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Death-Associated Protein Kinase is Underexpressed in High-Grade Oral Squamous Cell Carcinoma

La Expresión de la Proteína Quinasa Asociada a Muerte Celular (DAP Quinasa) está Reducida en Carcinoma de Células Escamosas Oral de Alto Grado

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SUMMARY: An immunohistochemical analysis of 40 cases of oral squamous cell carcinoma was performed to evaluate the relationship between the expression pattern of death-associated protein kinase (DAPk) positive cells with the histological malignancy grading of these lesions. According to our results, eleven cases (27.5%) were high-grade malignancy tumours and 29 (72.5%) were low-grade ones. We found that 92.86% of the low-grade tumours were positive to anti-DAP kinase antibody whereas only 7.14% of the high-grade tumours presented positivity, and this difference was statistically significant ($p<0.01$). Sixteen (55.2%) of the low-grade carcinomas exhibited moderate immunoreactivity whereas ten cases (34.5%) showed weak staining and three cases (10.3%) were negative tumours. Immunostaining was lacking in nine (81.8%) of the high-grade carcinomas and "weak" in the two (18.2%) remaining cases. Thus, DAPk expression is significantly decreased in high-grade oral carcinomas, and evidences indicate that it might be related to the severity of cytological atypia.

KEY WORDS: Immunohistochemistry; DAP kinase; Apoptosis; Squamous cell carcinoma.

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common malignant tumour occurring in the oral cavity. In the majority of the cases, it is diagnosed at an advanced stage and consequently present with a poor prognosis (Biazevic *et al.,* 2006; Borges *et al.*, 2008). In the last few years relevant advancements in the diagnosis and treatment of oral cancer has been observed. Nevertheless, despite this recent improvement, there are still many difficulties in evaluating the prognosis of OSCC. Thus, various studies attempting to establish the role of protooncogenes, antioncogenes and apoptosis-regulating genes in the tumoral progression have recently been performed in order to find more significant information to evaluate and predict the biological behavior of this neoplasm (Albuquerque *et al*., 2003).

The Death-associated protein kinase (DAP kinase) is a 160kDa cytoskeletal-associated calcium/calmodulin-dependent serine/threonine kinase which plays an important role in programmed cell death (Chen *et al.*, 2006; Zalckvar *et al*., 2009). Overexpression of DAP kinase has been demonstrated to induce apoptosis in various cell line (Yamamoto *et al*., 2002; Pelled *et al.*, 2002; Pulling *et al.*, 2004). On the other hand, research has demonstrated that DAP kinase protein expression is frequently reduced or absent in tumour cell lines (Raveh & Kimchi, 2001; Bai *et al.,* 2004). In addition, low expression of this protein, particularly in response to hypermethilation, has been associated to high invasiveness or metastatic potential of malignat neoplastic cells (Simpson *et al.*, 2002; Lehmann *et*

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al., 2002; González-Gómez *et al.*, 2003; Voso *et al.*, 2004; Lévy *et al.,* 2004).

There are only few reports looking at the possible role of DAP kinase in the pathogenesis of oral or head and neck cancer (Rosas *et al*., 2001; Ogi *et al*., 2002). Thus the goal of this study is to investigate the relation between the immunohistochemical expression of DAP kinase and the histological grading of malignancy in squamous cell carcinoma of the oral mucosa.

