International Journal of Science and Research (IJSR)
ISSN: 2319-7064
SJIF (2022): 7.942

Assessment of Human Telomerase Reverse Transcriptase Expression in Different Grades of Oral Epithelial Dysplasias and Oral Squamous Cell Carcinomas: An Immunohistochemical Study

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Abstract: ***Context: Oral cancer is a highly relevant problem of global public health, especially for dental surgeons. Many oral squamous cell carcinomas (OSCCs) develop from potentially malignant disorders (PMDs), characterized histopathologically by dysplastic changes, which manifest as red and white lesions. A specialized ribonucleoprotein complex called telomerase stabilizes telomeres by addition of 'TAG' repeats to the ends of chromosomes. It has been postulated that telomerase reactivation appears to play an important role in the process of cell immortalization, promoting the accumulation of genetic abnormalities and accelerating the process of tumorigenesis. Aims: The aim of the study was to assess human Telomerase Reverse Transcriptase (hTERT) expression in different grades of OEDs and OSCCs and to establish its role as a diagnostic marker of OSCC. Settings and Design: The present study was conducted in Department of Oral and Maxillofacial Pathology, Maitri College of Dentistry and Research Centre, Durg, Chhattisgarh, India. Methods and Material: The study group comprised 5 specimens of non - inflamed normal oral mucosa collected from extraction sockets of patients as a control group, 60 cases of clinically diagnosed leukoplakia/erythroplakia and histopathologically diagnosed with various grades of dysplasia and 60 cases of clinically and histopathologically diagnosed Oral Squamous Cell carcinoma. hTERT staining within each cell was studied in respect of localization of stain within the nucleus and cytoplasm and the percentage of cells stained. The various types of nuclear staining examined were dot - like (1–3 small discrete dots within the nucleus), granular (>3 coarse dots), smooth (homogenous brown). Statistical analysis used: All data obtained were analyzed by Pearson's Chi - square test (p=0.05). Results: The results revealed a predominance of dot - like nuclear staining of both oral epithelial dysplasia samples as well as oral squamous cell carcinoma samples as compared to normal oral mucosa (p=0.0). Conclusions: From our study, it was concluded that hTERT expression, along with advanced tools like RT - PCR and FISH, can be used as an effective diagnostic marker of OSCC.***

Keywords: Oral Squamous Cell Carcinoma, Oral Epithelial Dysplasia, human Telomerase Reverse Transcriptase

1. Introduction

Oral cancer is a highly relevant problem of global public health, especially for dental surgeons. According to a study in 2015, it was found that oral cancer is located within the top 10 ranking incidence of cancers and despite the progress in research and therapy, survival has not improved signifi-cantly in the last years, representing a continuing challenge for biomedical science.1 According to the report by the American Cancer Society in 2017, oral cancer is the most common cancer in males and second most common in females and accounts for the maximum number of deaths worldwide. In fact, there has been an increased prevalence of oral cancer, particularly among young individuals. The incidence of oral cancer as recorded by International Agency for Research on Cancer of the World Health Organization (IARC– WHO), is expected to increase from 10 million cases per year in 2000 to 16 million in 2020. One of the most common cancers of the oral cavity is Oral Squamous Cell Carcinoma (OSCC). Despite the ready accessibility of the oral cavity to direct examination, these malignancies are still detected at a later stage; thus, the survival rate for oral cancer has primarily remained unchanged over the past 3 decades. The 5 - year relative survival rates for oral cancers are 83% for cancer that has not spread, 62% for cancer that has spread to nearby lymph nodes and 38% for cancer that has spread to distant parts of the body.3

It is noteworthy that many oral squamous cell carcinomas develop from potentially malignant disorders (PMDs), characterized histopathologically by dysplastic changes which manifest clinically as red and white lesions within the oral cavity. These lesions encompass a histological continuum between the normal mucosa at one end through high grade dysplasia/carcinoma *insitu*to full - blown squamous cell carcinoma at the other, establishing a model of neoplastic progression.4

