hTERT in the early event of malignant transformation of OSMF. Telomerase could be used as a potent diagnostic marker to identify high-risk group of OSMF.

Keywords Biomarkers · Carcinogenesis · Immunohistochemistry · Oral submucous fibrosis · Squamous cell carcinoma · Telomerase

✉ Shankargouda Patil
dr.ravipatil@gmail.com

Lizbeth Raju K
lizbethraju17@gmail.com

Vanishri C. Haragannavar
vani.haragannavar@gmail.com

Roopa S. Rao
drroopasrao1971@gmail.com

Tejavathi Nagaraj
tejavathi.6@gmail.com

Dominic Augustine
dominicgerman@gmail.com

Sowmya S. Venkatesiah
drsowmya@yahoo.co.in

Shwetha Nambiar
drshwethas83@gmail.com

[1] Department of Oral Pathology and Microbiology, Faculty of Dental Sciences, Ramaiah University of Applied Sciences, M S R Nagar, Bangalore, Karnataka 560054, India

[2] Department of Maxillofacial Surgery and Diagnostic Sciences, Division of Oral Pathology, College of Dentistry, Jazan University, Jazan, Saudi Arabia

[3] Department of Oral Medicine and Radiology, Sri Rajiv Gandhi College of Dental Sciences & Hospital, Cholanagar, R.T. Nagar post, Bangalore, Karnataka 560 032, India

Introduction

Among neoplasias, head and neck neoplasias ranks as the 6th most commonly occurring cancer across the globe [1]. Oral squamous cell carcinoma comprises of more than 90% of cancers that occur in upper aerodigestive tract. More than 180,000 cases of mouth neoplasms occur every year in the South and South-East Asian population, of which the majority are habit related i.e. smoking and tobacco chewing [2]. Despite advances in medicine, mortality and morbidity of oral cancer remains unchanged. However, early detection and treatment has been proved to be promising.

Oral squamous cell carcinoma (OSCC) is most often preceded by potentially malignant disorders such as leukoplakia, erythroplakia or oral submucous fibrosis (OSMF). OSCC arising from OSMF are considered as a distinct disease clinicopathologically, owing to the differential mechanism of tumorigenesis when arecanut is involved. The malignant transformation rate of OSMF was assessed to be 7% to 13% [3, 4]. Studies have reported that OSCC arising from OSMF were clinically more aggressive with a relatively poorer prognosis than frank OSCC without a history of OSMF. The exact mechanism of malignant transformation of OSMF to OSCC is yet to be elucidated. To explore the possible mechanisms of malignant transformation, attempts have been made to come up with a panel of molecular markers that aid in early diagnosis and equally have therapeutic pertinence.

Carcinogenesis is a complex process involving multiple genetic and epigenetic alterations. Irrespective of the etiologic factor, most malignant cell transformation is dictated by changes in various fundamental cell physiology - hallmarks of cancer [5]. One among these hallmarks is the limitless replicative potential governed by "Telomeres". Telomeres are nucleoprotein complexes present at the chromosomal ends consisting of multiple TTAGGG repeats. Normal cells have limited replicative potential due to the end replication problem piloted by shortening of the telomeres. Telomerase is a specific enzyme that prevents shortening of telomeres by maintenance and de novo synthesis. Telomerase consists of catalytic subunit hTERT (human Telomerase reverse transcriptase) and an RNA unit (hTR) which acts as a prime for telomeric repeat synthesis. Expression levels of hTR and hTERT have been looked upon in order to assess the activity of telomerase. hTR expression has been seen to be positive in most of the cells thus rendering it non-specific for assessing telomerase activity. On the other hand, hTERT correlated with the telomerase activity in a cell and higher expression has been observed in germ cells, embryonic cells as well as cancer cells.

Increased telomerase activity is noted in many cancers including oral squamous cell carcinoma as well as potentially malignant disorders such as oral epithelial dysplasia. Studies have suggested increased telomerase activity as an early event of oral carcinogenesis and thus serve as a diagnostic marker. Limited studies have been done on assessing the telomerase activity on oral submucous fibrosis and cancers arising from the later. Expression of hTERT have been primarily assessed in OSCC tissue samples by means of PCR based TRAP (Telomerase repeat amplification protocol) assay. Despite its high sensitivity, it does not allow the evaluation of cellular localization of hTERT. Moreover, usage of TRAP assay is limited to fresh samples thus making it inapplicable for retrospective studies using archived tissue samples. The aim of the current study was to evaluate the expression levels of hTERT in archives of OSMF, OSCC in the background of OSMF and OSCC cases by means of immunohistochemistry and to correlate the expression with clinicopathologic parameters.

Methods and Methodology

Tissue Samples A total of 50 histopathologically diagnosed cases of 10 healthy oral mucosa,20 OSMF and 20 OSCC which included 5 OSCC in the background of OSMF, were retrieved from the archives (November 2015–March 2018) of Department of Oral Pathology, Faculty of Dental Sciences, Ramaiah University of Applied sciences, Bengaluru, India. OSMF and OSCC samples were graded based on histopathological criteria given by Khanna et al. [6] and Broder's criteria [7] respectively. The cases of OSMF and OSCC with a chronic habit history of smokeless tobacco and/or betel quid chewing for more than 5 years were included. Recurrent cases of OSCC, patients with any systemic disorders such as diabetes, hypertension or other metabolic disorders were excluded. The normal oral mucosa samples (control) were obtained from subjects without any oral habits during extraction of permanent impacted mandibular third molars with due patients consent. The study protocol was reviewed and approved by the institutional ethical clearance board (FDS/EC/2014–16/PGST/29).

Immunohistochemistry Immunohistochemical analysis using peroxidase labelled streptavidin-biotin technique was performed to evaluate the expression of hTERT in the selected formalin fixed paraffin embedded tissue samples. Serial sections of 4 μm were cut and mounted on glass slides coated with poly-L-lysine (Biogenex). Sections were then sequentially deparaffinised with xylene and rehydrated with decreasing grades of ethanol. Slides were further immersed in 0.3% of hydrogen peroxide in methanol in order to quench the endogenous peroxidase activity. After washing the slides in running water and phosphate buffered saline, antigen retrieval was carried out by submerging in 0.01 M citrate buffer (pH 6.0) and microwaving for 10 min. This was followed by washing and incubating with 1% goat serum albumin to impede non-specific binding. Sections were then incubated with anti hTERT overnight at 4 °C with a dilution of 1:25 (NCL-hTERT; Novo Castra, Newcastle, UK). After rinsing the

sections in PBS, they were subjected to biotinylated secondary antibodies (Novo Castra Peroxidase Detection system) followed by incubation with streptavidin-peroxidase conjugate. The reaction compounds were then visualized by treating with 0.02% of DAB (diaminobenzidine hydrochloride) which imparted a brown color. Sections were further counterstained lightly with Mayers hematoxylin, mounted in xylene based medium and observed under light microscope.

