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Original Article

Clinical implications of Cytokeratin 19 expression in patients with oral squamous cell carcinoma

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Summary

Objective. This study was done to quantify the prevalence of high cytokeratin (CK) 19 expression in Indonesian oral squamous cell carcinoma (OSCC) patients and explore the prognostic role of CK19 in OSCC.

Methods. Clinical data and samples from 61 patients diagnosed with OSCC at a tertiary national referral hospital in Jakarta, Indonesia were analyzed in this retrospective cohort study. Immunohistochemical staining of CK19 was performed on all patients and its expression was scored using the H system. All patients were followed up for a minimum of 36 months after diagnosis. Comparative and survival analyses were performed.

Results. Twenty six point two percent of Indonesian OSCC patients had high CK19 expression. There were no differences in clinicopathological characteristics between patients with low and high CK19 expression. The 3-year overall survival (OS) of our cohort was 11.5%. Patients with high CK19 expression had lower 3-year OS compared to patients with low CK19 expression, even if the difference in OS was not statistically significant. Keratinization was an independent prognostic factor for survival in multivariate regression analysis.

Conclusions. Data obtained here indicate a possible prognostic role of CK19 in OSCC. This prognostic role should be confirmed in larger series.

Key words: cytokeratin 19, Indonesia, oral squamous cell carcinoma, survival

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Introduction

Cancer remains the leading cause of death in most countries throughout the world. In the International Agency for Research on Cancer's most recent GLOBOCAN estimates of cancer incidence and mortality, oral cancer ranked 20th in number of estimated new cases with an estimated 377,713 new cases and 177,757 cancer deaths [1]. Oral cancer may arise from the epithelium lining the lips, gums, tongue, mouth, or palate. Keratinocytes comprise a majority of this epithelium, and 90-95% of oral cancer are oral squamous cell carcinomas (OSCCs) [2].

Cytokeratins (CKs) are intermediate filament proteins found in the cytoplasm of eukaryotic cells to maintain the cytoskeletal framework. They are specifically expressed in epithelial tissues. The expression of CKs depends on epithelium type and differentiation. CK19 is a low-molecular-weight and acidic cytokeratin expressed in simple ductal epithelium, mesothelium, and pseudostratified epithelium along with CK7 [3]. CK19 is abundant in the basal cell layer of normal adult simple and stratified squamous epithelial tissues [4]. The expression of CK19 in suprabasal stratified squamous epithelial cells of the oral mucosa may be induced by pathologic alterations such as inflammation and dysplasia, as observed

in oral potentially malignant disorders [3-5]. Additionally, CK19 is expressed in OSCC with increased expression according to the grade and stage of cancer [6,7]. Other studies have also found increased expression of CK19 in human papillomavirus (HPV)-positive OSCC compared to HPV-negative OSCC [8,9].

The role of CK19 as a prognostic marker in OSCC has not been widely studied. The five biomarkers most frequently studied with regard to the prognosis of OSCC were p53, Ki-67, p16, VEGFs, and cyclin D1 [10]. This study was done to quantify the prevalence of high CK19 expression in Indonesian OSCC patients and explore the role of CK19 as a prognostic marker in OSCC.

Materials and methods

Sample collection

This retrospective cohort study involved tissue sections from 61 patients diagnosed with OSCC. All patients who underwent histopathological examination and were diagnosed with OSCC at the Department of Anatomical Pathology Dr. Cipto Mangunkusumo Hospital in Jakarta, Indonesia from 2016 to 2018 was included in the study. Patients with incomplete data, change of diagnosis following reassessment, and tissue sections not adequate for evaluation were excluded from the study. Clinical data including age, sex, tumor location, and staging were obtained from medical records. Two independent pathologists reassessed patients' tissue sections to confirm the diagnosis and obtain histopathologic data including differentiation, keratinization, lymphovascular invasion, perineural invasion, and histological grading of OSCC according to Bryne's grading system. New sections were cut when necessary. The primary endpoint of this study was OS measured in months from diagnosis to death from any cause. All patients were followed up for a minimum of 36 months after diagnosis.

