Keratins 17 and 19 expression as prognostic markers in oral squamous cell carcinoma

B.A. Coelho[1], G.T. Peterle[2], M. Santos[3], L.P. Agostini[2], L.L. Maia[2], E. Stur[2], C.V.M. Silva[2], S.O. Mendes[2], C.C.J. Almança[2], F.V. Freitas[2], A.R. Borçoi[2], A.B. Archanjo[2], A.M.C. Mercante[1], F.D. Nunes[4], M.B. Carvalho[1], E.H. Tajara[5], I.D. Louro[2] and A.M.A Silva-Conforti[2,6]

[1]Laboratório de Biologia Molecular, Hospital Heliópolis, São Paulo, SP, Brasil
[2]Programa de Pós-Graduação em Biotecnologia,
Universidade Federal do Espírito Santo, Vitória, ES, Brasil
[3]Escola Multicampi de Ciências Médicas do Rio Grande do Norte,
Universidade Federal do Rio Grande do Norte, Caicó, RN, Brasil
[4]Departamento de Patologia Bucal, Faculdade de Odontologia,
Universidade de São Paulo, São Paulo, SP, Brasil
[5]Departamento de Biologia Molecular, Faculdade de Medicina,
São José do Rio Preto, SP, Brasil
[6]Departamento de Biologia, Universidade Federal do Espírito Santo,
Alegre, ES, Brasil

Corresponding author: A.M.A. da Silva-Conforti
E-mail: adriana.biomol@gmail.com

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ABSTRACT. Five-year survival rates for oral squamous cell carcinoma (OSCC) are 30% and the mortality rate is 50%. Immunohistochemistry panels are used to evaluate proliferation, vascularization, apoptosis, HPV infection, and keratin expression, which are important markers of malignant progression. Keratins are a family of intermediate filaments predominantly expressed in epithelial cells and have an essential role in mechanical support and cytoskeleton formation, which is essential for the structural integrity and stability of the cell. In this study, we analyzed the

expressions of keratins 17 and 19 (K17 and K19) by immunohistochemistry in tumoral and non-tumoral tissues from patients with OSCC. The results show that expression of these keratins is higher in tumor tissues compared to non-tumor tissues. Positive K17 expression correlates with lymph node metastasis and multivariate analysis confirmed this relationship, revealing a 6-fold increase in lymph node metastasis when K17 is expressed. We observed a correlation between K17 expression with disease-free survival and disease-specific death in patients who received surgery and radiotherapy. Multivariate analysis revealed that low expression of K17 was an independent marker for early disease relapse and disease-specific death in patients treated with surgery and radiotherapy, with an approximately 4-fold increased risk when compared to high K17 expression. Our results suggest a potential role for K17 and K19 expression profiles as tumor prognostic markers in OSCC patients.

Key words: Keratin 17; Keratin 19; Oral squamous cell carcinoma; Prognostic marker

INTRODUCTION

Head and neck cancer (HNC) is the sixth most common type of cancer worldwide and is associated with high mortality and morbidity rates. The latest world estimation showed an annual incidence of 600,000 new cases and 300,000 deaths due to this disease. Head and neck squamous cell carcinoma (HNSCC) shows a 5-year survival rate of 30% and a 50% mortality rate (Saman, 2012). The most important prognostic factor for HNC is the presence of lymph node metastasis, which results in a 50% reduction in survival rates. Primary etiological factors for HNSCC are alcohol and tobacco consumption (Ferlay et al., 2010). Currently, there is a need for the identification of biomarkers of HNC so that clinicians can prescribe the best treatment options. Although several molecular changes in HNC have been described in the literature, immunohistochemistry (IHC) is currently the only technique in use to choose treatment alternatives (Pradal et al., 1999). IHC panels are used to evaluate proliferation (Ki-67), vascularization (CD34), p53, and bcl-2 expression (Spafford et al., 1996), and human papillomavirus (HPV) infection (de Souza et al., 2013). Recently, keratin expression has also been used to evaluate malignant progression (Polachini et al., 2012).

