

Decreased Expression of DARPP-32 in Oral Premalignant and Malignant Lesions

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Abstract. *Background:* DARPP-32 is a neuronal protein that plays a central role in dopaminergic neurotransmission. Although DARPP-32 may contribute to the pathogenesis of several human malignancies, its expression has never been investigated in oral premalignant and malignant lesions. *Materials and Methods:* DARPP-32 expression was examined using immunohistochemistry in 14 normal oral mucosa, 5 normal lower lip mucosa, 41 oral leukoplakia (OL), 30 oral squamous cell carcinoma (OSCC) and 20 lower lip squamous cell carcinoma (LLSCC) specimens. Differences of its expression between groups were analyzed. *Results:* OSCC and OL with moderate or severe dysplasia showed lower DARPP-32 expression in relation to normal oral mucosa. LLSCC showed lower DARPP-32 expression than normal lower lip mucosa and OSCC. *Conclusion:* The decreased expression of DARPP-32 in oral premalignant and malignant lesions suggests a tumor suppressor role for this protein in the tumorigenesis of these lesions.

Oral squamous cell carcinoma (OSCC) is one of the 10 most common malignancies worldwide and represents approximately 90% of all malignant tumors of the oral cavity (1, 2). Despite considerable advances in diagnostic and therapeutic possibilities, the prognosis of squamous carcinoma in the oral cavity is still very poor. It is generally accepted that OSCC is a multi-step process of accumulated genetic damage leading to cell dysregulation with disruption

in cell signaling, DNA-repair and the cell-cycle, which are fundamental to homeostasis. Although frequent genetic abnormalities have been demonstrated in OSCC and in oral dysplastic epithelium (3-5), the precise molecular mechanisms of development and/or progression of OSCC still remain unclear.

Oral leukoplakia (OL) is the most frequent potentially malignant lesion of the oral mucosa (6). A follow-up study of a hospital-based population of 166 patients with OL revealed a 2.9% annual malignant transformation rate (7). The parameters associated with an increased risk of transformation were female gender, absence of smoking habits in women and a non-homogeneous clinical aspect.

Lower lip squamous cell carcinoma (LLSCC) is a form of squamous cell carcinoma with a distinct epidemiology and etiopathogenesis (8, 9). Although it is well-known that the most relevant risk factor for LLSCC is exposure to ultraviolet radiation (8, 9), its definitive pathogenic pathway remains unclear and few studies have investigated the molecular basis of its development and behavior (8).

DARPP-32 is a neuronal protein that plays a central role in the dopamine signaling pathway in dopaminergic neurotransmission and is a major factor in the functioning of dopaminoceptive neurons (10, 11). DARPP-32 presents various phosphorylation sites and is also the only known bifunctional protein that can act as a protein phosphatase 1 (PP1) inhibitor or as a protein kinase A (PKA) inhibitor. It orchestrates the degree of phosphorylation in a variety of molecular targets in the cell membrane and cytoplasm (12).

Recently, some studies have shown that DARPP-32 and a truncated isoform, t-DARPP, were expressed at various levels in several types of normal epithelial tissues outside the central nervous system, such as stratified squamous epithelium and breast epithelial cells (13). Overexpression of DARPP-32 and t-DARPP in carcinomas of the breast, prostate, colon (13), stomach (14), and decreased expression in thyroid carcinoma (15) have already been reported.

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Table I. Clinical characteristics of patients included in the study.

	No. of cases	Male:	Age (years)	
		female ratio	Mean	Range
Normal oral mucosa	14	1.33:1	37.8	14-52
OL with or without mild dysplasia	24	0.41:1	47.2	29-82
OL with moderate or severe dysplasia	17	2.40:1	48.0	18-73
OSCC	30	3.28:1	57.6	35-79
Normal lower lip mucosa	5	0.25:1	53.6	40-61
LLSCC	20	2.33:1	60.3	27-86

OL: oral leukoplakia; OSCC: oral squamous cell carcinoma; LLSCC: lower lip squamous cell carcinoma.

