Original Research

Immunoexpression of CD44, p16 and VEGF in oral cancer

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Abstract

Objectives: The aim of the present study was to examine the immunoexpression of CD44, p16 and VEGF in oral squamous cell carcinoma (OSCC) and correlate them to clinicopathological parameters and survival outcomes in order to clarify their prognostic impact.

Material and Methods: A total of 68 individuals with OSCC recruited between 2011 and 2015 from two referral centres were enrolled in the study. The samples were placed on silanized glass slides and subjected to immunohistochemistry using anti-p16, anti-CD44 and anti-VEGF antibodies. The H Score was used for p16 and VEGF, while CD44 was scored according to the percentage of stained cells. Chi-square tests and Fisher's exact probability tests were used to compare clinicopathological characteristics according to the immunohistochemical expression, while overall survival and disease-free survival were estimated and compared using the Kaplan-Meier method and log-rank test, respectively. For all hypothesis tests, the level of significance was set at $P \leq 0.05$.

Results: No correlation was observed between the expression of tumour VEGF, p16 and CD44, and the clinicopathological characteristics analysed. Patients with high stromal VEGF expression had better disease-free survival than patients with low VEGF expression ($P = 0.023$).

Conclusion: In summary, P16, CD44 and tumour VEGF did not prove to be good prognostic biomarkers. Stromal VEGF expression is suggested to be a good candidate prognostic biomarker, although additional studies are needed.

Keywords: Biomarker, oral squamous cell carcinoma, prognosis, VEGF

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INTRODUCTION

Oral cavity cancer is a public health problem and an important cause of worldwide morbidity and mortality. In 2020, a global incidence of about 377,000 cases was estimated, with more than 177,000 deaths.[1] In Brazil, oral cavity cancer rates equivalent to 15,190 new cases were estimated for 2022, with a prevalence rate of 2.8 men to one woman.[2]

Although there has been an improvement in the treatment adopted, the five-year survival of patients with oral squamous cell carcinoma (OSCC) remains in the range

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of 50 to 60%. Currently, the TNM system is the main prognostic indicator, while the depth of invasion and extranodal extension have been added to improve the predictive capacity of this system.[3] However, there is still a need for other biomarkers to predict the risk of recurrence, resistance to therapy and metastasis, favouring the stratification of patients in order to apply more individualized therapies. In this respect, there is great interest in defining biomarkers associated with different stages of the carcinogenic process, such as vascular endothelial growth factor (VEGF), p16 and CD44.

VEGF is a cytokine secreted by both the tumour and stromal cells, such as macrophages, endothelial cells and fibroblasts. VEGF has several functions in the tumour microenvironment, including the primary stimulus of angiogenesis, sprouting of endothelial cells and increased vascular permeability. VEGF can also inhibit the anti-tumour immune response by recruitment of regulatory T cells (Treg).[4] Positivity for VEGF is related to lymph node metastasis and therefore represents a poor prognostic factor for cancer.[5,6]

The p16 protein, a cell cycle regulatory protein, is responsible for the control of the transition from the G1 to the S phase. In normal cells, p16 is expressed at low levels, and in the epithelium, it is usually found in the basal and parabasal layers, in which there is higher proliferative activity. The loss of p16 expression is related to increased cell proliferation due to loss of cell cycle control[7] The expression of p16 can be altered by inactivation of the CDKN2 gene by mutation, deletion or hypermethylation, with consequent cell immortalization.[8,9] However, human papillomavirus (HPV), when involved in carcinogenesis, encodes the viral oncoprotein E7, binds to pRb and promotes overexpression of p16 through the E7-pRb complex owing to loss of negative feedback with pRb.[10]

CD44 is a transmembrane glycoprotein related to cell division, migration, adhesion and signalling. CD44, as an adhesion molecule, enables cell communication through cell-cell and cell-matrix signal transduction and mediates human epidermal growth factor receptor signal transduction and common cell signalling pathways that regulate tyrosine kinases. It acts as a platform for some growth factors and for proteoglycan heparan sulphate. CD44 is expressed in almost all cell types of the body, such as leukocytes and fibroblasts, and is also found in many cancer stem cells.[11] The aberrant expression of CD44 and its variant forms has been associated with the invasive and metastatic potential of cancer cells, which leads to a worse prognosis.[12,13]

Thus, the aim of this work was to evaluate the applicability of VEGF, p16 and CD44 as prognostic biomarkers in OSCC.

