DAP1 high expression increases risk of lymph node metastases in squamous cell carcinoma of the oral cavity

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ABSTRACT. Death-associated protein 1 (DAP1) is a member of the DAP family. Its expression is associated with cell growth and normal death of the neoplastic cells, regulated by the mammalian target of the rapamycin protein. Activated DAP1 negatively regulates autophagy, which has been associated with the development and progression of several diseases, such as cancer, and with prognosis and survival of diverse tumor types. Therefore, in this study we analyzed DAP1

expression in 54 oral squamous cell carcinoma tumor samples and in 20 non-tumoral margins by immunohistochemistry. The results showed that DAP1 is more frequently expressed in tumor tissues compared with marginal non-tumoral cells. Additionally, high DAP1 expression is associated with a 4-fold increase in the risk of lymph node metastases. Our results suggest that the DAP1 protein can be used as a potential marker of lymph node metastases predisposition, helping define the best therapy for each patient to minimize risk of developing metastases.

Key words: DAP1 expression; Lymph node; Prognostic marker; Oral cancer

INTRODUCTION

Head and neck squamous cell carcinoma (SCC) is a significant cause of morbidity and mortality worldwide, with approximately 780,000 cases and 391,000 deaths reported every year (Perez-Ordoñez et al., 2006; Heroui et al., 2013).

When diagnosed during the initial stages, this disease has a disease-free life expectancy varying from 60 to 90%. However, when the disease is diagnosed at advanced stages, only 50% of the tumors are operable with a survival rate of 40-50%. Non-operable cases show an even worse prognosis, with survival varying from 10 to 40% over 5 years (AJCC, 2002; Jemal et al., 2007; Adrien et al., 2014).

Molecular tests can provide a better understanding of tumor behavior and improve prognostic predictions, increasing life expectancy in patients with oral SCC to approximately 80% (Forastiere et al., 2001; McCarthy et al., 2004).

Disease aggressiveness is directly related to tumor cell adaptation to harsh microenvironment conditions, and the ability to metastasize (Mawrin et al., 2006; Stadler et al., 2008).

Several proteins have been investigated for their possible association with carcinogenesis and prognosis in SCC. Among these, the death-associated protein (DAP) family is particularly interesting. The DAP family includes DAP1, DAP2 (DAP kinase), DAP3, DAP4, and DAP5, which share pro-apoptotic domains. These proteins are related to cell death mediated by tumor necrosis factor alpha and the Fas ligand (Cohen et al., 1999; Jia et al., 2014; Wazir et al., 2014).

DAP1 protein expression is related to cell growth and death regulation, including in tumor cells, through negative regulation of autophagy (Jia et al., 2014). DAP1 is regulated by the mammalian target of rapamycin (mTOR), being functionally silenced in cells present in nutrient-rich environments by the inhibitory phosphorylation of mTOR on amino acid residues Ser3 and Ser51. During nutrient starvation, mTOR is inactivated, resulting in a rapid reduction of DAP1 phosphorylation, causing its activation and promoting inhibition of autophagy (Koren et al., 2010).

Autophagy is essential for cellular homeostasis, as dysregulation of this process has been associated with several diseases, such as cancer, autoimmune and cardiovascular diseases, neurodegeneration, and cell senescence (Kondo et al., 2005; Koren et al., 2010).

Autophagy dysregulation has been associated with breast (Wazir et al., 2014) and colorectal cancer (Jia et al., 2014), suggesting its role as a molecular marker of tumor suppression, survival, and disease prognosis. For these reasons, autophagy and DAP1 have become important

targets for new cancer therapies (Jia et al., 2014).

In a previous study, our group investigated the expression of DAP1 in more aggressive oral tumors (tumors with premature metastasis; T1/T2, N+), as well as less aggressive tumors (advanced tumors without metastasis; T3/T4, N0) (Severino et al., 2008), through microarray experiments.

Based on these results, the present study aimed to evaluate the association of DAP1 with oral SCC development and prognosis.

MATERIAL AND METHODS

Ethics

This study was approved by the Committee of Ethics in Research of the Heliópolis Hospital (No. 386) and written informed consent was obtained from all patients enrolled.

Samples

Samples were collected by the Head and Neck Genome Project (GENCAPO), a collaborative consortium created in 2002 with more than 50 researchers from nine institutions in São Paulo State, Brazil, with the aim of developing clinical, genetic, and epidemiological analysis of head and neck SCC. For this study, samples from 54 tumors and 20 non-tumoral surgical margins were obtained and used for immunohistochemical analysis of the DAP1 protein. The samples were taken from a total of 54 patients with oral SCC that were receiving surgical treatment at the Head and Neck Surgery Department of Heliópolis Hospital, São Paulo, Brazil, between January 2002 and December 2008. The clinical follow-up lasted for at least 24 months after surgery. Previous surgical or chemotherapeutic treatment, distant metastasis, cervical lymph nodes, and positive surgical margins were exclusion criteria. 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 (7th edn; UICC, 2009).