Immunohistochemical Evaluation hTERT is basically a nuclear stain. However, cytoplasmic expression was also considered as positive in the current study since literature review suggests the presence of expression in both nucleus and cytoplasm in cancer cells [8]. The slides were blinded and interpreted using a semi-quantitative method by two observers. A minimum of 5 random high power fields were selected for evaluation using criteria given by Luzar et al., 2004 [9], wherein several parameters were considered as shown in Table 1. Sections exhibiting an interobserver variation of greater than 10% were re-examined by a third observer to arrive at a consensus.

Statistical Analysis The mean hTERT LSs for all the samples were compared among each other by "Analysis of variance" (ANOVA) and pairwise comparison in each group was done by Student t-test. Fishers exact test was employed to compare LI, SI, cellular localization and tissue localization among the groups. Correlation between clinicopathological parameters and LS of hTERT was analysed by Chi-square test. A p value of less than 0.05 was considered to be statistically significant. The statistical tests were executed using "Statistical Package for the Social Sciences" (SPSS)- version 22.

Table 1 Criteria for staining evaluation [9]

Parameters	Microscopic findings/ score
Cellular localisation of the stain	Nuclear
	Cytoplasmic
	Both
Staining intensity (SI)	0- Negative
	1- Weakly positive
	2- Moderately positive
	3- Strongly positive
Percent of cells positive (Labelling index -LI)	Grade 0- Negative
	Grade 1- <10%
	Grade 2–10-30%
	Grade 3–30-60%
	Grade 4- > 60%
Labelling score (LS)	LI x SI
Tissue localization (OSMF and NOM)	Basal and Supra basal
	Entire epithelium

Results

Demographic Details A summary of the demographic details of the patients considered for the study is given in Table 2. Of 15 OSCC cases,5/15(33.3%) each were well differentiated, moderately differentiated and poorly differentiated squamous cell carcinomas. Among the OSMF cases, 6/20 (40%) were early OSMF, 6/20(40%) were moderately advanced and 8/20 (60%) were advanced OSMF. Among 5 OSCC cases that occurred in the background of OSMF, 3 were well differentiated, 1 poorly differentiated and 1 was early invasive squamous cell carcinoma.

Immunohistochemical Expression of hTERT in Tissue Samples The immunostained sections were evaluated based on the selected criteria. Both nuclear and cytoplasmic staining of hTERT were considered positive in OSMF as well as OSCC samples. Figures 1 and 2 illustrated the hTERT staining in OSMF and OSCC tissue samples.

OSCC The mean LS for OSCC was 8.67 ± 2.22. Mean LS increased from WDSCC to PDSCC, with a statistically significant difference between WDSCC and PDSCC (p = 0.031). Grade IV labelling indices was seen in 80% (12/15) cases and 20% cases showed Grade III LI with no significant difference among the grades. Moderate staining intensity was seen in 73.3% (11/15) cases and the rest 26.7% (4/15) cases demonstrated intense staining intensity (Table 3). hTERT staining was localized to the cytoplasm in majority of cases, i.e. 86.7% (13/15) and combined nuclear + cytoplasmic staining was observed in only 13.3% (3/15) of OSCC cases.

OSMF The mean LS for OSMF was 6.15 ± 1.98 which increased with advancing histologic grade (Table 3). Whatsoever, differences were not statistically significant (p = 0.60). Evaluation of Labelling indices revealed no significant differences among grades where 60% (12/20) had Grade IV LI and 40% (8/20) cases exhibited Grade III LI. 30% (6/20) cases of OSMF displayed a mild staining intensity and 70% (14/20) cases showed moderate staining intensity. Combined

Table 2 Demographic details of selected samples

Groups	Mean age ± Std.Dv	Gender	
		Female n (%)	Male n (%)
Normal	40.20 ± 23.61	8(80)	2(20)
OSCC	52.07 ± 13.77	8(53.3)	7(46.7)
OSMF	40.10 ± 12.39	6(30)	14(70)
OSMF with OSCC	48.20 ± 7.63	2(40)	3(60)

