Evaluating the expression of p16 and p27 in oral epithelial dysplasias and oral squamous cell carcinoma: A diagnostic marker for carcinogenesis

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Abstract

Objective: Immunohistochemical evaluation of the degree of expression of p16 and p27 in oral epithelial dysplasia and different histological grades oral squamous carcinoma.

Materials and Methods: The study consisted of 5 cases of oral squamous cell carcinoma (OSCC), 5 cases of low-risk potentially malignant disorders (PMDs), 5 cases of high-risk PMDs and 5 cases of normal epithelium. Five micrometer thickness sections on a positively charged slide were subjected to immunohistochemical staining for the localization of p16 and p27. The expression of p16 and p27 was assessed in 10 random high-power fields (×40). Staining intensity was graded, and the data were subjected to statistical analysis.

Results and Conclusion: OSCC and high-grade PMDs showed decreased intensity for both p16 and p27. In our study, we concluded that p16 and p27 could be used as a diagnostic marker for predicting carcinogenesis in epithelial dysplasia.

Keywords: Oral squamous cell carcinoma, p16, p27, potentially malignant diseases

INTRODUCTION

Oral cancer is the sixth most common malignancy and is one of the major causes of cancer morbidity and mortality worldwide. Cancer is caused due to a series of alteration in genetic and epigenetic factors that occur in multiple steps and is influenced by the genetic predisposition of the individual and by exogenous environmental factors. These factors result in a series of molecular alteration, including inactivation of tumor suppressor genes. These genes play an important role in various cell division processes such as regulation of gene expression, cell cycle control, apoptosis and genomic stability.

Regulation of the cell cycle is an important factor in carcinogenesis. Cell cycle activities are governed by cyclins, cyclin-dependent kinases (CDKs) and their inhibitors. The function of cyclins is to activate the CDKs, and their levels drop after they execute this function. The activity of cyclin-CDK complexes is regulated by CDK inhibitors. There are two main categories of inhibitors: the...
Cip/kip family and the INK-4/ARF family. In the Cip/Kip family, p21, p27 and p53 stand out as major regulators; in the INK-4/ARF family, p16 and p14 are the most prominent. These inhibitors work as tumor suppressors and are frequently altered in tumors. These inhibitors attach to the cyclin-CDK complexes and inactivate them. P16 tumor suppressor gene, also known as p16\textsuperscript{INK4a}, is a member of the INK4 family of CDK inhibitors. It blocks the entry of cells into the G1 phase of the cell cycle by binding cyclin D-dependent kinases (CDK4 and CDK6) and causes inactivation/phosphorylation of Rb (Retinoblastoma gene). p16 acts as negative regulator of cell proliferation. Altered or loss of p16 expression has been noticed in oral premalignant lesions and tumor oral cavity. p27 regulates the proliferation of cells by binding and inhibiting G1 cyclin-CDK complexes and negatively regulating progression through G1 and S phases of cell cycle. Reduced levels of P27 have been reported in a number of human tumors, and loss of this inhibition has been associated with aggressive biological behavior. Alterations in p27 expression appear to precede the invasive stages of oral tumorigenesis.

Early diagnosis helps to reduce mortality and incidence of invasive carcinomas and improves treatment of smaller lesions with lower morbidity. With the help of a more specific diagnostic biomarkers, identification of premalignant and malignant lesion can be done more precisely.

The present study aims to evaluate the expression of p16 and p27 proteins in oral epithelial dysplasia and different histological grades of oral squamous cell carcinoma (OSCC).

MATERIALS AND METHODS

The study was comprised of 20 paraffin-embedded tissue samples retrieved from archives from Department of Oral Pathology and Microbiology, MR Ambedkar Dental College and Hospital, Bengaluru.

Histopathologically diagnosed cases of:
- Low-risk PMDs – 14 cases
- High-risk PMDs – 20 cases
- OSCC – 8 cases
- Normal epithelium – 5 cases.

All cases were analyzed for p16 and p27 marker.

Evaluation of the hematoxylin and eosin stained slides
Hematoxylin and eosin stained sections were studied and were reclassified into normal mucosal epithelium, low-risk PMDs, high-risk PMDs and OSCCs. Based on the above diagnosis, further sections were taken in for the immunohistochemistry (IHC) procedure.

Immunohistochemistry staining
Two section each of 4 \( \mu \text{m} \) thickness of each paraffin-embedded tissues were taken on positively charged slides and stained for p16 and p27 antibody by IHC (p16\textsuperscript{INK4a} biocare [ACR 3007 A, C]) (p27 Kip antibody Thermofisher [AHZ0452]).