MATERIAL AND METHOD

Forty cases of OSCC were retrieved from the files of the Oral Pathology Service of the School of Dentistry of the University Tiradentes (Aracaju, Brazil). The tumors were graded as high or low score according to the criteria of histological malignancy grading established by Bryne et al. (1989). For immunohistochemical reactions, 3mm thick sections were prepared from formalin-fixed paraffin-embedded surgical specimens. Immunohistochemical staining was carried out using the Labelling Streptoavidin Biotin method (LSAB) (Large Volume Detection System antipolyvalent, Dako, LSAB2 kit peroxidase, Dako Corporation, Capinteria, CA, USA). The sections were deparaffinized in toluene and submitted to antigen recovery by immersion of the slides in citrate buffer, 0.01M, pH 6.0 treated in microwave oven (three cycles for five minutes each at 650W). After rehydratation in phosphate buffered saline (PBS, pH 7.2), the sections were incubated with the primary antibody anti – DAP kinase (clone V55, Abgent, CA, USA, 1:50, 18h), followed by biotinylated antirabbit immunoglobulins (Dakopatts) and finally with streptoavidin – peroxidase conjugate (Detection Kit – peroxidase/DAB, Dakopatts). The last two incubations were performed for 30 min at room temperature. The reaction was revealed with 0.03% diaminobenzidine (Sigma) in buffered solution for 12 min and then the sections were counterstained with Meyer's haematoxilin. Non-specific reactivity of the antibodies was checked by omitting the primary antibody.

Two observers evaluated all slides independently, and the immunostaining pattern of the antibody anti-DAP kinase was analyzed by light microscopy, considering the following parameters: (+++), when more than 50% of the tumour cells were labeled, (++) when 10 to 50% were positive; and (+) when less than 10% of positive cells could be seen. Carcinomas lacking immunostained cells were considered negative (-). After the immunohistochemical analysis, these data were correlated with the malignancy histological grading of the tumours. Significance was statistically determined using c2 statistics. A p-value < 0.05 was regarded as significant.

RESULTS

Morphological analysis showed that eleven cases (27.5%) were high-grade malignancy tumours and twenty-nine (72.5%) were low-grade malignancy tumors. Positive cytoplasmatic immunolabelling could be verified in twenty-eight cases (70.0%). Two (7.14%) of the twenty-eight DAP kinase-positive carcinomas were high-grade tumors whereas twenty-six (92.8%) were low-grade tumours. On the other hands, nine (75%) of the twelve DAP kinase-negative carcinomas were high-grade tumours and three of them low-grade lesions (Fig. 1).

Sixteen (55.2%) of the low-grade carcinomas exhibited moderate immunoreactivity whereas ten cases (34.5%) showed weak staining. Three cases (10.3%) were negative tumours. Immunostaining was lacking in nine (81.8%) of the high-grade carcinomas and weak in the two remaining cases (18.2%) (Table I, Fig. 2). Our results indicated an inverse relationship between DAP kinase immunoreactivity and the histological grading of tumoral malignancy ($p<0.01$). Positive staining was found in isolated cells or small cell groups within larger tumoral islands and sheets. Immunoreactivity showed to be more intense in cells adjacent to keratinization areas (diskeratotic cells and keratin pearls) but extensively absent in areas of severe cytological atypia.

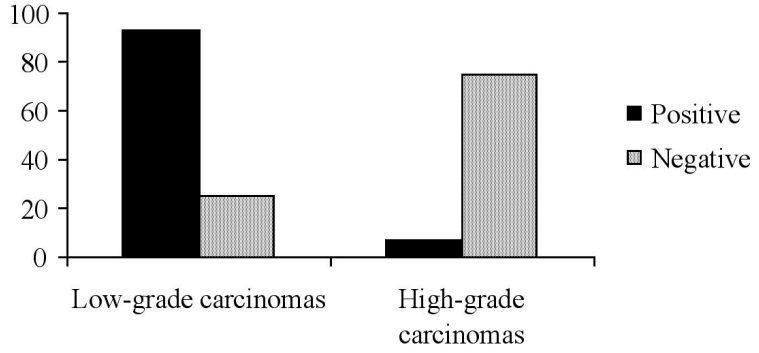

Fig. 1. Expression of death-associated protein kinase correlated with the histological malignancy grading of oral squamous cell carcinoma low and high grade.