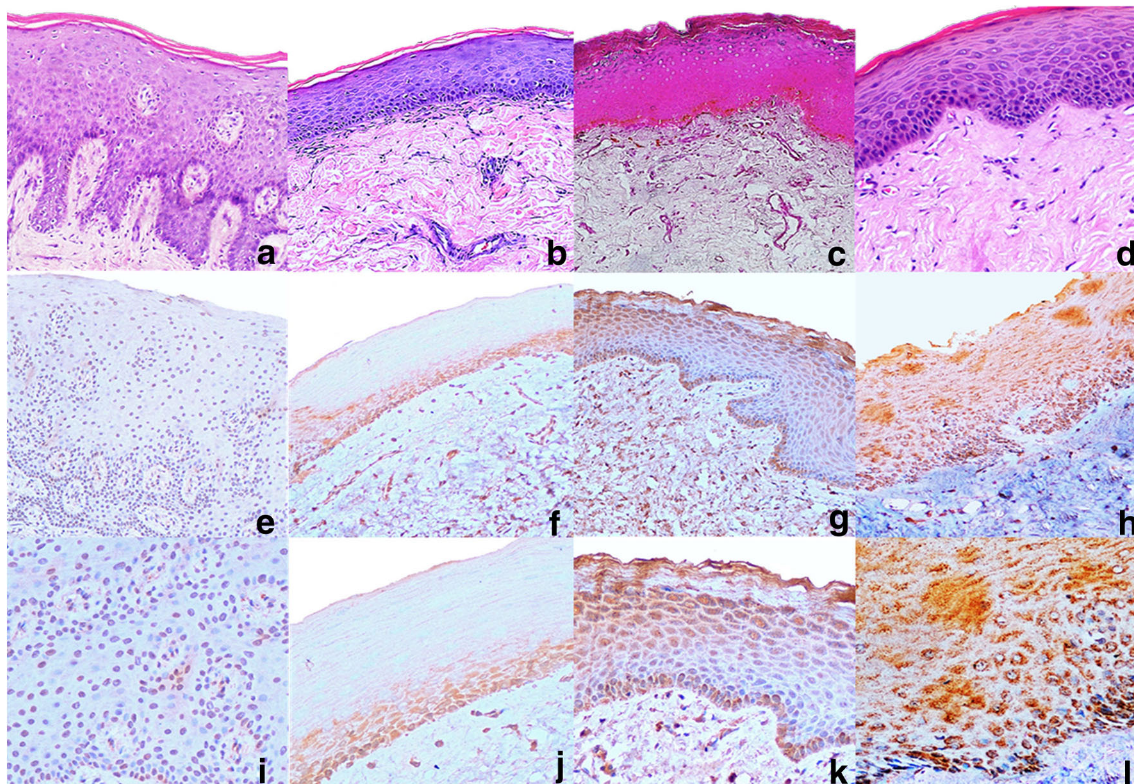


Fig. 1 **h** & **e** and hTERT immunostained photomicrographs of Normal oral mucosa (**a**, **e** and **i**), Early OSMF (**b**, **f** & **j**), Moderately advanced OSMF(**c**, **g** & **k**) and Advanced OSMF (**d**, **h** & **l**). Normal mucosa shows weak nuclear positive staining restricted to the basal and suprabasal layers. Early OSMF tissues shows a weak to moderate positive staining restricted to the basal and supra basal layers. Moderately advanced OSMF and Advanced OSMF shows a moderate nuclear and cytoplasmic staining throughout the epithelium

nuclear and cytoplasmic hTERT staining was observed in 85% (17/20) cases and the remaining 3 cases showed cytoplasm staining. On evaluating tissue localization of hTERT, 85% of the cases showed expression throughout the

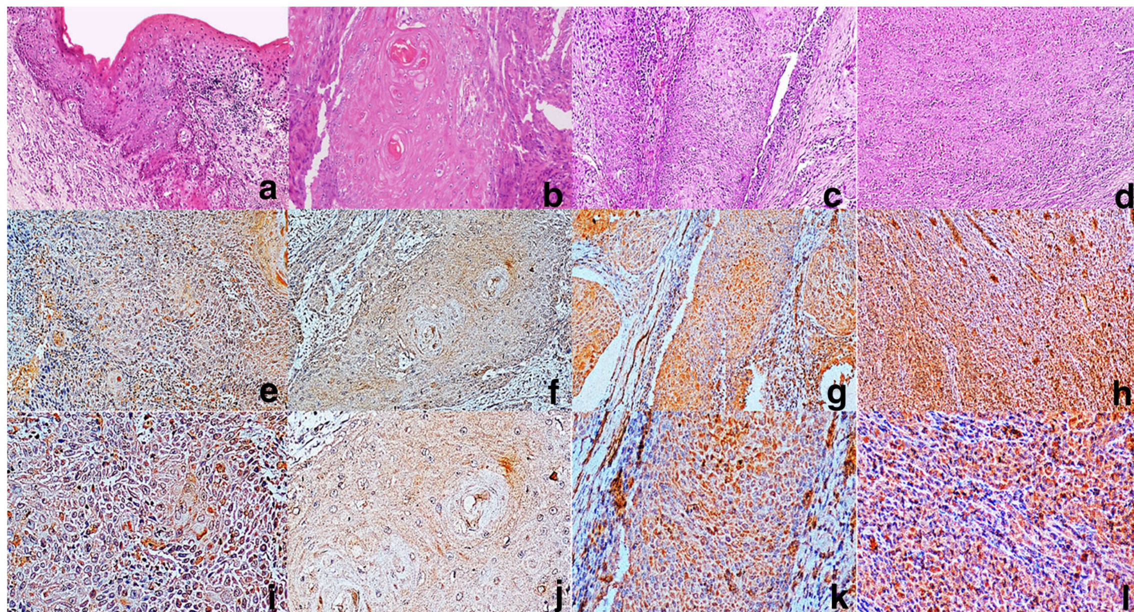


Fig. 2 **h** & **e** and hTERT immunostained photomicrographs of OSCC in the background of OSMF (**a**, **e** and **i**), WDSCC (**b**, **f** & **j**), MDSCC (**c**, **g** & **k**) and PDSCC (**d**, **h** & **l**). Tissues of OSCC in the background of OSMF showed a weak moderate nuclear and cytoplasmic immunostaining the tumor islands. WDSCC shows a predominant weak to moderate cytoplasmic staining in the tumor islands. MDSCC and PDSCC showed a moderate to strong cytoplasmic immunostaining in tumor tissue

Table 3 Means of hTERT expression in tissue samples

Groups		hTERT labelling score		p value
	No. of cases	Mean	Std. Deviation	
Normal	10	2.60	.548	
OSCC	15	8.67	2.225	0.000
OSMF	20	6.15	1.981	
OSMF with OSCC	5	7.20	1.095	

thickness of the epithelium and the rest displayed expression restricted to basal and suprabasal layers. Moderately advanced and advanced cases of OSMF demonstrated hTERT staining throughout the epithelial thickness as opposed to early OSMF where staining restricted to basal and suprabasal layers were noted in 50% of cases($p = 0.035$).

OSCC with OSMF Only five cases of OSCC with OSMF were taken due to the scarcity of such cases and a mean LS of 7.20 ± 1.095 was observed. Grade III LI was seen in 2 cases and the other 3 cases showed Grade IV LI. Moderate staining intensity with combined nuclear and cytoplasmic expression was seen in all the five cases.

Normal Oral Mucosa The mean Labelling score was 2.60 ± 0.548 with all the cases showing mild nuclear hTERT expression restricted to the basal and suprabasal layers.

Comparison of hTERT Expression among the Different Groups The expression levels of hTERT increased from normal through OSMF, OSCC in the background of OSMF and OSCC samples with a statistically significant difference in the mean LS ($p = 0.000$) (Fig. 3, Table 4). The mean LS of OSCC and OSMF differed significantly among each other ($p = 0.002$). Whatsoever they did not differ significantly with OSCC arising from OSMF. Similar findings were observed for staining intensity. All the lesional tissues showed a significant difference in SI when compared to normal wherein the later exhibited weak staining intensity. Marked difference of SI was also noted between OSCC and OSMF but not with the transforming cases. This indicates the role of hTERT in multistage carcinogenesis arising from OSMF. On evaluation of LI, majority of lesion tissues showed a higher grade of LI when compared to normal, where Percent of cells stained ranged from 0 to 10%. No significant difference in LI was observed among lesional tissues. Another intriguing find was the statistically significant change in subcellular localization of hTERT stain among the normal and diseased