Immunohistochemistry staining

Immunohistochemistry staining was carried out on 4 µm paraffin-embedded tissue sections mounted on poly-L-lysine coated slides. They were marked with diamond pencil, deparaffinized with graded xylol for 3-5 days, and rehydrated with alcohol of descending concentrations. Endogenous peroxidase was blocked by incubating sections in hydrogen peroxide 0.5% for 30 minutes. Antigen retrieval was done by heating sections in a decloaking chamber with tris EDTA of pH 9. Non-specific proteins were blocked with a universal background sniper (Starr Trek Universal HRP Detection Kit Biocare®) for 15 minutes. Subsequently, sections were incubated with the mouse monoclonal primary antibody CK19 (Biocare®) with a dilution of 1:300 for 60 minutes followed by the secondary antibody (Trekie Universal Link®) for 15 minutes. Finally, sections were incubated in diaminobenzidine for 1 minute, counter-stained with Mayer's hematoxylin, dehydrated with alcohol of ascending concentrations, immersed in xylol for clearing, and mounted under a cover slip for assessment.

Assessment of CK19 expression

Two independent pathologists, blinded to clinical data and outcomes, assessed the expression of CK19 using a Leica microscope. Tumour cells with cytoplasmic and membranous staining were captured and analysed using the ImageJ® software. The immunohistochemical expression of CK19 was scored using the H system calculated by multiplying the intensity of staining (0-3) with the percentage of stained tumor cells (0-100%). The intensity of staining was scored as negative (0), faint (1), moderate (2), and strong (3). The number of CK19-stained tumor cells was expressed as a percentage of 500 tumor cells or the maximum number of tumour cells found in tissue sections. H scores of > 150 were defined as high CK19 expression and vice versa.

Statistical analysis

Differences in clinicopathological characteristics between OSCC patients with high CK19 expression and low CK19 expression were observed. Continuous variables were compared using the t-test. Categorical variables were compared using the Chi-square test or its alternative test, Fisher's exact test. Kaplan-Meier method was used to generate 3-year OS curves and the log-rank test was applied to compare the curves. Univariate and multivariate Cox regression analyses were performed. P-values under 0.05 were regarded as statistically significant. Statistical analyses mentioned above were carried out using the Statistical Package for Social Sciences software version 25.0.

Results

The mean age of our patients was 47.84 ± 12.271. Most of the patients were male (54.1%) with tumors arising from the tongue (85.2%) of advanced stage (stage III 9.8% and stage IV 83.6%) and treated with chemotherapy alone (65.6%). Most tumors were well differentiated (60.7%), had good histological grade according to Bryne's grading system (59.0%), and had keratinization (88.5%). No lymphovascular invasion and perineural invasion were observed in the histopathological assessment of most patients (93.4% and 98.4% respectively). Characteristics of

the 61 patients included in this study are presented in Table I.

Assessment of CK19 expression revealed that 45 patients (73.8%) had low CK19 expression while 16 patients (26.2%) had high CK19 expression. The median H score was 69.8 with the minimum H score being 0 and the maximum H score being 300. CK19 has various expressions in normal, hyperplastic and dysplastic squamous epithelium while low and high CK19 of OSCC expressions are shown in Figure 1. Characteristics of patients according to CK19 expression can be seen in Table II. There were no significant differences in clinicopathological characteristics between patients with low CK19 expression and high CK19 expression. 54 patients (88.5%) and all patients with high CK19 expression died within 3 years after diagnosis. The 3-year OS rate was 11.5% and the median survival time from diagnosis was 8 months. The minimum survival time was 1 month and the maximum survival time was 64 months. Patients with low CK19 expression had a superior 3-year OS compared to patients with high CK19 expression, though not statistically significant ($p = 0.396$). Mean survival times for patients with low and high CK19 expression were 16.156 ± 3.146 and 9.063 ± 1.588 months respectively. Meanwhile, median survival times for patients with low and high CK19 expression were 9 and 6 months, respectively. Figure 2 shows survival curves stratified by expression of CK19.

