

Role of p16 and p53 in Oral Potentially Malignant Disorders and Oral Squamous Cell Carcinoma: A Study in Malaysia

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Abstract

Ninety-five percentages of oral cancer are classified as oral squamous cell carcinoma (OSCC). p16 and p53 are protein in human cell cycle regulation that can play role as tumour suppressor genes. The purpose of the research is to detect p16 and p53 expressions in oral potentially malignant disorders (OPMDs) and OSCC using immunohistochemistry (IHC). A total of 87 formalin-fixed paraffin embedded tissue were selected comprising of OPMDs cases (n=32), OSCC cases (n=46) and normal oral mucosa (n=9). The cases were retrieved from the archives of the Oral Pathology Laboratory, Faculty of Dentistry, UKM, Institute of Medical Research and Hospital Tunku Mizan. The IHC staining was manually performed using p16 antibody (1:1000) (Abcam) and p53 antibody (clone DO-7, Dako, Denmark) following manufacturer's instruction and assessed semi-quantitatively (positivity and staining intensity) between all groups. Positive and negative controls were used to validate the IHC run. All data were then analysed using SPSS version 22.0 and p values <0.05 were considered significant. The p16 were found positive in OSCC (96.7%) and in OPMDs (84.38%). There is significantly higher p16 positivity in OSCC compared to normal oral mucosa ($p<0.001$). The p53 expression was detected in OPMDs and OSCC. There are significant differences between p53 expression among in NOM and OSCC ($p<0.01$) as well as OPMDs and OSCC ($p<0.05$).

In this present study, p16 and p53 expressions were increased following degree of malignancy. The finding suggests that p16 and p53 can be used as a potential marker for oral malignancy detection.

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Introduction

Oral cancer carries the 11th position in the worldwide cancer incidence with oropharyngeal cancer rank as the first.¹ Incidence of oral cancer remains low in UK and USA, approximately only about 2% of the population. Contrast with that condition, the incidence of oral cancer is higher in developing countries such as India and Sri Lanka, approximately 40% of all the cancer there.² According to the World Health Organization (WHO) in April 2011, oral cancer deaths in

Malaysia reach 1587 cases or 1.55% of total deaths. The age adjusted death rate is 7.72 per 100,000 of population ranks Malaysia number 14 in the world. National Cancer Registry Malaysia reported that in 2007, oral cancer is ranked 21st most common cancer in the general population and 17th most frequent cancer in males and 16th in females.³

The exact aetiology for oral cancer is controversial, probably because of the variability of the cancer statistic quality and multifactorial with the complexity of the malignancy aetiology.² Some of aetiological risk factor for oral cancer are tobacco and tobacco products, alcohol consumption, human papillomavirus (HPV), nutritional factors (e.g. low intake of fruits and vegetables, iron deficiency, lack of vitamin A, C, E and riboflavin deficiency, impaired immunity (HIV, EBV and renal allograft recipients), and

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potentially malignant disorders.⁴ Over sunlight exposure is the risk factor for lip carcinoma, while carcinoma on buccal mucosa seems related with betel quid chewing. There is no evidence that chronic irritation will develop into oral cancer.⁵

Squamous cell carcinoma is a malignant tumour of the squamous epithelium which is the most common primary malignancy of the oral cavity. Oral squamous cell carcinoma (OSCC) usually presents as an exophytic ulcerative mass, but in early stage may occurs as white and plaque-like (leukoplakia), erythematous (red) and plaque-like (erythroplakia) or mixture from all of it (speckled leukoplakia). OSCC may occur anywhere in the oral cavity, but mostly on the buccal mucosa, floor of the mouth, ventrolateral tongue, soft palate and tonsillar pillar. The majority of OSCC found in patients over 40 years of age.

