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RESEARCH ARTICLE

Immunohistochemical detection of Def6 protein in oral cancer

Nyi Mas Siti Purwaningsih [a], Khor Goot Heah [a, d,*], Nik Mohd Mazuan Nik Mohd Rosdy [b], Effat Omar [c]

[a] Department of Preclinical Science, Faculty of Dentistry, Universiti Teknologi MARA, Selangor, Malaysia
[b] Department of Oral and Maxillofacial Diagnostic and Medicine, Faculty of Dentistry, Universiti Teknologi MARA, Malaysia
[c] Department of Pathology, Faculty of Medicine, Universiti Teknologi MARA, Selangor, Malaysia
[d] Oral Cancer and Maxillofacial Cancer Research Group, Universiti Teknologi MARA, Shah Alam, Selangor, Malaysia

* Corresponding author: gootheah@salam.uitm.edu.my

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Abstract

Nowadays, oral cancer becomes global health issue and reaches 21st rank in Malaysian population. Def6 or IRF-4-binding proteins (IBP) that mostly produced in lymph node and thymus are associated with cell survival and cell proliferation. Def6 protein expression is often found in autoimmune diseases, but current studies also found Def6 in some cancers. In this study, we would like to detect Def6 protein expression in oral cancer. 36 formalin-fixed paraffin embedded tissues were selected for both test group (oral cancer) and control group (normal oral mucosa). The cases were retrieved from the archives of Faculty of Dentistry, University of Malaya, Kuala Lumpur. Immunohistochemistry (IHC) staining was manually performed using Def6 antibody (Abcam 1:300) and assessed quantitatively (positivity and staining intensity). Positive and negative controls were used to validate the IHC run. All data were then analysed using SPSS version 25.0. Control group was negative for Def6 expression. 29 cases (82.86%) of oral cancer were Def6 positive with 2.86% weak staining, 5.71% moderate staining and 74.28% strong staining intensity. There was significant difference between Def6 expression in normal oral mucosa and oral cancer (p<0.05). Thus, we postulate that Def6 expression plays a big role in carcinogenesis of human oral cancer.

Keywords: Def6, immunohistochemistry, oral cancer, protein expression

INTRODUCTION

Cancer in oral cavity is one of the tenth most common human malignancies, not only in developing countries but also in developed countries (Montero & Patel 2015; Rivera 2015). The cause of oral cancer is different in each genetically susceptible individual since this is a multifactorial disease involving many aetiological factors. Aetiological factors for oral cancer are altered host immunity and metabolism, angiogenesis, chronic inflammation exposure, malnutrition, viruses (HPV, EBV and HIV), tobacco, hormones, alcohol consumption, physical irritant and premalignant lesion (Neville *et al*., 2016; Rhodus *et al.,* 2014; Stock *et al*., 2013).

The highest mortality from oral cancer is in the first 2 years since the patient harboured with cancer, the disease then continues to claim victims but a slower rate, and those few that survive for 10 years have a reasonable chance of having been cured. As a guide to survival rates, nearly 90% of males with the early-stage disease survive the first year, then about 65% of them survive for up to 5 years while only 55% survive for 10 years. For males with late-stage disease, around 45% survive the first year, about 16% survive for 5 years, and 12% survive for 10 years. As to the site of growth, the extremes are seen in the lip, where the 5-year survival rate for males is over 77%. For cancer in the tongue, it is 26%, and in the nasopharynx, it is only 17.6% (Odell 2017).

Def6 proteins are also known as IRF-4-binding proteins (IBP) or SWAP 70-like adaptor of T cells (SLAT). After the first study by Marc Hotfilder et al. in 1999, Def6 was believed to contribute towards the DNA recombination for T and B cell function and lymphoid development and/or function. They also observed that Def6 in human was highly expressed in lymph node, thymus and peripheral leucocytes, but in mouse, it was highly expressed in spleen (Hotfilder *et al*., 1999). Further study in 2003 placed Def6 as a guanine nucleotide exchange factor for Rho-family GTPases signalling pathways, an intracellular protein located in human lymphoid tissue, chromosome number 6, p arm 21 number 31 (p21,31) that highly expressed in B and T cells and played critical role in the immune system (Gupta *et al*., 2003; Jian *et al*., 2012; Z. Zhang *et al*., 2014). Def6 is also predicted to be associated with cell survival. Def6 deficiency displays disability of T cells in performing apoptosis through a cell autonomous pathway (Fanzo *et al*., 2006).