These findings suggest that DARPP-32 may play a role in epithelial cell signaling and may contribute to the pathogenesis of several human malignancies (13). Considering that various malignant tumors present alteration of DARPP-32 protein expression, together with the paucity of data concerning its investigation in oral lesions, we were prompted to make the first investigation of DARPP-32 protein expression in oral and lower lip malignant and potentially malignant lesions.

Materials and Methods

Tissue samples. Tissue samples of 14 normal oral mucosa, 5 normal lower lip mucosa, 41 cases of OL (24 with or without mild dysplasia and 17 with moderate or severe dysplasia), 30 cases of OSCC and 20 cases of LLSCC were included in the study. None of the patients with malignant lesions were submitted to radiotherapy or chemotherapy at the time of the biopsy. The age and gender distributions of the patients in each group are summarized in Table I. The present study was approved by the local Ethics Committee. The grade of epithelial dysplasia was established as described elsewhere (16).

Immunohistochemistry. Tissue sections were stained with DARPP-32 antiserum (Clone H-62: sc-11365; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Briefly, 4 µm-thick paraffin-embedded sections were dewaxed in xylene and hydrated with graded ethanol. Heat-induced epitope retrieval was performed with 10 mM citrate buffer pH 6.0 for 30 min in a steamer at 96°C. Endogenous peroxidase activity was blocked with 3% H₂O₂ in water for 10 min. Primary polyclonal rabbit anti-DARPP-32 antiserum was used at a 1:250 dilution (in BSA 0.5%) for 18 h at 4°C. This was followed by incubation with the labeled streptavidin-biotin (LSAB) Kit (DakoCytomation California Inc, Carpinteria, CA, USA). Peroxidase activity was developed with DAB (Sigma, St. Louis, MI, USA) with timed monitoring using a positive control sample. The sections were then counterstained with hematoxylin, dehydrated and mounted. Omission of the primary antibody was used as negative control.

Only sections containing sufficient epithelium to assess the antibody reactivity were considered for this study. Two experienced independent pathologists examined multiple fields and the percentage of positively stained cells was obtained for each case, regardless of staining intensity, after counting 1,000 epithelial cells.

Table II. Expression pattern of DARPP-32 in each group.

	Decreased expression	Normal expression	P-value*
Normal oral mucosa	-	14	
OL with or without mild dysplasia	-	24	n.s. ^a
OL with moderate or severe dysplasia	5	12	<0.05 ^a
OSCC	15	15	<0.05 ^a
Normal lower lip mucosa	-	5	
LLSCC	18	2	<0.05 ^b

^aVs. normal oral mucosa; ^bVs. normal lower lip mucosa; n.s., not significant; *P-values were obtained from the Fisher exact test. Decreased expression: 75% or less of DARPP-32 positive cells; Normal expression: more than 75% of DARPP-32 positive cells. OL: oral leukoplakia; OSCC: oral squamous cell carcinoma; LLSCC: lower lip squamous cell carcinoma.

Table III. Percentage of DARPP-32-positive cells in each group.

	n	Median	Range	P-value*
Normal oral mucosa	14	96.95	76.00-99.60	
OL with or without mild dysplasia	24	93.80	86.80-97.80	n.s. ^a
OL with moderate or severe dysplasia	17	84.10	15.70-99.00	<0.05 ^a
OSCC	30	78.05	1.10-97.80	<0.05 ^a
				<0.05 ^b
				n.s. ^c
Normal lower lip mucosa	5	98.00	92.12-100	n.s. ^a
LLSCC	20	13.45	0.30-96.40	<0.05 ^d
				<0.05 ^e

^aVs. normal oral mucosa; ^bVs. OL without or with mild dysplasia; ^cVs. OL with moderate or severe dysplasia; ^dVs. OSCC; ^eVs. normal lower lip mucosa; n.s. not significant; *p-values were obtained from the Mann-Whitney test. OL: oral leukoplakia; OSCC: oral squamous cell carcinoma; LLSCC: lower lip squamous cell carcinoma.

As all oral and lower lip normal mucosas presented more than 75% of cells positive to DARPP-32, the DARPP-32 expression pattern was classified as 'normal' expression (samples with more than 75% of labeled cells) and 'decreased' expression (samples with 75% or less of positive cells).