High CK19 expression was not significantly associated with 3-year OS (univariate analysis: hazard ratio (HR) 1.274, 95% confidence interval (CI) 0.707-2.298, $p = 0.420$; multivariate analysis: HR 1.187, 95% CI 0.646-2.182, $p = 0.581$). In univariate Cox regression analysis, no variables were significantly associated with OS. However, multivariate analysis revealed that the presence of keratinization was significantly associated with better 3-year OS (HR 0.375, 95% CI 0.145-0.970, $p = 0.043$). Univariate and multivariate Cox regression analyses of 3-year OS are presented in Table III.

Table I. Characteristics of 61 patients.

Characteristics	Distribution	n (%) (n = 61)
Mean age (SD)	47.84 (12.271)	
Sex	Male	33 (54.1)
	Female	28 (45.9)
Tumor location	Tongue	52 (85.2)
	Buccal region	5 (8.2)
	Gingiva	1 (1.6)
	Mandible and maxilla	3 (4.9)
Size of primary tumor (T)	T1	1 (1.6)
	T2	7 (11.5)
	T3	2 (3.3)
	T4	51 (83.6)
Involvement of lymph nodes (N)	N0	21 (34.4)
	N1	18 (29.5)
	N2	22 (36.1)
Distant metastasis (M)	M0	58 (95.1)
	M1	3 (4.9)
AJCC staging	I	1 (1.6)
	II	3 (4.9)
	III	6 (9.8)
	IV	51 (83.6)
Therapy	Chemotherapy	40 (65.6)
	Radiotherapy	2 (3.3)
	Surgery	9 (14.8)
	Chemotherapy and radiotherapy	6 (9.8)
	Surgery and chemotherapy	3 (4.9)
	Surgery and radiotherapy	1 (1.6)
Tumor differentiation	Well differentiated	37 (60.7)
	Moderately differentiated	16 (26.2)
	Poorly differentiated	8 (13.1)
Bryne's grading system	Good grade	36 (59.0)
	Moderate grade	20 (32.8)
	Poor grade	5 (8.2)
Keratinization	Present	54 (88.5)
	Absent	7 (11.5)
Lymphovascular invasion	Present	4 (6.6)
	Absent	57 (93.4)
Perineural invasion	Present	1 (1.6)
	Absent	60 (98.4)
CK19 expression	Low expression (H-score ≤ 150)	45 (73.8)
	High expression (H-score > 150)	16 (26.2)
3-year overall survival	Dead	54 (88.5)
	Alive	7 (11.5)

AJCC: American Joint Committee on Cancer

Discussion

To our knowledge, this is the first study reporting CK19 expression and survival in OSCC patients from Indonesia. However, this study has some notable limitations as follows. This study involved only a small number of patients with the majority of patients being advanced-stage OSCC (stage III and IV) from a single centre. Furthermore, there is currently no established criterion to assess CK19 expression. In this study, we used the H score to differentiate between high and low CK19 expression consistent with the most recently published study on CK19 expression in oral and oropharyngeal squamous cell carcinoma.[9] Another limitation is that we did not evaluate worst pattern of invasion (WPOI).