Elevated Def6 expressions are commonly observed in autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE), autoimmune uveitis, systemic lupus erythematosus (SLE) and psoriasis vulgaris (Canonigo-Balancio *et al*., 2009; Chandrasekaran *et al*., 2016; Fanzo *et al.,* 2006; Ni *et al*., 2012; Subramanian *et al*., 2005; Vistica *et al.,* 2012). Recently, Def6 overexpressions are also found in many malignancy diseases such as renal cell carcinoma, extraskeletal myxoid chondrosarcoma, colorectal cancer, breast cancer, ovarian carcinoma and also in oral squamous cell carcinoma (Jian *et al.,* 2012; Khor *et al*., 2014; Li *et al*., 2009; Liew *et al.,* 2016; Otsubo *et al.,* 2014; Z. Zhang *et al*., 2014; Zhujun Zhang *et al.,* 2009).

EXPERIMENTAL

Materials

In this research, the exclusion criteria would focus on patients with factors that might modify the immune response (medically compromised patients or those currently on medications) and undergo

treatment would be excluded from the study. Inclusion criteria would be patients who were clinically and histologically diagnosed with oral cancer using established TNM classification of carcinomas of the oral cavity by World Health Organization (WHO) (Barnes *et al*., 2005).

35 oral cancers for test group and 1 normal mucosa for control which were formalin fixed paraffin embedded tissues (FFPETs) specimens and relevant data were obtained from the Malaysian Oral Cancer Database and Tissue Bank (MOCDTBS) in conjunction with the Oral Cancer Research and Coordinating Centre (OCRCC) University of Malaya. Ethical approval for this study was taken from the Medical Ethics Committee of the Faculty of Dentistry, University of Malaya (Ethic reference no: DF OP1101/0049(L)). Samples were processed in Dental Research Laboratory UiTM for immunohistochemistry detection.

Immunohistochemistry staining

Sample section was cut with 4µm thickness from FFPET block and placed on poly-L-lysine coated glass slides. Before starting on immunohistochemistry procedure, sample sections were deparaffinised in the oven at 60°C overnight and put in PT Link approximately for 1 hour filled with target retrieval solution. After PT Link process was done, sample sections were incubated in hydrogen peroxidase for 5 minutes and rinsed with phosphate buffer saline (PBS). Then, sections were added with Def6 primary antibody (Abcam) in 1:300 dilution. After Def6 overnight incubation, sample sections were rinsed thoroughly with PBS and added with secondary antibody (DAKO), before being incubated in room temperature for 20 minutes. Next, sample sections were stained with 3,3' diamino benzidine tetrahydrochloride chromogen solution (DAB) for 3 minutes. Sections were washed several times with PBS in between all steps.

After antibody incubation, sections were counterstained by hematoxilin eosin for 3 minutes and rehydrated in alcohol and xylene. Last step, sections were mounted with slip glass cover using mounting agent. Positive control was followed the same procedure by using human tonsil tissue. Negative control slides without the primary antibody were included in staining. The results of the immunohistochemistry staining were examined and graded in at least 10 high powered (40x) fields using microscope Nikon eclipse 50i. IHC image was captured with Nikon camera which connected with microscope and using Nikon Imaging System Basic Research (NIS-BR) software. All steps are summarised in Fig 1.

Semi-quantitative analysis

Semi-quantitative analysis was taken by referring to Liew et al. (2016). Positive IHC expression was defined by granular brown staining cells in cytoplasm. The percentage of positive cells was assessed by 2 independent observers and scored as 0= less than 5%, 1= weak staining, 5-24% or total cells show positive staining, 2= moderate staining, 25-75% of the cells show positive, 3= strong staining, more than 75% cells show positive staining.