Among the individuals analyzed, the mean age was 54.6 ± 10.1 years, 47 (87.0%) being men and 7 (13.0%) women (Table 1). With regards to the anatomical tumor sub-sites, 19 (35.2%) were on the tongue, 11 (20.4%) were on the gum, 19 (35.2%) were on the floor of the mouth, and 5 (9.3%) were in the retromolar area.

Tissue microarray

Tissue microarrays were made using buffered, formalin-fixed, paraffin-embedded tissue sections from 54 primary oral and oropharyngeal SCC patients treated at the Head and Neck Surgery Department of Heliópolis Hospital, São Paulo, SP. The sections were used for immunohistochemistry analysis. Histological characterization of all samples was carried out by hematoxylin and eosin staining, followed by immunohistochemistry analysis of tissue microarrays. Two 1-mm cylinders were used to represent each sample in the tissue microarrays slide (Beecher Instruments®, Silver Spring, MD, USA). Tumor histological characteristics were evaluated by regular microscopy.

Epidemiological features	Total	
	No.	(%)
Gender		
Female	7	(13.0)
Male	47	(87.0)
Age (in years)		
Median ± SD	54.6 ± 10.1	
Smoker	38	(70.4)
Alcoholic	32	(59.3)
Tumor sub-sites		
Tongue	19	(35.2)
Gum	11	(20.4)
Floor of mouth	19	(35.2)
Retromolar area	5	(9.3)
Tumor stage		
I, II	16	(29.6)
III	11	(20.4)
IV	27	(50.0)
Total	54	(100.0)

Table 1. Epidemiological features.

Immunohistochemistry

Anti-DAP1 monoclonal antibody (Abcam®) was used in the IHC reaction, at a 1:50 dilution (Rimm et al., 2001; Hedvat et al., 2002; Hsu et al., 2002). Positive and negative controls (absence of primary antibody) were used for reaction quality control. Sample scoring was performed by semi-quantitative microscopic analysis, considering the number of stained tumor cells and signal intensity. Two spots were evaluated for each sample and a mean score was calculated. Considering the percentage of DAP1 immune-positive tumor cells, a score of 1 was given when ≤10% of cells were positive; 2 when 11-50% of cells were positive, and 3 when >50% of cells were positive. The signal intensity was scored as weak 1), moderate 2), and strong 3). Both scores were multiplied (dos Santos et al., 2012) and the resulting score was used to categorize DAP1 expression as negative (0), positive low (1-4), and positive high (≥4).

Statistical analysis

Chi-square and Fisher exact tests were used for association analysis and confirmation was obtained by the Lilliefors test (results were considered to be significant when $P < 0.05$). Multivariate logistic regression was used to calculate odds ratios and 95% confidence intervals. Survival time was considered to be the number of months between surgery and death for each patient, or the last appointment in cases where the patient was still alive. To calculate local disease-free survival, the time endpoint was the date of local disease relapse. The Kaplan-Meier model was used for survival analysis, using the Wilcoxon P value and Cox proportional hazards to adjust P values and obtain hazard ratios. Statistical calculations were performed using the Epi-Info® v3.4.3, 2007 and StatSoft Statistica® v7.0.61.0 softwares.

RESULTS

DAP1 expression was examined in 54 tumors, of which 51 were positive (94.4%)

and only three were negative (5.6%). In non-tumoral surgical margins, DAP1 expression was positive in six (30.0%) and negative in 14 (70.0%). DAP1 expression was different in tumors and non-tumoral samples (P < 0.001; Table 2).

Table 2. Frequency of death-associated protein 1 (DAP1) expression in primary tumor cells and surgical margins.

DAP1 expression	Tumor		Surgical margin		P value
	No.	(%)	No.	(%)	
Negative	3	(5.6)	14	(70.0)	<0.001
Positive	51	(94.4)	6	(30.0)	
Low	17	(31.5)	2	(10.0)	0.661
High	34	(63.0)	4	(20.0)	
Total	54	(100.0)	20	(100.0)	

DAP1 expression did not show a significant association with tumor characteristics such as size (P = 0.699), differentiation grade (P = 0.342), lymphatic invasion (P = 0.192), and perineural invasion (P = 0.842), but the expression was significantly associated with lymph node status (P = 0.029; Table 3). Multivariate analysis showed that high DAP1 expression was an independent marker for lymph node metastases (OR = 4.27, 95%CI = 1.10-16.59; Table 4).