Tumour suppressor genes have big role in physiological processes, such as signal transduction pathways for growth, differentiation and programmed cell death. p16 and p53 are classified as tumour suppressor genes that have function in cell cycle control.⁶

Immunohistochemistry (IHC) is popularly used in medical research, especially in detecting carcinoma tissues. Immunohistochemistry analysis of p16 in Head and Neck Squamous Cell Carcinoma (HNSCC) biopsies has been shown to work as a surrogate marker to identify HPV in sample from HNSCC.⁷ p16 or p16^{Ink4A} is one of protein in cell cycle regulation. Expression of p16 significantly showed the correlation with improved outcomes in head and neck squamous cell carcinoma according to Lewis (2010) and Harris *et al.* (2011).^{8,9} While according to Antonsson *et al.* (2015) positive p16 expression was only detected in 28% of HNSCC cases.¹⁰

The purpose of this research is to detect the expression of p16 and p53 in OPMDs and OSCC within Malaysian population and relate it to socio-demographic variables.

Materials and methods

This is a retrospective study with total of 87 formalin-fixed paraffin embedded tissues (FFPET) selected comprising of test group OSCC cases (n=46), OPMDs (n=32) together with control group NOM (n=9) from Oral Pathology Laboratory UKM, Institute for Medical Research (IMR), Kuala Lumpur and Hospital

Tunku Mizan, Malaysia. Insufficient tissues and samples with no definite diagnosis of OSCC and OPMDs will be excluded from the research. Inclusion criteria are samples come from patients who are clinically and histologically diagnosed with OSCC using established TNM classification of carcinomas of the oral cavity by World Health Organization in 2005 and samples come from new cases and the patients never undergo with any anticancer treatment before. Specimens which are FFPET were processed in Oral Pathology UKM laboratory for immunohistochemistry staining. An ethical approval was brought from PPUKM Medical Research and Ethics Committee, Universiti Kebangsaan Malaysia with approval number UKM 1.5.3.5/244/DD/2015/013(2).

p16 and p53 Immunohistochemistry Procedures

4µm thickness tissues were cut from FFPET blocks and placed on poly-L-lysine coated glass slides. Before starting immunohistochemistry, sections were deparaffinized in the oven at 60°C for overnight and put in PT Link (Dako) machine approximately for 1 hour filled with target retrieval solution. After PT Link process done, sections were incubated in hydrogen peroxidase for 5 minutes and rinsed with phosphate buffer saline (PBS). Sections were then reacted with anti-p16 primary antibody (1:1000, Abcam, England) and anti-p53 primary antibody (clone DO-7, Ready to use, Dako, Denmark) and incubated at room temperature for 45 minutes. After 45 minutes, sections were incubated with secondary antibody, amplified with streptavidin-peroxidase and stained with 3,3' diaminobenzidine tetrahydrochloride chromogen solution (DAB) for 3 minutes. Sections washed for several times with PBS in between all steps. After antibody incubation, the sections were counterstained by hematoxylin eosin for 3 minutes and rehydrated in alcohol and xylene. The sections were then mounted with slip glass cover using mounting agent. For negative control, slides were stained without the primary antibody included for staining.

We examined the results of the immunohistochemistry staining in at least 400x magnification using microscope. Semi quantitative analysis was taken referring to Abrahao *et al.* and Tarakji *et al.* (2010).^{11,12} Positive IHC expression was defined by granular brown staining cells in nuclear and cytoplasm of

epithelial cells. The percentage of positive cells were assessed and scored as 0= no expression, 1= weak staining, 0-25% or total cells show positive staining, 2= moderate staining, 25-75% of the cells show positive staining, 3= strong staining, >75-100% cells show positive staining. Analysis were done by two observer that has already meet the Kappa Agreement with *K* value = 0.07.

All data were coded and analysed by using SPSS for windows version 22 with 95% confidence interval ($p < 0.05$). The Kolmogorov-Smirnov analysis was used to test the normality of the data. For immunohistochemistry, significant differences in the positive staining cell ratios between each of three groups were analysed using Kruskal-Wallis test with $p < 0.05$ were considered significant followed by Mann-Whitney test as post hoc test.