The clinical concept of malignant transformation in oral mucosa has been proposed for more than 100 years. Sir James Paget first described malignant transformation of an oral lesion into tongue carcinoma in 1870.5 Schwimmer also reported the same finding in 1877.6Several years later, the term "Potentially Malignant Disorders" (PMDs) was defined by World Health Organization (WHO) as the risk of malignancy being present in a lesion or condition either during the time of initial diagnosis or at a future date. WHO also classified PMDs into two subgroups as follows: a) *precancerous lesion*, a benign lesion with morphologically altered tissue, which has a greater than normal risk of transforming into malignancy; b) *precancerous condition*, a

disease or patients' habit that does not necessarily alter the clinical appearance of local tissues but is associated with a greater than normal risk of transformation into precancerous lesion or cancer development in that tissue.[7, 8] Although little information is available regarding the real prevalence of PMDs in the general population, a commonly accepted prevalence of 1–5% has been reported.7 Average age of patients with PMDs is 50–69 years, which is 5 years before occurrence of oral cancer. Unfortunately, in recent years 5% of PMDs have been observed in persons under 30. Premalignant disorders are usually found on the buccal mucosa, followed by gingivae, tongue and floor of the mouth.

A specialized ribonucleoprotein complex called telomerase stabilizes telomeres by addition of 'TAG' repeats to the ends of chromosomes. The telomerase complex consistsof RNA template (hTR), a catalytic sub - unit called human Telomerase Reverse Transcriptase (hTERT) and associated protein (hTP - 1).9 hTERT expression (molecular weight ~130 kDa) is one of the critical determinants of telomerase activity.9Telomerase is an RNA - dependent DNA polymerase that synthesizes short tandem repeats of TTAGGG telomeric DNA sequences which universally provides the molecular basis for unlimited proliferative potential.1[0]Telomerase is an enzyme best known for its role in maintaining the integrity of the telomere. Telomerase reverse transcriptase (TERT), as the catalytic subunit of telomerase, has the capacity to limit the activity of telomerase to avoid the erosion of telomere. As telomeres become shorter with each cycle of replication and reach a critical limit; Most cells die or enter into a stage of replicative senescence. Telomere length maintenance by telomerase is required for all the cells that exhibit limitless replicative potential.[10]

It has been postulated that telomerase reactivation appears to play an important role in the process of cell immortalization, thus promoting the accumulation of genetic abnormalities and increasing genomic instability that contributes to malignant transformation.1[0]Telomerase activity is up regulated in many tumors and studies have shown that increased telomerase expression is an early event in oral carcinogenesis.9 It is the hTERT component of telomerase that is most reactive in dysplastic and malignant lesions of the oral cavity, for the reason which immunohistochemical studies on these tissue samples are chiefly directed towards hTERT.

Therefore, this study was conducted to assess Telomerase/ human Telomerase Reverse Transcriptase (hTERT) expression in different grades of Oral Epithelial Dysplasias and Oral Squamous Cell Carcinomas with an aim to determine the malignant potential of PMDs. Furthermore, an attempt has been made towards elucidating the role of hTERT as a diagnostic marker of OSCC.

2. Materials and Methods

The study group comprised 5 specimens of non - inflamed normal oral mucosa collected from extraction sockets of patients as a control group (GROUP I), 60 patients clinically diagnosed with leukoplakia/erythroplakia (GROUP II) and 60 patients diagnosed clinically with Oral Squamous Cell carcinoma (GROUP III). The present study was conducted in Department of Oral and Maxillofacial Pathology, Maitri College of Dentistry And Research Centre, Durg, Chhattisgarh, India. Samples derived from various study group were stained with hTERT immunostain using standard immunohistochemical procedures.

The following parameters were used to evaluate hTERT staining[10]:

1) ***Tissue localization of the stain***: hTERT staining was limited either to basal and parabasal layers of the epithelium, extended up to the spinous layer or was seen throughout all the layers of epithelium.
2) ***Cellular location of the stain***: hTERT staining within each cell was either localized to the nucleus or was present both in the nucleus and the cytoplasm.
3) ***Nature of stain***: The pattern of positive hTERT immunostain was both nuclear as well as cytoplasmic. The various types of nuclear staining examined were dot - like (1–3 small discrete dots within the nucleus), granular (>3 coarse dots), smooth (homogenous brown). The cytoplasmic staining was either smooth or Granular.