Statistical analysis. Differences in the expression pattern between the groups were analyzed using the Fisher exact test. Differences in the percentage of positive cells between the groups were analyzed using the Mann-Whitney test. The values were considered significantly different when the p-value was less than 0.05.

Results

DARPP-32 expression in different groups is shown in Figure 1 and Tables II and III. Normal oral and lower lip mucosa showed more than 75% of cells positive to DARPP-32. While

OL with or without mild dysplasia presented similar results to normal oral mucosa, OL with moderate or severe dysplasia showed lower expression than normal oral mucosa. OSCC showed lower expression in relation to normal oral mucosa and OL without or with mild dysplasia. LLSCC showed lower expression in relation to normal lower lip mucosa and OSSC.

Discussion

DARPP-32 (dopamine- and cyclic AMP-regulated phosphoprotein, of relative molecular mass 32,000) is a protein that plays a central role in neuronal pathways, especially dopaminergic. It is a central signaling molecule activated by an array of neurotransmitters such as dopamine, glutamate, serotonin and GABA. When phosphorylated on threonine 34 by protein kinase A (PKA), DARPP-32 has a potent inhibitory effect on protein phosphatase 1 (PP-1) which regulates the activation of receptors, channels and transcriptional factor (12). DARPP-32 knockout mice showed no abnormalities in brain morphology (17) but it was demonstrated that DARPP-32 is involved in kidney and nephron development, thyroid differentiation and apoptosis (15, 18, 19). Alterations in DARPP-32 levels in schizophrenia have also been demonstrated (20). Several studies have reported the presence of dopamine receptors and alteration in its signal transduction in human tumoral cell lines (21-24).

Aside from many studies in the brain, the potential function of DARP-32 outside of the nervous system has only recently been investigated. The importance of DARPP-32 in the differentiation of cells was initially demonstrated by Garcia-Jimenez *et al.* (2005) (15). They showed that DARPP-32 is expressed in normal thyroid gland and thyroid cell lines, but is lost in transformed thyroid cells. This provided evidence that DARPP-32 is essential for the maintenance of thyroid cell differentiation. In contrast, in gastric (14) and esophageal cancer (25), and in some common adenocarcinomas (13), DARPP-32 is overexpressed compared to their normal untransformed parental cell types. These data show the complexity of this protein in different cancer entities.

In the present study, OSCC cases showed lower expression of DARPP-32 compared to normal oral mucosa and OL with or without mild dysplasia. As OL with moderate or severe dysplasia presented lower expression than normal oral mucosa, a DARPP-32 down-regulation would seem to occur early during OSSC development. Our study also demonstrates that DARPP-32 expression in LLSCC is lower than in OSCC. This is in accordance with the distinct etiopathogenesis of LLSCC in comparison with OSCC (8, 9). It has been reported that while OSCC present genetic changes characteristic of DNA damage caused by

tobacco smoke, mutations in LLSCC reveal a UV-light signature (26). Taken together, these findings suggest that DARPP-32 has a tumor suppressor gene role in OSCC and LLSCC and it may operate in the maintenance of squamous cell differentiation in the same fashion as in thyroid and breast tissues.

A recent study demonstrated that DARP32 protein was either reduced or lost in various breast cancer cell lines (27). Furthermore, the authors found that re-expression of DAPR-32 impaired migration of breast tumor cells. Considering that patients with esophageal cancer expressing DARP-32 have a better prognosis than those who are DAPR-32 negative (25), we can suggest that DARP-32 modulation could be a new target for effective anti-metastatic cancer therapy of the oral cavity and lower lip.

Conclusion

For the first time, we have shown decreased expression of DARPP-32 occurs in potentially malignant and malignant lesions of the oral and lower lip mucosa, which suggests that the neuromodulator DARPP-32 may have an important role in OSCC and LLSCC tumorigenesis.

