

Association of P16 Expression with Clinicopathological Features of Oral Cavity Squamous Cell Carcinoma Patients in Bali

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Background: Oral cavity cancer is considered as a major health problem worldwide and has been associated with high recurrence rate and poor prognosis. Oral cavity squamous cell carcinoma (OCSCC) in Sanglah General Hospital Denpasar contributed to 71.7% of all head and neck cancer from 2002-2011 with an increasing trend annually. Advances in our understanding of OCSCC have not improved the outcome in OCSCC management significantly. Therefore, many studies have focused on the roles of biomolecular markers in OCSCC. One of biomolecular marker that has been the focus of many OCSCC studies is p16. **Method:** This was an analytic cross sectional study with 31 samples to determine the association of p16 expression with age group, tumor location, stage, and grade in OCSCC. Data was analyzed descriptively and the association between variables were evaluated with Chi-Square or Fisher's Exact Test with a p value <0.05 was considered significant. **Results:** From 31 samples collected, the mean age was 56.19±15.197 years old. 17 samples (54.8%) were female and 17 samples (54.8%) were from the ≤60 years old age group. Majority of the tumor was located on the tongue (14 samples; 45.2%) with postero-inferior group location (19 samples; 61.3%). Almost half of the samples were in Stage IVA-B (15 samples; 48.4%) and within high grade group (24 samples; 77.4%) meanwhile 21 samples (67.7%) were in low p16 expression group. There was a significant association between p16 expression with tumor grade group (p=0,044). **Conclusion:** p16 expression was significantly associated with tumor grade in OCSCC patients.

Keywords: Oral Cavity Squamous Cell Carcinoma, p16, clinicopathological features.

DOI: 10.15562/bmj.v5i3.205

Cite This Article: Ampur, O., Sudarsa, I., Suryawisesa, I. 2016. P16 Expression Correlation with Clinicopathological Features of Oral Cavity Squamous Cell Carcinoma Patients in Bali. *Bali Medical Journal* 5(3). DOI:10.15562/bmj.v5i3.205

INTRODUCTION

Oral cavity cancer (OCC) is a malignancy which arises from the mucosal epithelium lining the oral cavity, the organs of the oral cavity and salivary glands (especially the minor salivary glands) which were located on the wall of the oral cavity. The scope of the organs in the oral cavity were the upper and lower lips, tongue anterior two-thirds, buccal mucosa, floor of mouth, gingiva, maxilla and mandible, trigonum retromolare, palatum durum and molle triangule.^{1,2}

Oral cancer was the sixth most found cancer in the world with an estimated 500,000 new cases each year. Frequency and risk factors differ in different geographic regions. OCC contributed to 3-5% of all human malignancy.³

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OCC was a major health problem and was associated severe morbidity. The mortality rate was 120,000 deaths annually.^{4,5} The incident is estimated at 270,000 cases in men (5% of all cancers) and 119,000 (2.5%) in women. Two-thirds of the cases (67%) were reported from developing countries with a ratio of male and female 3-4:1.³ Locally, the incidence of Oral Cavity Squamous Cell Carcinoma (OCSCC) based of epidemiological data in Sanglah Hospital Denpasar was approximately 71.7% (2002 - 2011) and there has been significant increase annually.⁶

More than 90% of malignancy of the oral cavity was squamous cell carcinoma. Despite many progress in diagnostic and therapy for OCSCC, current modality still unable to detect precancerous lesions (leukoplakia and erythroplakia) which is the reason why majority of patients come with advanced disease (stages III and IV). Furthermore, approximately 20-50% cases had unknown clinical lymph node metastases to the neck and primary tumor recurrence within one year after treatment with poor prognosis.

Current consideration in determining oral cancer treatment decisions (the tumor node metastasis, histopathological type and grade) were considered unsatisfactory. Therefore, biomolecular markers were considered important for diagnostic and prognostic indicators.²

p16 is a tumor suppressor gene which produced p16 protein that binds to CDK4/CDK6, causing cell cycle arrest. Normal p16 activity is required for cell cycle regulation from the G1 phase to the S phase.⁸ Mutations in p16 gene produce mutant p16 protein which is unable to bind CDK4/CDK6, therefore causing uncontrolled cell cycle and results in cellular transformation.⁹