Statistics Used:
Descriptive statistical analysis was carried out in the present study wherein the various grades of Oral Epithelial Dysplasia (mild, moderate, severe) and Oral Squamous Cell Carcinoma (Grade I, Grade II, Grade III) was expressed as a percentage of the total number of samples in each group. The three parameters for assessing hTERT, tissue localization, cellular localization and nature of stain was compared between the various grades of Oral Epithelial Dysplasia and Oral Squamous Cell Carcinomas and the data obtained was compared with that in normal oral mucosa using Pearson's Chi - Squared Test. A p - value less than 0.05 was considered significant.

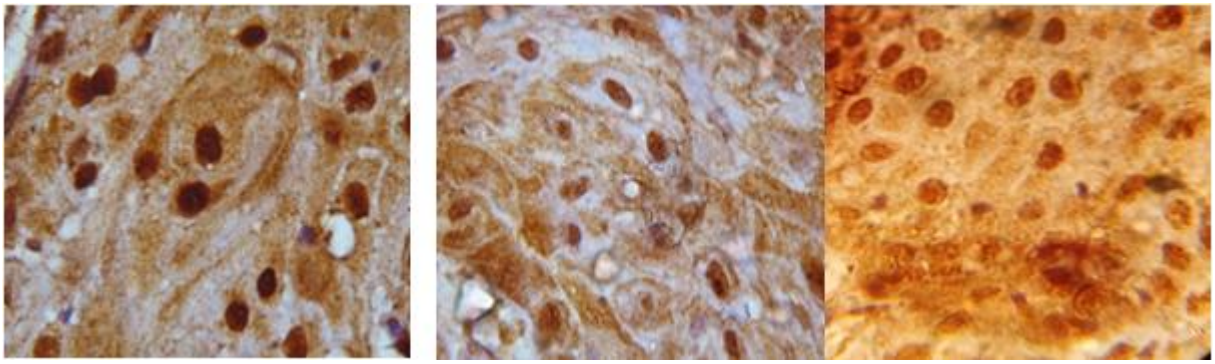

Figure 1: (left) Immunostain with hTERT in moderate oral epithelial dysplasia (100X) showing granular and dot - like staining of the nucleus. (middle) Immunostaining in well differentiated squamous cell carcinoma (100X), showing predominant dot - like staining of the nucleus of malignant cells. (right) Immunistaining in poorly - differentiated squamous cell carcinoma showing homogenous cytoplasmic stain and predominantly granular nuclear stain of neoplastic cells.

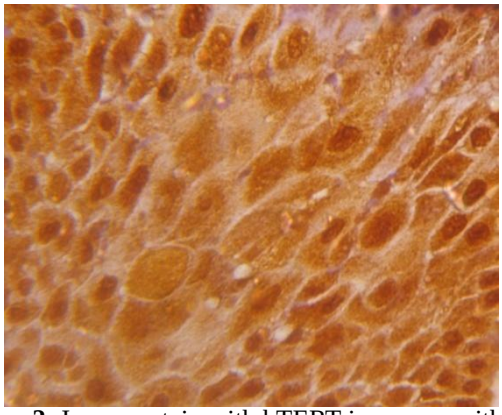

Figure 2: Immunostain with hTERT in severe epithelial dysplasia (40X) showing smooth nuclear and cytoplasmic staining

3. Results and Observations

The evaluation of expression of human Telomerase Reverse Transcriptase staining and subsequent quantitative analysis was based on the number of cells showing positive nuclear staining and the percentage of cells stained per 100 cells in a field. The number of cells showing positive immunostaining was evaluated based on the number of cells showing nuclear localization in different study groups. The results revealed a predominance of dot - like nuclear staining of both oral epithelial dysplasia samples as well as oral squamous cell carcinoma samples as compared to normal oral mucosa. The results were significant (p=0.0).

