

Prognostic Value of Cell Cycle Proteins in Squamous Cell Carcinomas of the Oral Cavity

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Abstract

The cell cycle is under strict regulation. Cyclin-dependent kinases (CDKs) at the G1-S and G2-M checkpoints are positively regulated by cyclins and negatively regulated by CDK inhibitors. Oral squamous cell carcinoma (OSCC) is a common malignancy that is characterized by a high degree of local aggression and lymph node metastasis. We examined positive regulators of the cell cycle (cyclin A, cyclin B1, and cyclin D1), cell cycle inhibitors (p16, p21, p27, p53, and Rb), and the proliferation markers Ki-67 and topoisomerase IIA by immunohistochemistry in a tissue microarray, comprising 136 cases of OSCC. Expression of cyclin A, cyclin B1, cyclin D1, topoisomerase IIA, p53, and Rb was positively associated with Ki-67. Overexpression of cyclin D1 correlated with the presence of lymph node metastasis. Overexpression of cyclin A, cyclin D1, topoisomerase IIA, and Rb and downregulation of p21 and p27 were associated with reduced overall survival in OSCC patients. Cyclin A, p21 and p27, were implicated an independent prognostic factor, by multivariate analysis, in OSCC patients. In conclusion, cyclin A, cyclin D1, p21 and p27 expression can be a valuable marker of poor prognosis and tumor aggressiveness in OSCC.

Keywords: Cell cycle proteins; Oral squamous cell carcinoma; Immunohistochemistry; Prognosis

Introduction

One of the hallmarks of oral squamous cell carcinoma (OSCC) is its capacity to invade locally and metastasize to regional lymph nodes. The identification of cervical lymph node metastasis is the chief prognostic factor of patients with OSCC and influences the selection of the appropriate treatment [1].

The pathogenesis of OSCC is believed to be a multistep phenomenon in which several genetic alterations can be detected in the early stages of the disease. Dysregulation of molecular pathways of cellular differentiation, apoptosis, cell adhesion, angiogenesis, metastasis, and the cell cycle regulates OSCC carcinogenesis [2].

The cell cycle is governed by cyclin-dependent kinases (CDKs), which are positively regulated by cyclins and negatively regulated by CDK inhibitors (CKIs) [3]. The cell cycle is tightly controlled throughout its phases-G1, S, G2, and M. Individual cyclins, specific for each phase of the cell cycle, accumulate and activate CDKs at the appropriate times during the cell cycle and subsequently are degraded, causing kinase inactivation. CKIs, which inhibit certain cyclin/CDK complexes, also rise and decline at specific times during the cell cycle [4].

Different cyclin-CDK complexes regulate various cell cycle transitions. Cyclin D interacts with CDK4/6 and is necessary for the G1-S transition. Cyclin E interacts with CDK2 for the G1-S transition. Cyclin A interacts with CDK2 for S phase progression, and cyclin-A/B associates with CDC2 for entry into M phase [5,6].

CKIs are classified into 2 major groups. The CIP/KIP family comprises p21, p27, and p57, which inhibit all CDKs, and the INK4 family contains p16, p15, p18, and p19, which specifically inhibit G1 phase, cyclin D, CDK4, and CDK6. p21 is regulated by wild-type protein p53 in response to DNA damage and contributes to G1 cell cycle arrest under these circumstances [7,8].

Activation of specific CDKs through complex formation with their cyclin partners drives cell cycle progression by phosphorylation of cellular substrates, which include the retinoblastoma protein (Rb). Phosphorylation of Rb during the G1 phase of the cell cycle allows G1-S transition through release of transcription factor (E2F) important in the induction of S-phase. Initial Rb phosphorylation events are mediated by cyclin D1/CDK4 and/or cyclin D1/CDK6 complexes [9,10].

Several studies have reported associations between overexpressed cyclins and CKIs and OSCC [11-18]. Yet, this finding is controversial, because other groups have not observed such correlations [19-22].