Results

In the present study, OSCC cases were more dominant in woman (60.87%) and most populated among Malays (36.95%) compared to Indian (36.95) and Chinese (10%). p16 expression in normal oral mucosa (NOM) were detected in basal layer of the epithelium and less in supra basal layer, while in other hand, for OSCC cases, p16 expression were detected more intense in term of cell numbers and located not only in basal layer and supra basal layer of epithelium, but also in the connective tissue. The p16 were found positive in OSCC (97.83%) and in NOM (22.22%). In OSCC, 26.08% were shown weak p16 staining, 34.79% were moderate staining and 39.13% of p16 strong staining intensity.

p16 positivity	NOM	OPMDs	OSCC	<i>p</i> value
Positive	2 (22.22%)	27 (84.38%)	45 (97.83%)	<0.001**
Negative	7 (77.78%)	5 (15.62%)	1 (2.18%)	
p16 staining intensity				
Weak	0 (0%)	9 (28.12%)	11 (23.91%)	0.001**
Moderate	2 (22.22%)	9 (28.12%)	16 (34.78%)	
Strong	0 (0%)	9 (28.12%)	18 (39.13%)	
Total	9 (100%)	32 (100%)	46 (100%)	

Table 1. Result of p16 positivity.

The p16 were found positive in OSCC (97.83%), OPMDs (84.38) and NOM (22.22%) as shown in Table 1. In this present study, there are no significant differences between gender and p16 positivity and p16 staining intensity, and between races and p16 positivity and p16 staining intensity, respectively ($p > 0.05$) as shown

in Table 3. There is significant difference between tumour site and p16 expression, where lateral tongue is the most common site with p16 positivity. In p53, we found significant difference ($p < 0.05$) between p53 and malignancy grading, where moderately differentiated squamous cell carcinoma is the first rank with p53 positivity. There are no significant differences between p53 and sociodemographic variables and tumour site (Table 3).

p53 positivity	NOM	OPMDs	OSCC	<i>p</i> value
Positive	0 (0%)	8 (25%)	23 (50%)	0.018*
Negative	9 (100%)	24 (75%)	23 (50%)	
p53 staining intensity				
Weak	0 (0%)	2 (6.26%)	12 (26.09%)	0.037*
Moderate	0 (0%)	3 (9.37%)	8 (17.39%)	
Strong	0 (0%)	3 (9.37%)	3 (6.52%)	
Total	9 (100%)	32 (100%)	46 (100%)	

Table 2. Result of p53 positivity.

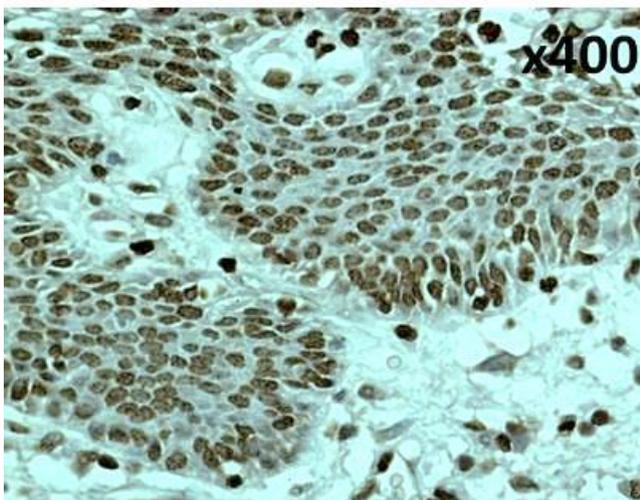
Parameters	p16		<i>p</i> value	All OSCCs		<i>p</i> value
	negative	positive		p53 negative	p53 positive	
Gender						
Female	0	28	0.392	14	14	1.00
Male	1	17		9	9	
Race						
Malay	0	18	0.452	7	11	0.470
Chinese	0	10		6	4	
India	1	17		10	8	
Age						
< 20	0	0	0.675	0	0	0.393
21-40	0	4		1	3	
41-59	0	16		7	9	
≥ 60	1	25		15	11	
Tumour site						
Buccal mucosa	0	15	0.000**	8	7	0.431
Lateral tongue	0	20		9	11	
Tip of the tongue	1	0		0	1	
Alveolar ridge	0	6		2	4	
Lip	0	2		2	0	
Floor of the mouth	0	1		1	0	
Palatal	0	1		1	0	
	0	1		1	0	
Malignancy grading						
Well-differentiated	0	23	0.515	17	6	0.005**
Moderately differentiated	1	19		5	15	
Poorly differentiated	0	3		1	2	