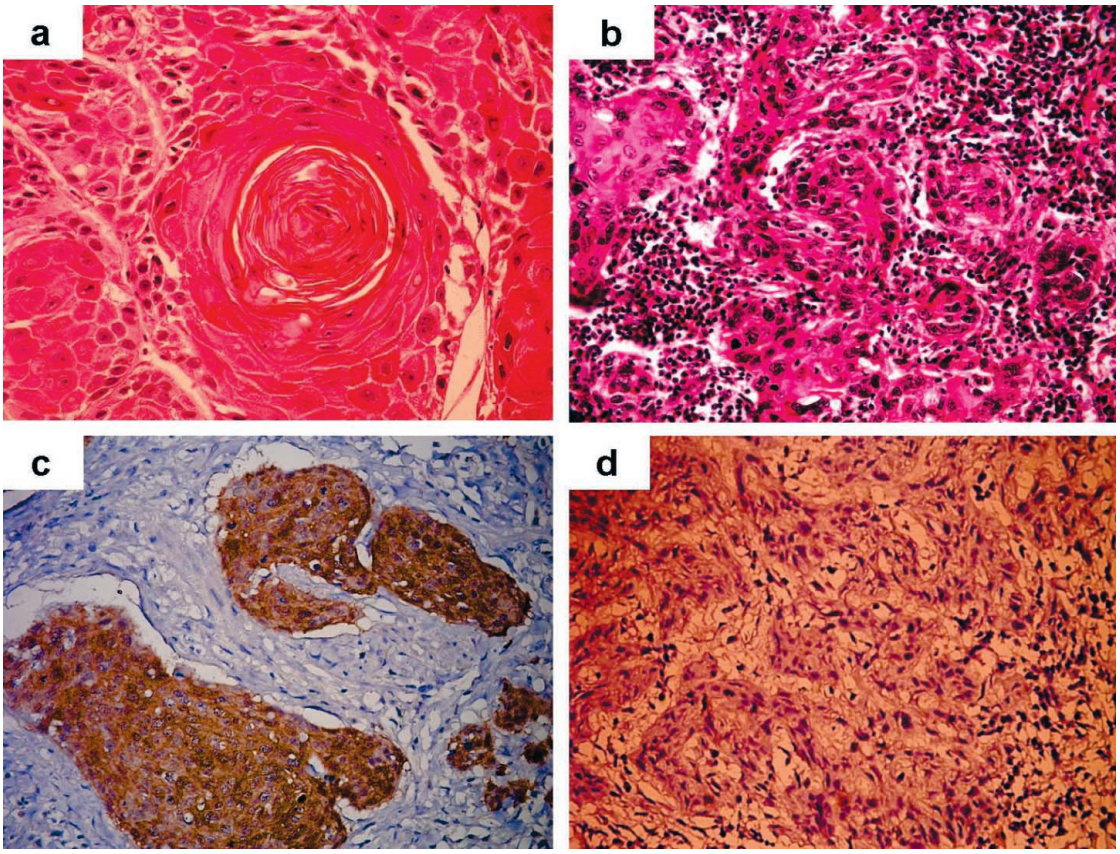

Fig. 2. (a) Low-grade squamous cells carcinoma presenting islands of keratinizing well-differentiated epithelial cells (HE, 400x). (b) High-grade squamous cells carcinoma exhibiting small nests of poorly differentiated pleomorphic epithelial cells (HE, 400x). (c) Strong immunohistochemical positivity for DAP kinase in the cytoplasm of tumoral cells in a low-grade squamous cells carcinoma (LSAB, 400x). (d) Lack of immunostaining in tumoral cells of a high-grade squamous cells carcinoma (LSAB, 400x).

Table I. Relationship among histological malignancy grading of the oral squamous cells carcinomas and the intensity of the immunostaining. (++) moderate, (+) weak, (-) negative or absent.