The aim of this study was to analyze the association of positive cell cycle regulators (cyclin A, cyclin B1 and cyclin D1,) and cell cycle inhibitors (p16, p21, p27, p53, and Rb) with clinicopathological parameters of the OSCC patients. Ki-67 and topoisomerase IIA was also examined as a cellular proliferation marker in OSCC.

Materials and Methods

Tissue samples and patient characteristics

Tissue samples were obtained by surgical resection of 136 OSCCs from 1992 to 2005, stored in files in the Department of Anatomical Pathology, Hospital A. C. Camargo, São Paulo, Brazil. None of the

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patients had received radiotherapy or chemotherapy before surgical excision.

The study was performed with approval from the local ethics committee (Study No. 986/07). The clinicopathological characteristics of the OSCC patients are summarized in Table 1.

Tissue microarray (TMA)

Cases were chosen based on the evaluation of 2 observers; selected blocks of each tumor and normal tissue were used to construct the TMA.

The areas that were used to generate the TMAs were marked on the slide and the donor block. The tissues that corresponded to the selected areas were sampled using a manual arraying instrument (Manual Tissue Arrayer 1, Beecher Instruments Micro-array Technology, Silver Spring, MI, USA), drawing 1-mm cores from different areas of the tumor from a single case of OSCC, placed at specified coordinates. Each case was spotted in duplicate.

Immunohistochemistry (IHC) analysis

Four-micron sections were deparaffinized, rehydrated, and subjected to antigen retrieval. Details on the antigen retrieval methods and primary antibody clones, sources, and titers are listed in Table 2 (supplementary material). Slides were placed in 3% hydrogen peroxide for 15 min to quench endogenous peroxidase activity. The sections were blocked with Protein Block Serum-Free (Dako, Carpinteria CA, USA) at room temperature for 20 min to suppress nonspecific binding of subsequent reagents. The sections were rinsed with PBS after each incubation step.

Clinicopathological parameters	IHC Total (n) (%)
Sex Male Female	108 (79) 28 (21)
Tobacco smoking No Yes Not available	15 (12) 110 (88) 11
Alcohol consumption No Yes Not available	25 (20) 100 (80) 11
Clinical stage I and II III and IV	36 (27) 100 (73)
Lymph nodes N0 N1-3 No lymph node dissection	57 (46) 66 (54) 13
Perineural invasion No Yes Not available	67 (55) 54 (45) 14
Vascular embolization No Yes Not available	71 (58) 51 (42) 14
Histological grade Well-differentiated Poorly/moderately differentiated Not available	105 (79) 28 (21) 03
Recurrence No Yes	83 (64) 49 (36)

 Table 1: Clinical and pathological characteristics of patients with OSCC.



Figure 1: Positive examples of each cell cycle protein analyzed by immunohistochemistry in oral squamous cell carcinoma: cyclin A, cyclin B1, cyclin D1, p16, p21, p27, p53, Rb, topoisomerase IIA and Ki-67.

The sections were incubated with the primary antibodies at room temperature in a humidified chamber for 2 hours. After being washed with PBS 3 times for 5 min each and incubated for 1h using an indirect dextran polymer detection system (Leica, Newcastle, UK). The slides were stained by incubation in 3,3' diaminobenzidine tetrachloride (Dako, Carpinteria CA, USA). The slides were lightly counterstained with hematoxylin, dehydrated, and mounted with coverslips using a permanent mounting medium.

Negative controls were generated by substituting the primary specific antibodies with non-immune serum, and positive controls were implemented according to the primary serum manufacturer's datasheet. All IHC reactions were performed in quadruplicate (2 cores per slide, 2 stained slides).