METHODS

Patients

This was an observational longitudinal prospective analytical study conducted at two Brazilian centres: Hospital Santa Rita de Cassia and Hospital Universitário Cassiano Antônio de Moraes, both located in Espírito Santo. The study was approved by the clinical centre Ethics Committees and by the National Commission on Ethics in Research (Centro Integrado de Atenção à Saúde, Vitória, ES - Protocol 318/2011). Written consent was given by each patient prior to his or her participation in the study.

A total of 68 individuals diagnosed with OSCC between 2011 and 2015 were enrolled in the study. The inclusion criteria were individuals with oral cavity tumours (C02.0–C02.3, C02.8, C02.9, C03.0, C03.1, C03.9, C04.0, C04.1, C04.8, C04.9, C05.0, C05.8, C05.9, C06.0–C06.2, C06.8, C06.9) according to the International Classification of Diseases version 10 (ICD-10), who had not undergone any previous antineoplastic treatment. All cases were reviewed by an experienced pathologist.

Clinical and pathological data (i.e., age, sex, tumour site, TNM stage, alcohol consumption and tobacco exposure) were obtained by interview and from the medical records. The clinical stage of the tumours was categorized as early (clinical stages I and II) or advanced (clinical stages III and IV) according to the seventh edition of the TNM classification of the American Joint Committee on Cancer.[14]

Patients were submitted to the initial interview at diagnosis and followed up 6 to 18 months after the initial interview (first follow-up), 6 to 18 months after the first follow-up (second follow-up) and 30 and 60 months after the initial interview. Data such as alcohol and tobacco consumption, response to treatment and relapse were analysed. After receiving patient consent, formalin-fixed paraffin-embedded (FFPE) tumour blocks were retrieved from the Pathology archives at both centres.

Immunohistochemistry

FFPE tumours were cut into 3 μm sections and placed on silanized glass slides. Deparaffinization was performed with xylene and rehydration with descending alcohol dilution. Immunohistochemical stains for VEGF and CD44 were performed using the LSAB Kit (Dako, Glostrup,

Denmark) according to the manufacturer's protocol. Anti-VEGF (VEGF A-20, Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) and anti-CD44 (HCAM DF1485, Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) antibodies were applied to tissue sections at respective dilutions of 1:100 and 1:50, followed by a secondary biotinylated antibody and streptavidin-HRP conjugate complex. Immunohistochemistry for the anti-p16 antibody (clone E6H4, CINtec p16INK4a Histology Kit, Roche MTM Laboratories AG, Heidelberg, Germany) was performed using NovoLink Polymer detection systems (Novocastra Laboratories Ltd, Newcastle Upon Tyne, UK). After washing in buffer, the chromogen diaminobenzidine was applied, followed by a counterstain with Harris hematoxylin. Kidney tissue samples, oropharyngeal squamous cell carcinoma known to be positive for p16 and tonsil samples were used as positive controls for VEGF, p16 and CD44, respectively. Omission of the primary antibody was used as a negative control.

Microscopic evaluation

The slides were read independently by two trained researchers blinded. VEGF and p16 expression were determined using the H Score. The highest intensity of staining present in the tumour was scored on an ordinal score of 0 to 3 (0 for negative, 1 for weak, 2 for intermediate and 3 for strong staining), relative to the intensity of the positive (score 3) and negative (score 0) controls. The H Score resulted from the cross-product of the intensity (0 to 3) and percentage of the stained cells at each intensity (0 to 100%) and ranged from 0 to 300.

High p16 expression was considered if strong and diffuse nuclear and cytoplasmic tumour staining was present, with a cut-off point of ≥60 for the H Score according to.[15] VEGF expression was scored as high if diffuse cytoplasmic staining was observed in tumour and stroma, with a cut-off point for the H Score of ≥251 and ≥76 for tumour and stroma, respectively, using the median values as a cut-off point

CD44 expression was scored as high if cell membrane staining was present, with 26 to 100% of the tumour cells showing positive staining and was scored as low if 0 to 25% of the tumour cells showed positive staining.

Statistical analysis

The SPSS statistical software for Windows, version 20 (Statistical Package for the Social Sciences, Chicago, USA) was used for data analysis. Chi-square tests and Fisher's exact probability tests were used to compare the clinicopathological characteristics according to the immunohistochemical expression and disease recurrence or death. Overall survival (OS) and disease-free survival (DFS) were estimated and compared by the Kaplan-Meier method and log-rank test, respectively. The level of statistical significance was accepted at $P < 0.05$.