Keratins (K) are a family of intermediate filament proteins predominantly expressed in epithelial cells and have a basic structural function in forming the cytoskeleton, which is essential for cell structure integrity and stability (Flitney et al., 2009). Keratins are also involved in protein synthesis and epithelial cell growth (Kim et al., 2006), signaling (Alam et al., 2011), organelle transport (Planko et al., 2007), and cell mobility and proliferation (Chung et al., 2012). There are 54 genes that encode type I (acidic) and type II (basic) keratins, which form heterodimers and show tissue-specific expression (Flitney et al., 2009). Keratins 17 and 19 (K17 and K19) are type I keratins and their varying expression patterns have been implicated in cancer. Prognostic associations between K17 and K19 have been made for gastric adenocarcinoma, breast cancer, intraepithelial cervical cancer, ovarian cancer, hepatocellular carcinoma, and lung, larynx, thyroid,and oral cancer (Cohen-Kerem et al., 2004; Ikeda et al., 2008; Safadi et al., 2010; Ide et al., 2012; Kitamura et al., 2012; Alshareeda et al., 2013; Bugalho et al., 2013; Kaczka et al., 2013; Lee et al., 2013; Wang et

al., 2013). In the present study, we evaluated the differential expression of K17 and K19 in tumoral and non-tumoral tissue. In addition, we have examined the correlation between K17 and K19 protein expression with clinicopathological features and prognosis of patients with oral squamous cell carcinoma (OSCC).

MATERIAL AND METHODS

Patient samples

Samples were collected by the Head and Neck Genome Project (GENCAPO), a collaborative consortium created in 2002 by more than 50 researchers in Brazil. In this study, 67 tumoral tissue samples and 67 non-tumoral surgical margin tissues were obtained and used for immunohistochemical analysis of K17 and K19. These patients were surgically treated at the Head and Neck Surgery Department of the Heliópolis Hospital (São Paulo, Brazil) during the period January 2002 to December 2008. The clinical follow-up was at least 24 months after surgery. Previous surgical or chemotherapy treatment, distant metastasis, no removal of cervical lymph nodes, and positive surgical margins were criteria for exclusion. Histopathological slides were reviewed by a senior pathologist to confirm the diagnosis and select appropriate areas for immunohistochemical analysis. Tumors were classified according to the TNM system (Sobin et al., 2009). Among the analyzed individuals, the mean age was 55.0 years (degree of freedom was ± 10.8) and consisted of 57 men (85.1%) and 10 women (14.9%). This study was approved by the Research Ethics Committee of the Heliopolis Hospital (Brazil) on December 14, 2007 (CEP #446) and informed consent was obtained from all patients.

Tissue microarray

Formalin-fixed, paraffin-embedded tissue sections of 67 primary OSCCs treated at the Head and Neck Surgery Department of Heliópolis Hospital (São Paulo, Brazil) were used for IHC analysis. Histological characterization of all samples was done by hematoxylin and eosin staining, followed by IHC analysis of tissue microarrays (TMA). Two 1mm cylinders were used to represent each sample in the TMA slide (Beecher Instruments, Silver Spring, MD, USA).

Immunohistochemistry

Anti-K17 monoclonal antibody and anti-K19 monoclonal antibody (Santa Cruz Biotechnology, USA) were used in the IHC reaction, at a 1:40 and 1:100 dilution, respectively (Rimm et al., 2001; Hsu et al., 2002). Positive and negative controls were used. Sample scoring was performed by semi-quantitative microscopic analysis, considering the number of stained cells and signal intensity. Two spots were evaluated for each sample and a mean score was calculated. Considering the percentage of immune-positive tumor cells, a score of 1 was given when ≤10% of cells were positive, 2 when 10-50% of cells were positive, and 3 when ≥50% of cells were positive. Signal intensity was scored as negative (0), weak (1), moderate (2) or strong (3). Both scores were multiplied (Soini et al., 2000; Campos et al., 2009) and the resulting score was used to categorize K17 and K19 expression as negative (<3), positive weak (3-6) or positive strong (>6).