Positive nuclear staining was measured in percentage using the Automated Cellular Imaging Systems-ACIS III, version 3.1 (Dako, San Juan Capistrano, CA, USA). The ACIS III consists of an automated

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Primary Serum	Clone	Туре	Source	Titer	Antigen Retrieval
Cyclin A		Monoclonal	NEOMARKERS	1:200	CITRATE pH 6.0
Cyclin B1	V152	Monoclonal	DAKO	1:1500	EDTA+TRIS pH 9.0
Cyclin D1	RBT-14	Monoclonal	BIO-SB	Pre-diluted	EDTA+TRIS PH 9.0
p16	F12	Policlonal	SANTA CRUZ	1:400	CITRATE pH 6.0
p21	SX118	Policlonal	DAKO	1:50	CITRATE pH 6.0
p27	SX53G8	Monoclonal	DAKO	1:200	CITRATE pH 6.0
p53	DO-7	Monoclonal	DAKO	1:2000	CITRATE pH 6.0
Topoisomerase 2A	SWT-3D1	Monoclonal	DAKO	1:800	CITRATE pH 6.0
KI-67	MIB-1	Monoclonal	DAKO	1:300	CITRATE pH 6.0
Rb	NCL-RB358	Monoclonal	NOVOCASTRA	1:300	CITRATE pH 6.0

 Table 2: Primary serum, clones, source, titer and antigen retrieval.

		Clinical	stage	Histological grade		Lymph node status		Vascular embolization		Perineural infiltration		Recurrence	
		I — II	III – IV	Well differentiated	Poor/moderated differentiated	Negative	Positive	No	Yes	No	Yes	No	Yes
Cyclin A	N	30	84	88	24	48	55	58	44	55	46	75	39
	Mean	8.49	9.53	8.62	11.18	9.50	10	9.25	9.82	8.88	10	9.99	7.84
	SD	10.54	9.77	9.42	11.20	11.47	9.26	10.86	9.83	10.15	10.51	11.1	7.13
	P value	0.376		0.311		0.349		0.543		0.551		0.788	
Cyclin B1	N	34	99	103	27	56	65	69	50	64	54	87	46
	Mean	12.61	14.16	13.01	16.73	15.23	13.20	12.72	15.32	14.67	12.70	14.51	12.37
	SD	14.16	14.17	13.63	16.16	14.99	13.94	12.83	15.68	14.98	12.90	14.36	13.74
	P value	0.517		0.287		0.533		0.626		0.540		0.224	
Cyclin D1	N	34	99	104	26	56	65	68	51	64	54	87	46
	Mean	33.60	40.34	37.77	42.66	33.96	44.63	38.18	41.05	38.49	40.28	38.42	38.98
	SD	20.76	20.55	21.01	17.97	20.47	20.06	21.69	18.90	20.76	19.98	19.39	23.29
	P value	0.084		0.264		0.006		0.298		0.534		0.876	
Topo IIA	N	34	99	103	27	57	64	68	51	64	54	87	46
	Mean	23.13	25.16	23.23	28.92	25.27	24.29	26.21	24.60	26.51	24.37	24.40	25.11
	SD	17.77	16.80	16.36	18.52	16.87	17.38	16.89	17.14	16.69	17.04	17.48	16.26
	P value	0.456		0.163		0.693		0.562		0.402		0.613	
Ki-67	N	33	99	102	27	56	65	69	50	64	54	86	46
	Mean	33.70	31.61	31.88	32.80	33.23	31.37	33.34	31.64	33.71	31.02	32.09	32.22
	SD	14.71	15.17	13.58	18.46	14.73	15.66	16.47	13.75	14.49	16.33	14.22	16.60
	P value	0.457		0.806		0.420		0.919		0.265		0.956	
P16	N	35	99	104	27	57	65	69	51	65	54	88	46
	Mean	41.67	42.19	41.43	41.49	43.96	40.20	41.20	43.03	43.33	40.98	44.16	38.02
	SD	23.77	23.10	23.55	21.44	25.24	22.27	23.58	23.84	24.40	22.40	23.01	23.24
	P value	0.925		0.918		0.382		0.667		0.575		0.185	
P21	Ν	28	87	90	23	47	58	57	45	57	45	75	40
	Mean	15.64	11.90	12.37	15.29	13.34	12.21	10.90	14.03	12.83	11.32	13.93	10.71
	SD	12.37	10.32	10.94	10.85	11.20	10.79	9.96	10.94	11.30	9.48	11.13	10.32
	P value	0.142		0.189		0.668		0.129		0.639		0.124	
P27	Ν	29	87	89	26	47	59	57	46	57	45	77	39
	Mean	31.48	28.34	28.41	31.91	30.04	28.03	31.13	26.41	30.25	28.26	31.12	25.19
	SD	17.70	19.22	18.70	19.67	18.00	19.46	17.03	20.24	18.75	18.10	19.35	17.31
	P value	0.321		0.389		0.474		0.108		0.652		0.124	
P53	Ν	30	80	90	26	48	60	58	47	58	46	77	41
	Mean	49.00	46.28	45.47	52.27	49.33	48.53	49.87	46.94	46.36	50.24	48.77	43.60
	SD	33.50	32.57	33.86	27.07	33.35	32.74	31.11	34.47	32.28	33.69	32.27	33.58
	P value	0.462		0.389		0.961		0.709		0.596		0.432	
Rb	Ν	31	95	98	26	53	62	65	49	65	48	82	44
	Mean	42.52	52.42	52.81	44.47	49.37	52.54	53.26	50.58	52.92	50.48	49.60	52.80
	SD	25.94	24.35	24.17	25.87	25.26	24.28	25.64	23.80	24.39	25.27	23.91	26.61
	P value	0.269		0.127		0.596		0.593		0.697		0.471	