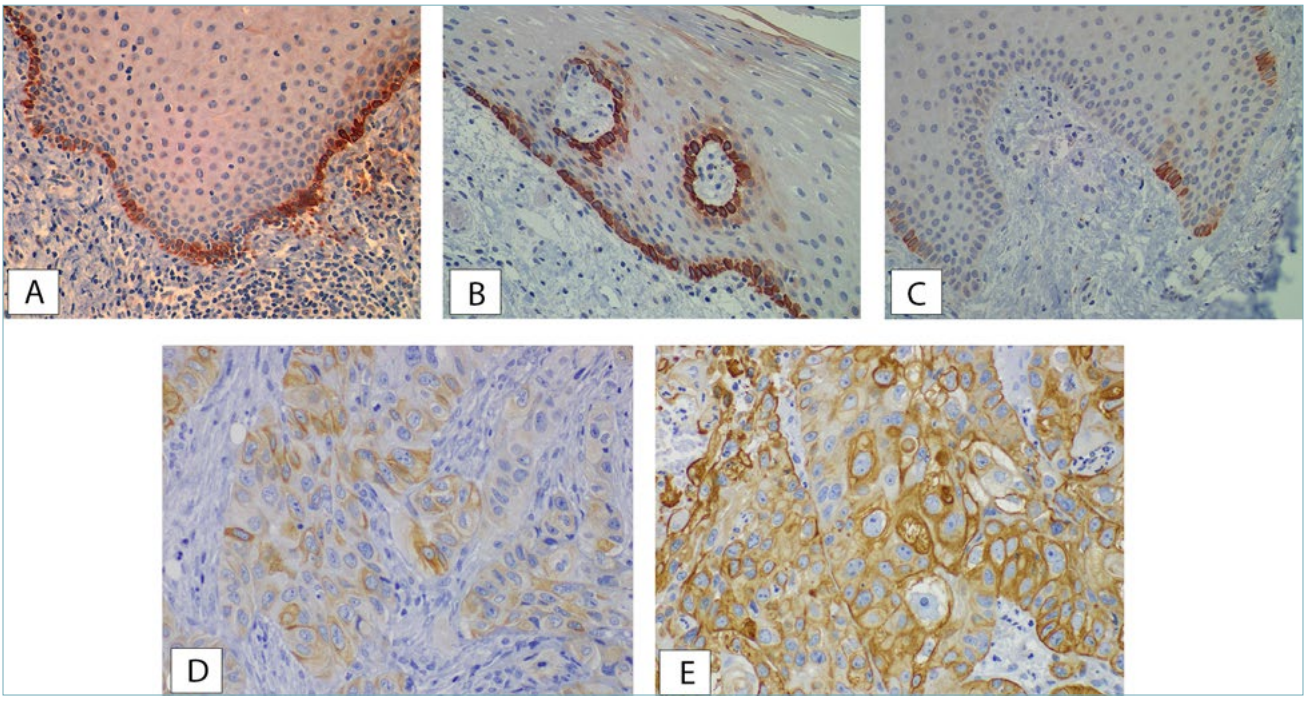

Figure 1. Immunohistochemical expression of CK19. CK19 expression in normal and hyperplastic epithelium showed no difference, respectively. CK19 positive on the basement membrane of the squamous epithelium (A, B). However, in mild-moderate dysplastic epithelium, there is a decrease in CK19 expression (C). Low (D) and high (E) immunohistochemical expression of CK19 in OSCC.