The paraffin-embedded tissues sectioned at 4 \( \mu \text{m} \) thickness were deparaffinized in xylene and rehydrated in graded alcohols and distilled water. Endogenous peroxidase activity was blocked for 30 min in methanol containing 0.3% hydrogen peroxide. Antigens were retrieved in ethylenediaminetetraacetic acid (EDTA) solution with heat using a Declocking chamber (95°C) for 40 min. The slides were then washed twice in tris buffer. Slides were kept in a humid chamber and were incubated with protein block for 10 min following which the sections were washed with tris buffer twice. One section of each tissue specimen was incubated with primary antibody against p16, and the other section of tissue specimen was incubated with primary antibody against p27 at 37°C temperature for 45 min in a moist chamber. Subsequently, the sections were incubated with mouse probe (for binding nonspecific antigen) for 10 min and washed with tris buffer. Secondary antibody conjugated with horseradish peroxidase (MRH538 L10) was added to the slide at room temperature and kept for 30 min. The sections were washed in tris buffer twice for 4 min. The sections were treated with diaminobenzidine (BRB900B) solution for 5 min to observe the reaction products. The section was counterstained with Harris hematoxylin for 1 min, washed with tap water for 1 min, air dried, cleared with xylene and coverslipped using dibutyl phthalate in xylene.

- Tris buffer solution was prepared by adding 8 g of sodium chloride and 0.6 g of tris buffer in 1000 ml of distilled water and pH maintained to 7.2–7.6 using hydrochloric acid.
- EDTA solution was prepared by adding 2.5 ml of EDTA solution in 250 ml of distilled water.

Quantitative and qualitative assessment of p16 and p27
The IHC-stained sections (normal mucosa, PMDs and OSCC cases) were analyzed and assessed in 5 random high-power fields under \( \times 40 \) magnification using research microscope (Olympus-BX53-progress software) by author 1 and author 2, and the grading was based on the intensity of brown color and areas of positive staining. The cells from the highly positive-stained areas were selected and scored. The percentage of positive cells was classified as follows:
The nuclear expression of p16 and p27 were analyzed and assessed in 5 random high-power fields according to the scoring criteria mentioned in Table 1. The intensity of staining obtained was graded. The data collected were analyzed.

Evaluation of the immunohistochemical staining
IHC revealed that the p16 and p27 protein showed nuclear staining. The intensity of staining was highest in cases of normal epithelium and decreased in intensity from low- to high-grade PMDs and OSCCs.

The extent of staining for p16 and p27 gene was scored depending on the level of the epithelium involved. The data were scored on a grade of I-IV as mentioned above.

RESULTS

Based on the above scores which were obtained as results, graph was obtained and results were tabulated [Tables 2 and 3 and Graphs 1 and 2].

p16 and p27 expression was assessed based on both the intensity of nuclear staining within the basal, parabasal and suprabasal layers of the epithelial cells and the percentage of cells that were positive.

By immunohistochemical analysis, it was demonstrated that 60% of normal epithelium was diffusely positive for p16 and p27. In cases of low-risk PMDs, 42% of cases demonstrated diffuse positivity for p16 and 57% of cases demonstrated diffuse positivity for p27. In cases of high-risk PMDs, 45% of cases showed diffuse positivity for p16 and 30% of cases showed diffuse positivity for p27. None of OSCC showed diffuse positivity either for p16 and p27.

DISCUSSION

OSCC is the sixth most frequently diagnosed malignancy with a high incidence of mortality and morbidity worldwide.[6] Abnormalities in various components of cell cycle have been found in several types of human cancer, including oral cancer.[7] The late G1 checkpoint is the most important step in cell cycle which is governed by many tumor suppressor gene[8] which in turn is regulated by cyclin/CDK complexes and CDK inhibitors.[9]

OSCC is generally believed to be preceded by potentially malignant disorders (PMDs). The main purpose of identification of PMDs is to prevent malignant transformation by initiating adequate intervention. In our study, p16 and p27 expression was immunohistochemically analyzed in PMDs and in OSCCs.[10]