SD:standard deviation

Table 3: Correlation between the expression of cell cycle proteins and clinicopathological parameters in patients with OSCC by IHC.

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Markers	Expression	Cutoff value	Five-year Overall survival (%)	P (log rank)	Ten-year Overall survival (%)	P (log rank)
Rb	Downregulation Overexpression	> 54.4	64.7 47.5	0.057	54.9 33.2	0.027
Cyclin D1	Downregulation Overexpression	> 37.7	66.3 48.1	0.049	58.1 35.6	0.023
Cyclin B1	Downregulation Overexpression	≤8.6	53.3 61.7	0.296	40.0 53.0	0.202
Cyclin A	Downregulation Overexpression	> 3.4	69.6 49.7	0.047	54.8 38.3	0.041
Ki-67	Downregulation Overexpression	> 28.1	71.3 46.5	0.010	54.5 40.0	0.045
Торо 2А	Downregulation Overexpression	> 34.2	64.5 43.4	0.026	51.8 37.2	0.081
p21	Downregulation Overexpression	≤18.4	48.0 77.1	0.013	39.1 69.0	0.013
p53	Dowregulation Overexpression	> 77.2	60.6 47.8	0.203	46.6 47.8	0.592
p27	Downregulation Overexpression	≤8.6	31.3 61.0	0.002	23.4 50.5	0.004
p16	Downregulation Overexpression	≤41.3	51.2 64.8	0.137	40.0 55.5	0.121

Table 4: Cutoff determined by ROC analyses and comparison of five and ten-year overall survival in oral squamous cell carcinoma.

Variable	Category	n	HR	95% CI (HR)	P value
Lymph node metastasis	No	43	1.0	Reference	-
	Yes	52	4.2	2.1 – 8.3	< 0.001
Cyclin A	Downregulation	32	1.0	Reference	-
	Overexpression (> 3.4)	63	2.3	1.2 – 4.4	0.017
p 21	Downregulation	70	2.4	1.1 – 5.3	0.037
	Overexpression (> 18.4)	25	1.0	Reference	-
p 27	Downregulation	13	2.8	1.3 – 5.8	0.007
	Overexpression (> 8.6)	82	1.0	Reference	-
Treatment	Surgery	35	1.0	Reference	-
	Surgery + radiotherapy	60	0.8	0.4 – 1.6	0.541
Age	(continuos variable)	95	0.99	0.96 - 1.02	0.544

Table 5: Risk factors for death according to Cox model (final model adjusted for treatment and age).

brightfield microscope with image and proprietary processing analysis software for evaluating cells or tissue sections on glass microscope slides that are stained by IHC to detect, count, and classify cells based on levels of hue (color), saturation (density), and luminosity (darkness). This signal is converted into a numerical continuous density measurement.