Table 3. Association between sociodemographic and histopathologic parameters and p16 and p53 expression among OSCC samples.

p53 expression were found increasing from NOM, OPMDs and OSCC with percentages of 0%, 25%, and 50% respectively as shown in Table 2. From Kruskal Wallis test, we found significant differences between p16 expression among all groups ($p < 0.001$) and between p53 expression among all groups ($p < 0.05$). Using Mann-Whitney test as a post hoc test, we found significant differences between p16 expression in NOM and OPMDs ($p < 0.01$), NOM and OSCC ($p < 0.01$) as well as OPMDs and OSCC ($p < 0.05$). We also found significant differences between p53 expression in NOM and OSCC ($p < 0.01$) as well as OPMDs and OSCC ($p < 0.05$).

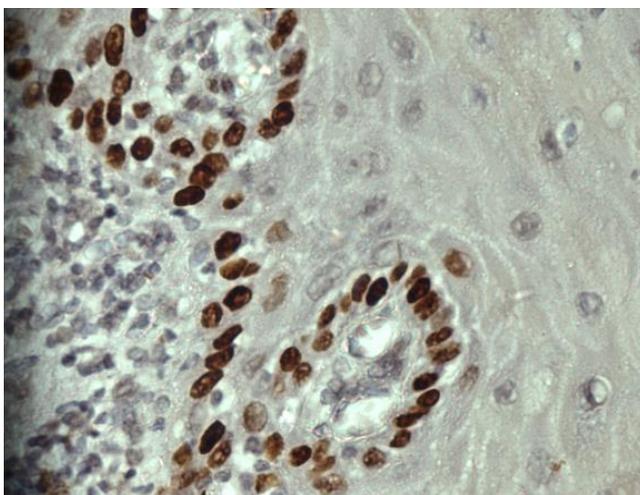


1.a

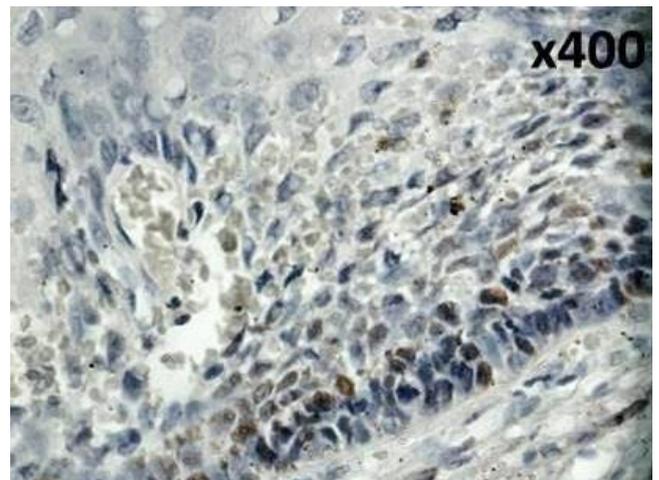


1.b

Figure 1. a,b shown p16 expression in OPMDs and OSCC



2.a



2.b

Figure 2. ab shown p53 expression in OPMDs and OSCC.

In our study, p16 were found mostly in basal layer with strong intensity, but in OSCC p16 were spread almost in whole epithelium layer with moderate staining (Figure 1a,b). p53 in OPMDs were detected restricted in the basal layer of the epithelium and almost in all epithelium layer in OSCC (Figure 2a,b).