Table 3. Clinical and pathological tumor features and their association with death-associated protein 1 (DAP1) expression.

Features	Total		DAP1 expression				P value
			Low		High		
	No.	(%)	No.	(%)	No.	(%)	
Tumor size (T)[a]							
pT1, pT2	21	(41.2)	7	(41.2)	14	(41.2)	0.699
pT3	9	(17.6)	4	(23.5)	5	(14.7)	
pT4	21	(41.2)	6	(35.3)	15	(44.1)	
Lymph node metastases (N)[a]							
Absent	25	(49.0)	12	(70.6)	13	(38.2)	0.029
Present	26	(51.0)	5	(29.4)	21	(61.8)	
Differentiation							
Good	21	(41.2)	9	(52.9)	12	(35.3)	0.342
Moderate	28	(54.9)	8	(47.1)	20	(58.8)	
Poor	2	(3.9)	0	(0.0)	2	(5.9)	
Lymphatic invasion							
Absent	15	(29.4)	7	(41.2)	8	(23.5)	0.192
Present	36	(70.6)	10	(58.8)	26	(76.5)	
Perineural invasion							
Absent	23	(45.1)	8	(47.1)	15	(44.1)	0.842
Present	28	(54.9)	9	(52.9)	19	(55.9)	
Disease relapse							
No	17	(33.3)	3	(17.6)	14	(41.2)	0.083
Yes	34	(66.7)	14	(82.4)	20	(58.8)	
Local disease relapse							
No	29	(56.9)	6	(35.3)	23	(67.6)	0.027
Yes	22	(43.1)	11	(64.7)	11	(32.4)	
Disease-specific death							
No	20	(39.2)	6	(35.3)	14	(41.2)	0.747
Yes	26	(51.0)	9	(52.9)	17	(50.0)	
Not available[b]	5	(9.8)	2	(11.8)	3	(8.8)	
Total	51	(100.0)	17	(33.3)	34	(66.7)	

[a]TNM classification 7th edn. [b]Not available (not considered in the statistical calculations).

Table 4. Multivariate analysis of the relationship between clinical pathological tumor features and survival with death-associated protein 1 (DAP1) expression.

Features	Multivariate analysis				Cox proportional	
	Lymph node metastases		Local disease relapse		Local disease-free survival	
	OR (95%CI)	P value	OR (95%CI)	P value	HR (95%CI)	P value
DAP1 expression						
Low	1		1		1	
High	4.27 (1.10-16.59)	0.036	0.31 (0.08-1.19)	0.087	0.45 (0.19-1.09)	0.076
Tumor size (T)[a]						
pT1, pT2	1		1		1	
pT3	1.98 (0.36-10.90)	0.432	7.93 (1.13-55.89)	0.038	2.47 (0.86-7.12)	0.094
pT4	5.53 (1.37-22.32)	0.016	1.46 (0.36-5.86)	0.597	1.96 (0.68-5.64)	0.210
Irradiated						
No	-	-	1		1	
Yes	-	-	0.29 (0.08-1.09)	0.068	0.43 (0.17-1.09)	0.077

OR = odds ratio; HR = hazard ratio; CI = confidence interval. [a]TNM classification 7th edn. Values were adjusted by multivariate analysis.

DAP1 expression levels were associated with local disease relapse (P = 0.027; Table 3). However, multivariate analysis did not confirm DAP1 as an independent marker of local disease relapse (OR = 0.31, 95%CI = 0.08-1.19; Table 4). DAP1 expression was not correlated with disease relapse and disease-specific death (P = 0.083 and P = 0.747, respectively; Table 3).

Local disease-free survival was significantly correlated with DAP1 expression (P = 0.040). According to a 12-month post-surgery follow-up, approximately 25% of patients with low DAP1 expression presented local disease relapse, whereas in the same period, 50% of patients with high expression relapsed for local disease (Figure 1A). However, multivariate analysis did not confirm DAP1 as an independent marker of local disease-free survival (HR = 0.45, 95%CI = 0.19-1.09; Table 4). In addition, DAP1 expression was not associated with disease-specific survival (P = 0.546; Figure 1B).

DISCUSSION

Autophagy is a well-conserved system for maintaining cellular homeostasis, and is activated by several factors, such as hypoxia, nutrient and growth factor starvation, oxidative stress, and DNA damage (Jung et al., 2010). It is also the mechanism by which apoptotic cells are removed (Levine et al., 2011; Oh and Lee, 2012).