Statistical analysis

The chi square and Fisher exact tests were used for association analysis and confirmation was obtained by the Lilliefors test (significance considered when $P < 0.05$). Multivariate logistic regression was used to obtain the odds ratio (OR) and confidence intervals (CI 95%). Survival was calculated by the number of months between surgery and death for each patient or the last follow-up appointment if the patient was alive. In order to calculate disease-free survival, the endpoint time was the date of disease relapse. The Kaplan-Meier model was used for survival analysis, using the Wilcoxon P value and the Cox Proportional Hazards to adjust P values and obtain hazard ratios (HR). Statistical calculations were performed using the Epi Info v3.4.3, 2007 and Statsoft Statistica v7.0.61.0 software.

RESULTS

Positive K17 expression

Positive K17 expression was examined in 67 tumors, of which 53 were positive (79.1%) and 14 were negative (20.9%). In non-tumoral surgical margin tissues, K17 expression was positive in 4 (6.0%) and negative in 63 (94.0%) of the samples analyzed. The K17 expression was different between tumoral and non-tumoral samples ($P < 0.001$; Table 1). Positive K17 expression did not significantly correlate with tumor characteristics such as size ($P = 0.670$) or differentiation grade ($P = 0.075$), but positive expression was significantly associated with lymph node metastasis ($P = 0.004$; Table 2). Multivariate analysis showed that positive K17 expression was an independent marker for lymph node metastasis (OR = 6.46, CI = 1.45-28.86; Table 3). Positive K17 expression was not significantly associated with disease relapse or disease-specific death ($P = 0.381$ and $P = 0.156$, respectively; Table 2). K17 expression also did not correlate with the prevalence of disease-free or disease-specific survival ($P = 0.984$ and $P = 0.755$, respectively).

Table 1. Analysis of keratins 17 and 19 expression between tumor and non-tumor tissues.

Expression	Tissue analyzed				P
	Non-tumor		Tumor		
	N	(%)	N	(%)	
Keratin 17					
Negative	63	(94.0)	14	(20.9)	<0.001
Positive	4	(6.0)	53	(79.1)	
Keratin 19					
Negative	67	(100.0)	59	(88.1)	0.004
Positive	0	(0.0)	8	(11.9)	
Total	67	(100.0)	67	(100.0)	

K17 expression levels

The K17 expression level was not associated with tumor size ($P = 0.871$), lymph node metastasis ($P = 0.784$), or differentiation grade ($P = 0.191$). Disease relapse and disease-specific death did not correlate with K17 expression level ($P = 0.261$ and $P = 0.201$, respectively; Table 2). The expression level of K17 also did not correlate with disease-free and disease-specific survival ($P = 0.110$ and $P = 0.101$, respectively; Figure 1A and B). However, when cases were stratified by

Table 2. Epidemiological, clinical, and pathological tumor features and their association with K17 and K19 expressions.

Clinical and pathological features	Total		Keratin 17 expression									Keratin 19 expression				
			Negative		Positive		P	Low		High		P	Negative		Positive		P
	N	(%)	N	(%)	N	(%)		N	(%)	N	(%)		N	(%)	N	(%)	
Tumor size (T)[a]																	
T1+T2	28	(41.8)	7	(50.0)	21	(39.6)	0.670	8	(44.4)	13	(37.1)	0.871	24	(40.7)	4	(50.0)	0.830
T3	13	(19.4)	3	(21.4)	10	(18.9)		3	(16.7)	7	(20.0)		12	(20.3)	1	(12.5)	
T4	26	(38.8)	4	(28.6)	22	(41.5)		7	(38.9)	15	(42.9)		23	(39.0)	3	(37.5)	
Lymph node metastasis (N)[b]																	
Negative	30	(44.8)	11	(78.6)	19	(35.8)	0.004	6	(33.3)	13	(37.1)	0.784	26	(44.1)	4	(50.0)	0.520
Positive	37	(55.2)	3	(21.4)	34	(64.2)		12	(66.7)	22	(62.9)		33	(55.9)	4	(50.0)	
Differentiation																	
Well	30	(44.8)	6	(42.9)	24	(45.3)	0.075	11	(61.1)	13	(37.1)	0.191	27	(45.8)	3	(37.5)	0.808
Moderate	32	(47.8)	5	(35.7)	27	(50.9)		7	(38.9)	20	(57.1)		28	(47.5)	4	(50.0)	
Poor	5	(7.5)	3	(21.4)	2	(3.8)		0	(0.0)	2	(5.7)		4	(6.8)	1	(12.5)	
Disease Relapse																	
No	28	(41.8)	7	(50.0)	21	(39.6)	0.381	5	(27.8)	16	(45.7)	0.261	24	(40.7)	4	(50.0)	0.478
Yes	37	(55.2)	6	(42.9)	31	(58.5)		12	(66.7)	19	(54.3)		33	(55.9)	4	(50.0)	
Not available*	2	(3.0)	1	(7.1)	1	(1.9)		1	(5.6)	0	(0.0)		2	(3.4)	0	(0.0)	
Disease-Specific Death																	
No	27	(40.3)	3	(21.4)	24	(45.3)	0.156	10	(55.6)	14	(40.0)	0.201	25	(42.4)	2	(25.0)	0.256
Yes	37	(55.2)	9	(64.3)	28	(52.8)		7	(38.9)	21	(60.0)		31	(52.5)	6	(75.0)	
Not available*	3	(4.5)	2	(14.3)	1	(1.9)		1	(5.6)	0	(0.0)		3	(5.1)	0	(0.0)	
Total	67	(100.0)	14	(20.9)	53	(79.1)		18	(26.9)	35	(52.2)		59	(88.1)	8	(11.9)	

