RESEARCH ARTICLE

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Protein Expression of MAGEB2 in Normal Oral Mucosa, Oral Epithelial Dysplasia, and Oral Squamous Cell Carcinoma and Its Association with Clinicopathological Parameters

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Abstract

Objective: To compare the expression of the MAGEB2 antibody in normal oral mucosa (NOM), oral epithelial dysplasia (OED), and oral squamous cell carcinoma (OSCC) patients, and to evaluate the association of *MAGEB2* expression with clinicopathological characteristics and overall survival in OSCC patients. **Methods:** Immunohistochemical staining with the MAGEB2 antibody was performed on 20 NOM, 40 OED, and 60 OSCC samples. The Kruskal–Wallis test was used to compare *MAGEB2* expression between NOM, OED, and OSCC tissues. The diagnostic accuracy of MAGEB2 in distinguishing NOM, OED, and OSCC tissues, and the prognostic accuracy of MAGEB2 in relation to socio-demographic and clinicopathological characteristics, were determined using receiver operating characteristic (ROC) curve analysis. Kaplan–Meier survival analysis was used to determine the association between *MAGEB2* expression and overall survival (OS) in OSCC patients. **Result:** *MAGEB2* expression was observed in 61.7% of OSCC, 27.5% of OED, and 20.0% of NOM tissues. *MAGEB2* expression was significantly higher in OSCC compared to OED and NOM tissues ($p < 0.05$). However, no significant difference was found between *MAGEB2* expression in NOM and OED. MAGEB2 was able to distinguish OSCC from OED tissue with 63.3% sensitivity and 72.5% specificity. A significant association between *MAGEB2* expression and age was observed, whereas no associations were found with other socio-demographic or clinicopathological characteristics, or with overall survival (OS) in OSCC patients. However, a trend toward better OS was noted in OSCC patients with high *MAGEB2* expression. **Conclusion:** MAGEB2 is a potential biomarker in cancer whereby its protein expression is seen highest in oral cancer.

Keywords: Cancer testes antigens- MAGEB2- immunohistochemistry- oral squamous cell carcinoma

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Introduction

Oral cancer, a subset of head and neck cancer, is a heterogeneous disease with a known multifactorial aetiology. In Malaysia, oral cancer (ICD-10 codes C00–C06) is not among the ten most common cancers overall. However, according to the Malaysian National Cancer Registry (Azizah, Nor Saleha, Noor Hashimah, Asmah, & Mastulu, 2016), it ranks as the fourth most common cancer among Indian females and the eighth among Indian males. The alarmingly high incidence among Indian populations is mainly due to betel quid chewing habits. Oral squamous cell carcinoma (OSCC) represents more than 90% of cancers diagnosed in the oral cavity [1].

Despite advancements in diagnostic and therapeutic approaches to oral cancer, the overall survival (OS) of OSCC patients in Malaysia is still low compared to other developed countries. A study in England reported that 5-year OS as 56% in OSCC patients [2]. In contrast, the 5-year OS in Malaysia was 13.4%, which was three times worse than the OS for OSCC patients in England [3]. As a result, there is an urgent need to develop new alternatives for oral cancer detection and treatment. A major drawback of OSCC is the failure of early detection, as most of the patients are diagnosed at advanced stages [4].

A biomarker is defined as a biological molecule found in blood, other body fluids, or tissues that indicates a

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sign of a normal or abnormal process or disease, such as cancer [5]. Several previous studies demonstrated specific targets for immunotherapeutic approaches that could be useful in controlling cancer progression [6]. However, there have been limited studies carried out on head and neck cancer. The relevance of studying biomarkers such as *CTA*s lies in their potential to guide targeted therapy and immunotherapy. Identifying tumour-specific antigens can help develop personalised treatment strategies that enhance the body's immune response against cancer cells while minimising damage to normal tissues [7]. In head and neck cancers, exploring such therapeutic targets is particularly important, as current treatment options often lead to significant morbidity and recurrence [8].

Cancer testes antigens (*CTA*) are proteins that are expressed in various malignant tumours, but expression in normal tissue is restricted to the testes and occasionally placental trophoblasts [9]. Thierry Boon and his colleagues first discovered *CTA* in 1991, when they successfully cloned the first antigen that made a significant advancement in tumour immunology [10]. To date, more than 200 *CTA*s have been identified [11].

Many *CTA*s have been shown to evoke cellular and serological immune responses in humans [12]. The *CTA*s are considered potential targets for cancer vaccines due to their presence in various malignant tumours and their capability to initiate an immune response. Several clinical trials have been and are being conducted using *CTA*s as vaccine targets to stimulate autologous immune responses in patients with various tumours [13]. Consequently, there is a rapidly expanding body of global research that examines various aspects of *CTA* in cancer, but relatively little is known about them in head and neck cancer, particularly in oral malignancies.