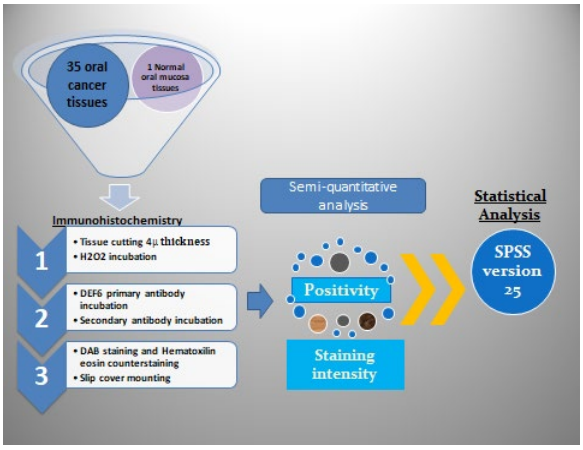

Fig. 1 Schematic diagram for Def6 detection in oral cancer.

Statistical analysis

All data was coded and analysed by using SPSS for windows version 25 with 95% confidence interval ($p<0.05$). The Kolmogorov-Smirnov analysis was used to test the normality of the data. Significant differences in the positive staining cell ratios between each groups were analysed using chi square test with $p<0.05$ considered to be significant.

RESULTS AND DISCUSSION

Demographic and pathological variables of samples

We assessed Def6 expression in 35 oral cancer tissues and 1 normal oral mucosa tissue by immunohistochemical staining. Table 1 shows demographic and pathological variables in 35 oral cancer patients. Oral cancer most occurred in age ≥ 60 years old (60%). 71.42% of oral cancer patients were come from Indian race. Female patients were dominant in our samples which was 68.75%. Buccal mucosa was the most frequent site for oral cancer (45.71%), followed by tongue (31.43%). Most etiological factors for oral cancer revealed in our samples were betel quid chewing and alcohol consumption which were 60% and 34.28%, respectively. Moderate-differentiated oral cancer was the most common case that reported in our samples, as shown in Table 1.

Table 1 Demographic and pathological data of oral cancer patients.

Variables		Percentage
Age	<30	1/35 (2.86%)
	30-59	12/35 (34.28%)
	≥60	21/35 (60%)
Ethnics	Malay	5/35 (14.28%)
	Indian	25/35 (71.42%)
	Chinese	5/35 (14.28%)
Gender	Female	24/35 (68.57%)
	Male	11/35 (31.43%)
Habits	Betel-quid chewing	21/35 (60%)
	Smoking	6/35 (17.14%)
	Alcohol	12/35 (34.28%)
Tumour site	Buccal mucosa	16/35 (45.71%)
	Tongue	11/35 (31.43%)
	Gingiva	7/35 (20%)
	Alveolar ridge	1/35 (2.86%)
Tumour grading	Poor-differentiated	0/35 (0%)
	Moderate-differentiated	22/35 (62.86%)
	Well-differentiated	13/35 (37.14%)

Def6 was not expressed in normal oral mucosa as shown in Table 2. In contrast, Def6 was overexpressed in 29 oral cancer samples (82.86%). Only 6 samples were negative for Def6 (17.14%). These results clearly showed that Def6 expression was strongly expressed in oral cancer tissues, but not in normal oral mucosa tissue. This result was in good agreement with study done by Jian *et al*. (2012) that found 46.15% Def6 in OSCC, but not in normal oral mucosa epithelium. Moreover, Def6 protein was detected in oral cancer cytoplasm as demonstrated in the picture above (Fig. 2).

Human tonsil tissues were used for negative and positive control (Fig. 2(a),(b)). As expected, Def6 was not found in normal oral mucosa tissue as shown in Fig. 2(c). Fig. 2 (d), (e), and (f) demonstrated weak, moderate and strong immunohistochemistry staining intensities in oral cancer tissues that obtained with 400x magnification. Positive Def6 in oral cancer was found mostly in basal cell layer of epithelium compared to supra basal layer (Fig. 2(e),(f)).