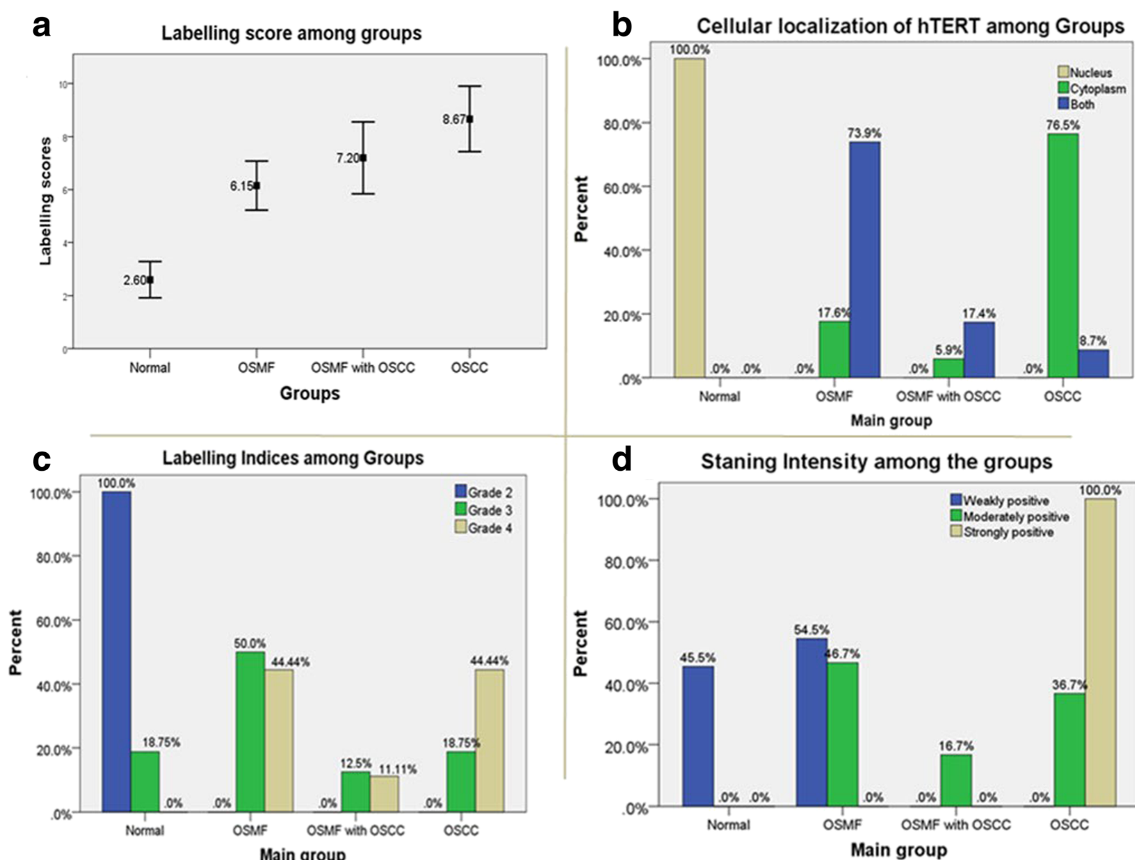


Fig. 3 **a** Box-plot graph showing labelling score among each group. **b**, **c** & **d** Bar graph depicting cellular localization of stain, labelling index and staining intensity among the groups respectively

Table 4 Group wise comparision of hTERT labelling scores in tissue samples

Group 1 vs Group 2		Mean difference	*p* value
Normal	OSMF	−3.550	.003*
	OSMF with OSCC	−4.600	.003*
	OSCC	−6.067	.000*
OSMF	OSMF with OSCC	−1.050	.693
OSMF with OSCC	OSCC	−1.467	.455
OSCC	OSMF	2.517	.002*

tissues ($p = 0.000$). hTERT staining was localized to the nucleus in normal mucosa, which changed to combined nuclear and cytoplasmic stain in OSMF and transforming cases, followed by OSCC tissues exhibiting a predominant cytoplasmic staining.

Correlation of hTERT Expression with Clinicopathological Parameters hTERT expression in OSMF did not differ significantly with age, gender or histological grades (Table 5). In OSCC, no statistically significant correlation was seen between hTERT and few clinicopathological parameters such as age, gender, tumor stage and lymph node metastasis (Table 6). However, hTERT expression differed markedly in cancers arising from gingivo-buccal sulcus (10 ± 2.828) as compared to those arising from the tongue (8.80 ± 1.789) and buccal mucosa(7.20 ± 1.033) ($p = 0.30$). In addition to the site, histological grading affected the hTERT expression substantially. PDSCC exhibited an increased LS when compared to WDSCC and Invasive carcinoma ($p = 0.047$).

Discussion

Telomeres are specialized DNA strands positioned at the ends of chromosomes composed of 5′-TTAGGG-3' repeats [10]. The constantly replicating cells such as those in germ layers and the bone marrow, overcome the wearing away

Table 5 Clinico-pathologic correlation of hTERT expression in OSMF

	Mean hTERT LS ± SD	p value
Age		
<50 (*n* = 17)	6.35 ± 1.869	0.287
≥50 (n = 3)	5.00 ± 2.646	
Gender		
Male (*n* = 14)	6.43 ± 1.910	0.350
Female (n = 6)	5.50 ± 2.168	
Histological grades of OSMF		
Early OSMF (n = 6)	5.50 ± 2.168	0.600
Moderately Advanced OSMF (*n* = 6)	6.17 ± 2.229	
Advanced OSMF (n = 8)	6.63 ± 1.768	

Table 6 Clinico-pathologic correlation of hTERT expression in OSCC

	Mean hTERT LS ± SD	p value
Age		
<50 (n = 9)	8.67 ± 2.646	0.491
≥50 (*n* = 11)	8.00 ± 1.549	
Gender		
Male (n = 10)	8.80 ± 2.348	0.295
Female (*n* = 10)	7.80 ± 1.751	
Location		
Gingivo-buccal sulcus (*n* = 5)	10.00 ± 2.828	0.030*
Buccal mucosa (n = 10)	7.20 ± 1.033	
Tongue (*n* = 5)	8.80 ± 1.789	
T status		
T1 + T2 (*n* = 12)	8.33 ± 2.389	0.933
T3 + T4 (*n* = 8)	8.25 ± 1.669	
N status		
N0 (n = 17)	8.47 ± 2.183	0.397
N1 + N2 + N3 (*n* = 3)	7.33 ± 1.155	
Clinical staging		
Stage 1 + 2 (*n* = 11)	8.55 ± 2.382	0.574
Stage 3 + 4 (*n* = 9)	8.00 ± 1.732	
Histology of SCC		
Invasive Carcinoma (n = 1)	6.0 ± 0.00	0.047*
WDSCC (n = 8)	7.25 ± 1.035	
MDSCC (n = 5)	8.40 ± 2.191	
PDSCC (*n* = 7)	10.00 ± 2.191	

of telomere by a ribonucleoprotein complex called telomerase. Telomerase activity is looked upon by the presence of two major subunits namely hTERT and hTR. Most somatic cells and cancer cells show hTR expression, whereas hTERT expression is almost entirely constrained to cancer cells and stem cells. Moreover, hTERT expression shows a strong correlation with telomerase activity [11]. Somatic cells have a decreased telomerase expression [12]. Studies have noted that mere addition of hTERT gene into telomerase negative cells induce activation of telomerase, thereby causing immortalization of cells which would have otherwise undergone cellular senescence [13, 14].