Using ACIS III software, threshold values for each parameter were optimized to allow color intensity-based spectral resolution via a colorspace transformation. The areas that were analyzed within each core section were manually selected using the ACIS III region selection tools. For each case, a final numerical value, corresponding to the mean value of the 4 cores analyzed was generated.

Statistical analysis

Comparisons of mean values of protein expressions according to the clinicopathological parameters were evaluated by Mann-Whitney test.

Because there are no standard cutoff values for biomarkers evaluated in this study, in our survival rate analysis, the optimal cutoff points were defined using a nonparametric receiver operating characteristic (ROC) analyses. According to these cutoff values, the 5-year and 10-year overall survival rates were analyzed by Kaplan-Meier and the curves were compared by log rank test. The multivariate analysis was performed by using the Cox proportional hazard model.

The correlation between the expression of cyclins, CKIs, p53,

J Mol Biomark Diagn ISSN:2155-9929 JMBD an open access journal Rb, topoisomerase IIA, and Ki-67 was examined using Spearman's correlation coefficient.

All p-values were based on two-tailed statistical analysis and p<0.05 indicated statistical significance. Statistical analysis was performed using the SPSS 17.0 statistical package (SPSS, Inc, Chicago, IL).

Results

Cyclin B1, cyclin A, p16, p21, p27, p53, topoisomerase IIA, Ki-67, and Rb were detected in the nuclei. Figure 1 shows representative immunohistochemical stains of these molecules in OSCC samples. Although cyclin B1 and D1 stained primarily in the nucleus, cytoplasmic staining was also observed sporadically in OSCC samples (Figure 1). In the statistical analysis, only nuclear measurements were considered.

The expression of cell cycle proteins and their association with clinicopathological parameters by Mann-Whitney test are shown in (Table 3). Cyclin B1, cyclin A, p16, p21, p27, p53, Rb, Ki-67, and topoisomerase IIA were not associated with the clinicopathological parameters that were studied (clinical stage, histological grade, perineural invasion, vascular embolization, and lymph nodes status).

Cyclin D1 expression and lymph node metastasis associated significantly. Cyclin D1-positive cells were more frequent in tumors with lymph node metastases than in tumors without lymph node metastases (p=0,006). Other parameters were not associated with expression cyclin D1 protein.

P=0.045 P=0.041 0.8 Cumulative survival Ki-67 downresulated yclin A downregula Cumulative survival 0,6 0.6 Ki-67 positive Cyclin Apositive 0.2 0.1 20.0 Follow-up period (120 months) Follow-up period (120 months) 1,0 P=0.027 P=0.023 0, Cyclin D1 downregulated Rb downregulated Cumulative survival Cumulative survival 0,4 Rb positive Cyclin D1 positive 0. 0. 80.08 80.00 100.00 Follow-up period (120 months) Follow-up period (120 months) P=0.026 Topoisomerase downregulated

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Figure 2: Kaplan-Meier curves showing 10-year overall survival in patients with OSCC, comparing the cutoff points of A: cyclin A, B: cyclin D1, C: Ki-67, D: Rb, E: topoisomerse II A.

