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Preliminary Correlation of the Immunoexpression of Cathepsin B and E-Cadherin Proteins in Vocal Fold Leukoplakia

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SUMMARY: Objectives. Early identification of vocal fold leukoplakia (VFL), which has a risk of progressing to malignant transformation, remains a controversial topic. The identification of biological markers for diagnosing these lesions would lead to a more effective treatment. We aimed to analyze the immunoexpression of cathepsin B and E-cadherin in VFL and correlate it with clinical and epidemiological data and disease prognosis.

Methods. Thirty-two patients with VFL treated with microsurgery were retrospectively evaluated. The patients were distributed according to the histological results into Group A (low grade) and Group B (high grade). The expression of markers was quantitatively determined as per their staining intensity and tissue distribution using ImageLab. The index of expression (IE) of each marker was correlated with tobacco and alcohol consumption, signs of laryngopharyngeal reflux, and local recurrence of the lesion.

Results. The correlation between the IE of markers and variables within the two groups (A and B) demonstrated that patients in Group B with local recurrence had a higher IE of cathepsin B. When all patients (A + B) were included, the same analysis demonstrated that the IE of cathepsin B was higher among smokers and patients who did not show signs of reflux and that the IE of E-cadherin was higher only in patients with recurrence.

Conclusion. Patients with moderate to severe dysplasia and carcinoma *in situ* who smoked as well as had a high IE of cathepsin B were more prone to local recurrence. Regardless of the type of histological lesion, patients with signs of laryngopharyngeal reflux had a lower IE of cathepsin B. The IE of E-cadherin was higher among patients with VFL who relapsed after initial treatment.

Key Words: Larynx—Reflux—Early glottic cancer—Laryngology.

INTRODUCTION

Leukoplakia is a strictly clinical term that refers to small white mucosal lesions that cannot be detached, and it is characterized in the same manner as any other condition. The most frequent site of leukoplakia in the larynx is the vocal fold; however, other anatomical sites, such as the vestibular fold, and the interarytenoid space, can be affected.[1,2]

Leukoplakia is a response of tissues to repeated aggressions and chronic irritation, usually because of tobacco smoking, alcohol consumption, and laryngopharyngeal reflux; there is a direct relationship between the levels of exposure to and consumption of these irritants with the progression of the dysplasia and the consequent increase in the malignancy risk.[3–6]

The transformation rate of leukoplakia into invasive carcinoma varies considerably in the literature, ranging between 2% and 12% in cases of leukoplakia without histological evidence of dysplasia and between 7% and 31% in patients with different degrees of dysplasia.[7–9]

The detection of leukoplakia in the vocal fold before it progresses to invasive carcinoma may mean the preservation of speech organs. Thus, identifying tumor markers that determine the biological behavior of these lesions is important for the early diagnoses of premalignant lesions with a high risk of malignant transformation.[8–10]

In particular, cathepsin B, and E-cadherin are biological markers that have been investigated in different types of cancer because of their roles in tumor local growth and metastatic processes.[11,12]

Cathepsin B is a proteolytic enzyme reported in lysosomes. It is one of the 11 human cathepsins (B, C, F, H, L, K, O, S, V, W, and X/Z) and plays an important role in biochemical processes. Increased E-cadherin levels is a predictive factor of poor prognosis owing to its participation in tumor progression; this has been well documented in malignant tumors of the brain, oral cavity, nasopharynx, larynx, esophagus, stomach, lung, pancreas, ovary, prostate, bladder, colon/rectum, and thyroid.[13–19]

E-cadherin is a transmembrane glycoprotein that has been implicated as one of the important proteins involved in

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cell adhesion. Decreased levels of E-cadherin are associated with disruption of normal tissue architecture and progression to malignancy and are well documented in lung, breast, colorectal, and bladder carcinomas; prostate adenocarcinoma; and head and neck squamous cell carcinoma.[20–25]

The aim of this study was to analyze the immunoexpression of cathepsin B and E-cadherin in vocal fold leukoplakia (VFL) and to correlate it with clinical, epidemiological, and histopathological data and with disease prognosis.