Role of Telomerase in Carcinogenesis Telomerase reactivation occurs in 85–90% of cancer, thus making it an eminent cancer biomarker and therapeutic target [15]. In particular, evidence show OSCC cell lines and tissue samples to have a significantly increased telomerase activity [8, 16–23]. Malignant cells might possibly offset the shortening of telomeres by means of increasing telomerase activity rendering its need for unlimited replicative potential. The exact molecular mechanism behind such activation is not understood. However, recent findings suggest mutations in telomerase promoter genes (228C > T and 250C > T) to be frequently noticed in cancers and correlated with high telomerase activity [24].

These mutations extricate cellular differentiation and silencing of telomerase, eventually leading to increased telomerase transcriptional activity. OSCC, Laryngeal SCC and tongue carcinomas have shown significant TERT promoter mutations [25–27]. Furthermore, TERT expression is induced in cancer cells by several transcription factors including NF- B [28], β-catenin [29] and c-myc [30]. Additionally, wild type TP53 down-regulates TERT by forming complexes with its transcription activator Sp1 [31]. Any mutations in TP53 (seen in most cancers) could possibly increase TERT expression. Goessel et al. recently created a cellular model of oro-oesophagal carcinogenesis by inducing genetic alterations in genes such as cyclin d1, c-myc, dnp53 and EGFR in oral keratinocytes. These transfections led to malignant transformation by means of p53 inactivation and telomerase activation via EGFR and PI3K/AKT pathway. Telomerase, therefore, could play a major role in the process of malignant transformation.

Effect of Carcinogens in Arecanut and Tobacco on Telomerase Activity Since the cases of OSMF, OSCC and OSCC in the background of OSMF taken in the present study had a habit history of tobacco and/or betel quid chewing, the role of these factors in the pathophysiology cannot be ignored. Carcinogens such as N-nitrosamines derived from smokeless tobacco as well as betel quid are known chemical carcinogens in humans wherein they cause various genetic alterations that ultimately lead to carcinogenesis [32–34]. Genetic alterations occur as a result of p450 enzyme activation. These enzymes culminate in the production of highly reactive pyridoxobutyl diazonium ions that react with DNA causing miscoding and mutation [35]. Persistent DNA adducts and evasion of cellular repair mechanisms lead to permanent DNA damage. Mutations in pivotal regions of DNA such as p53 and RAS cause cancer development [5]. Additionally, evidences suggest tobacco and betel quid related carcinogens to perturb various pathways that affect the cell cycle [33, 34]. Alveolar epithelial cells have shown P450 activation to concomitantly increase hTERT expression [36]. Tobacco and areca associated carcinogens produce a significant amount of reactive oxygen species (ROS) that eventually damages DNA. Telomeres are rich in guanine, making them one of the most susceptible areas for oxidative damage. ROS reacts with telomere to form 8-oxoguanine (8-oxoG) thereby promoting telomerase activity [37]. Additionally, increased telomerase activity was observed on exposing normal oral keratinocytes to arecoline and nicotine [38]. This could possibly explain the increased telomerase expression seen in in the epithelium of OSMF and OSCC tissue samples in the current study. However, the exact molecular mechanism behind the effect of chemical carcinogens on hTERT activity remains to be an unexplored area of research.

hTERT in Multistage Carcinogenesis Arising from Oral Submucous Fibrosis Studies recapitulating tobacco-induced carcinogenesis on *in vivo* and *in vitro* models have observed hTERT to be frequently up-regulated during multistep carcinogenesis [39, 40]. Increased hTERT expression in OPMDs such as oral epithelial dysplasias, OSMF as well as OSCC has been observed in various studies suggesting its involvement as an early event in carcinogenesis [8, 16, 20–22, 41–43]. However, meagre studies have been conducted on evaluating expression levels of hTERT in OSMF and OSCC [21]. Among OPMDs, OSMF is characterized by extensive fibrosis in the lamina propria followed by epithelial atrophy. Pathogenesis of OSMF is primarily attributed to betel quid chewing. Areca nut extracts have been shown to increase expression levels of hTERT in oral keratinocytes in a dose-dependent manner. Nicotine present in smokeless tobacco had a synergistic effect on hTERT expression in conjunction with areca nut [38]. Such increase in hTERT expression in OSMF could be due to chemical carcinogen-induced p450 enzyme activation as mentioned earlier. To add-on, various transcription factors that are known activators of hTERT (NF- B, β-catenin, c-myc) are found to be up-regulated in OSMF tissues [16, 44, 45]. Immunohistochemical evaluation showed a consistent increase in hTERT levels from normal mucosa to OSMF to OSCC tissue samples. This was on par with the studies conducted by Palani et al. wherein statistically significant differences were observed [21]. On evaluation of LI, they found OSMF cases to have a lower LI when compared to normal mucosa in contradiction to our results where LI increased from normal to OSMF. Such disparity of result could be owed to the fact that: 1) selection of cases in the aforementioned study were restricted to those OSMF cases having atrophic epithelium whereas we incorporated all histopathologic grades of OSMF 2) Method of evaluation LI was different wherein they counted the percentage of cells stained using eyepiece graticule under high power objective.

Pathogenesis of OSMF is a complex process involving multiple molecular pathways. Though the involvement of hTERT in pulmonary fibrosis has been known, its role in the disease process of OSMF is yet to be decoded [46]. Despite it being a pathology of the connective tissue characterized by extensive fibrosis, it poses a high risk of developing into an epithelial carcinoma. The downright mechanism for such transformation is still an active area of research. Epithelial-Mesenchymal Transition (EMT) is thought to be the central mechanism liable for invasion and metastasis of various cancers. Role of EMT in the malignant transformation of OSMF has also been noted [47]. Zhao et al. demonstrated the capacity of hTERT to exhibit characteristics of EMT in primary human oral epithelial cells. Upregulation of hTERT induced spindle-like morphology in cultured oral epithelial cells. On further probing, cells with increased hTERT expression showed a reduction in E-cadherin and upregulated expression of

vimentin and EMT transcription factors such as Slug and Twist1 [16]. Telomerase regulates the expression of NF- B target genes like MMP 9, IL-6, IL-8 and TNF- α by directly interacting with NF-kB p65 subunit. These molecules in turn activate a cascade of events involving JAKSTAT3-SNAIL and AKT signalling pathways thereby inducing EMT [48]. Likewise, telomerase modulates MMP-2, MMP-9 and cathepsin D which subsequently degrades collagen type IV and extracellular matrix that are crucial for integrity and stability of basement membrane [49]. This could ultimately cause a breach in the basement membrane promoting invasion of neoplastic epithelial cells into the connective tissue; onsetting squamous cell carcinoma.