50.00

Topoisomerase positive

100.00

Follow-up period (60 months)

150.00

200.00

Cumulative survival

0.00

To assess whether protein expression levels correlated with survival rates, cutoff points was established for all markers by ROC curve [23] (Table 4). The 5-year overall survival rate was significantly lower in patients who overexpressed positive cell cycle regulators than in low expression-cyclin A (49.72% versus 69.6%, p=0.047), cyclin D1 (48.1% versus 66.3%, p=0.049), Ki-67 (46.5% versus 71.3%, p=0.010), Rb (47.5 versus 64.7%, p=0.057) and topoisomerase IIA (43.4% versus 64.5%, p=0.026) (Table 4). Similar results were observed with regard to the 10-year overall survival rate for cyclin A (38.3% versus 54.8%, p=0.041), cyclin D1 (35.6% versus 58.1%, p=0.023), Ki-67 (40.0% versus 54.5%, p=0.045), and Rb (33.3% versus 54.9%, p=0.027) (Figures 2A, 2B, 2C, and 2D) (Table 4).

In contrast, patients who overexpressed the cell cycle inhibitors p21 and p27 had higher 5-year and 10-year overall survival rates compared with patients in whom they were downregulated-p21 (5-year, 77.1%

versus 48%, p=0.013; 10-year, 69.0% versus 39.1%, p=0.013); for p27 (5-year, 61.0% versus 51.2%, p=0.002; 10-year, 50.5% versus 23.4%, p=0.004) (Figure 3A, 3B). No significant correlations were observed between cyclin B1, p53 and p16 and overall survival rate in OSCC patients (Table 4).

By univariate analysis, we observed a significant correlation between 10-year overall survivals and clinical stage (P=0.002), lymph node metastasis (P<0.001), treatment (P=0.041), and vascular embolization (P=0.001). Further, our multivariate analysis, based on a Cox proportional hazard model, demonstrated that lymph node metastasis, cyclin A, p21, and p27, adjusted for treatment and age, were independent prognostic factors of overall survival in OSCC patients (Table 5).

Figure 4 shows the expression profile of the cell cycle molecules that we evaluated, categorized by cutoff of the ROC curve.

P=0.013 p21positive 18 Cumulative survival p21 downregulated 0.0 0.00 20.00 40.00 60.00 80.00 100.00 120.00 Follow-up period (120 months) P=0.004 0.8 P27 positive Cumulative survival P27 downregulated 0.3 0.0 80.00 0,00 20,00 40,00 60,00 100.00 120.00 Follow-up period (120 months)





Cellular proliferation was assessed based on Ki-67 expression; we observed a positive correlation between Ki-67 expression and cyclin A (rho=0.392, p<0.001), cyclin B1 (rho=0.304, p<0.001), cyclin D1 (rho=0.202, p=0.021), topoisomerase IIA (rho=0.600, p<0.001), Rb (rho=0.453, p<0.001), and p53 (rho=0.190, p=0.042).

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Discussion

Aberrant expression of cell cycle proteins has been reported in several cancers, including oral carcinomas. We examined the expression of cell cycle proteins (cyclin D1, cyclin B1, cyclin A, p16, p21, p27, p53, and Rb) and the proliferation markers Ki-67 and topoisomerase IIA by IHC in 136 OSCC patients in a single TMA to determine their prognostic value.

The study population was primarily male (n=108, 79%), which could be attributed to the higher consumption of alcohol and tobacco by men versus women. In our group, 94% and 91% of men reported use of tobacco and alcohol, respectively. Our sample was also characterized by a high number of well-differentiated tumors, showing lymph node metastasis and high clinical stage. Our institution is a reference center for cancer, and generally, due to the precariousness of the Brazilian public health service system, patients are referred to such centers after the disease has advanced.

Gene rearrangements and amplification of cyclin D1, which can lead to its overexpression, have been reported in many human neoplasms, including breast [24], hepatocellular [25], colon [26], and oral squamous cell carcinoma [6,14]. Overexpression of cyclin D1 leads to the propagation of unrepaired DNA damage, accumulation of genetic errors, and abnormalities in many tumors, conferring a selective proliferative advantage to altered cells [9,27]. Shintani et al. [18] suggest that cyclin D1 regulates the development of OSCC by endowing severe epithelial dysplasia with a growth advantage.