Discussion

Prakash et al. (2013) found that 57% cases of leukoplakia were positive for p16 expression while 71.01% cases of OSCC were positive of p16.¹³ Montebugnoli et al. (2011) found that p16 was absent in normal epithelium, 35 patients from 56 (64%) were positive for p16 in oral lichen planus, while in leukoplakia 28% (10/36) were found positive for p16.¹⁴

In OSCC, p16 expression were found in connective tissues as well as in supra basal and basal layer of the oral squamous epithelium. p16 expression increased in OSCC compared in OPMDs. Neufcoeur et al. (2010) found only 9% of p16 positive in hypopharyngeal carcinoma that contrast with our findings.¹⁵ In the present study, almost 100% of OSCC samples were positive for p16. The different result may be due to different sites used in the research. We took OSCC sample from buccal mucosa, lip, palate and tongue sites while Neufcoeur et al. (2010) used hypopharyngeal carcinoma for the sample of malignancy. Angiero et al. (2008) demonstrated p16 increasing in higher grades of dysplasia and invasive OSCC compared to normal and

hyperplastic mucosa which is also consistent with our finding.¹⁶

In our study, p53 in OPMDs were detected restricted in basal layer of the epithelium which comparable with Arreaza et al. (2013) and Alvarez et al. (2013) that observed positive p53 expression in OPMDs limited in the basal layer of the epithelium.^{17,18} Ara et al. (2011) found 60% of OPMDs positive with p53 expression and 67% in OSCC.¹⁹ The frequencies were higher but comparable to our results in OSCC, but not in OPMDs. Here, p53 expression in OSCC is higher than in OPMDs which comparable with Ara et al. (2011); Panjwani et al. (2008) and Alvarez et al. (2013).^{18,19,20} The finding was contrast with Levya-Huerta et al. (2012) that found p53 expression were higher in OPMDs compared to OSCC.²¹ It might occur due to uneven distribution of OPMDs and OSCC cases were used in the research.

In the present study, p53 expression was found 0% in NOM, 25% in OPMDs and 50% in OSCC that similar with Swaninathan et al. (2012) that found the amount of p53 expression increasing from normal to OSCC.²² This consistent result may occur due the same primary antibody that used in study (Clone DO-7, Dako). The finding was contrast with Abrahao et al. (2011) that found p53 expression were higher in premalignant than in malignant lesions.¹¹ In this research, we found no significant differences between NOM and OPMDs. It might occur due to the limitation in a number of normal oral mucosa (NOM) samples compared to other groups (OPMDs and OSCC).

By using Pearson chi-square test and Fisher's exact test, we found that sociodemographic parameters of gender, race and age were not associated with p16 and p53 expression in OSCC ($p>0.05$). There is a strong association between p16 expression and tumour site ($p<0.005$). We also found an association between p53 expression and malignancy grading ($p<0.05$) as shown in Table 3.

Conclusions

The p16 expression was detected in almost of OSCC cases compared with OPMDs and normal oral mucosa (NOM). p16 expression is associated with degree of oral malignancy. The percentages of p53 expression are increasing

from normal oral mucosa, potentially malignant disorders to oral squamous cell carcinoma. p16 and p53 play role in oral tumor progression. The findings suggest that p16 and p53 together can be used as potential markers for early detection in oral malignancy.

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Declaration of Interest

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References

1. Syrjänen, S. Human papillomavirus and head and neck cancer- a global view. 30th International Papillomavirus Conference 2015. Lisbon.
2. Cawson, R.A. & Odell, E.W. Cawson's Essentials of Oral Pathology and Oral Medicine 8th edition. 2008; 277-89.
3. National Cancer Registry Report. Malaysia. 2007. 54.
4. Kalu, U.E. & Bingham, C. 2012. Overview of Oral Cancer. US.
5. Ibsen, O. & Phelan, J. A. Oral Pathology for the Dental Hygienist 6th edition. 2014.
6. Warshawsky, D. & Landolph, J. Molecular Carcinogenesis and The Molecular Biology of Human Cancer. 2006.
7. Mendelsohn, A. H., C.K. Lai, I P. Shintaku, D. A. Elashoff, S. M. Dubinett & E. Abemayor. Histopathologic findings of HPV and p16 positive HNSCC. Laryngoscope. 2010; 120: 1788-94.
8. Lewis, J.S., Thorstad, W.L., Chernock, R.D., Haughey, B.H., Yip, J.H., Zhang, Q. & El-Mofty, S.K. p16 positive oropharyngeal squamous cell carcinoma: an entity with a favorable prognosis regardless of tumour HPV status. Am J Surg Pathol. 2010;34: 1088-96.
9. Harris, S.L., Thorne, L.B., Seaman, W.T., Hayes, N.N., Couch, M.E. & Kimple, R.J. Association of p16 overexpression with improved outcomes in young patients with squamous cell cancer of the oral tongue. Head Neck. 2010; 33:1622-27.
10. Antonsson, A., Neale, R.E., Boros, S., Lampe, G., Coman, W.B., Pryor, D.I., Porceddu, S.V. & Whiteman, D.C. Human papillomavirus status and p16INK4A expression in patient with mucosal squamous cell carcinoma of head and neck in Queensland, Australia. Cancer Epidemiol. 2015.
11. Abrahao, A.C., Bonelli, B.V., Nunes, F.D., Dias, E.P. & Cabral, M.G. Immunohistochemical expression of p53, p16 and hTERT in oral squamous cell carcinoma and potentially malignant disorders. Braz Oral Res. 2010; 25(1):34-41.
12. Tarakji, B., Kujan, O. & Nassani, M.Z. Immunohistochemical expression of p53 in pleomorphic adenoma and carcinoma ex pleomorphic adenoma. J Cancer Epidemiol. 2010.