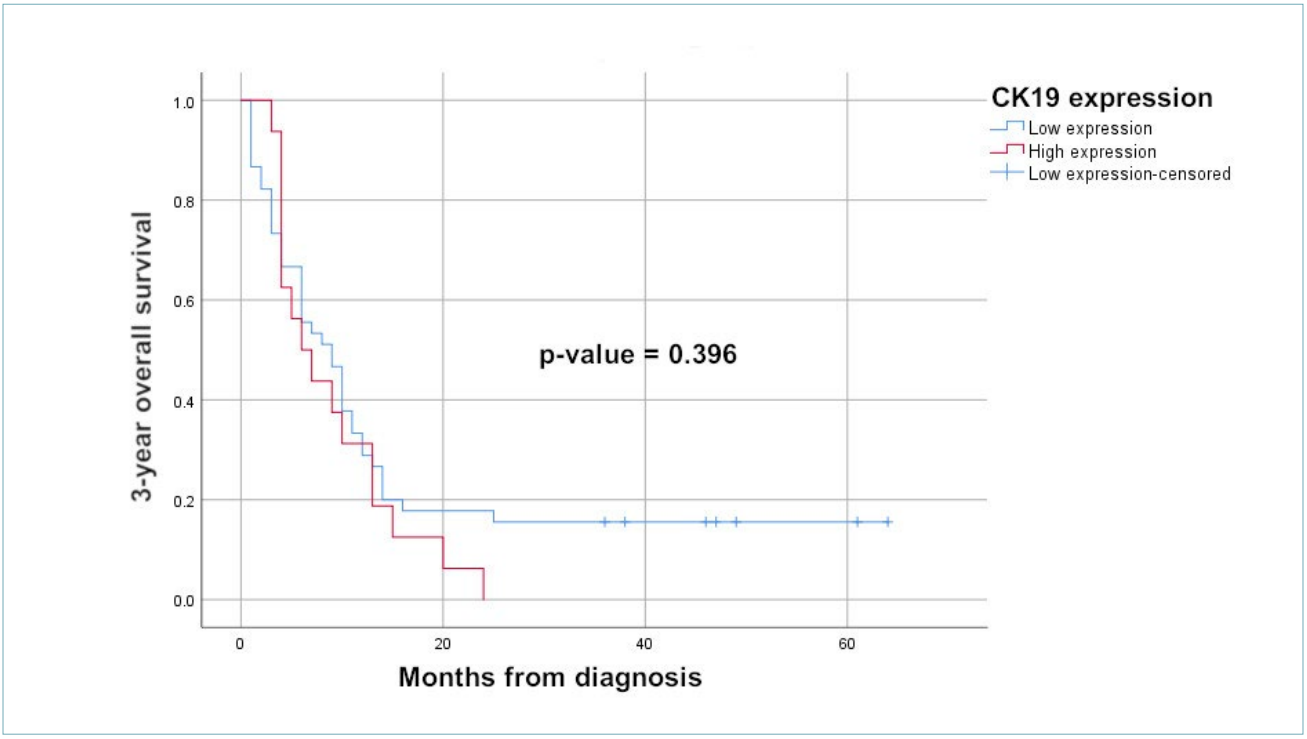

Figure 2. Kaplan-Meier curves stratified by CK19 expression.

Table II. Characteristics of 61 patients according to CK19 expression.

Characteristics	Low CK19 expression n (%) (n = 45)	High CK19 expression n (%) (n = 16)	p-value
Mean age (SD)	47.91 (12.319)	47.63 (12.532)	0.937 [1]
Sex			
Male	22 (48.9)	11 (68.8)	0.171 [2]
Female	23 (51.1)	5 (31.3)	
Tumor location			
Tongue	39 (86.7)	13 (81.3)	0.686 [3]
Non-tongue	6 (13.3)	3 (18.8)	
Size of primary tumor			
T1-T2	5 (11.1)	3 (18.8)	0.422 [3]
T3-T4	40 (88.9)	13 (81.3)	
Involvement of lymph nodes			
N0	15 (33.3)	6 (37.5)	0.763 [2]
N1-N2	30 (66.7)	10 (62.5)	
Distant metastasis			
M0	43 (95.6)	15 (93.8)	1.000 [3]
M1	2 (4.4)	1 (6.3)	
AJCC staging			
I-II	2 (4.4)	2 (12.5)	0.279 [3]
III-IV	43 (95.6)	14 (87.5)	
Tumor differentiation			
Well differentiation	28 (62.2)	9 (56.3)	0.674 [2]
Moderate-poor differentiation	17 (37.8)	7 (43.8)	
Bryne's grading system			
Good grade	26 (57.8)	10 (62.5)	0.741 [2]
Moderate-poor grade	19 (42.2)	6 (37.5)	
Keratinization			
Present	40 (88.9)	14 (87.5)	1.000 [3]
Absent	5 (11.1)	2 (12.5)	
Lymphovascular invasion			
Present	3 (6.7)	1 (6.3)	1.000 [3]
Absent	42 (93.3)	15 (93.8)	
Perineural invasion			
Present	1 (2.2)	0	1.000 [3]
Absent	44 (97.8)	16 (100.0)	
3-year overall survival			
Dead	38 (84.4)	16 (100.0)	0.174 [3]
Alive	7 (15.6)	0	

AJCC: American Joint Committee on Cancer. [1]T-test; [2]Chi-square test; [3]Fisher's exact test.