Cyclin D1 overexpression is associated with a poor prognosis. Our results are consistent with studies that have linked cyclin D1 with an increased risk of lymph node metastasis in oral cancer [12,14,24,28,29]. In contrast, others studies have failed to observe a correlation between cyclin D1 and clinicopathological parameters or survival rates in OSCC patients [17,20].

In this study, we noted an association between cyclin D1 expression and poor prognosis in OSCC. Overexpression of cyclin D1 was associated with lymph node metastases, which can be attributed to evidence that alterations in cyclin D1 levels are related to intense proliferation and increased invasiveness. We also observed a positive correlation between the expression of cyclin D1 and Ki-67, suggesting that increased proliferative activity increases cyclin D1 expression. Our study also demonstrated reduced overall survival of patients who overexpressed cyclin D1, which is consistent with previous findings, in which expression of this marker was linked to reduced disease-free survival and overall survival in OSCC patients [30-34]. However, in our study, cyclin D1 was not an independent prognostic factor in OSCC by multivariate analysis.

Cyclin A and cyclin B1 regulate the G2-M transition of the cell cycle. Some groups have claimed that they are useful markers of proliferation and potential indicators of poor clinical outcomes in oral carcinoma patients [15,22,35-37].

In our study, we did not observe any association between cyclin A and cyclin B1 expression with clinicopathological parameters in OSCC. Consistent with our result, other groups noted no linked between

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Overexpression

cyclin A and clinicopathological parameters in oral or head and neck carcinoma [21,38]. Watanabe et al. [39] correlated tumor differentiation with cyclin B1 expression but not with other clinicopathological features or overall survival.

In contrast, we observed an association between cyclin A overexpression and poor survival by multivariate analysis, which implicated as an independent prognostic factor in OSCC patients. High cyclin A expression has also been linked to short survival rates by Chen et al. [13], but in contrast to our study, they noted an association with clinical and pathological parameters in OSCC. We found that the expression of cyclins A and B1 correlated positively with Ki-67 expression, consistent with others reports [11,36,39]. These data suggest that increased cyclin A and B expression reflects increased tumor proliferation.

The CKIs p21, p27, and p16 control the cell cycle directly. Reduced p21 expression has been linked to a poor prognosis in OSCC [40,41]. Downregulation of p27 is associated with early development and invasiveness of cancer in OSCC [18,42-44].

In this study, p21 or p27 expression was not associated with clinical or pathological parameters. However, we observed that their downregulation indicated reduced overall survival and, therefore, a poor prognosis. Our multivariate analysis suggested that p21 and p27 are independent prognostic factors in OSCC patients. Several studies have demonstrated an association between the loss of p21 or p27 expression and decreased survival rate [41,45,46], suggesting that the loss of CKIs, p21, and p27 influence the critical transition from G1 to S phase. In contrast, other groups did not observe a significant relationship between p27 or p21 expression and survival rates [19,47,48].

Our results demonstrate that p16 expression is not associated with prognosis in OSCC, similar to Pande et al. [47] and Goto et al. [40]. However, other studies have linked the loss of p16 function to poor prognosis [16,17,31].

p53 ensures genomic integrity by regulating cell cycle arrest and apoptosis. Alterations in its expression have been well described in many human cancers, including OSCC [16,17,49-51]. However, its prognostic value is unknown. Some studies have reported that the loss of p53 function is significantly associated with reduced overall survival (16, 52), tumor stage, and metastasis to lymph node [17,53,54], whereas other studies failed to demonstrated the prognostic significance of p53 in head and neck tumors [41,55,56].

In our study, p53 expression did not influence the prognosis of patients with oral cancer, but we observed a positive correlation between p53 and Ki-67 expression, suggesting that p53 expression regulates intense proliferation. Lavertu et al. [57] noted that coexpression of p53 and Ki-67 was associated with lower disease-free and overall survival. Balz et al. [58] reported that most cases of head and neck cancers lacked normal p53 function, concluding that inactivation of p53 *per se* is not a suitable prognostic or molecular marker.