In the current study, hTERT activity increased significantly from well differentiated to poorly differentiated SCC indicating the presence of more number of immortal cells in PDSCC correlating with its clinical aggressiveness. However, the correlation between hTERT with tumor staging and lymph node metastasis did not render significant difference. Results of studies executed by Pannone et al. and Lee et al. were also in parallel with our results where no significant correlation was found between hTERT expression and clinic-pathologic parameters [23, 50]. Nevertheless, it has also been of note that increased hTERT expression positively correlated with clinicopathologic parameters of OSCC such as histologic grade, tumor staging and lymph node involvement or extracapsular lymph node metastasis [51]. Hence, studies using larger sample size and sensitive methodologies are warranted to address such disparities. Prediction of the outcome by observing telomerase activity have also been administered for OSCC tissues and increased telomerase correlated with the reduced response to treatments and reduced survival rates. Association between higher telomerase levels and aggressiveness of OSCC could be justified by 1) telomere ability to maintain telomere length, 2) the non-canonical functions of telomerase; interactions with other cancer-associated signalling cascades like Wnt/β-catenin and NF- B [48].

Intracellular hTERT Trafficking during Malignant Transformation of OSMF into OSCC Yet another interesting find in the current study was the shift in the localization of the hTERT staining from normal through OSMF to OSCC. In normal epithelium, staining was restrained to the nucleus, unlike OSMF where majority showed combined nuclear and cytoplasmic staining. Cells of OSCC almost entirely showed staining restricted to the cytoplasm. These findings were in line with Chen et al., where a decrease in nuclear hTERT and increase in cytoplasmic hTERT was noted in OSCC sample unlike OED samples [8]. Telomere maintenance is controlled by proper assembling of hTR and hTERT into a ribonucleoprotein alongside regulation of several cofactors that aid in maturation, stability and subcellular localization of telomerase. During G0 phase, although hTERT and hTR are contained in the nucleoli, they do not share the same compartment. hTERT is restrained to the nucleolar foci whereas hTR is present in the Cajal bodies. As the cell goes into the S phase, hTERT is observed to be physically associated with hTR, and their assembly is strictly modulated by chaperones, Hsp90 [52]. Once assembled, they interact with telomere, thus maintaining its length. However, cellular injury induced by ROS evokes GTPase dependent nuclear exportation of hTERT via nuclear pores into the cytoplasm of the cell [53]. Chemical carcinogens in smokeless tobacco and betel quid are frequently associated with increased ROS production with subsequent DNA damage. ROS has been noticed to play a role in the pathogenesis of OSMF [54, 55]. The possible reason for the change in localization of hTERT staining from nuclear to cytoplasm could be pinned to ROS that is most often found to be increased in OSMF tissues. Most of the OSCC cases showed a homogenous cytoplasmic hTERT staining. According to Akiyama et al., this could be due to alternative splicing of hTERT and failure of variant hTERT proteins to translocate to the nucleus mediated by NF- B p65 subunit and TNF-α [56]. Moreover, genotoxic compounds seen in areca nut are suggested to increase the cytoplasmic expression of hTERT in OSCC tumor cells [8]. Further investigation is required to delineate the exact mechanism behind such a shift. These findings suggest hTERT activity to be a rate-limiting step in malignant transformation of OSMF into OSCC and could be used as a potent diagnostic marker.

Conclusion

Though the neoplastic transformation rate of potentially malignant disorders have been analysed, the prediction of high-risk lesion continues to be difficult. Therefore it’s essential to come up with biomarkers aiding in detecting cases with increased risk for carcinoma development. The increase in hTERT expression from normal through OSMF, OSCC in the background of OSMF and OSCC observed suggests its involvement in early stages of malignant transformation of OSMF. The current study aided in shedding light on the potential use of hTERT as a diagnostic marker in identifying high-risk cases of OSMF. One of the limitations in our study was the lack of a sufficient number of OSCC in the background of OSMF cases. Additionally, though various studies have utilized immunohistochemistry for detection of hTERT in different tumours, different commercially available antibodies show slight differences in hTERT expression in terms of specificity and localization. Hence, methods that are more sensitive and at the same time allow visualization of cellular localization such as PCR based in-situ hybridization should be utilized on a larger sample size to validate the results. Profound strategies have been put forward in the field of translational medicine to curb telomerase enzyme activity. Now

that the role of telomerase has been proposed in malignant transformation of OSMF, development of therapeutic agents against telomerase could possibly prevent the onset of carcinogenesis in OSMF patients.

Acknowledgements We personally thank Dr. Saman Warnakulasurya, Kings College, London for his valuable guidance in churning out this study.

Compliance with Ethical Standards

Declaration of Conflict of Interest The authors declare no conflict of interest.