RESULTS

A total of 68 patients, 55 males and 13 females with a mean age at diagnosis of 59.71 years (range: 34–83 years; standard deviation: 11.93 years) were included in the analysis. The mean follow-up time of patients was 21.23 months (range: 1.1–51.5 months).

Immunohistochemical analysis demonstrated that most tumours had a low expression of p16 and tumour and stromal VEGF (82.26%, 57.58% and 62.12%, respectively). High CD44 expression was detected, equivalent to 58.82% of the tumours analysed. The immunohistochemical expression observed for p16, VEGF and CD44 is shown in Figure 1.

No significant association was observed between p16, VEGF and CD44 immunostaining and characteristics such as tumour stage, tumour size (T category), nodal metastasis (N category), history of alcohol and tobacco consumption or primary tumour location. These data are summarized in Table 1 and Table 2.

In order to confirm sample homogeneity and study reliability, we analysed the association between death and relapse events with staging, T and treatment adopted. We observed a significant association between staging, tumour size, treatment adopted and death [Table 3]. These data reveal that there is a higher occurrence of death associated with advanced staging and tumours of larger dimensions and that the surgical removal of the tumour with adjuvant radiotherapy is associated with fewer relapses. These significant associations were expected and were confirmed by survival curve analysis since tumour size, the occurrence of lymph node metastasis and distant metastasis are variables used to determine disease staging, just as staging is one of the determining factors for the treatment line to be adopted for the patient.

The mean OS time was 25.8 months (95% confidence interval: 21.30–30.31). Overall survival analysis revealed that patients with the highest survival rates were those with smaller tumours in the initial stage, without lymph node metastases and undergoing surgery. Thus, these data agree with the literature, reflecting the effectiveness

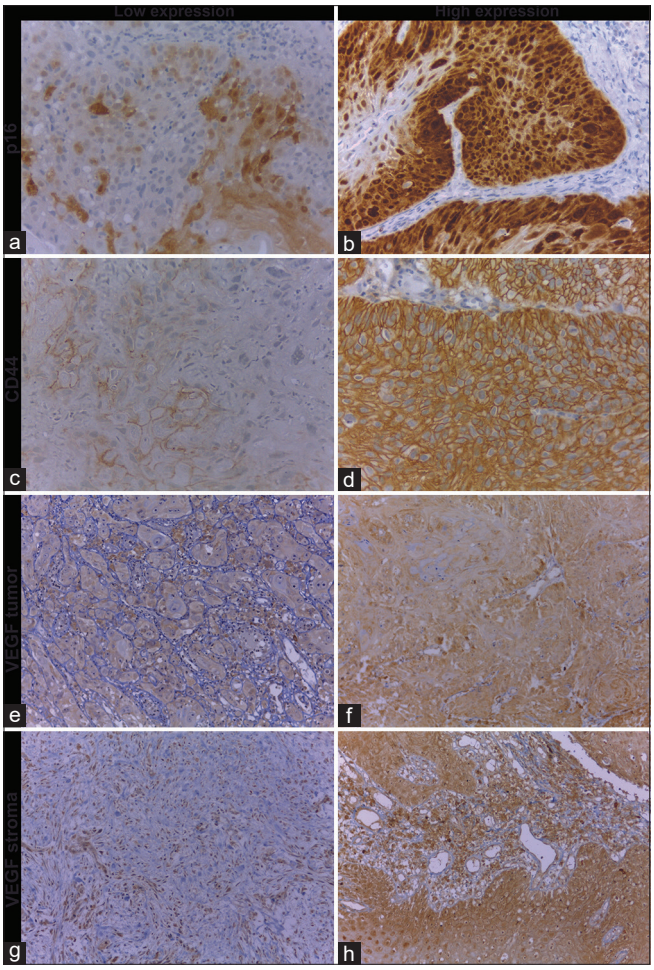

Figure 1: Immunohistochemical staining of OSCC according to the expression of p16 (a and b), CD44 (c and d), tumour VEGF (e and f) and stromal VEGF (g and h). Original magnification: 40x (a-d) and 20x (e-h)

of the sampling performed as representative of the population. No significant relationship with risk factors or immunohistochemical staining of p16, CD44, tumour VEGF or stromal VEGF was detected.