DAP1-mediated dysregulation of autophagy has been associated with tumoral progression due to accumulation of damaged cells, altered proteins, mitochondrial damage, increased oxidative stress that causes DNA damage, and consequently tumor genetic changes (Karantza-Wadsworth et al., 2007; Eisenberg-Lerner and Kimchi, 2009; Yang et al., 2011).

In the present study, positive DAP1 expression was more frequently observed in tumor samples compared with normal tissue around the tumor. In a colorectal cancer study, DAP1 was underexpressed in tumor cells compared with normal adjacent cells (Jia et al., 2014). Autophagy has been associated with tumor suppression, with a strong influence on tumorigenesis. Therefore, higher DAP1 expression inhibits autophagy and helps tumor development (Mathew et al., 2007; White and DiPaola, 2009).

In addition, our results suggest that strong DAP1 expression is an independent risk

factor for lymph node metastases development, increasing risk by over four times. There is a lack of data in the literature concerning the association of DAP1 expression and lymph node metastases. Autophagy inhibition may increase tumor cell life, augmenting genetic variation and leading to a worse prognosis, as described for breast cancer (Wazir et al., 2014) and colorectal cancer (Jia et al., 2014).
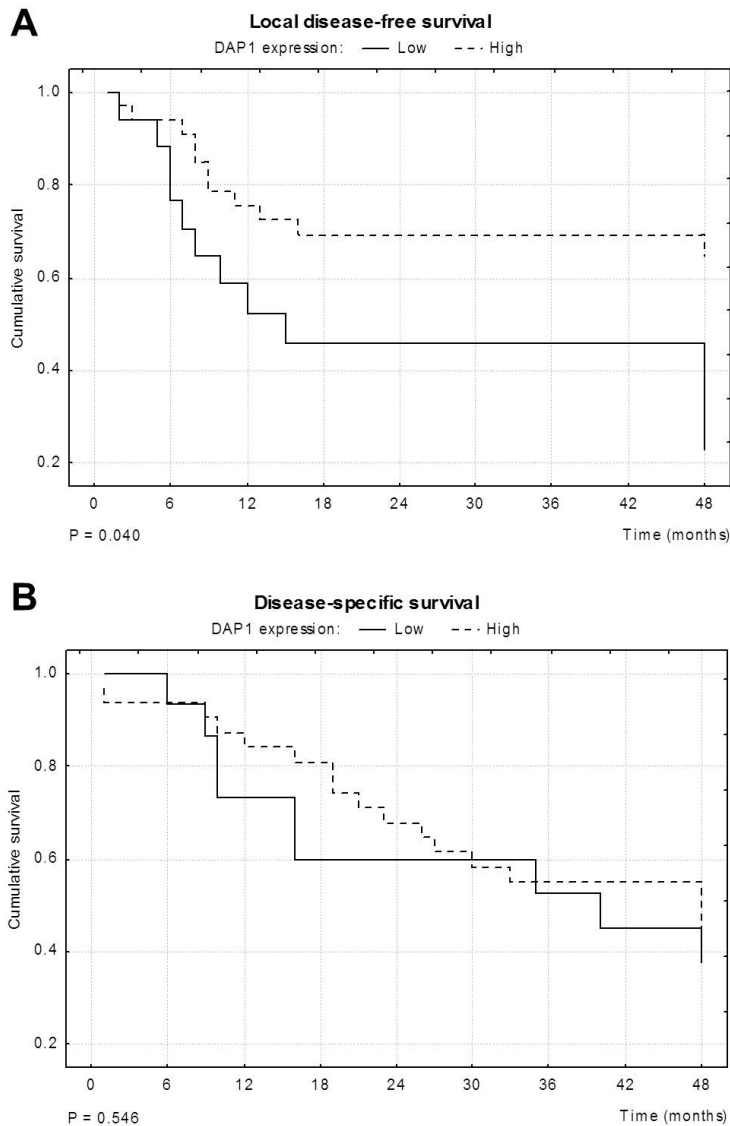
Figure 1. Survival plots. **A.** Local disease-free survival and **B.** disease-specific survival according to positive death-associated protein 1 (DAP1) expression.

Owing to its important role in autophagy, DAP1 has been associated with cell growth and death, as well as tumor development and prognosis (Koren et al., 2010).

In conclusion, our results suggest that DAP1 is a promising marker of lymph node metastases, helping clinicians select patients who are prone to metastasis and determine a more efficient therapy for them.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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