Altered or loss of p16 expression has been noticed in oral premalignant lesions and tumor of the oral cavity.[11] In our study, there was decreased expression of p16 in high-risk PMDs and OSCC [Figures 1 and 2] when compared with low-risk PMDs and normal epithelium [Figures 3 and 4]; these results were in accordance with study conducted by Papadimitrakopoulou et al. who demonstrated that there was decreased immunohistochemical expression of p16 in oral premalignant lesions. Study conducted by Muirhead et al. and Shah et al. showed decreased expression of p16 in OSCC when compared with dysplasia and normal epithelium. P16 is a tumor suppressor gene which inhibits phosphorylation of Rb and promotes the formation of an Rb-E2F repressive transcriptional complex, which blocks cell cycle progression at the G1-S restriction point. Decreased expression of p16 in dysplasia and OSCC can be explained by p16 gene inactivation which may occur due to

Table 1: Scoring Criteria

| Qualitative score | Staining |
|-------------------|----------|
| GRADE I           | Negative, no stained cells |
| GRADE II          | Focally positive, fewer than 25% of stained cells (+) |
| GRADE III         | Moderately positive, more than 25% and fewer than 50% of stained cells (++) |
| GRADE IV          | Diffusely positive, more than 50% of stained cells (+++) |

Table 2: Shows p16 gene expression in different study groups

| Case               | Grade 0 | Grade 1 | Grade 2 | Grade 3 |
|--------------------|---------|---------|---------|---------|
| Normal epithelium  | 0       | 0       | 0       | 3       |
| Low risk PMD S     | 0       | 3       | 0       | 2       |
| High risk PMD S    | 0       | 0       | 2       | 3       |
| OSCC               | 0       | 3       | 1       | 1       |

Table 3: Shows p27 gene expression in different study groups

| Case               | Grade 0 | Grade 1 | Grade 2 | Grade 3 |
|--------------------|---------|---------|---------|---------|
| Normal epithelium  | 0       | 0       | 2       | 3       |
| Low risk PMD S     | 0       | 2       | 1       | 2       |
| High risk PMD S    | 0       | 2       | 3       | 0       |
| OSCC               | 0       | 3       | 2       | 0       |

Graph 1: P16 expression in different study groups
homozygous gene deletion, mutation and hypermethylation of promoter region of p16 gene which was observed at high frequency in severe dysplasia and OSCC.[1][i]

p27 is a cyclin-dependent kinase inhibitor; it has an ability to block the activity of cyclin E/cdk2 and cyclin A/cdk2 in cells arrested in G1 phase. A large

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**Figure 1:** Expression of p16 in high-risk potentially malignant disorders

**Figure 2:** Expression of p16 in oral squamous cell carcinoma

**Figure 3:** Expression of p16 in normal oral epithelium

**Figure 4:** Expression of p16 in low-risk potentially malignant disorders

**Figure 5:** Expression of p27 in low-risk potentially malignant disorders

**Figure 6:** Expression of p27 in high-risk potentially malignant disorders
number of studies have examined the diagnostic and prognostic significance of p27 expression in various tumors.\[^{15}\] Reduced p27 expression was also associated with increasing lymph node metastasis and stage of tumor and resulted in a poor prognosis for patients with OSCC. Downregulation of p27 protein in cancers promotes metastasis as well as cell proliferation and hence represents as a powerful prognostic marker for survival rate determination in OSCC patients.\[^{16}\] A significant decrease in expression of p27 with the progressive grades of dysplasia was observed in our study which was in accordance with the study conducted by Ramasubramanian \etal,\[^{10}\] who suggested that reductions in p27 protein may contribute to, or reflect, the increased cell proliferation seen in any progression toward oral carcinoma and early carcinogenesis. Our study also demonstrated decreased expression of p27 in PMDs and OSCC [Figures 5-7] when compared to normal epithelium [Figure 8]; this is in accordance with Lloyd \etal,\[^{17}\] who showed reduced expression of p27 protein in OSCC. Downregulation of p27 protein in OSCC may be explained by increased ubiquitin–proteasome-mediated degradation, Skp2-specific factor for the ubiquitination and consequent degradation of p27. Skp2 overexpression was frequently observed in epithelial dysplasia and OSCC.\[^{16}\]

**CONCLUSION**

We concluded from our study that both p16 and p27 expression was significantly high in normal epithelium when compared to PMDs and OSCC. Decreased expression of both the proteins in high-risk PMDs was seen when compared to low-risk PMDs and normal epithelium, thus giving a substantial proof that p16 and p27 play an important role in carcinogenesis and are good prognostic markers for predicting the malignant transformation of oral epithelial dysplasia. However, p27 is a better marker and has an accurate prediction as a prognostic marker of OSCC. Therefore, these proteins can be used as an effective tool to evaluate the progression of PMDs to dysplastic changes in the epithelial leading to OSCC.

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**Conflicts of interest**

There are no conflicts of interest.