References

1. Warnakulasuriya S (2009) Global epidemiology of oral and oropharyngeal cancer. Oral Oncol 45:309–316. https://doi.org/10.1016/j.oraloncology.2008.06.002
2. Johnson NW, Amarasinghe HK (2011) Epidemiology and aetiology of head and neck cancers. In: Head and neck cancer. Springer New York, New York, pp 1–40
3. Wanninayake Mudiyanselage Tilakaratne RPE (2013) Oral submucous fibrosis: review on mechanisms of pathogenesis and malignant transformation. J Carcinog Mutagen. https://doi.org/10.4172/2157-2518.S5-002
4. Hsue S-S, Wang W-C, Chen C-H, Lin CC, Chen YK, Lin LM (2007) Malignant transformation in 1458 patients with potentially malignant oral mucosal disorders: a follow-up study based in a Taiwanese hospital. J Oral Pathol Med 36:25–29. https://doi.org/10.1111/j.1600-0714.2006.00491.x
5. Vinay K, Abbas AK (2010) Robbins & cotran pathologic basis of disease, 9th edn. Saunders Elsevier, Philadelphia
6. Bhagvan More C, Gupta S, Joshi J, Verma SN (2012) Classification system for oral submucous fibrosis. J Indian Acad Oral Med Radiol 24:24–29. https://doi.org/10.5005/jp-journals-10011-1254
7. Bhargava A, Saigal S, Chalishazar M (2010) Systems in oral squamous cell carcinoma : a review. J Int Oral Heal 2:1–10
8. Chen H-H, Yu C-H, Wang J-T, Liu BY, Wang YP, Sun A, Tsai TC, Chiang CP (2007) Expression of human telomerase reverse transcriptase (hTERT) protein is significantly associated with the progression, recurrence and prognosis of oral squamous cell carcinoma in Taiwan. Oral Oncol 43:122–129. https://doi.org/10.1016/j.oraloncology.2006.01.011
9. Luzar B, Poljak M, Marin IJ, Eberlinc A, Klopcic U, Gale N (2004) Human telomerase catalytic subunit gene re-expression is an early event in oral carcinogenesis. Histopathology 45:13–19. https://doi.org/10.1111/j.1365-2559.2004.01892.x
10. Sharpless NE, DePinho RA (2004) Telomeres, stem cells, senescence, and cancer. J Clin Invest 113:160–168. https://doi.org/10.1172/JCI20761
11. Ito H, Kyo S, Kanaya T, Takakura M, Inoue M, Namiki M (1998) Expression of human telomerase subunits and correlation with telomerase activity in urothelial cancer. Clin Cancer Res 4:1603–1608
12. Kim NW, Piatyszek MA, Prowse KR, Harley C, West M, Ho P, Coviello G, Wright W, Weinrich S, Shay J (1994) Specific association of human telomerase activity with immortal cells and cancer. Science 266:2011–2015
13. Kapanadze B, Morris E, Smith E, Trojanowska M (2010) Establishment and characterization of scleroderma fibroblast clonal cell lines by introduction of the hTERT gene. J Cell Mol Med 14:1156–1165. https://doi.org/10.1111/j.1582-4934.2009.00773.x
14. Wieser M, Stadler G, Jennings P, Streubel B, Pfaller W, Ambros P, Riedl C, Katinger H, Grillari J, Grillari-Voglauer R (2008) hTERT alone immortalizes epithelial cells of renal proximal tubules without changing their functional characteristics. Am J Physiol Renal Physiol 295:F1365–F1375. https://doi.org/10.1152/ajprenal.90405.2008
15. Shay JW, Bacchetti S (1997) A survey of telomerase activity in human cancer. Eur J Cancer 33:787–791. https://doi.org/10.1016/S0959-8049(97)00062-2
16. Zhao T, HU F, Qiao B et al (2015) Telomerase reverse transcriptase potentially promotes the progression of oral squamous cell carcinoma through induction of epithelial-mesenchymal transition. Int J Oncol 46:2205–2215. https://doi.org/10.3892/ijo.2015.2927
17. Astuti AK, Prasetyo I, Yunita JN et al (2011) Profile of human TERT and P73 in oral squamous cell carcinoma cell lines compared to normal human oral mucosa: a preliminary study. J Dent Indones 18:10–16. https://doi.org/10.14693/jdi.v18i1.55
18. Rai A, Naikmasur VG, Sattur A (2016) Quantification of telomerase activity in normal oral mucosal tissue and oral squamous cell carcinoma. Indian J Med Paediatr Oncol 37:183–188. https://doi.org/10.4103/0971-5851.190350
19. Miyazaki Y, Yoshida N, Nozaki T, Inoue H, Kikuchi K, Kusama K (2015) Telomerase activity in the occurrence and progression of oral squamous cell carcinoma. J Oral Sci 57:295–303. https://doi.org/10.2334/josnusd.57.295
20. Abrahao AC, Bonelli BV, Nunes FD, Dias EP, Cabral MG (2011) Immunohistochemical expression of p53, p16 and hTERT in oral squamous cell carcinoma and potentially malignant disorders. Braz Oral Res 25:34–41
21. Palani J, Lakshminarayanan V, Kannan R (2011) Immunohistochemical detection of human telomerase reverse transcriptase in oral cancer and pre-cancer. Indian J Dent Res 22:362–363. https://doi.org/10.4103/0970-9290.84281
22. Kannan S, Tahara H, Yokozaki H et al (1997) Telomerase activity in premalignant and malignant lesions of human oral mucosa. Cancer Epidemiol Biomark Prev 6:413–420
23. Pannone G, De Maria S, Zamparese R et al (2007) Prognostic value of human telomerase reverse transcriptase gene expression in oral carcinogenesis. Int J Oncol 30:1349–1357
24. Weinhold N, Jacobsen A, Schultz N, Sander C, Lee W (2014) Genome-wide analysis of noncoding regulatory mutations in cancer. Nat Genet 46:1160–1165. https://doi.org/10.1038/ng.3101
25. Chang K-P, Wang C-I, Pickering CR, Huang Y, Tsai CN, Tsang NM, Kao HK, Cheng MH, Myers JN (2017) Prevalence of promoter mutations in the TERT gene in oral cavity squamous cell carcinoma. Head Neck 39:1131–1137. https://doi.org/10.1002/hed.24728
26. Vinothkumar V, Arunkumar G, Revathidevi S, Arun K, Manikandan M, Rao AKDM, Rajkumar KS, Ajay C, Rajaraman R, Ramani R, Murugan AK, Munirajan AK (2016) TERT promoter hot spot mutations are frequent in Indian cervical and oral squamous cell carcinomas. Tumor Biol 37:7907–7913. https://doi.org/10.1007/s13277-015-4694-2
27. Qu Y, Dang S, Wu K, Shao Y, Yang Q, Ji M, Shi B, Hou P (2014) TERT promoter mutations predict worse survival in laryngeal cancer patients. Int J Cancer 135:1008–1010. https://doi.org/10.1002/ijc.28728
28. Yin L, Hubbard AK, Giardina C (2000) NF-κB regulates transcription of the mouse telomerase catalytic subunit. J Biol Chem 275:36671–36675. https://doi.org/10.1074/jbc.M007378200
29. Zhang Y, Toh L, Lau P, Wang X (2012) Human telomerase reverse transcriptase (hTERT) is a novel target of the Wnt/β-catenin pathway in human cancer. J Biol Chem 287:32494–32511. https://doi.org/10.1074/jbc.M112.368282
30. Greenberg RA, O'Hagan RC, Deng H et al (1999) Telomerase reverse transcriptase gene is a direct target of c-Myc but is not

functionally equivalent in cellular transformation. Oncogene 18: 1219–1226. https://doi.org/10.1038/sj.onc.1202669