13. Prakash, P., Khandare, M., Kumar, M., Khanna, R., Singh, P.G., Nath, G. & Kumar, A. Immunohistochemical detection of p16 in leukoplakia and oral squamous cell carcinoma. *J Clin Diagn Res.* 2013; 7 (12):2793-5.
14. Montebugnoli, L., Venturi, M., Gissi, D.B. Leonardi, E., Farnedi, A. & Foschini, M.P. Immunohistochemical expression of p16 protein in oral lichen planus. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* 2011; (112):222-7.
15. Neufcoeur, P.E., Afara, M., Decaestecker, C., Duray, A., Remmelink, M., Leroy, X., Herfs, M., Somja, J., Depuydt, C.E., Delvenne, P. & Saussez, S. Combined analysis of HPV DNA, p16, p21 and p53 to predict prognosis with stage IV hypopharyngeal carcinoma. *J Cancer Res Clin Oncol.* 2011; 137:173-181.
16. Angiero, F., Berenzi, A., Benetti, A., Rossi, E., Del Sordo, R., Sidoni, A., et al. Expression of p16, p53, and Ki-67 protein in the progression of epithelial dysplasia of the oral cavity. *Anticancer Res.* 2008; 28(5A):2535-9.
17. Arreaza, A., Rivera, H. & Correnti, M. p53 expression in oral lichenoid lesions and oral lichen planus. *Oral Med Oral Diagn Oral Pathol.* 2013:69-72.
18. Alvarez, M., Jimenez-Gomez, R. & Ardila, M. C. Immunoexpression of p53 in oral squamous cell carcinoma and oral dysplastic lesions in patients with the habit of reverse smoke. *Int J Odontostomat.* 2013; 7(2):185-91.
19. Ara, N., Atique, M., Bukhari, S. G. A., Akhter, F., Jamal, S., Sarfraz, T. & Khadim, T. Immunohistochemical expression of protein p53 in oral epithelial dysplasia and oral squamous cell carcinoma. *Pak Oral Dent J.* 2011; 31(2):296-99.
20. Panjwani, S. & Sadiq, S. p53 expression in benign, dysplastic and malignant oral squamous epithelial lesions. *Pak J Med Sci.* 2008; 24(1):130-135.
21. Leyva-Huerta, E.-R., Ledesma-Montes, C., Rojo-Botello, R.-E. & Vega-Memije, E. p53 and bcl-2 immunoexpression in patients with oral lichen planus and oral squamous cell carcinoma. *Med Ora, Patol Oral Y Cirugia Bucal.* 2012; 17(5):e745-50.
22. Swaminathan, U., Joshua, E., Rao, U. K. & Ranganathan, K. Expression of p53 and Cyclin D1 in oral squamous cell carcinoma and normal mucosa: An Immunohistochemical study. *J Oral Maxillofac Pathol.* 2012; 16(2):172-7.