[a,b]TNM classification 7th edition (UICC, 2009). *Not available (not considered in statistical calculations).

Table 3. Multivariate analysis of the relationship between clinical and pathological features and K17 expression with lymphnode metastasis.

Clinical and pathological features	Logistic regression Lymph node metastasis (N)[b]	
	OR (95%CI)[c]	P[d]
Keratin 17 expression		
Negative	1	
Positive	6.46 (1.45-28.86)	0.015
Tumor size (T)[a]		
T1+T2	1	
T3	0.76 (0.18-3.15)	0.701
T4	4.66 (1.30-16.77)	0.019
Lymphatic invasion		
Absent	1	
Present	1.62 (0.49-5.41)	0.433

OR = odds ratio; CI = confidence interval. [a,b]TNM classification 7th edition (UICC, 2009). [c,d]Values adjusted by multivariate logistic regression.

the treatment modality, the level of K17 expression did show significant correlation with disease-free survival and disease-specific death of patients who received surgery and radiation therapy (P = 0.003 and P = 0.002, respectively; Figure 2A and B). This correlation was not seen in patients that only received surgery (P = 0.775 and P = 0.648, respectively; Figure 2C and D). According to a 24 month post-surgical follow-up, approximately 90% of patients who received surgery and radiotherapy accompanied bylow expression of K19 experienced disease relapse, as compared to approximately 30% of disease recurrence in patients treated identically with high expression of K17 (Figure 2A). Additionally, according to a 36 month post-surgical follow-up, approximately 90% of patients who received surgery and radiotherapy with low K17 expression died of cancer, as

compared to 30% of patients who received the same treatment with high expression of K17 (Figure 2B). Multivariate analysis revealed that low expression of K17 was an independent marker for early disease relapse and disease-specific death in patients treated with surgery and radiotherapy, with an approximately 4-fold increased risk when compared to high K17expression (HR = 4.11, CI = 1.17-14.42 and HR = 4.75, CI = 1.33-16.91, respectively; Table 4).