Several studies have shown reduced or absence of p16^{INK4} expression in premalignant lesions of the oral cavity and OSCC.¹⁰⁻¹⁴ A study in Japan in 1999 showed changes in α transcripts of p16 gene in all OSCC examined and obtained multiple patterns of gene inactivation.¹⁵ Naggar et.al found reduced or absence of p16 expression in 89% of OSCC cases. Nakahara et.al reported that 67.9% of p16 is under expressed in OSCC. Meanwhile, a study with adequate tools to assess oral epithelial dysplasia and reduced expression of p16 was conducted by Greer et.al. He found reduced expression of p16 in three early stages of OSCC. The same study also reported Smokeless tobacco keratosis (STK) and alveolar ridge keratosis (ARK) was very weak but relatively strong in all SCC.¹⁶

Yuen et.al had 229 head and neck squamous cell carcinomas patients in which 48% had low expression of p16 and a higher incidence of p16 under expression was found in the larynx and pharynx compared to the pharynx and oral cavity.¹⁷ Yuen et al found that p16 under expression was significantly associated with the tumor location, size, and history of radiotherapy. They found a weak correlation between p16 expression with younger age, higher tumor grade, as well as a more posterior location. Nevertheless, there has been no established consensus about p16 expression with clinicopathological features of oral cavity squamous cell carcinoma with majority of researches showed mixed results for p16 correlation with clinicopathological features of OSCC.^{10,14,18}

Given the high incidence of OSCC in Indonesia (especially Bali), generally unsatisfactory clinical outcome, and variations of results between literatures, we interested to investigate the association of p16 expression with clinicopathological features (age, tumor location, tumor stage, and tumor grade) of OSCC in Bali.

MATERIAL AND METHOD

A cross-sectional analytical study was conducted to determine the correlation of p16 protein expression with clinicopathological features

of OSCC patients in Bali. The sampling method was retrospective which includes OSCC patients treated at the Department of Surgery Udayana University / Sanglah General Hospital in Denpasar Bali, from January 1st 2010 until March 31st 2013.

The data of tumor location, histopathologic type, tumor stage and tumor grade were collected from OSCC patient's medical records in the Department of Pathology Anatomy Udayana University/Sanglah General Hospital. Paraffin block were cut into 5 μ m thickness, de-waxed for 15 minutes in xylene and rehydrated with ethanol. Slides were treated with 3% hydrogen peroxidase solution for 30 minutes at room temperature. After being washed three times with phosphate buffered saline, microwave antigen retrieval was performed in citrate buffer (pH 6) for 5 minutes. We used a 1/250 dilution, followed by immunohistochemical staining of p16 (Avidin biotin complex immunoperoxidase and Anti-CDKN2A/p16INK4a antibody [EPR1473] ab108349 (Abcam).

Sample age was determined by age listed in medical records at the time of diagnosis. In this study, samples were grouped into two groups: ≤ 60 years and >60 years of age. Tumor location was determined from intraoperative tumor location. Oral cavity organ locations were the lips, tongue, buccal, gingival maxilla and mandible, hard and soft palate. Tumor locations were grouped into: antero-superior location (lip, palate and maxillary gingiva) and postero-inferior location (tongue, buccal, and mandibular gingiva). Tumor stage was determined by AJCC tumor staging system (2002). Stage I, II, III were categorized as low stage and stage IV A-B and IV C were categorized as advanced stage. Well differentiated tumors were categorized as low grade, while moderate and poorly differentiated tumors were categorized as high grade.

Descriptive analysis was conducted to determine samples baseline characteristics as well as proportion of variables. The association between p16 and other variables was evaluated by chi-square or Fisher's exact test. P-value of <0.05 was considered significant.

RESULTS

Of the 36 samples collected, five samples did not meet the inclusion criteria. Two with no paraffin blocks, two samples showed normal histopathologic features, and one sample with damaged paraffin block. Of the 31 patients who were the subjects of research, the youngest was 19 years old and the oldest was

80 years old with a mean age of 56.19 ± 15.197 years. In this study there were 14 samples (54.8%) within ≤ 60 years age group and 14 samples (45.2%) within >60 years age group. A total of 23 samples (74.2%) were from Bali and 8 samples (25.8%) were Non Balinese.