**REFERENCES**

1. Fregonesi PA, Teresa DB, Duarte RA, Neto CB, de Oliveira MR, Soares CP, \etal. P16(INK4A) immunohistochemical overexpression in premalignant and malignant oral lesions infected with human papillomavirus. J Histochem Cytochem 2003;51:1291-7.
2. DE Almeida MR, Pérez-Sayáns M, Suárez-Peñaranda JM, Somoza-Martín JM, García-García A. P27Kip1 expression as a prognostic marker for squamous cell carcinoma of the head and neck. Oncol Lett 2015;10:2675-82.
3. Patel V, Jakus J, Harris CM, Ensley JF, Robbins KC, Yeudall WA, \etal. Altered expression and activity of G1/S cyclins and cyclin-dependent
kinases characterize squamous cell carcinomas of the head and neck. Int J Cancer 1997;73:551-5.
4. Queiroz AB, Fochi G, Dobo C, Gomes TS, Ribeiro DA, Oshima CT, et al. Expression of p27, p21(WAF/Cip1), and p16(INK4a) in normal oral epithelium, oral squamous papilloma, and oral squamous cell carcinoma. Anticancer Res 2010;30:2799-803.
5. Shintani S, Nakahara Y, Mihara M, Ueyama Y, Matsumura T. Inactivation of the p14(ARF), p15(INK4B) and p16(INK4A) genes is a frequent event in human oral squamous cell carcinomas. Oral Oncol 2001;37:498-504.
6. Kale AD, Mane DR, Babji D, Gupta K. Establishment of field change by expression of cytokeratins 8/18, 19, and MMP-9 in an apparently normal oral mucosa adjacent to squamous cell carcinoma: A immunohistochemical study. J Oral Maxillofac Pathol 2012;16:10-5.
7. Chin L, Pomerantz J, DePinho RA. The INK4a/ARF tumor suppressor: One gene – Two products – Two pathways. Trends Biochem Sci 1998;23:291-6.
8. Lee JK, Kim MJ, Hong SP, Hong SD. Inactivation patterns of p16/INK4A in oral squamous cell carcinomas. Exp Mol Med 2004;36:165-71.
9. Saito T, Nakajima T, Mogi K. Immunohistochemical analysis of cell cycle-associated proteins p16, pRB, p53, p27 and Ki-67 in oral cancer and precancer with special reference to verrucous carcinomas. J Oral Pathol Med 1999;28:226-32.
10. Ramasubramanian A, Ramani P, Sherlin HJ, Premkumar P, Natesan A, Thiruvengadam C, et al. Immunohistochemical evaluation of oral epithelial dysplasia using cyclin-D1, p27 and p63 expression as predictors of malignant transformation. J Nat Sci Biol Med 2013;4:349-58.
11. Vairaktaris E, Yapijakis C, Psyrri A, Syrroididou S, Yannopoulos A, Lazaris A, et al. Loss of tumour suppressor p16 expression in initial stages of oral oncogenesis. Anticancer Res 2007;27:1799-84.
12. Papadimitrakopoulou V, Izzo J, Lippman SM, Lee JS, Fan YH, Clayman G, et al. Frequent inactivation of p16INK4a in oral premalignant lesions. Oncogene 1997;14:1799-803.
13. Muiirhead DM, Hoffman HT, Robinson RA. Correlation of clinicopathological features with immunohistochemical expression of cell cycle regulatory proteins p16 and retinoblastoma: distinct association with keratinization and differentiation in oral cavity squamous cell carcinoma. J Clin Pathol 2006;59:711-5.
14. Shah NG, Trivedi TI, Tankshali RA, Goswami JV, Jerly DH, Shukla SN, et al. Prognostic significance of molecular markers in oral squamous cell carcinoma: A multivariate analysis. Head Neck 2009;31:1544-56.
15. Shintani S, Mihara M, Nakahara Y, Kiyota A, Ueyama Y, Matsumura T, et al. Expression of cell cycle control proteins in normal epithelium, premalignant and malignant lesions of oral cavity. Oral Oncol 2002;38:235-43.
16. Kudo Y, Kitajima S, Ogawa I, Miyauchi M, Takata T. Down-regulation of Cdk inhibitor p27 in oral squamous cell carcinoma. Oral Oncol 2005;41:105-16.
17. Lloyd RV, Erickson LA, Jin L, Kulig E, Qian X, Cheville JC, et al. P27kip1: A multifunctional cyclin-dependent kinase inhibitor with prognostic significance in human cancers. Am J Pathol 1999;154:313-23.