Among the various *CTA* families, the melanoma antigen gene (MAGE) family has been widely studied for its diagnostic and therapeutic potential in melanoma, lung, breast, and head and neck cancers. MAGEB2, a member of the MAGE-B subfamily, is classified as a "testis-restricted" *CTA* and has been reported to show mRNA expression in nearly half of HNSCC cases while being absent in normal tissues [14]. This selective tumour-associated expression highlights MAGEB2 as a promising biomarker and potential immunotherapeutic target.

A previous protein array study conducted by our team at the Oral Cancer Research and Coordinating Centre, Faculty of Dentistry, Universiti Malaya (OCRCC-UM), identified several upregulated tumour-associated antibodies (TAAs) in sera from OSCC patients. Among the top candidates were *TP53, MAGEA4, MAGEB2, NRIP3*, and *SH2B1,* of which *MAGEB2* was significantly associated with favourable patient outcomes after adjustment for clinico-pathological parameters (unpublished data). These preliminary findings suggest that *MAGEB2* may play a role in tumour immunogenicity and patient prognosis.

However, despite evidence of *MAGEB2* involvement at the serological and mRNA levels, its protein expression in OSCC has not been investigated to date. Understanding its tissue-level expression may provide important insights into its biological relevance and clinical utility. Therefore, the present study aimed to examine and compare the protein expression of MAGEB2 in normal oral mucosa (NOM), oral epithelial dysplasia (OED), and OSCC, and to evaluate its association with socio-demographic and clinico-pathological parameters as well as overall survival (OS).

Materials and Methods

Study design and ethical approval

As this was an exploratory cross-sectional study to investigate the expression of the MAGEB2 antibody in NOM, OED, and OSCC, a formal sample size calculation was not performed. The formalin-fixed paraffin-embedded (FFPE) tissues used were retrieved from the archival records of the Malaysian Oral Cancer Database and Tissue Bank System (MOCDTBS), coordinated by OCRCC-UM. This research was approved by the Medical Ethics Committee of the Faculty of Dentistry, Universiti Malaya [Ethics Committee/IRB reference number: DF OS1910/0043 (P)].

This was an exploratory cross-sectional study,
Patient selection

For the OSCC group, patients who underwent surgical treatment and/or received adjuvant chemoradiotherapy and had FFPE tissue samples available together with complete socio-demographic and clinico-pathological information and their survival status for analysis were included in the study. The OED cases were further categorised into mild, moderate, and severe epithelial dysplasia. Conversely, patients with co-existing other types of malignancies or incomplete socio-demographic and clinico-pathologic information were excluded.

Data collection

The 20 NOM, 40 OED, and 60 OSCC cases were selected randomly from 2008 to 2019. FFPE, together with patients' data and information, were retrieved from MOCDTBS, OCRCC-UM archival records, and databases. Data extracted includes (i) socio-demographic characteristics such as age, gender, ethnicity, and risk habits (alcohol, smoking, betel quid chewing) and (ii) clinico-pathologic characteristics of OSCC such as tumour site, tumour grading, pathological tumour size (pT), pathological lymph node metastases (pN), pathological tumour staging (pTNM), pattern of invasion (POI), perineural invasion (PNI), lymphovascular invasion (LVI), bone invasion, lymphocytic host response (LHR), surgical margin status, treatment, recurrence, and survival status.

Laboratory procedures

A total of 120 FFPE tissue blocks (20 NOM, 40 OED, and 60 OSCC) were retrieved. Two sections with a 4-micrometre thickness were sectioned from FFPE blocks, and one section was mounted on the poly-L-lysine coated slides. One tissue section was stained with Hematoxylin and Eosin (H&E), and another tissue section was stained with an anti-MAGEB2 antibody for immunohistochemistry (IHC). The H&E slides were examined to assess the availability of the tissue prior to the commencement of the IHC procedure.

Immunohistochemistry (IHC) procedures

Human testes tissue was used in the IHC procedure as a positive control for the anti-MAGEB2 staining. All the tissues sections were deparaffinized, rehydrated, and subjected to heat-induced epitope retrieval in microwave oven at 99°C for 20 min in citrate buffer (pH 6.0). Endogenous peroxidase was blocked, and protein blocking was performed using the background sniper for 10 min (Biocare Medicals, US). The primary antibody, rabbit anti-MAGEB2 antibody (NBP2-62688) (Novus Biologicals Inc, US) was diluted at 1:100 and applied to the tissues for 60 minutes at room temperature. This was followed by a secondary antibody with HRP using the DAKO REALTM EnVisionTM kit (Dako, Denmark) for 30 minutes at room temperature. The DAB substrate was used for 5 minutes for the detection, with counterstaining using Harris hematoxylin. The slides were dehydrated, cleared, and mounted with dibutylphthalate polystyrene xylene (DPX). This process allowed the visualization of the MAGEB2 protein expression in the tissues.