Histological malignancy grading	Intensity of immunostaining			Total
	++ n (%)	+ n (%)	- n (%)	
Low-grade tumours	16 (55.2)	10 (34.5)	3 (10.3)	29 (72.5)
High-grade tumours	-	2 (18.2)	9 (81.8)	11 (27.5)
Total	16 (40)	12 (30)	12 (30)	40 (100)

DISCUSSION

Various studies have demonstrated that DAPk gene transcription is involved in apoptosis phenomenon and therefore may play a relevant role in suppressing oncogenic transformation (Raveh & Kimchi, 2001; González-Gómez *et al*.; Bai *et al*.). DAPk protein downregulates the expression of integrins, and consequently blocks cellular adhesion, thereby activating a particular pathway of apoptosis known as anoikis (Wang *et al.,* 2002). Nevertheless, the capacity to promote apoptosis through DAPk pathway is frequently lost in anoikis-resistent cells, resulting in anchorage independence, a hallmark of malignant tumour cells closely associated to metastasis (Frisch & Screaton, 2001). Loss of

DAP kinase RNAm and protein expression was found in human cancer, often as a result of silencing by DNA hypermethylation (Lehmann *et al*.; Simpson *et al.*; Pulling *et al*.). In addition, DAPk appears to be underexpressed in metastatic cell lines (Lehmann *et al*.; Simpson *et al*.; Pulling *et al*.).

We investigated the relationship between the immunohistochemical expression of DAPk and the histological grading of malignancy in forty cases of squamous cell carcinoma of the oral mucosa. According to our results, the intensity of the immunohistochemical expression of DAPk is inversely proportional to the histological malignancy grading of oral squamous cell carcinoma. This theory is supported by the fact that immunoreactivity was more intense in cells adjacent to keratinizatinized areas, hallmarks of well-differentiated tumors, but lacked in areas of severe cytological atypia. Likewise, loss of DAPk expression has been reported in a wide range of tumour types (Raveh & Kimchi; Lehmann *et al.*; Simpson *et al*.; Bai *et al*.). Surprisingly, three cases of low-grade tumours failed in expressing DAPk. However, it must be emphasized that all those tumors presented marked cytological atypia and minimum keratinization, despite they were classified as low-grade tumors. Thus, these findings seem to point at a link between the phenomenon of "dedifferentiation" and loss of DAPk expression in OSCC. Nevertheless, the mechanism responsible for silencing DAPk gene is not completely clear yet. It has been suggested that hypermethylation of CpG islands (segments of DNA within the gene promoter characterized by extensive repetition of residues of cytosine followed by residues of guanine) might suppress gene transcription (Lehmann *et al*.; González-Gómez *et al.*; Pulling *et al*.), but its role in the carcinogenesis of oral carcinoma is still unknown. In addition, the relationship between loss of DAK expression and worse overall survival prognosis was recently demonstrated in breast cancer (Lévy *et al*.) but further investigations are necessary to assess the role of DAPk in evaluating the survival rates and prognosis of carcinomas of the oral mucosa.

In conclusion, we have found that DAPk expression decreased significantly in high-grade oral carcinomas, which means that this expression might correlate with the histological malignancy grading of these tumour.

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RESUMEN: Fue realizado un análisis inmunohistoquímico de 40 casos de carcinoma oral de células escamosas para evaluar la relación entre el patrón de expresión de la proteína quinasa (DAPK) asociada a la muerte celular y la clasificación histológica de malignidad de estas lesiones. Según nuestros resultados, 11 casos (27,5%) eran tumores de alto grado de malignidad y 29 (72,5%) de bajo grado. Se encontró que 92,86% de los tumores de bajo grado de malignidad fueron eran positivos para anticuerpos anti-DAP-quinasa, mientras que sólo 7,14% de los tumores de alto grado presentaron positividad, esta diferencia fue estadísticamente significativa ($p < 0,01$). En 16 casos (55,2%) los carcinomas de bajo grado de malignidad mostraron inmunorreactividad moderada mientras que 10 casos (34,5%) mostraron una tinción débil y 3 casos (10,3%) fueron negativos. La inmunotinción estuvo ausente en 9 (81,8%) de los carcinomas de alto grado y "débil" grado de malignidad en los 2 (18,2%) casos restantes. Así, la expresión DAPK se redujo significativamente en los carcinomas orales de alto grado y las evidencias indican que podría estar relacionado con la severidad de la atipia citológica.

PALABRAS CLAVE: Inmunohistoquimica; DAP kinasa; Apoptosis; Carcinoma de células escamosas.

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