Table 1: Comparison of Number of Cells showing positive nuclear immunostaining in Oral Epithelial Dysplasia and Oral Squamous Cell Carcinoma with that in Normal Oral Mucosa

Nature of stain	*Number of cells stained*			*p Value*
	OED	*OSCC*	*CONTROL*	
Dot - like	35	40	0	0.0
Granular	25	20	1	
No Stain	0	0	4	
Total	60	60	5	

Pearson's Chi - Squared Test. * p value ≤ 0.05 was considered statistically significant

Table 2: Percentage of cells stained positive with immunostain in various grades of oral epithelial dysplasias and oral squamous cell carcinoma

Grades of OED and OSCCs	*Percentage of cells stained*
Mild	65
Moderate	83
Severe	86
Well - Differentiated	73.3
Moderately - Differentiated	86.9
Poorly - Differentiated	84

4. Discussion

Oral Squamous Cell Carcinoma is the most common malignant neoplasm of the oral cavity, with the highest rate of metastasis to distant structures. It is one of the major causes of death within the Indian population. OSCCs mostly arise due to excessive use of tobacco in both smoking as well as smokeless forms, cumulated with chronic consumption of alcohol. However, genetics seem to play a prior role in determination of initiation of the disease. In fact, with each progressing day newer concepts are being added to the genetic basis of carcinogenesis. The underlying molecular mechanism of this malignant disease, along with the various molecular catalysts involved in its initiation and progression, however well - documented, is very difficult to elucidate. Thus, proper recognition of the stage of the disease and its early interception through various diagnostic aids are very important to improve the quality of life of individuals suffering from this dreadful ailment.

As has been mentioned earlier, malignant transformation within the oral cavity initiates as red and white lesions of the overlying oral mucosa, known as Potentially Malignant Diseases (PMDs), which are histopathologically characterised by various grades of epithelial dysplasia. Samples of pre - malignant lesions used in this study were leukoplakia and erythroplakia, which are classical "Red and White Lesions" of the oral cavity. The advantage of selecting these samples for assessing immunostaining in this study is that they are available in abundance on the Indian subcontinent, due to high predilection of tobacco and alcohol use here, not only in males but nowadays increasingly in females as well. Study of the pattern of immunostaining in these PMDs not only helps in judging the severity of the disease but also gives a clear overview about

those PMDs which have a greater risk of transformation into malignancy.

A striking characteristic of cells undergoing malignant transformation is their unlimited replicative potential, which renders them immortal. The telomere, which is the terminal part of any chromosome, is the region which delimits mitotic division in a cell, in that the length of the telomere goes on decreasing as a cell continues division and finally a stage is reached when the cell reaches a phase of replicative senescence. In this stage, there is complete stoppage of cell division and a cell can no longer divide. It is this phenomenon which is disrupted in malignant cells i. e. the telomeres in cancerous cells acquire unlimited replicative potential which is chiefly attributed to the enzyme telomerase reverse transcriptase. It has been postulated that telomerase reactivation plays an important role in the process of cell immortalization, thus promoting the accumulation of genetic abnormalities and increasing genomic instability that contributes to malignant transformation. This is a change which is noted early in any cell undergoing malignant transformation. The main motto of this study was to choose such a marker of early malignant transformation, which could be easily isolated and could be an early predictor of malignant change. Very recently, human Telomerase Reverse Transcriptase (hTERT) has emerged as an excellent marker for detecting malignant transformation in various tissues, including the oral cavity and further research is going on in validating its role as a diagnostic and prognostic marker of oral malignancy. Thus, human Telomerase Reverse Transcriptase (hTERT) has been chosen human Telomerase Reverse Transcriptase (hTERT) in this study.

hTERT is a nuclear protein generally repressed in normal cells and upregulated in immortal cells, suggesting that hTERT is the primary determinant for the enzymatic activity in normal and cancerous cells; increased hTERT expression is predominantly observed in cancerous cells. Moreover, it is also elevated, apart from malignant cells, in those somatic cells which have a high rate of metabolic activity e.g. embryonic stem cells and germ cells. The regulation of telomerase activity occurs at various levels, including transcription, mRNA splicing, maturation and modifications of hTR and hTERT, transport and subcellular localization of each component, assembly of active telomerase ribonucleoprotein and accessibility and function of the telomerase ribonucleoprotein on telomeres.[11]