Scoring of IHC staining

The IHC scoring was carried out by two assessors (AR and NAAH). The clinico-pathological information and the diagnosis were concealed from the assessors. The immunoreactive score (IRS), a semi-quantitative scoring approach, was chosen for this study. Final IRS is a product of multiplication between intensity of staining (0 = no; 1 = mild; 2=moderate; 3=strong) and percentage of positive cells (0 = no positive cells; 1 = <10% of positive cells; 2 = 10–50% positive cells; 3 = 51–80% positive cells; 4 = >80% positive cells). The IRS value ranges from 0 to 12 (Remmele, 1987). A total score of 0–1 indicates negative expression; 2-3 indicates mild positivity; 4–8 indicates moderate positivity; 9–12 indicates strong positivity. The average score for each sample was obtained. Intra- and inter-observer agreement was evaluated before the IRS scoring of the immunostaining.

Statistical analysis

Intraclass coefficient (ICC) was used to evaluate intra- and inter-observer agreement between the two assessors. To explore the association between socio-demographic and clinico-pathological parameters, Fisher's Exact Test was applied when the expected frequency in any cell was less than 5, while the Chi-Square Test was used otherwise. Kaplan-Meier analysis was performed for overall survival (OS). Statistical analyses were performed by SPSS software (version 26, IBM).

Results

Socio-demographic characteristics of total samples

A total of 120 cases were included in this study, comprising 20 NOM, 40 OED, and 60 OSCC patients. Table 1 depicts the distribution of total cases based on socio-demographic parameters. In summary, the age ranged from 18 to 94 years, with a mean age of 56.45 (±18.1). The median age was 60 years and was used as the cut-off value for age groups in this study. In this study, 62.5% of the total sample was female. About half of the

Table 1. Socio-Demographics and Clinico-Pathological Characteristics of All Cases Included in This Study (n=120)

Characteristics	Overall n=120 (%)	NOM n=20 (%)	OED n=40 (%)	OSCC n=60 (%)
Age group (years)				
≤ 59	58 (48.3)	19 (95.5)	17 (42.5)	22 (36.7)
> 59	62 (51.7)	1 (5.0)	23 (57.5)	38 (63.3)
Gender				
Male	45 (37.5)	7 (35.0)	11 (27.5)	27 (55.0)
Female	75 (62.5)	13 (65.0)	29 (72.5)	33 (45.0)
Ethnicity				
Malay	23 (19.2)	7 (35.0)	4 (10.0)	12 (20.0)
Chinese	34 (28.3)	11 (55.0)	9 (22.5)	14 (23.3)
Indian	63 (52.5)	2 (10.0)	27 (67.5)	34 (56.7)
Smoking				
Yes	22 (18.3)	2 (10.0)	1 (2.5)	19 (31.7)
No	98 (81.7)	18 (90.0)	39 (97.5)	41 (68.3)
Alcohol				
Yes	17 (14.2)	1 (5.0)	2 (5.0)	14 (23.3)
No	103 (85.8)	19 (95.0)	38 (95.0)	46 (76.7)
Betel-quid				
Yes	31 (25.8)	0 (0.0)	12 (30.0)	19 (31.7)
No	89 (74.2)	20 (100.0)	28 (70.0)	41 (68.3)
OED				
Mild ED			13 (32.5)	
Moderate ED			14 (35)	
Severe ED			13 (32.5)	
OSCC site				
Tongue				23 (38.3)
Buccal mucosa				16 (26.7)
Alveolus				8 (13.3)
*Others				13 (21.7)
Tumour grading				
Well				6 (10)
Moderate				51 (85)
Poor				3 (5)
Tumour size (pT)				
pT1 & pT2				28 (46.7)
pT3 & pT4				32 (53.3)
Lymph node metastasis (pN)				
No				35 (58.3)
Yes				25 (41.7)
Tumour staging				
Early				21 (35)
Advanced				39 (65)
Depth of invasion (mm)				
≤10				38 (63.3)
>10				22 (36.7)
Pattern of invasion				
Cohesive				2 (3.3)
Non-cohesive				58 (96.7)
Margin status (mm)				
Clear (>5)				14 (23.3)
Close (1-5)				17 (28.3)
Involved (<1)				29 (48.3)