METHODS

This was a retrospective analysis of the medical records of 32 patients with VFL examined in an otolaryngology outpatient clinic and treated by microsurgery of the larynx in a hospital surgical center between 2000 and 2004. All participants were clinically followed for a period of six years after surgical treatment. These patients were maintained outpatient evaluation by the same specialist until 2010. During this period, clinical data were properly noted in their medical records and cases that presented local recurrence, after performing the first surgical treatment, were referred for a new treatment surgical and subsequent clinical follow-up. This study was approved by the Research Ethics Committee under registration number 0338/06. All patients were previously interviewed and examined by the same specialist physician, and their primary risk factors for premalignant and malignant lesions of the larynx were included in their medical records: use (+) of tobacco or not (−); use (+) of alcohol or not (−); and presence (+) or absence (−) of signs suggesting laryngopharyngeal reflux (epigastric pain, globus pharyngeus, hoarseness, throat clearing, pyrosis, regurgitation, chronic dry cough, interarytenoid hyperemia, retrocricoid edema, vocal fold edema, subglottic edema, diffuse larynx edema, and thick endolaryngeal mucus).[6,26]

Tobacco use (+) was defined as the consistent consumption of tobacco up to the first visit. Conversely, the non-use of tobacco (−) was defined as not smoking or having quit smoking 10 years or more before the interview. Similar definitions were established for alcohol consumption.

The diagnosis of VFL was made using a rigid laryngeal telescope or a flexible nasal fiberscope by the same physician who was first consulted by the patient. Initially, the patients were clinically treated for 30–40 days, and referred to surgery if clinical treatment was unsuccessful. In cases of local recurrence after surgery, they were classified as positive (+) in the medical records, and treated accordingly.

The inclusion criteria were as follows: patients without previous surgical treatment of the vocal fold; patients with VFL who were unresponsive to the initial clinical treatment and who underwent complete excision of the lesion by microsurgery of the larynx; and patients with correctly filled medical records, with complete and up-to-date information regarding risk factors, treatments, recurrence, and current patient clinical status.

The exclusion criteria were as follows: patients who had lesions with signs of ulceration or infiltration evident on examination or with paralysis of the vocal fold; patients with previous radiotherapy treatment; and patients whose histopathological specimens were inadequately preserved or were too small for preparing additional slides.

The patients were distributed into two groups according to the histopathological diagnosis:

- Group A: Low grade (n = 16) − Patients with simple epithelial hyperplasia and mild dysplasia.
- Group B: High grade (n = 16) − Patients with moderate to severe dysplasia and carcinoma *in situ*.

Histological evaluation was performed according to the 2005 World Health Organization guidelines, in which vocal fold leukoplakia is divided into the following categories: squamous cell hyperplasia without dysplasia; mild dysplasia; moderate dysplasia; severe dysplasia; carcinoma in situ, and squamous cell carcinoma.[27]

The vocal fold tissue samples, obtained from patients treated with microsurgery, were fixed in 10% formalin and sent for histopathological study. The material was processed and stained with the hematoxylin and eosin method and embedded in paraffin blocks to obtain additional slides.[28] These slides were then classified histologically a single highly experienced pathologist who was blinded to clinical information re-examined all the samples and marked the reading points for the measurement of the studied markers. Immunohistochemical analysis was performed using 4-μm-thick histological sections obtained from this material and mounted on slides previously silanized with 4% organosilane (3-aminopropryltriethoxysilane; Sigma, St. Louis, MO, USA) solution in acetone and subjected to successive washes in xylene and 100% ethanol (Synth, Diadema, State of São Paulo, Brazil) for complete deparaffinization and dehydration. Hydration was subsequently performed with running water and distilled water.