Table 1. OCSCC Sample Baseline Characteristics

Variable	n
Age	
≤60 years	17 (54,8%)
>60 years	14 (45,2%)
Sex	
Male	14 (45,2%)
Female	17 (54,8%)
Tumor Location	
Tongue	14 (45,2%)
Gingiva	6 (19,4%)
Palatum	4 (12,9%)
Buccal	5 (16,1%)
Lip	2 (6,5%)
Tumor Location Group	
Antero-superior	12 (38,7%)
Postero-inferior	24 (61,3%)
Stadium	
Stadium II	1 (3,2%)
Stadium III	14 (45,2%)
Stadium IV A-B	15 (48,4%)
Stadium IV C	1 (3,2%)
Tumor Grade	
Well Differentiated	7 (22,6%)
Moderately Differentiated	22 (71,0%)
Poorly Differentiated	2 (6,5%)
P16 expression	
+1	15 (48,4%)
+2	6 (19,4%)
+3	7 (22,6%)
+4	3 (9,6%)

12 samples (38.7%) had tumor located in the antero-superior region and 19 samples (61.3%) had a postero-inferior tumor location. Meanwhile, 15 samples (48.4%) had a low stage, 16 samples (51.6%) with advanced stage, 7 samples (22.6%) with low-grade and 24 samples (77.4%) with a high grade. A total of 21 samples (67.7%) showed weak p16 expression and 10 samples (32.3%) showed strong p16 expression. Overall, the characteristics of the sample are presented in **Table 1**.

Association between p16 Expression with Oral Cavity Cancer Age Group

There were 17 samples in the ≤60 years age group with 12 samples (70.6%) showed weak expression of p16 and 5 samples (29.4%) showed strong expression of p16. From 14 samples in >60 years age group there were 9 samples (64.3%) with weak expression of p16 and 5 samples (35.7%) showed strong expression. Fisher's exact test showed no significant association between p16 expression with age in this study group (**Table 2**).

Relationship between p16 Expression with Oral Cavity Cancer Location

There were 12 samples with tumor located in the antero-superior region, as many as 7 samples (58.3%) showed weak expression of p16 and 5

samples (41.7%) showed strong expression of p16. The postero-inferior tumor group contained 19 samples of which 14 samples (73.7%) showed weak expression of p16 and 5 samples (26.3%) showed strong expression of p16 (**Table 3**).

Table 2. p16 Expression Correlation with Age Group

	Age ≤60 years	Age >60 years	p
Weak p16 expression	12 (70,6%)	9 (64,3%)	0,503
Strong p16 Expression	5 (29,4%)	5 (35,7%)	
Total	17 (100%)	14 (100%)	

Table 3. Association of p16 Expression with Tumor Location

	Antero-Superior	Postero-Inferior	p
Weak p16 expression	7 (58,3%)	14 (73,7%)	0,308
Strong p16 Expression	5 (41,7%)	5 (26,3%)	
Total	12 (100%)	19 (100%)	

Fisher's exact test results demonstrate no significant association between p16 expression with tumor location in this study (p = 0.308).

Association of p16 Expression with Oral Cavity Cancer Stage

From 15 samples with low stage, as many as 10 samples (66.7%) showed weak expression of p16 and 5 samples (33.3%) showed strong expression of p16.

Table 4. Association of p16 Expression Correlation with Tumor Stage Group

	Low Stadium	Advanced Stadium	p
Weak p16 expression	10 (66,7%)	11 (68,8%)	0,602
Strong p16 Expression	5 (33,3%)	5 (31,3%)	
Total	15 (100%)	16 (100%)	

Within the group of advanced stage tumors, there were 16 samples of which 11 samples (68.8%) showed weak expression of p16 and 5 samples (31.3%) showed strong expression of p16 (**Table 4**). Fisher's exact test results demonstrate no significant association between p16 expression with tumor stage in this study (p=0.602).

The study also found no significant association between p16 expression with variables T, N, and M staging system (**Table 5**).

Table 5. Association of p16 Expression with T-N-M Status in OCSCC

Variable	Weak p16 expression	P
T	T ₀₋₃ 12 (57,1%) 9 (42,9%)	0,597 (Fisher's exact test)
	T ₄	
N	N ₀ 6 (28,6%) 15 (71,4%)	0,483 (Fisher's exact test)
	N ₁₋₂	
M	M ₀ 20 (95,2%) 1 (4,8%)	0,548 (Fisher's exact test)
	M ₁	

Association between p16 Expression with Oral Cavity Cancer Grade

From 7 samples with low-grade tumors, a total of 7 samples (100.0%) showed weak expression of p16 and no sample showed strong expression of p16. In the group of high-grade tumors, there were 24 samples of which 14 samples (58.3%) showed weak expression of p16 and 10 samples (41.7%) showed strong expression of p16 (Table 6). Fisher's exact test results demonstrate the value of $p = 0.044$ ($p < 0.05$), so that there was a significant association between p16 expression with tumor grade in this study.