WPOI at the tumor-host interface is one of the prognostic parameters for patient survival in early-stage OSCC and early oral tongue squamous cell carcinoma (OTSCC) [11]. There are five types of WPOI as follows: broad pushing tumor front; finger-like pushing invasion; large tumor island with more than 15 cells; small tumor island with less than 15 cells; and satellite tumor nodule at least 1 mm away from the primary tumor, respectively [12].

CK19 is a unique type I cytokeratin that is expressed in specific epithelial cell types and is frequently found in cancers derived from these cells. CK19 has been extensively studied as a marker of differentiation, stem cell, premalignant, malignant, and metastatic, as well as a diagnostic and prognostic marker [13].

CK19 expression was found in the epithelial cells of most glandular organs in normal tissues, but it was restricted to the basal layer of nonkeratinizing squamous epithelium and was unavailable in the skin [13]. According to a study conducted by Rejeswari et al, CK19 expression was found to be elevated in oral epithelial hyperplasia and associated with increasing grades of inflammation in both types of epithelia. Its expression was reduced in mild and moderate dysplasia, but increased dramatically in severe dysplasia. There was no evidence of a progressive increase in CK19 expression with increasing grades of dysplasia. Safadi et al., on the other hand, found a progressive increase in CK19 expression with increasing grades of dysplasia in samples obtained from different grades of dysplasia using an automated color deconvolution program [14].

Table III. Univariate and multivariate Cox regression analyses of 3-year OS.

Variable	Univariate			Multivariate		
	HR[1]	95% CI	p-value	HR[1]	95% CI	p-value
pT3-4	1.254	0.566-2.780	0.578	1.658	0.517-5.314	0.395
pN1-2	1.497	0.838-2.676	0.173	1.639	0.826-3.250	0.158
pM1	2.695	0.810-8.965	0.106	2.840	0.771-10.453	0.117
Stage III-IV	1.577	0.490-5.071	0.445	0.440	0.071-2.712	0.376
Moderate-poor differentiation	0.906	0.524-1.569	0.725	0.825	0.397-1.716	0.607
Moderate-poor grade	0.822	0.475-1.422	0.483	0.739	0.349-1.566	0.430
Presence of keratinization	0.544	0.243-1.218	0.139	0.375	0.145-0.970	0.043
Presence of LVI	0.545	0.170-1.752	0.309	0.520	0.112-2.427	0.406
Presence of PNI	0.871	0.120-6.348	0.892	3.563	0.260-48.747	0.341
High CK19 expression	1.274	0.707-2.298	0.420	1.187	0.646-2.182	0.581

HR: hazard ratio, CI: confidence interval, LVI: lymphovascular invasion, PNI: perineural invasion. [1]Cox regression analysis

The 5-year OS of OSCC was 64.4 [15]. Our study reported a 3-year OS of 11.5%. This number is low compared to the aforementioned 5-year OS and to 3-year OS reported in other studies. The 3-year OS was 58.6% among OSCC patients from North Portugal [16], 68% among OSCC of the tongue in patients from Egypt [17], and 77% among young OSCC patients from China [18]. Differences in OS mentioned above may be attributed to the characteristic of our cohort in which 93.4% had advanced stage III and IV OSSC. Meanwhile, advanced-stage OSCC was only diagnosed in 54.7%, 78%, and 51% of the Portuguese, Egyptian, and Chinese cohorts, respectively [16-18].

Only 26.2% of our patients had high CK19 expression. A prior study that, like ours, evaluated CK19 expression using the H score reported that 29.9% of their OSCC patients had high CK19 expression [9]. Other studies with different thresholds for assessing CK19 expression reported positive CK19 expression in 31.8% [19], 56% [20], 58% [6], 62.2% [7], 90.9% [21], and 100% [22] of OSCC patients respectively. A uniform criterion to assess CK19 expression should be established in further studies.