Table 1. Continued

Characteristics	Overall n=120 (%)	NOM n=20 (%)	OED n=40 (%)	OSCC n=60 (%)
Dysplasia at margin				
No ED				45 (75)
Mild ED				7 (11.7)
Moderate ED				4 (6.7)
Severe ED				4 (6.7)
Perineural invasion				
No				40 (66.7)
Yes				20 (33.3)
Lymphovascular invasion				
No				53 (88.3)
Yes				7 (11.7)
Lymphocytic host response				
Weak				7 (11.7)
Intermediate				38 (63.3)
Strong				15 (25)

*Others, floor of the mouth, gingiva, hard palate, angle of the mouth

cases involved Indians. About two-thirds of the cases did not practise any risky habits like smoking, drink alcohol, and chew betel-quid. A total of 20 NOM was retrieved in this study. Gingiva (12/20) was the most common site, followed by buccal mucosa (4/20), tongue (1/20), and lip (1/20). From 40 OED cases, 47.5% were taken from the buccal mucosa, followed by the tongue (27.5%), soft palate (7.5%), alveolus (7.5%), and others. About 14 cases were moderate epithelial dysplasia, while 13 each were mild and severe epithelial dysplasia. Meanwhile, the most common sites for OSCC were the tongue, buccal mucosa, and alveolus.

Table 2. Pairwise Comparisons of Anti-*MAGEB2* Expression with Disease Status

Disease group pair	Standard. Error	p-value†
NOM Vs OED	9.41	1
NOM Vs OSCC	8.88	0.01
OED Vs OSCC	7.02	0.001

† Kruskal-Wallis ANOVA, two-sided p-value <0.05

Immunohistochemical staining and distribution of MAGEB2 in NOM, OED, and OSCC

The IHC staining for the MAGEB2 antibody was localised in the cytoplasm and nucleus of epithelial cells. The intraclass coefficient (ICC) was performed to determine the intra- and inter-observer agreement in scoring the expression of MAGEB2. Both intra- and inter-observer agreement for each study group was above 0.8, indicating good agreement. Figure 1 illustrates the photomicrograph of anti-MAGEB2 in control tissue of human testes, NOM, and OSCC tissue. Regarding the distribution of *MAGEB2* expression in NOM, OED, and OSCC, about 43% of all tissue samples showed immunopositivity towards MAGEB2. The percentage of tissue with positive *MAGEB2* expression in OSCC tissue was 61.7%. MAGEB2 protein expression was also observed in NOM (20.0%) and OED (27.5%). In terms of intensity of staining, about one third of all immunopositive tissue exhibits mild positivity (36.5%). About 25% (15/60) of OSCC tissue and 10% (4/40) of OED tissue demonstrate mild positivity for MAGEB2. On the other hand, moderate MAGEB2 positivity was observed in 36.7%, 17.5%, and 20% of OSCC, OED, and NOM, respectively. However, none of the tissue samples showed strong positivity.