Mean DFS was 40.65 months (95% confidence interval: 35.45-45.85). DFS analysis revealed a significantly higher survival rate for patients with high stromal VEGF expression (P = 0.023). No significant relationship with clinicopathological characteristics, risk factors or immunohistochemical staining of p16, CD44 or tumour VEGF was detected. Data related to OS and DFS are available in Table 2 and can be visualized in Figure 2.

DISCUSSION

The staging system, which considers tumour size and the presence of lymph node and distant metastasis, is the main predictor of the prognosis of head and neck cancer. Recently, other parameters were included in the new American Joint Committee on Cancer staging manual (8th edition), such as the expression of p16 in oropharyngeal tumours, depth of invasion in the OSCC and extranodal extension. The addition of these parameters improved the predictive capacity of the system; however, new biomarkers are still needed to facilitate accurate patient risk stratification, to guide treatment selection and to predict the response.[3,16]

In this study, p16 and CD44 expression were not associated with survival outcomes and clinicopathological parameters

Table 1: Association of clinicopathological characteristics and risk factors with immunoexpression of VEGF in OSCC

Characteristic	Stromal VEGF-IHC expression		*P**	Tumour VEGF-IHC expression		*P**
	High *n* (%)	Low *n* (%)		High *n* (%)	Low *n* (%)	
Total	28 (42.42)	38 (57.58)		25 (37.88)	41 (62.12)	
Tumour Stage						
I-II	10 (15.15)	10 (15.15)	0.431	7 (10.61)	13 (19.70)	0.790
III-IV	18 (27.27)	28 (42.43)		18 (27.27)	28 (42.42)	
T category						
1-2	11 (16.67)	12 (18.18)	0.604	7 (10.61)	16 (24.24)	0.431
3-4	17 (25.75)	26 (39.40)		18 (27.27)	25 (37.88)	
N category						
1-3	10 (15.15)	14 (21.21)	1.000	9 (13.64)	15 (22.73)	1.000
0	18 (27.27)	24 (36.37)		16 (24.24)	26 (39.39)	
Smoking history[a]						
Ever smoker	18 (27.27)	28 (42.43)	0.574	17 (25.76)	29 (43.94)	0.584
Never smoker	9 (13.64)	9 (13.64)		8 (12.12)	10 (15.15)	
Alcohol use history[b]						
Ever use	18 (27.27)	24 (36.37)	1.000	15 (22.73)	27 (40.91)	0.407
Never use	8 (12.12)	12 (18.18)		10 (15.15)	10 (15.15)	
Primary tumour site						
Tongue	19 (28.79)	19 (28.79)	0.320	12 (18.18)	26 (39.39)	0.468
Floor of the mouth	3 (4.54)	8 (12.12)		5 (7.58)	6 (9.09)	
Others[d]	6 (9.09)	11 (16.67)		8 (12.12)	9 (13.64)	

Chi-square test or Fisher's exact test. [a]Smoking data missing for 2 patients. [b]Alcohol use data missing for 4 patients. [c]Relapse data missing for 2 patients. [d]Others primary sites analysed: hard palate, retromolar area, gum, buccal vestibule and cheek

Table 2: Association of clinicopathological characteristics and risk factors with immunoexpression of p16 and CD44 in OSCC

Characteristic	p16-IHC expression		*P**	CD44-IHC expression		*P**
	High *n* (%)	Low *n* (%)		High *n* (%)	Low *n* (%)	
Total	11 (17.74)	51 (82.26)		40 (58.82)	28 (41.18)	
Tumour Stage						
I-II	6 (9.68)	13 (20.97)	0.077	13 (19.12)	8 (11.77)	0.794
III-IV	5 (8.06)	38 (61.29)		27 (39.70)	20 (29.41)	
T category						
1-2	7 (11.29)	16 (25.81)	0.082	16 (23.53)	9 (13.24)	0.612
3-4	4 (6.45)	35 (56.45)		24 (35.29)	19 (27.94)	
N category						
1-3	4 (6.45)	21 (33.87)	1.000	13 (19.12)	12 (17.65)	0.448
0	7 (11.29)	30 (48.39)		27 (39.70)	16 (23.53)	
Smoking history[a]						
Ever smoker	10 (16.13)	33 (53.23)	0.155	27 (39.70)	21 (30.88)	0.785
Never smoker	1 (1.61)	16 (25.81)		11 (16.18)	7 (10.29)	
Alcohol use history[b]						
Ever use	9 (14.51)	30 (48.39)	0.142	23 (33.82)	21 (30.88)	0.275
Never use	1 (1.61)	18 (29.03)		14 (20.59)	6 (8.82)	
Primary tumour site						
Tongue	6 (9.68)	28 (45.16)	0.990	25 (36.76)	13 (19.12)	0.317
Floor of the mouth	2 (3.22)	10 (16.13)		5 (7.35)	7 (10.29)	
Others[d]	3 (4.84)	13 (20.97)		10 (14.71)	8 (11.77)	