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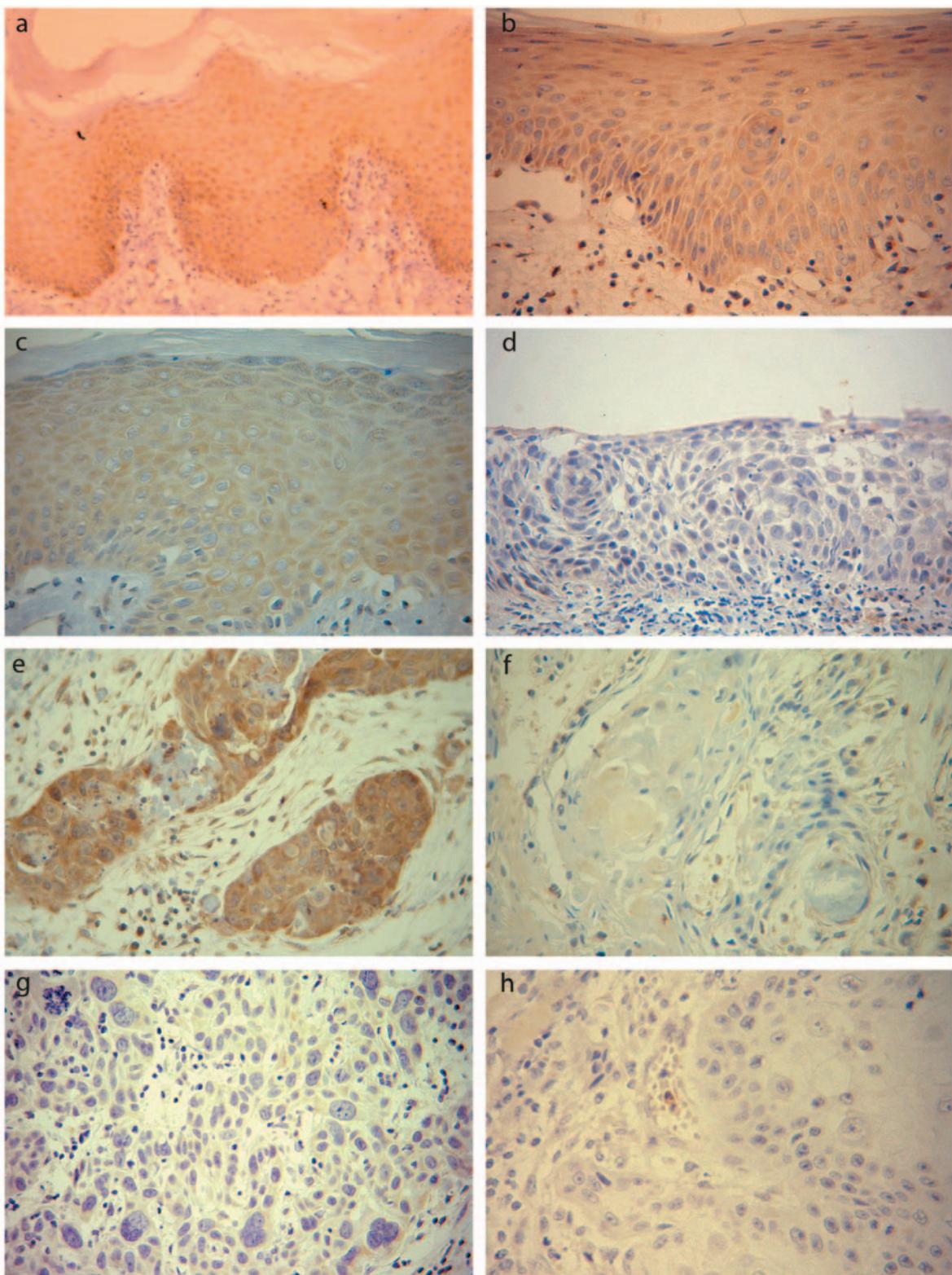


Figure 1. Immunohistochemical staining for DARPP-32. Normal oral mucosa (a) and normal lower lip mucosa (b) showing more than 75% positively stained cells (normal expression). Oral leukoplakia with mild dysplasia (c) demonstrating normal expression. Oral leukoplakia with severe dysplasia (d) showing reduced expression. Oral squamous cell carcinoma with normal (e) and reduced (f) expression. Lower lip squamous cell carcinoma with normal (g) and reduced (h) expression (original magnification: x100 in (a) and x400 in (b-h)).

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