Use of IHC is a useful, reliable method of localizing the hTERT protein in tissue sections.[12, 13] Telomerase activity is conventionally studied using modified polymerase chain reaction, the telomeric repeat amplification protocol (TRAP) assay. TRAP assay, however, does not permit cellular localization of telomerase activity, which is better demonstrated with techniques like IHC/in situ hybridization. Cellular localization of the Immunostain in epithelial dysplasia and squamous cell carcinoma can be defined as being either nuclear or cytoplasmic or both. hTERT immunostaining can be localized diffusely in the nucleoplasm or more strongly in the nucleoli of cancer cells. It is important to detect cellular localization as it helps us identify the cells on the path of malignant transformation. In this study, immunohistochemical expression of hTERT protein was assessed and compared among normal mucosa as a control, oral epithelial dysplasia and OSCC samples. Also, an attempt was made to elucidate the role of human Telomerase Reverse Transcriptase as a diagnostic and a prognostic marker of Oral Epithelial Dysplasias and Oral Squamous Cell Carcinoma. The grading of OEDs were done according to the criteria given by WHO in 2005. Furthermore, an attempt was made to grade OSCC according to the classification system given by Bryne in 1989.

In the later half of the 20th century, extensive research was conducted on the expression of telomerase in cervical cancers and prostate cancers among others. Though it was found that there was a higher expression of telomerase in those tissues on the path of malignant transformation, nothing conclusive could be said about the potency of telomerase as a diagnostic and prognostic marker of carcinomas. In the early half of the 21st century, research work was initiated on the expression of hTERT immunostaining of the oral and paraoral structures and an attempt was made to establish its efficacy as a diagnostic and prognostic marker of OSCC. Though many conclusive works are present in the literature regarding this, studies are still undergoing.

Mutirangura et al. (1991) reported telomerase expression in 43% (10/23) of leukoplakia; Kannan et al. (1997) reported that 75% (27/36) of leukoplakia expressed telomerase activity and Liao et al. (2000) showed that 54.5% (12/22) of leukoplakia samples had telomerase expression. Abrahao et al. (2011) reported 100% (15/15) hTERT expression in potentially malignant disorders (leukoplakia and erythroplakia). The results in this study were slightly different as compared to the study by Abrahao et. al. (2011) and Kumar et. al. (2005), where the percentage of cells showing positive hTERT staining were 65%, 83% and 86% in mild, moderate and severe dysplasia respectively and 73.3%, 86.9% and 84% in well - differentiated, moderately - differentiated and poorly - differentiated OSCC respectively. The present study was however, in accordance with other studies such as the one by Raghunandan et. al. (2016) where there was a steady increase in the expression of hTERT from normal oral mucosa to oral epithelial dysplasia to OSCC cases. Moreover, higher the grade of OED and OSCC, higher was the percentage of cells showing positive immunostaining.

When telomere becomes short reaching critical length, [14] telomerase is activated in premalignant and malignant cells. There is a steady increase in level of nuclear expression of hTERT in the oral epithelial dysplasia and OSCC cases than in normal oral mucosa. This indicates that an increased hTERT expression is to maintain chromosomal stability and to promote cell proliferation in oral epithelial dysplasia and OSCC lesions than in normal oral mucosa. According to Palani et. al. (2011) hTERT is considered to be the critical determinant of telomerase activity in neoplasias, ashTR and hTP - 1 are ubiquitously expressed by both normal and cancerous cells, whereas an increased hTERT expression is observed predominantly in cancerous cells. The results of this study were also in accordance with the study of Palani

and co - workers, where there was a noticeable increasing gradient in the expression of telomerase from normal oral mucosa to OED to OSCC. Some authors, particularly Sumida T et. al. (2001) and Falchetti et. al. (2000), have suggested that telomerase activation is an early event during neoplastic transformation in vivo and is frequently related to the proliferation rate of cancer cells. At later stages, many solid malignant tumors, most probably as a consequence of a critical size increase and insufficient vascularization, become necrotic in their central region and are associated with a marked down - regulation of hTERT gene expression.

Distribution of nuclear localization of immunostain was highly significant among the various grades of OED as well as OSCC (p Value=0.01). Thus, it can be concluded from this study that the nuclear localization of Immunostain is a good predictor of the aggressiveness of oral epithelial dysplasia and of those samples of OEDs which have a greater than normal risk of conversion into OSCC. These results were similar to the observations by Chen et. al. (2006). The localization of hTERT expression in cytoplasm which represents phosphorylated and inactive forms of the protein, probably awaiting nuclear translocation has been previously reported in carcinomas of breast (Kyo S et. al., 1997), cervix (Frost M, 2000) and larynx (Luzar B, 2003). However, in our study only the nuclear localization of stain has been considered as a parameter for assessing hTERT expression in pre - malugnancy and malignancy.