Chi-square test or Fisher's exact test. [a]Smoking data missing for 2 patients. [b]Alcohol use data missing for 4 patients. [c]Relapse data missing for 2 patients. [d]Others primary sites analysed: hard palate, retromolar area, gum, buccal vestibule and cheek

Table 3: Overall survival and disease-free survival according to the clinicopathologic characteristics, risk factors and immunohistochemical staining

Variable	Category	Overall Survival			Disease-Free Survival		
		Average survival (in months)	CI (95%)	*P* log-rank	Average survival (in months)	CI (95%)	*P* log-rank
Sex[a]	Male	25.57	20.83–30.30	0.847	-	-	0.151
	Female	27.14	15.21-39.08		-	-	
Age, years	≤ 60	26.03	20.16–31.91	0.997	39.01	31.74–46.27	0.528
	>60	25.43	18.42-32.45		40.2	34.35–46.04	
Smoke	Ever smoke	23.42	18.47–28.37	0.168	34.67	29.38–39.96	0.085
history	Never smoke	30.03	20.21–39.84		48.71	43.44–53.99	
Alcohol use	Ever use	24.66	19.25–30.06	0.971	37.47	32.20–42.74	0.816
history	Never use	24.95	16.64–33.26		40.91	30.42–51.40	
Primary	Tongue	24.70	18.60–30.81	0.742	42.14	35.08–49.20	0.384
tumour site	Floor of the mouth	26.60	16.96–36.24		32.53	23.20–41.86	
	Others[b]	27.65	19.03–36.27		40.75	33.65–47.85	
T category	1-2	36.52	30.00–43.04	0.001*	41.01	34.02–48.00	0.969
	3-4	19.38	14.44–24.33		38.16	32.03–44.30	
N category	0	29.46	23.79–35.14	0.017*	43.68	38.17–49.19	0.080
	1-3	19.38	12.57–26.18		32.58	24.17–40.99	
Tumour stage	I-II	37.38	30.31–44.46	0.001*	41.50	34.10–48.90	0.833
	III-IV	20.13	15.38–24.88		37.52	31.64–43.40	
Treatment[c]	Surgery	39.11	31.36–46.87	0.001*	-	-	0.172
	Surgery + Radiotherapy	32.14	24.56–39.72		-	-	
	Chemotherapy + Radiotherapy	23.76	15.98–31.54		-	-	
	Radiotherapy	11.07	7.23– 14.92		-	-	
p16	High expression	28.17	17.39-38.95	0.340	37.21	28.70-45.71	0.723
	Low expression	24.27	19.28-29.26		38.61	32.12-45.10	
Stromal VEGF	High expression	26.13	18.85–33.40	0.652	47.66	42.68–52.64	0.023*
	Low expression	24.56	18.89–30.26		33.11	26.64–39.57	
Tumour VEGF	High expression	21.64	14.42–28.87	0.321	36.41	27.14–45.68	0.796
	Low expression	27.40	21.90–32.91		40.69	34.73–46.66	
CD44	High expression	25.66	19.64–31.69	0.964	44.87	39.01–50.73	0.061
	Low expression	25.52	19.10–31.94		33.06	26.05–40.06	

Kaplan-Meier survival analysis. [a]On disease-free survival, there were no women with relapse. Then, no statistics were computed because all cases are censored. [b]Others primary sites analysed: hard palate, retromolar area, gum, buccal vestibule and cheek. [c]On disease-free survival, there were no relapsed patients who underwent radiation therapy. Then, no statistics were computed because all cases are censored

analysed. However, a strong correlation was found between VEGF expression, exclusively in the stroma and DFS.