We observed no differences between patients with low CK19 and high CK19 expression which was not consistent with prior studies. Babiker et al. reported significantly higher CK19 expression in males ≥ 50 years old [6]. Additionally, CK19 expression rises in line with the grade and stage of cancer, with poorly differentiated OSCC patients and stage IV OSCC patients displaying a higher percentage of high CK19 expression when compared to patients of other grades and stages [6]. Zhong et al., Bombeccari et al., and Tanaka et al. also found that CK19 expression was correlated with higher-grade tumors (moderately or poorly differentiated OSCC) [20,21,23]. Safadi et al. also reported significant correlations between higher CK19 expression and OSCC with distant metastasis as well as stage IV OSCC compared to OSCC without distant metastasis and stage I-III OSCC [24].

In our study, patients with high CK19 expression had inferior 3-year OS compared to patients with low CK19 expression though not statistically significant. Conflicting findings were found in prior studies analyzing the survival of OSCC patients with regard to CK19 expression. Tanaka et al. found no significant differences between the 5-year OS curves of patients when stratified into CK19 expression of < 5%, 5-77%, and ≥ 77% [23]. Meanwhile, Fillies et al. and Ernst et al. found that patients with high CK19 expression had poorer OS compared to their counterparts with significant differences between Kaplan-Meier curves [19,22]. A notable difference is that while Ernst et al. found that high CK19 expression was significantly associated with worse OS in multivariate regression analysis (HR 2.035, 95% CI 1.231-4.482, p = 0.010) [19], Fillies et al. did not [22]. High CK19 expression was not associated with OS in our multivariate regression analysis either. In line with Ernst et al., multivariate regression analysis by Safadi et al. showed that 5-year OS was significantly associated with decreased CK19 score (HR 0.84, 95% CI 0.72-0.98, p = 0.02) [24]. Results of several studies examining the relationship between CK 19 and OSCC are summarized in Table IV.

Keratinization is a histologic feature marked by the formation of keratin pearls by malignant squamous cells. Keratin pearls are whorled-shaped accumulations of keratin present in concentric layers between the squamous epithelium [25]. It is more likely to be observed in well or moderately differentiated SCC. Well differentiated SCC are comprised of cells that produce abundant keratinization meanwhile cells of moderately differentiated SCC usually produce less keratinization [26]. Multivariate regression analysis of our cohort showed that the presence of keratinization was significantly

Table IV. Summary of studies examining the relationship between CK 19 and OSCC.

No	Author, year	Country	Samples	Site of involvement	Outcome
1	Babiker AY, et al., 2014	Sudan	-190 patients of OSCC -90 patients of inflammatory lesions	Oral cavity	This discovery demonstrates the potential of p16 and CK19 as prognostic markers in OSCC and significant molecular events in the etiology of oral cancer [6].
2	Menz A, et al., 2021	Germany	-15,977 samples from 122 tumor types -608 samples of 76 normal tissue	-Skin -Head and neck -Lung, pleura, and thymus -Female genital tract -Breast -Digestive system -Urinary system -Male genital organs -Endocrine organs -Haemotopoetic and lymphoid tissues -Soft tissue and bone	In squamous epithelium, CK19 IHC may aid in the early diagnosis of neoplastic transformation [7].
3	Santoro A, et al., 2015	Italy	38 OSCC patients	Oral cavity	This study demonstrates CK19 as a new marker that can help differentiate between OSCCs and OPSCCs that have HPV + or HPV- [8].
4	Woods RSR, et al., 2022	Ireland	253 patients: -OPSCC (134) -SCC (22) -oral tongue SCC (97)	- Oropharyngeal - Oral tongue	Cytokeratin has a role in the etiopathogenesis of HPV-related OPSCC. This is supported by the higher expression of CK7 and CK19 in HPV-positive OPSCC as compared to HPV-negative [9].
5	Ernst J, et al., 2016	Switzerland	129 patients	Oral cavity	Patients with positive CK19 immunoreactivity had a worse outcome with regards to overall and disease-specific survival [19].
6	Bombeccari GP, et al., 2017	Italy	36 samples: -18 samples from OLP lesions -18 samples from OLP-related OSCC lesions	Oral cavity	The pathologic differentiation grade substantially correlates with the elevated CK19 protein expression in OSCC [20].
7	Zhong LP, et al., 2007	China	33 OSCC patients	Oral cavity	Expression of CK19 in distant tissue predicts increased recurrence rate and decreased survival rate. The increased expression of this protein correlates with the level of pathological differentiation [21].
8	Fillies T, et al., 2006	Germany	308 OSCC patients	Oral cavity	A poor clinical outcome is linked to CK19 expression [22].
9	Tanaka S, et al., 2020	Japan	100 OSCC patients	Oral cavity	These findings imply that CK19 is implicated in OSCC invasion and metastasis and may provide an unique biomarker for OSCCs that are incredibly invasive and have the potential to spread [23].
10	Safadi RA, et al., 2019	Jordan	100 OSCC patients	-Oral cavity -Oropharynx -Paranasal sinuses -Lips vermilion borders	Expression of cytokeratin can be a prognostic factor such as 5-year survival and other parameters [24].
11	Rajeswari P, et al., 2020	India	-10 samples normal oral mucosa -10 samples epithelial hyperplasia -10 samples oral epithelial dysplasias and OSCC	Oral cavity	The increasing expression of CK19 can be used to predict the malignant transformation of epithelium [13].