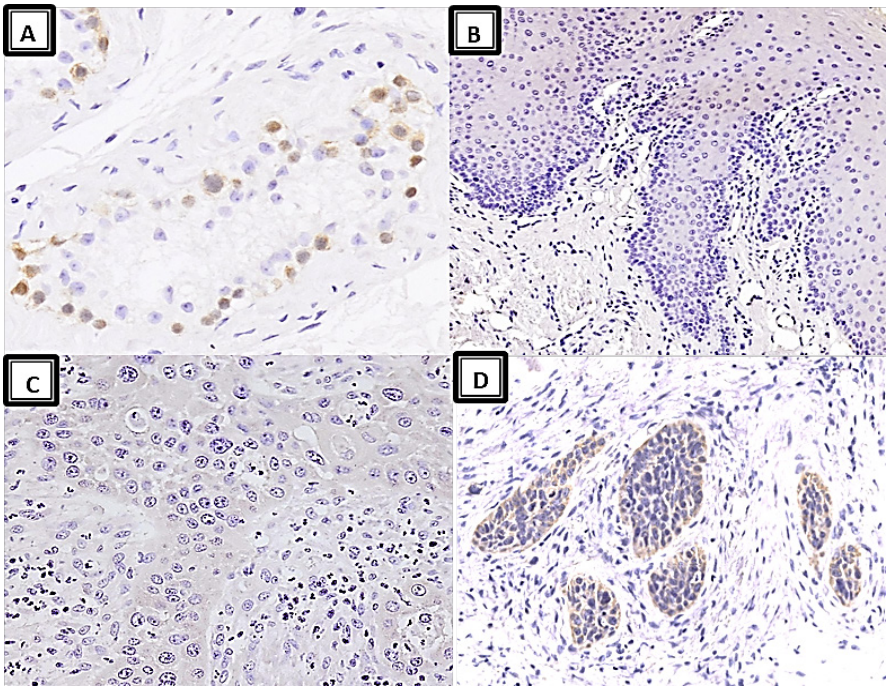

Figure 1. Photomicrographs Shows anti-MAGEB2 Protein Expression which is (A) strong in the nucleus and cytoplasm of spermatogonia cells of human testes tissue (seminiferous tubules) (Original magnification: 400x); (B) negative in the epithelium of normal oral mucosal (Original magnification: 100x); (C) mildly positive in the epithelium of oral epithelial dysplasia (Original magnification: 400x); (D) moderately positive in the oral squamous cell carcinoma (Original magnification: 400x).

Table 3. Association between Socio-Demographic, Clinico-Pathological Parameters and Anti-*MAGEB2* Expressions in NOM, OED, and OSCC patients.

Variables	Category	MAGEB2 expression		p-value†
		Negative n (%)	Positive n (%)	
Age group	≤ 59	39 (32.5%)	19 (15.8%)	0.02
	>59	29 (24.2%)	33 (27.5%)	
Ethnicity group	Indian	31 (25.8%)	32 (26.7%)	0.09
	Non-Indian	37 (30.8%)	20 (16.7%)	
Gender	Female	45 (37.5%)	30 (25.0%)	0.34
	Male	23 (19.2%)	22 (18.3%)	
Smoking	No	57 (47.5%)	41 (34.2%)	0.49
	Yes	11 (9.2%)	11 (9.2%)	
Drinking alcohol	No	61 (50.8%)	42 (35.0%)	0.16
	Yes	7 (5.8%)	10 (8.3%)	
Betel-quid chewing	No	53 (44.2%)	36 (30.0%)	0.28
	Yes	15 (12.5%)	16 (13.3%)	
Disease status	NOM	16 (80.0%)	4 (20.0%)	<0.01[a]
	OED	29 (72.5%)	11 (27.5%)	
	OSCC	23 (38.3%)	37 (61.7%)	
OED	Mild ED	10 (25.0%)	3 (7.5%)	0.90[a]
	Moderate ED	10 (25.0%)	4 (10%)	
	Severe ED	9 (22.5%)	4 (10%)	
OSCC site	Tongue	10 (16.7%)	13 (21.7%)	0.59
	Others	13 (21.7%)	24 (40.0%)	
Tumour grading	Well	1 (1.7%)	5 (8.3%)	0.33[a]
	Moderate	20 (33.3%)	31 (51.7%)	
	Poor	2 (3.3%)	1 (1.7%)	
Tumour size (pT)	pT1 & pT2	10 (16.7%)	18 (30.0%)	0.7
	pT3 & pT4	13 (21.7%)	19 (31.7%)	
Lymph node metastasis (pN)	No	15 (25.0%)	20 (33.3%)	0.43
	Yes	8 (13.3%)	17 (28.3%)	
Tumour staging (pTNM)	Stage 1 & 2 (early)	8 (13.3%)	13 (21.7%)	1.00
	Stage 3 & 4 (advanced)	15 (25.0%)	24 (40.0%)	
Depth of invasion, DOI (mm)	≤10	15 (25.0%)	23 (38.3%)	1.00
	>10	8 (13.3%)	14 (23.3%)	
Pattern of invasion, POI	Cohesive	1 (1.7%)	1 (1.7%)	1.00
	Non-cohesive	22 (36.7%)	36 (60.0%)	
Margin status (mm)	Clear (>5)	6 (10.0%)	8 (13.3%)	0.42[a]
	Close (1-5)	8 (13.3%)	9 (15.0%)	
	Involved (<1)	9 (15.0%)	20 (33.3%)	
Dysplasia at margin	No ED	17 (28.3%)	28 (46.7%)	0.86[a]
	Mild ED	2 (3.3%)	5 (8.3%)	
	Moderate ED	2 (3.3%)	2 (3.3%)	
	Severe ED	2 (3.3%)	2 (3.3%)	
Perineural invasion, PNI	No	14 (23.3%)	26 (43.3%)	0.58
	Yes	9 (15.0%)	11 (18.3%)	
Lymphovascular invasion, LVI	No	20 (33.3%)	33 (55.0%)	1.00
	Yes	3 (5.0%)	4 (6.7%)	
Lymphocytic host response, LHR	Weak	4 (6.7%)	3 (5.0%)	0.54[a]
	Intermediate	14 (23.3%)	24 (40.0%)	
	Strong	5 (8.3%)	10 (16.7%)	
Treatment	Surgery only	23 (38.3%)	35 (58.3%)	0.52
	Surgery & radiotherapy	0 (0.0%)	2 (3.3%)	
Survival status	Alive	12 (20.0%)	21 (35.0%)	0.84[a]