Although VEGF expression is associated with a worse prognosis in OSCC,[5,17] our study showed a positive

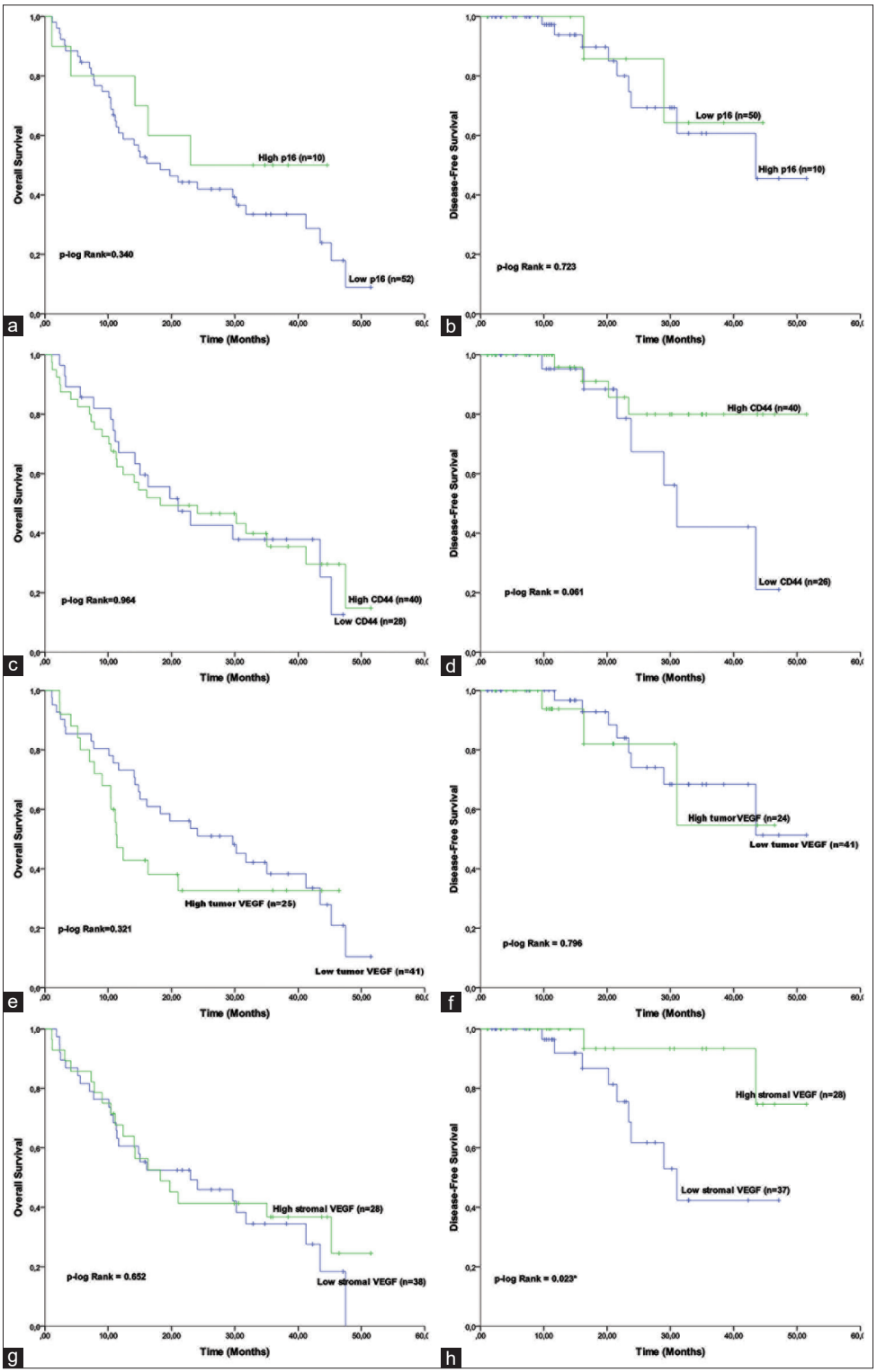

Figure 2: Kaplan-Meier survival curves according to the immunoexpression levels of p16 (a and b), CD44 (c and d), tumour VEGF (e and f) and stromal VEGF (g and h) in OSCC. (a, c, e, g) Overall survival. (b, d, f, h) disease-free survival

correlation between VEGF expression in the stroma and better DFS. Stromal VEGF expression is justified by the fact that the stromal microenvironment is active and important due to the information exchange between normal and tumour cells;[18] however, there are few studies about stromal VEGF expression in OSCC and its prognostic role remains controversial. A study of stromal VEGF-A and VEGFR-2 expression in prostate cancer reported that VEGF-A is a poor prognostic factor capable of predicting recurrence. In contrast, in the studies by

Khorana *et al*.[19] and Tayama *et al*.,[20] VEGF expression in stromal cells was associated with better OS and DFS, respectively, in colorectal cancer.