Several studies have shown that alterations in Rb regulate oral carcinogenesis; however, the prognostic significance of these alterations is unknown. Many groups have observed that loss of Rb or mutation in Rb is frequent in oral cancer [52,59]. Yet, increased Rb expression by IHC has been seen in OSCC [60,61], although its prognostic value in these cancers has not been demonstrated [17,62].

Our results show that Rb overexpression is associated with reduced overall survival rate, although it was not an independent prognostic factor in OSCC by multivariate analysis. In addition, increased levels of Rb correlated with Ki-67 expression. Increased Rb levels in these cases can be associated with greater protein phosphorylation recognized by antibody used in IHC assays. Phosphorylation of Rb during G1 phase permits the transition from G1 to S phase. Rb overexpression has been shown to block p53-mediated apoptosis [60]. We made an attempt to correlate Rb and p53 with survival; however, no correlation in 5-year and 10-year overall survival rate was observed (data not show).

Ki-67 and topoisomerase IIA are proliferation markers that can be used to assess the percentage of cycling cells in tumor tissues. Many studies have shown the importance of analyzing proliferation rates as a prognostic factor in head and neck carcinomas [12,17,63-66]. Their expression was examined to determine their prognostic significance in OSCC. We observed an association between Ki-67 and topoisomerase IIA overexpression and poor overall survival. Thus, our results suggest that tumor cell proliferation rates influence prognosis.

A general overview of the expression profile of the cell cycle molecules that we evaluated, categorized by cutoff of the ROC curve is observed in Figure 4. In it, we demonstrate that cell cycle positive regulator proteins showed overexpression in the majority of cases. On the other hand, negative regulator proteins, in its great majority, were downregulated. Taken together, these data suggest an unbalance of cell cycle control in OSCC.

Our results contradict those of other studies, perhaps due to the selection of different IHC antibodies, the quality of the specimen (frozen or paraffin-embedded), and the choice of arbitrary cutoff values for positive immunoreactivity. In this study, the cutoff point of numerical variables was determined by ROC (Receiver Operating Characteristic) curve analysis, which graphically represents the true positive rate according to the rate of false positives in different cutoff points of a numerical variable. It allows the definition of a most suitable cutoff point of a variable showing numerical distribution according to a dichotomous variable [23].

Notably, we based our study on a TMA, enabling us to analyze many tumor-associated proteins in a large number of samples by IHC and providing experimental uniformity, improving the quality of our statistical analysis. A critical concern regarding the TMA is whether the small tissue samples of heterogeneous tumors are representative of the donor tumor. To overcome these limitations, many cores were evaluated. Further, the objective measurements by automated cellular imaging systems define better standards for IHC analysis, due to the higher accuracy and sensitivity and better reproducibility of the data and quantification, preventing subjective interpretations. Our sample was relatively large, and all patients were treated in the same institution. Notably, all paraffin blocks were prepared under the same conditions, and the IHC assays were performed using standard protocols.

In summary, we have demonstrated that the expression of the cell cycle proteins cyclin A, B1, D1, Rb, p53 and topoisomerase IIA correlate with increased Ki-67 levels, suggesting that they exist in proliferating cells and regulate OSCC carcinogenesis. Although our results also show that overexpression of cyclin A, cyclin D1, topoisomerase IIA and Rb and downregulation of p21 and p27 are associated with reductions in survival rates of OSCC patients, only cyclin A, p21 and p27, were implicated an independent prognostic factor, by multivariate analysis, in OSCC patients Additionally, cyclin D1 is linked to aggressive tumors. We propose that cyclin A, cyclin D1, p21 and p27 expression can be valuable markers of poor prognosis and tumor aggressiveness in OSCC.

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