Table 6. Association of p16 Expression Correlation with Tumor Grade Group

	High Grade	Low Grade	P
Weak p16 expression	14 (58,3%)	7 (100%)	0,044
Strong p16 Expression	10 (41,7%)	0 (0,0%)	
Total	24 (100%)	7 (100%)	

DISCUSSION

In this study, 31 samples were selected from 36 patients included. A total of 23 samples (74.2%) were Balinese and 8 samples (25.8%) were Non Balinese. The youngest was 19 years of age and the oldest was 80 years of age. Fourteen samples (45.2%) were male and 17 samples (54.8%) were female. The mean age of the sample was 56.19 ± 15.197 years with 17 samples ≤ 60 years (54.8%) and 14 samples > 60 years (45.2%). Most tumors were located on the tongue (14 samples, 45.2%) with most at postero-inferior region (19 samples, 61.3%). Most samples were in Stage IVA-B (15 samples, 48.4%), the high-stage group (16 samples, 51.6%), and had a moderately differentiated grade (22 samples, 71.0%) within the high grade group (16 samples, 51.6%). A total of 21 samples (67.7%) showed a weak p16 expression.

In terms of age, we found the tendency for OCSCC to be younger (≤ 60 years, 17 samples,

54.8%) in this study, which was in accordance with data obtained by Westra and Curado.^{19,20} There was a difference in terms of male to female ratio in this study compared to others (14 male samples (45.2%) and 17 female samples (54.8%); 8,2:10). We suspect that other factors such as smoking, alcohol, tobacco chewing, oral sex, and education, which were not analyzed in this study contributed to this difference in result. We found that the OCSCC patients in Bali were relatively young, but the majority were women. OCSCC found in this study was mostly on the tongue (In accordance with the findings of Westra 2009) with the majority of the group located on the postero-inferior region. Therefore, further research on this matter is still needed.

The role of p16 in the carcinogenesis and clinicopathology of OCSCC has become a topic of research for the last two decades.²¹ Pande et al in 1998 found a significant correlation between tumor progression with tumor stage. However, there are still many differences between the results of studies on the role of p16 in OCSCC.^{14,15,22} Nakahara et al (2001) found that weak expression of p16 was found in 67.9% of squamous cell carcinoma samples, with similar results to that obtained in our study where weak p16 expression were found in 21 samples (67.7%). However, Yuen et al (2002) in his study on the expression of p16 in OCSCC found weak p16 expression by 48%. Differences between this study and Yuen et al (2002) is in terms of the number of samples, Yuen et al used 225 samples that may be more representative of the population there. During sample calculations, we used the proportional value of p16 under expression of 0.6, where the figure was obtained from the research Nakahara et al (2001). This value indicates that the difference in the proportion of p16 under expression on OCSCC likely differs between each population and requires further research with a larger sample size.

Significant correlation between p16 expression with OCSCC was not found in this study ($p = 0.602$), as well as between p16 expression with age group ($p = 0.503$). Vairaktaris et al (2007) found that p16 under expression was found at the beginning of the carcinogenesis process of OCSCC, but they did not find a significant association between p16 under expression with dysplasia phase in OCSCC carcinogenesis. In this study, we did not obtain any significant correlation between p16 protein expression with tumor location ($p = 0.595$). Yuen et al (2002) found that more frequent p16 under expression were found in laryngeal carcinoma compared to carcinoma of the oral cavity and pharynx. One weakness of this study is limited diagnostic facilities for laryngeal and pharyngeal carcinomas. Therefore, we limited the study only on oral cavity carcinoma. What was interesting in

this study is that although there was no significant correlation between p16 expression with T-N-M status ($p = 0.597$, $p = 0.483$, $p = 0.548$ respectively), we found a significant correlation between p16 expression with OCSCC grade ($p = 0.044$). However, we could not determine the value of the odds ratio because we did not find any samples which showed strong expression of p16 tumor with low grade.

CONCLUSION

A significant correlation was found between p16 expression with OCSCC grade. This study was a preliminary study; a more robust research has to be designed to better understand the role of p16 in OCSCC.

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