According to the study by Raghunandan and his coworkers, tissue and cellular localization of immunostain could be used as authentic parameters when comparing the nature of staining in normal oral mucosa, oral mucosa showing epithelial dysplasia and those showing squamous cell carcinoma. There was a significant high Immunostain localization from normal mucosa to OED and OSCC, which is suggestive of the fact that immunostaining localization increases as the degree of malignant transformation of the oral mucosa becomes increases. This was similar to the results of Palani et. al. (2011). However, in this study the tissue localization of stain was assessed by staining sections with H&E. It was not taken as a parameter while assessing immunostain. In contrary to the study by Chen et. al. (2007), where it was concluded that hTERT was overexpressed in patients having oral habits like smoking or chewing of tobacco, no such results were obtained in this study.
This study is a preliminary attempt at detecting human telomerase reverse transcriptase (hTERT) protein using IHC with a monoclonal hTERT antibody on paraffin embedded tissues samples representing multistage oral carcinogenesis. The study was entirely prospective in nature and was conducted in vivo. Antigen retrieval for hTERT is highly sensitive and depends on environmental conditions. Thus, a control experiment was set up, where slides of the same tissue section were retrieved for 1, 2, 3, 4 and 5 minutes before being immunohistochemically stained to evaluate optimum antigen retrieval time for the particular antibody being used. The dilution of the antibody was done with an assay dependent concentration. The intense expression of hTERT in potentially malignant lesions 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 to elucidate if hTERT may be a useful diagnostic or prognostic marker in oral potential malignant disorders and cancer.

5. Conclusion

The present study was focused on assessing Telomerase/ human Telomerase Reverse Transcriptase (hTERT) expression in different grades of Oral Epithelial Dysplasias and Oral Squamous Cell Carcinomas.

Staining in normal oral mucosa was not significant in this study. The expression of hTERT increased as the grade of Oral Epithelial Dysplasia increased. There was no significant correlation between the expression of hTERT and the grade of Oral Squamous Cell Carcinoma. There was no significant expression of hTERT in normal oral mucosa used as a control. The expression of hTERT was significantly increased in Oral Epithelial Dysplasia when compared with normal oral mucosa. The expression of hTERT was significantly increased in Oral Squamous Cell Carcinoma when compared with normal oral mucosa. The expression of hTERT increased significantly from normal oral mucosa to Oral Epithelial Dysplasia to Oral Squamous Cell Carcinoma.

These findings suggest the hTERT expression increased from normal mucosa to OED and was the highest in OSCC. The present finding clearly reiterates the role of hTERT expression in cell proliferation and the process of malignant transformation. As far as nuclear localization of stain was concerned, there was a greater propensity of both OED as well as OSCC for dot - like staining as compared to nuclear stain. Only nuclear and not the cytoplasmic localization of stain was considered as a parameter for the assessment of expression of hTERT staining. Moreover, higher the grade of OED and OSCC, higher was the percentage of cells stained with hTERT. This study also showed a greater propensity for mild and moderate epithelial dysplasias towards nuclear Immunostain localization, whereas severe ones showed an increase in both nuclear as well as cytoplasmic localization of Immunostain.

Furthermore, the present study revealed that cellular localization of stain within the nucleus and percentage of cells stained with immunostain per 100 cells in a field were effective parameters in determining the rate of malignant transformation of PMDs and can also be used to eliminate those PMDs which have a greater risk of malignant transformation than others.

Thus, quantitative analysis of hTERT expression can be used as an effective tool to assess Epithelial Dysplasias and OSCCs. However, histopathology still remains the gold standard for evaluating these lesions; henceforth histopathological grading using H&E along with the special stains and immunohistochemistry should be used to assess the thickness of the epithelial tissue involved in dysplastic lesions and risk of subsequent conversion into malignancy. As far as hTERT is concerned, RT - PCR and FISH can also be useful diagnostic aids for screening malignancy.

Source (s) of support: nil

Presentation at a meeting: nil

Conflicting Interest (If present, give more details): nil

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