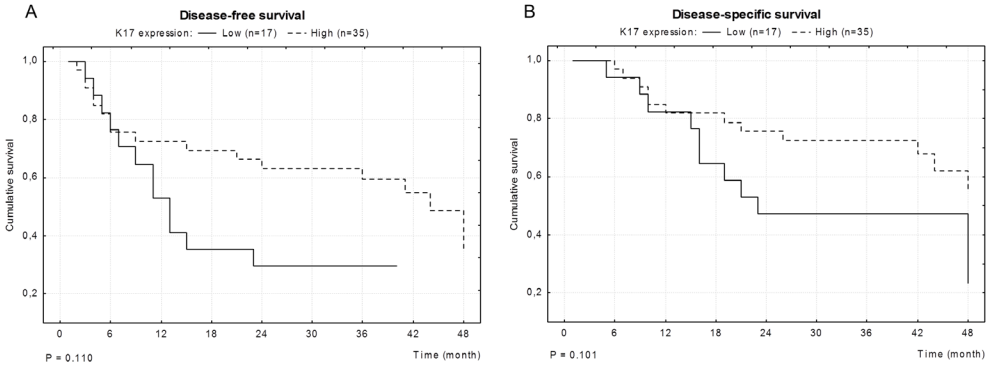

Figure 1. Cumulative survival of patients according to K17 expression level. Kaplan-Meier curves are shown for (**A**) disease-free survival and (**B**) disease-specific survival according to low (solid lines) or high (dashed lines) K17 expression levels.

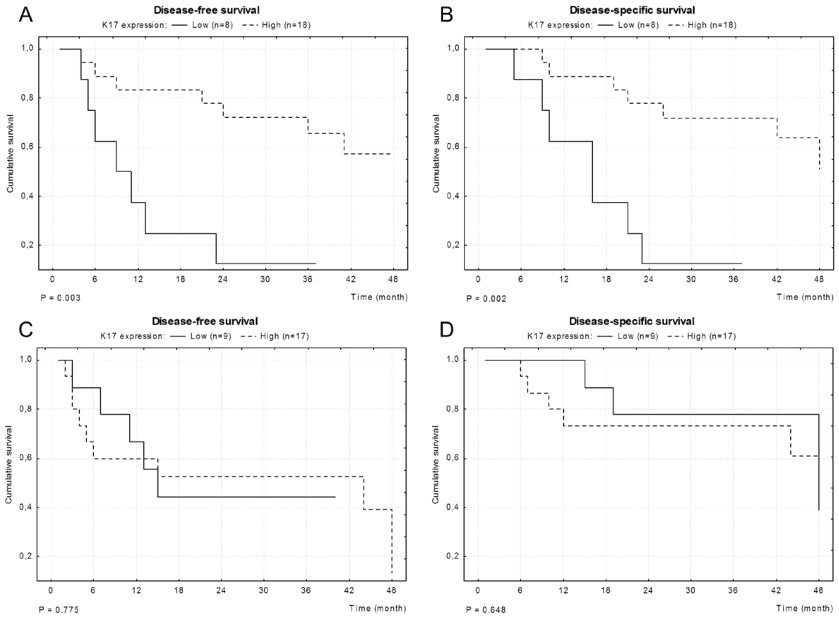

Figure 2. Cumulative survival of patients treated with surgery and/or radiotherapy according to K17 expression level. Kaplan-Meier curves are shown for (**A**) disease-free survival and (**B**) disease-specific survival in patients treated with surgery and radiotherapy according to low (solid lines) or high (dashed lines) K17 expression levels. Kaplan-Meier curves are shown for (**C**) disease-free survival and (**D**) disease-specific survival in patients treated only with surgery according to low (solid lines) or high (dashed lines) K17 expression levels.

Table 4. Multivariate analysis of the relationship between clinical and pathological features and K17 expression with survival of patients treated with surgery and radiotherapy.

Clinical and pathological features	Cox proportional			
	Disease-free survival		Disease-specific survival	
	HR (95%CI)[c]	P[d]	HR (95%CI)[c]	P[d]
Keratin 17 expression				
Positive high	1		1	
Positive low	4.11 (1.17-14.42)	0.027	4.75 (1.33-16.91)	0.016
Tumor size (T)[a]				
T1+T2	1		1	
T3	1.58 (0.29-8.50)	0.594	1.40 (0.26-7.47)	0.693
T4	1.27 (0.37-4.38)	0.708	0.98 (0.28-3.46)	0.979
Lymph node metastasis (N)[b]				
Absent	1		1	
Present	Undefined	0.987	Undefined	0.987

HR = Hazard ratio; CI = confidence interval. [a,b]TNM classification 7th edition (UICC, 2009). [c,d]Values adjusted by Cox proportional Hazard.