Table IV. *continues.*

No	Author, year	Country	Samples	Site of involvement	Outcome
12	Frohwitter G, et al., 2016	Germany	193 OSCC patients	Oral cavity	Physiologically CK19 is not expressed in normal squamous epithelium, but may be expressed during carcinogenesis.[29]
13	Kale AD, et al., 2012	India	20 cases of OSCC	Oral cavity	Increased expression of CK 8/18, CK 19, and MMP 9 in ANM can predict carcinogenesis.[30]

OSCCs: oral squamous cell carcinomas, CK: cytokeratin, IHC: immunohistochemistry, HPV: human papilloma virus, OPSCCs: Oro-Pharyngeal Squamous Cell Carcinomas, SCC: squamous cell carcinoma, OLP: oral lichen planus, ANM: apparently normal looking mucosa

associated with better 3-year OS. Consistent with our finding, Dissanayaka et al. showed that 5-year survival in OSCC patients was significantly associated with the degree of keratinization [27]. Wolfer et al. also demonstrated in their study that OSCC patients with no or low keratinization had decreased 5-year disease-free survival rate compared to patients with good or high keratinization. However, in the same study, keratinization was also an independent prognostic indicator for recurrence in OSCC [28]. Beside keratinization, CK19 has been reported to play a role as prognostic marker. Further studies need to be done to evaluate the values of these two variables in prognosis of OSCC patients [29-30].

Conclusions

OSCC patients with high CK19 expression demonstrated lower 3-year OS compared to OSCC patients with low CK19 expression, though the difference in OS was not statistically significant. Keratinization was an independent prognostic factor for survival but CK19 expression was not. The prognostic role of CK19 and keratinization in OSCC warrants further studies.

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The authors have nothing to acknowledge.

Conflicts of interest

All authors declare that they have no potential conflicts of interest.

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Ethical consideration

This study (protocol number 22-08-0861) was approved by the Medical Ethics Committee of the Faculty of Medicine, Universitas Indonesia/ Dr. Cipto Mangunkusumo Hospital under the following reference number KET-769/UN2.F1/ETIK/PPM.00.02/2022. This research was conducted ethically, with all study procedures being performed in accordance with the requirements of the World Medical Association's Declaration of Helsinki. An informed consent waiver was obtained from the Medical Ethics Committee.

Authors' Contribution

NR and S designed and conceived the study. DRH, MS, and EK acquired clinical data. NR and S analysed and interpreted histopathological data and then drafted the manuscript. DRH, MS, and EK revised the manuscript. All authors read and approved the final manuscript for publication.

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