†, Fischer's exact test; two-sided. [a], Chi square. p-value <0.05

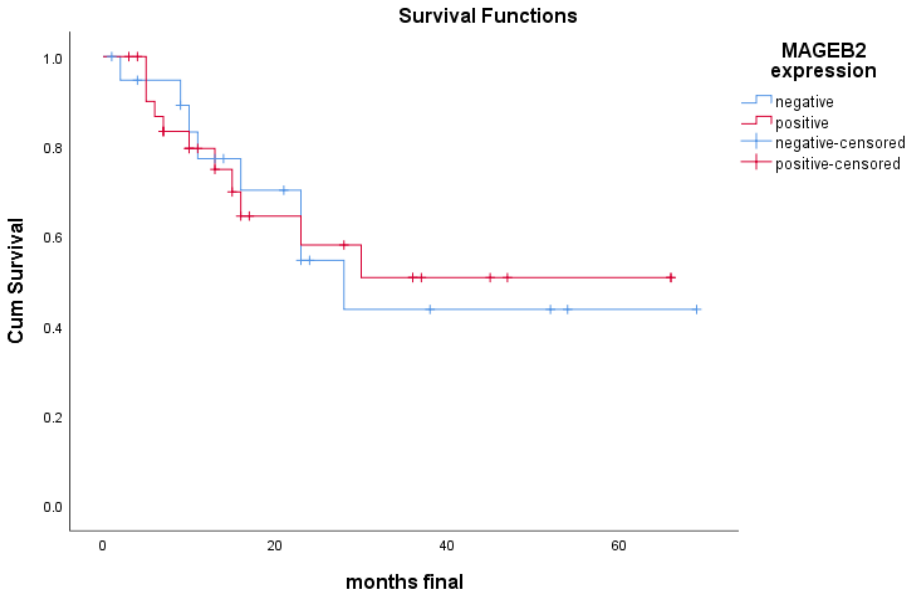

Figure 2. The Graph Shows Kaplan-Meier Overall Survival (OS) Plot for Anti-*MAGEB2* Expression (n=120)

The highest *MAGEB2* expression was observed in OSCC (mean = 3.1; 95% CI 2.6–3.6), followed by OED tissue (mean = 1.7; 95% CI 1.2–2.2), and NOM tissue (mean = 1.65; 95% CI 1.0–2.3).

Comparison of MAGEB2 expressions in NOM, OED, and OSCC

Table 2 shows a pairwise comparison of *MAGEB2* expressions in NOM, OED, and OSCC. Significant associations were observed between OSCC vs. NOM and OSCC vs. OED ($p< 0.05$). Hence, *MAGEB2* expressions in OSCC were significantly higher compared to OED and NOM. Inversely, no significant association was observed between OED and NOM ($p> 0.05$).

Diagnostic accuracy of MAGEB2 expression in distinguishing NOM and OED from OSCC

The diagnostic accuracy of *MAGEB2* expressions in distinguishing OED from OSCC was determined from the ROC curve. In this study, the ROC curve plot was above the 45-degree diagonal line, indicating that MAGEB2 was able to discriminate OED from OSCC (p-value<0.005; AUC = 0.72) and NOM from OSCC (p-value<0.005; AUC = 0.72). This study showed that both AUC values of 0.72 indicate that MAGEB2 is an acceptable diagnostic marker to discriminate OED from OSCC and NOM from OSCC tissue. In this study, MAGEB2 was able to distinguish NOM from OSCC tissue with 61.7% sensitivity and 80% specificity at a cut-off value of 2.75 and OED from OSCC tissue with 63.3% sensitivity and 72.5% specificity at the same cut-off point value. However, MAGEB2 was unable to distinguish NOM from OED tissue.

Association between MAGEB2 expressions with clinicopathological parameters in OSCC patients

In this study, a statistically significant association was found between *MAGEB2* expression and age and disease status ($p<0.01$). Positive *MAGEB2* expression was observed to be statistically higher in elderly patients older than 59 years and in the OSCC group ($p = 0.02$). There was no significant association between *MAGEB2* expression and other clinico-pathological parameters ($p > 0.05$) (Table 3).