Our study is the first to demonstrate the positive prognostic value of stromal VEGF in oral cancer. VEGF undergoes alternative splicing and produces different isoforms which have been organized into two families: VEGFxxxa, with pro-angiogenic activity and VEGFxxxb with anti-angiogenic activity.[21] Some studies have already reported the positive prognostic role of VEGEFxxxb expression in tumour cells[22,23] and in tumour stroma.[20] The prognostic value of VEGFxxxb has not been described in oral cancer; however, Nagasaki and cols.[24] showed that OSCC cells secrete VEGF165b in the stroma, where it inhibits gelatinase-expressing cells and activates the ability of fibroblasts to adhere to the endoplasmic reticulum membrane protein complex (EMC), contributing to the anti-angiogenic process. Therefore, we assume that the anti-angiogenic isoforms were present at higher concentrations in the tumour stroma of the studied population.

All isoforms contain exons 1-5, and the identity of each VEGF-A isoform is determined by exons 6a, 6b, 7a and 7b. The splicing at the distal and proximal sites of exon 8 generates the isoforms VEGFxxxa and VEGFxxxb, respectively, which therefore differ by the C-terminal.[25] In our analyses, we used the antibody that binds to the N-terminal portion of VEGF, common to the isoforms of both families, which supports our hypothesis. However, additional studies for the detection of mRNA should be performed to clarify this result.

P16 expression was not associated with clinicopathological characteristics analysed or OS and DFS in our study. However, low p16 expression tended to be related to advanced stages ($P = 0.077$), a fact that would agree with the literature, since p16 is related to cell cycle control. In a study by Zafereo and cols,[26] the overexpression of p16 was significantly related to younger patients with the primary tumour site in the tongue but was not related to better or worse OS or DFS. Schneider and colleagues[27] showed, in a large cohort, that p16 was not related to survival and is a poor biomarker for OSCC patients.

The p16 expression has been used as a surrogate marker for HPV infection, since studies have shown HPV-positive tumours behave similarly to p16-positive tumours when compared to negative or p16-negative HPV tumours, with similar survival.[28] However, the association between HPV and OSCC remains controversial. The overexpression of p16 can be found in the oral cavity, but the frequency of HPV is low.[26,29] For oropharyngeal squamous cell carcinoma, the use of p16 as a biomarker is established, but its application in OSCC requires further investigation.[28]

CD44 has been recognized as a cancer stem-like cell marker in oral cancer, and it seems to participate in tumour progression and metastasis.[13] Other studies have shown the potential prognostic role of CD44 in OSCC, and it was found that high expression of CD44 is associated with advanced stage and shorter OS and DFS.[12,30] These findings were not confirmed in our study. The expression of CD44 had no significant relation with any of the clinicopathological characteristics or with OS and DFS. However, a trend ($P = 0.061$) could be observed for DFS, which was better in patients with high CD44 expression. Future studies with larger numbers of patients could rule out or confirm this trend of CD44 rates regarding DFS. Overall, our study at first indicates that CD44 would not be a good candidate as a prognostic biomarker.

It is important to highlight some limitations of our study: the small sample size; VEGF-A labelling heterogeneity in tumour tissue and stroma in addition to its extensive distribution in different tissues and cell types; and the lack of distinction between VEGF isoforms, which could be determined by RT-PCR or *in situ* hybridization and could more accurately indicate the presence of isoforms with anti-angiogenic activity.

CONCLUSION

In conclusion, no correlation was observed between the expression of tumour VEGF, p16 and CD44 and the clinicopathological characteristics analysed, which suggests that these proteins would not be good candidates as prognostic biomarkers. However, patients with high stromal VEGF expression had better DFS than patients with low VEGF expression.

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Conflicts of interest

There are no conflicts of interest.

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