Def6 positivity and staining intensity

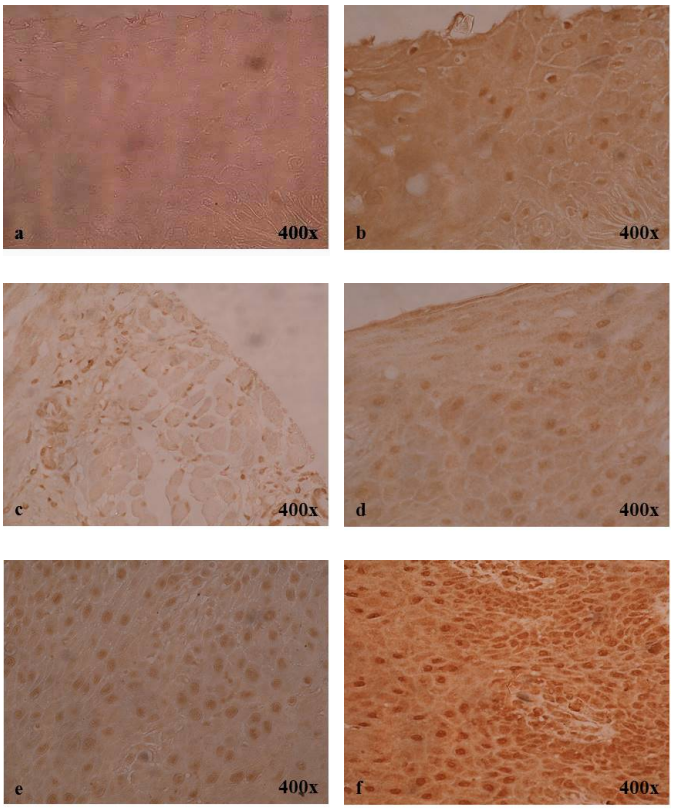

Fig. 2 (a) Negative control in human tonsil (b) Positive control in human tonsil (c) Def6 negative in normal oral mucosa (d) Weak staining of Def6 in oral cancer (e) Moderate staining of Def6 in oral cancer (f) Strong staining of Def6 in oral cancer.

Table 2 Def6 positivity and staining intensity.

Tissue	Negative staining	Weak staining	Moderate staining	Strong staining	*p* value
Normal mucosa	1 (100%)	0 (0%)	0 (0%)	0 (0%)	
Oral cancer	6 (17.14%)	1 (2.86%)	2 (5.71%)	26 (74.28%)	0.04

Def6 or IBP or SLAT, is a guanine nucleotide exchange factor for small GTPases, regulating Cd4 (+) T cell inflammatory responses by controlling Ca (2+)/NFAT signalling. Def6 protein expression can perform very critical roles in cytokine production, Bcl6 protein synthesis deregulation and actin reorganisation throughout inflammatory immune feedbacks (Hashimoto *et al*., 2017; Yi *et al.,* 2017). Besides it is found in cancers and some immunology diseases, Def6 is also found in chronic inflammatory diseases and contributed in its bone destruction through osteoclastogenesis mechanism (Binder *et al*., 2017).

Def6 protein is highly expressed in B and T cells and played a role in immune system. Regulatory T cell itself plays a crucial role in many types of tumour evasion of immune surveillance. Liu *et al.* (2016) found that regulatory T cells increased in oral cancer, suggesting that elements in T cells might be potential biomarker in oral cancer, including Def6 as one of proteins that oftenly found in T cells (Joshi *et al*., 2017). 82.86% of Def6 protein overexpression found in oral cancer and none in normal oral mucosa were similar with Jian *et al*., (2012) that suggested Def6 might be participated in oral cancer tumourgenesis and could be a potential therapeutic target for oral cancer.

CONCLUSION

In summary, our research showed that Def6 was expressed intensely in oral cancer. Def6 has a role in promoting cell proliferation by shortening the G1 interval in the human cell cycle, proving that Def6 could be a novel and potential growth factor in human oral cancer. Def6 detections in oral cancer might be used as a potential biomarker in defining oral cancer diagnostic and prognostic by clinician. In addition, Def6 protein could be a therapeutic target for oral cancer treatment in the future.

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