Association between MAGEB2 expressions with overall survival in OSCC patients

Based on Kaplan-Meier survival analysis, the 5-year OS for OSCC patients was 43%. The mean and median survival estimates were 39 months (95% CI: 30.3–48.0) and 30 months (95% CI: 18.5–41.5), respectively. In the current cohort of 60 OSCC patients, 19 patients were deceased, 33 patients remained alive throughout the study period, and the other eight patients were lost to follow-up. The mean OS estimates were 39 months. This study demonstrated a better OS in tissue with high *MAGEB2* expression, where the mean survival estimates for low *MAGEB2* expression were 37 months compared to 47 months for high *MAGEB2* expression but however without significance (Figure 2).

Discussion

The World Health Organisation (WHO) reported in 2015 that cancer is one of the leading causes of death. Late detection of cancer and frequent locoregional metastases are the main factors associated with treatment failure in cancer patients [15]. Although lip and oral cavity cancer is not as common globally as other cancers, the incidence is higher in Southeast Asia due to different cultural practises such as chewing betel quid and low socioeconomic background [16].

The recent discovery of tumour-targeted treatments using tumour antigens opened a new door for novel forms of cancer treatment. Immunotherapy is a growing cancer treatment due to the antitumoral effect caused by immunologic memory [14]. Hence, immunologic

intervention may assist in the control of cancer progression and recurrence. Recent FDA approval for Sipuleucel-T in prostate cancer and Ipilimumab in metastatic melanoma has triggered substantial interest in applying immunotherapy approaches to different types of cancer [17, 18]. Cancer testes antigens (*CTA*) were recognised as promising tumour antigens due to their high immunogenicity and specific expression pattern. *CTA*s have attracted attention as potential targets for tumour immunotherapy, but few reports are available in the literature about their expression, especially regarding the MAGE family in head and neck cancers [19].

MAGEB2 expression was highest in OSCC, followed by OED and NOM. It was significantly higher in OSCC compared with both OED and NOM, while no significant difference was observed between NOM and OED. ROC curve analysis indicated that MAGEB2 demonstrated a moderate ability to differentiate OSCC from OED and NOM, suggesting its potential as a supportive biomarker for distinguishing malignant from non-malignant lesions. Zamunér et al. [14] reported that MAGEB2 showed moderate sensitivity but perfect specificity in distinguishing HNSCC from normal tissue. They also demonstrated that combining MAGEB2 with other *CTA*s improved diagnostic accuracy, suggesting potential value for immunotherapy. In contrast, the present study found that MAGEB2 alone did not reliably differentiate OED from NOM, although its expression was notably higher in OSCC, supporting its possible role in malignant transformation.

To the best of our knowledge, this is the first study comparing protein expression of MAGEB2 in NOM, OED, and OSCC tissue. In this study, *MAGEB2* expression was detected in approximately 62% of OSCC cases. A comprehensive RT-PCR study on HNSCC demonstrated the expression of 11 *CTA* genes, including five testis-restricted *CTA*s which were *MAGEB2, MAGEA1, MAGEB6, SPANX-CD*, and *CXORF48* [14]. In that study, MAGEB2 mRNA was detected in 44.9% of HNSCC samples but was absent in normal tissues, highlighting its tumour-specific expression profile. However, in this study most OSCC exhibit mild to moderate positivity for the MAGEB2 protein expression, whereas only focal mild positive expression was observed in both OED and NOM. The variation in *MAGEB2* expression between studies may be explained by differences between mRNA and protein expression levels, which are not always correlated. A similar pattern was reported for MAGE genes in lung carcinoma [20]. Likewise, [21] found that MAGEB2 protein expression in human tissues was slightly lower (63%) than its mRNA expression (89%). Zamunér et al. [14] analysed nucleic acids extracted from fresh frozen HNSCC tissues from multiple sites, including the oral cavity, oropharynx, hypopharynx, and larynx, whereas the present study focused solely on oral cancers. Differences in tissue type, anatomical sites, and analytical methods may have influenced the outcomes, making direct comparison between studies difficult.

Scanlan et al. [12] reported that *MAGEB2* expression was present in various tumours but absent in normal tissues, except for the testes and placenta. Similarly, Zamunér et al. [14] found no MAGEB2 mRNA expression in normal control samples, supporting its classification as a "testis-restricted" *CTA*. In contrast, the present study detected low levels of MAGEB2 protein expression in a small proportion of NOM (3%) and OED (11%) tissues. However, the staining in these tissues was weak and focal compared to the higher and more diffuse expression observed in OSCC. This suggests that while MAGEB2 is predominantly tumour-associated, minimal expression may occasionally occur in non-malignant oral tissues. Interestingly, [14] also reported low-level expression (up to 10%) of other *CTA*s, including *PRAME, SPANX-CD, MAGEA1, MAGEC2*, and *CRISP2*, in normal samples.