K19 expression

In terms of K19 expression, 8 (11.9%) tumors showed positive expression, 59 (88.1%) showed negative expression, and all non-tumoral tissue samples were negative for expression. K19 expression was significantly different between tumoral and non-tumoral samples (P = 0.004; Table 1), and was not related to tumor size (P = 0.830), lymph node metastasis (P = 0.520) or differentiation grade (P = 0.808, Table 2). K19 expression was not significantly associated with disease relapse or disease-specific death (P = 0.478 and P = 0.256, respectively; Table 2) nor did it correlate with disease-free or disease-specific survival (P = 0.681 and P = 0.541, respectively; data not shown).

DISCUSSION

In this study, keratins 17 and 19 expression was analyzed by IHC in tumoral and non-tumoral surgical margins from OSCC patients. Our results show that these keratins are highly expressed in tumoral tissue compared to non-tumoral surgical margins. Other studies have shown the presence of K17 in gastric adenocarcinoma, Ewing sarcoma, OSCC, and ovary, breast, and cervical cancers (Maddox et al., 1999; Ide et al., 2012; Kitamura et al., 2012; Alshareeda et al., 2013; Sankar et al., 2013; Wang et al., 2013). K19 expression was shown in liver, breast, lung, ovary, thyroid and oral cancers (Safadi et al., 2010; Isic Dencic et al., 2013; Kong et al., 2013; Lee et al., 2013; Liu et al., 2013; Park et al., 2013). In a study with 56 OSCC samples and normal mucosal controls, 31 keratins were analyzed by high-density oligonucleotide microarrays (Toyoshima et al., 2008). In this study, increased expression of keratins 17, 19 and 20 was reported in tumor samples. Confirmation of these results was obtained by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (Toyoshima et al., 2009). Higher expression of K17 has also been reported in patients with stage I and II OSCC and in well-differentiated oral cavity carcinoma (Toyoshima et al., 2009; Kitamura et al., 2012).

In the present study, K17 immunopositive expression was associated with the presence of lymph node metastasis. Multivariate analysis confirmed this relationship showing a correlation of K17 expression in OSCC and an augmented risk of lymph node metastasis by approximately

6-fold. Corroborating our result, expression analysis has previously shown a relationship between K17 and lymph node metastasis in OSCC by RT-qPCR (Toyoshima et al., 2008; Toyoshima et al., 2009) and in gastric cancer by IHC (Ide et al., 2012). We have also found that low K17 expression is associated with worse prognosis in patients who received surgery and radiotherapy, which was confirmed by multivariate analysis (increased disease-related death by approximately 4-fold).

Keratin filaments are essential intracellular components that modulate intracellular signaling involved in cell growth and angiogenesis (Xu et al., 2009; Chung et al., 2012). Fibroblast and endothelial cell culture show that some keratins also have non-structural roles in intracellular signaling and vascular growth through angiogenesis (Katagata et al., 2002; Xu et al., 2009). Therefore, it may be possible that a worse radiotherapy response in patients with low K17 expression is due to less oxygen permeating tumor cells and consequently a lower production of oxygen reactive species (ROS), which are necessary for lethal damage after radiotherapy (Karar and Maity, 2009). It has been well-established that hypoxic cells require 2.5- to 3-fold higher doses of radiotherapy to achieve the same apoptotic levels of normoxic cells (Salnikow and Zhitkovich, 2008).

Several cytoskeletal proteins have functions in organelle mobility and specific nuclear events such as transcription, DNA repair and nuclear body formation (Kumeta et al., 2012). Biochemical properties (e.g. solubility) of nuclear keratins are different fromcytoplasmic variants, which may be related to their enzymatic roles (Kumeta et al., 2013). For example,K17 plays a role in Akt/mTOR signaling and cell growth (Kim et al., 2006), which may grant tumor cells higher organellar mobility and favor tumor growth and progression.

In conclusion, we have shown that K17 and K19 are more highly expressed in OSCC tumor tissues in comparison with non-tumoral surgical margins. K17 expression is associated with positive lymph node metastasis and disease-specific survival in patients who received surgery and radiotherapy. Moreover, it may be of particular clinical importance to investigate keratin expression in tumor vasculature and progenitor endothelial cells in an attempt to discover new targets for anti-tumoral drugs.

Conflicts of interest

The authors declare no conflict of interest.

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