31. Fabricius E-M, Kruse-Boitschenko U, Khoury R et al (2009) Immunohistochemical determination of the appropriate anti-hTERT antibodies for in situ detection of telomerase activity in frozen sections of head and neck squamous cell carcinomas and tumor margin tissues. Int J Oncol 34:1257–1279. https://doi.org/10.1038/sj.onc.1203890
32. Hoffmann D, Brunnemann KD, Prokopczyk B, Djordjevic MV (1994) Tobacco-specific N-nitrosamines and Areca-derived N-nitrosamines: chemistry, biochemistry, carcinogenicity, and relevance to humans. J Toxicol Environ Health 41:1–52. https://doi.org/10.1080/15287399409531825
33. Xue J, Yang S, Seng S (2014) Mechanisms of Cancer induction by tobacco-specific NNK and NNN. Cancers (Basel) 6:1138–1156. https://doi.org/10.3390/cancers6021138
34. Sharan RN, Mehrotra R, Choudhury Y, Asotra K (2012) Association of betel nut with carcinogenesis: revisit with a clinical perspective. PLoS One 7:e42759. https://doi.org/10.1371/journal.pone.0042759
35. Reed L, Arlt VM, Phillips DH (2018) The role of cytochrome P450 enzymes in carcinogen activation and detoxication: an in vivo-in vitro paradox. Carcinogenesis 39:851–859. https://doi.org/10.1093/carcin/bgy058
36. Shervington A, Mohammed K, Patel R, Lea R (2007) Identification of a novel co-transcription of P450/1A1 with telomerase in A549. Gene 388:110–116. https://doi.org/10.1016/j.gene.2006.10.010
37. Lee H-T, Bose A, Lee C-Y, Opresko PL, Myong S (2017) Molecular mechanisms by which oxidative DNA damage promotes telomerase activity. Nucleic Acids Res 45:11752–11765. https://doi.org/10.1093/nar/gkx789
38. Gao Y, Ling T, Yin X et al (2007) Effects of arecoline and nicotine on the expression of hTERT in oral keratinocytes. Zhonghua Kou Qiang Yi Xue Za Zhi 42:26–30
39. Kanojia D, Vaidya MM (2006) 4-Nitroquinoline-1-oxide induced experimental oral carcinogenesis. Oral Oncol 42:655–667. https://doi.org/10.1016/j.oraloncology.2005.10.013
40. Sumida T, Hamakawa H, Sogawa K, Bao Y, Zen H, Sugita A, Nezu K, Abe Y, Tanioka H, Ueda N (1999) Telomerase activation and cell proliferation during 7,12-dimethylbenz[a]anthracene-induced hamster cheek pouch carcinogenesis. Mol Carcinog 25:164–168
41. Kim H, Christensen R, Park N et al (2001) Elevated expression of hTERT is associated with dysplastic cell transformation during human oral carcinogenesis in situ. Clin Cancer Res 7:3079–3086
42. Raghunandan BN, Sanjai K, Kumaraswamy J et al (2016) Expression of human telomerase reverse transcriptase protein in oral epithelial dysplasia and oral squamous cell carcinoma: An immunohistochemical study. J Oral Maxillofac Pathol 20:96–101. https://doi.org/10.4103/0973-029X.180953
43. Mutirangura A, Supiyaphun P, Trirekapan S et al (1996) Telomerase activity in oral leukoplakia and head and neck squamous cell carcinoma. Cancer Res 56:3530–3533
44. Lin S-C, Lu S-Y, Lee S-Y, Lin CY, Chen CH, Chang KW (2005) Areca (betel) nut extract activates mitogen-activated protein kinasesand NF-κB in oral keratinocytes. Int J Cancer 116:526–535. https://doi.org/10.1002/ijc.21104
45. Srinivasan M, Jewell SD (2001) Quantitative estimation of PCNA, c-myc, EGFR and TGF-alpha in oral submucous fibrosis–an immunohistochemical study. Oral Oncol 37:461–467
46. Armanios M (2012) Telomerase and idiopathic pulmonary fibrosis. Mutat Res 730:52–58. https://doi.org/10.1016/j.mrfmmm.2011.10.013
47. Yanjia H, Xinchun J (2007) The role of epithelial–mesenchymal transition in oral squamous cell carcinoma and oral submucous fibrosis. Clin Chim Acta 383:51–56. https://doi.org/10.1016/j.cca.2007.04.014
48. Boscolo-Rizzo P, Da Mosto MC, Rampazzo E et al (2016) Telomeres and telomerase in head and neck squamous cell carcinoma: from pathogenesis to clinical implications. Cancer Metastasis Rev 35:457–474. https://doi.org/10.1007/s10555-016-9633-1
49. Park Y-J, Kim EK, Bae JY, Moon S, Kim J (2016) Human telomerase reverse transcriptase (hTERT) promotes cancer invasion by modulating cathepsin D via early growth response (EGR)-1. Cancer Lett 370:222–231. https://doi.org/10.1016/j.canlet.2015.10.021
50. Lee BK, Diebel E, Neukam FW et al (2001) Diagnostic and prognostic relevance of expression of human telomerase subunits in oral cancer. Int J Oncol 19:1063–1068
51. Boscolo-Rizzo P, Rampazzo E, Perissinotto E, Piano MA, Giunco S, Baboci L, Spinato G, Spinato R, Tirelli G, da Mosto MC, del Mistro A, de Rossi A (2015) Telomere shortening in mucosa surrounding the tumor: biosensor of field cancerization and prognostic marker of mucosal failure in head and neck squamous cell carcinoma. Oral Oncol 51:500–507. https://doi.org/10.1016/j.oraloncology.2015.02.100
52. Dey A, Chakrabarti K (2018) Current perspectives of telomerase structure and function in eukaryotes with emerging views on telomerase in human parasites. Int J Mol Sci 19:333. https://doi.org/10.3390/ijms19020333
53. Haendeler J, Hoffmann J, Brandes RP, Zeiher AM, Dimmeler S (2003) Hydrogen peroxide triggers nuclear export of telomerase reverse transcriptase via Src kinase family-dependent phosphorylation of tyrosine 707. Mol Cell Biol 23:4598–4610. https://doi.org/10.1128/MCB.23.13.4598-4610.2003
54. Shakunthala GK, Annigeri RG, Arunkumar S (2015) Role of oxidative stress in the pathogenesis of oral submucous fibrosis: A preliminary prospective study. Contemp Clin Dent 6:S172–S174. https://doi.org/10.4103/0976-237X.166823
55. Gupta S, Reddy MVR, Harinath BC (2004) Role of oxidative stress and antioxidants in aetiopathogenesis and management of oral submucous fibrosis. Indian J Clin Biochem 19:138–141. https://doi.org/10.1007/BF02872409
56. Akiyama M, Hideshima T, Hayashi T, Tai YT, Mitsiades CS, Mitsiades N, Chauhan D, Richardson P, Munshi NC, Anderson KC (2003) Nuclear factor-kappaB p65 mediates tumor necrosis factor alpha-induced nuclear translocation of telomerase reverse transcriptase protein. Cancer Res 63:18–21

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.