No significant association was found between *MAGEB2* expression and any socio-demographic, clinicopathological, or treatment parameters, except for age. Similar findings have been reported for other *CTA*s such as MAGE-A1, SSX-1, CTp11, and HCA587 in hepatocellular carcinoma [22]. Likewise, *CTA*s including NY-ESO-1, MAGE-A3, and KK-LC-1 in non-small cell lung cancer, and SPAG9 in epithelial ovarian cancer, have shown no significant association with clinicopathological parameters [22-24].

The 5-year overall survival (OS) rate among OSCC patients in this cohort was 43%, comparable to the 50% reported previously [25]. In contrast, a Malaysian study documented poorer outcomes, with 1-year and 5-year OS rates of 67.2% and 13.4%, respectively [26]. In the present study, *MAGEB2* expression showed no significant association with OS, although a trend toward better survival was observed in cases with higher protein expression. Similar favourable trends have been reported in other cancers, where *CTA* expression was associated with improved outcomes including CT10 in urothelial carcinoma [27], MAGE-A3/4 in lung adenocarcinoma [28], ACTL8, OIP5, XAGE3, and CTCFL in glioblastoma, and MAGE-C1 in epithelial ovarian cancer [29, 30].

Conversely, Zamunér et al. [14] observed an opposite trend, where higher MAGEB2 mRNA expression correlated with poorer OS, despite no statistically significant association. Differences between studies may relate to the molecular level examined (protein vs. mRNA), tumour site, or analytical methods. Likewise, *CTA* expression has been associated with poor prognosis in multiple myeloma, head and neck, ovarian, gastric, and lung cancers [31-35]. Zamunér et al. [14] also found that MAGEB6, CRISP2, and CXORF48 expressions were linked to worse outcomes, with MAGEA3/6 serving as an independent predictor of recurrence, suggesting that *CTA* expression in HNSCC may trigger immune responses influencing disease prognosis. Similarly, high *CTA* expression has been associated with poorer outcomes in multiple myeloma and myxoid liposarcoma, where NY-ESO-1 expression predicted reduced survival [36, 37].

In contrast, several studies have shown no association between *CTA* expression and survival, including MAGE expression in testicular cancer [21]. Other *CTA*s such as NY-ESO-1, MAGE-A3, and KK-LC-1 in non-small-cell lung cancer; MAGE-A1, CTp11, SSX-1, and HCA587 in hepatocellular carcinoma; and SPAG9 in epithelial ovarian cancer have also demonstrated no significant correlation

with OS [22-24]. These conflicting findings across tumour types may be attributed to variations in tumour composition, the proportion of undifferentiated cells, histological origin, and methodological differences [21].

Previous clinical trials in ovarian cancer have explored several cancer-testis antigens (*CTA*s) as potential targets for anticancer vaccines and immunotherapy [38]. In head and neck squamous cell carcinoma (HNSCC), a vaccine targeting MAGEA3 and HPV-16 antigens has also been evaluated [14]. Although these studies highlight the broader immunotherapeutic potential of *CTA*s, evidence regarding the specific role of MAGEB2 remains limited. Further research is required to elucidate its biological function and potential immunogenicity in OSCC.

In the present study, higher MAGEB2 protein expression was observed in OSCC compared to OED and NOM. However, its role in OSCC carcinogenesis and disease progression remains unclear. Understanding the underlying molecular mechanisms regulating *MAGEB2* expression. such as transcriptional control and post-translational modification, may help clarify its contribution to tumour behaviour.

While MAGEB2 and other MAGE family members have been investigated in preclinical models for their potential as vaccine candidates, the current findings are preliminary and do not provide direct evidence for prognostic, diagnostic, or therapeutic applications. Nevertheless, MAGEB2 warrants further investigation as a tumour-associated antigen with possible future relevance in targeted immunotherapy once its biological and immunological roles are better defined.

Author Contribution Statement

Conceptualization, A.R., and R.B.Z.; methodology, N.A.H., S.M., and A.R.; validation, N.A.H., and A.R.; formal analysis, N.A.H., and G.R.W.; resources, S.M., G.R.W., R.B.Z., and Z.Z. ; data curation, N.A.H., G.R.W., and A.R.; writing—original draft preparation, N.A.H., and G.R.W.; writing—review and editing, S.M., Z.Z., R.B.Z., and A.R.; visualization, N.A.H.; supervision, A.R., and R.B.Z.; project administration, G.R.W.; funding acquisition, S.M., and R.B.Z. All authors have read and agreed to the published version of the manuscript.

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Ethical Declaration

This study was approved by the Medical Ethics Committee of Faculty of Dentistry, Universiti Malaya [Ethics Committee/IRB reference number: DF OS1910/0043 (P)].

Conflict of Interest

The authors declare that there are no conflicts of interest.

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