The slides were then placed in a dark humidified chamber for 18 hours with the primary monoclonal antibodies anti-E-cadherin (1:150) and anti-cathepsin B (1:300) (Sigma, USA). Two slides were then prepared for each tissue sample, one slide for each antibody, and the dilution was determined by evaluating the positivity of known positive control slides with different concentrations to determine the optimal titer of the two antibodies.

Then, the slides were counterstained with Carrazi's hematoxylin for 2 minutes, washed with running water for 5 minutes, dehydrated in sequences of 100% ethanol and xylene, and mounted using Entelan (Merck). To quantify the markers, 8–10 fields with the strongest staining were selected on average from each slide, and all were analyzed by the same pathologist who prepared the slides. The slides were analyzed using a light microscope. Photomicrographs of the areas with the strongest immunostaining were prepared and transferred to a computer with a Pentium four processor running Windows XP. The areas that best represented immunostaining was chosen and analyzed by the same pathologist at 400x magnification (Figure 1).

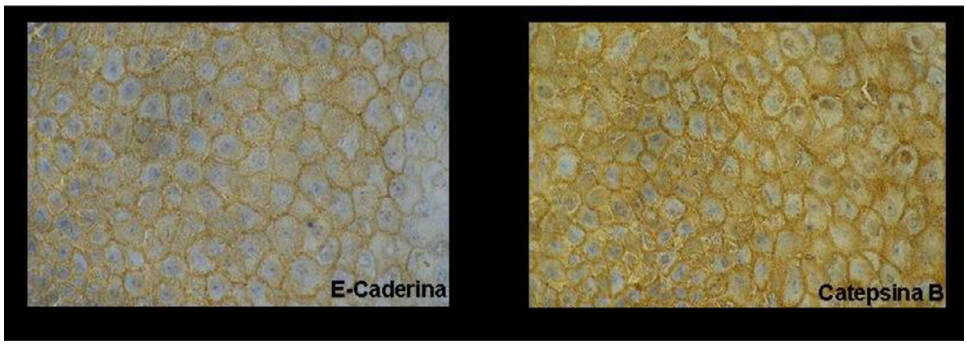

FIGURE 1. Photomicrograph of vocal fold epithelium stained with Cathepsin-B and E-cadherin markers at 400X magnification.

The quantification of cathepsin B and E-cadherin expression was performed using ImageLab image processing software (Softium Informática, São Paulo, Brazil) based on the intensity and tissue distribution of staining. The index of positivity (IP, represented by the number of positive cells, ie, those expressing the markers), intensity of expression (ItE, mean comparative color intensity in the selected region), and index of expression (IE) were thus determined for each case according to the method proposed by Matos et al (2006).[29]

IE was obtained by multiplying IP by ItE and dividing the product by 100, as per the following equation:

$$IE = \frac{IP \,.\, ItE}{100}$$

IE values varied between 0.0 and 320.7 OU/μm^2 and were directly proportional to the expression level of the antibody. The three indices and the method for the digital quantification of immunohistochemical reactions were standardized.[29]

The data were subjected to statistical analysis. The nonparametric Mann–Whitney *U* test was used to correlate the IE of cathepsin B and E-cadherin markers with the study variables (use or non-use of alcohol, use or non-use of tobacco, presence or absence of reflux, and presence or absence of recurrence) in each group. Student's *t* test was used to correlate the IE of markers with the study variables for all patients in the sample (A + B).

RESULTS

The sample included 32 patients with VFL treated at the outpatient otolaryngology clinic, 29 men, and three women, who were distributed into two groups. The mean age of the patients ranged between 27 and 84 years, with a mean of 57.8 years in Group A and 60.9 years in Group B. Within the sample, 62.5% of patients were smokers (Group A = 9; Group B = 10), 71.9% consumed alcohol (Group A = 13; Group B = 10), and 65.6% exhibited signs and symptoms suggesting laryngopharyngeal reflux (Group A = 10; Group B = 11). More than half of the patients in the sample relapsed after the initial treatment (56.3%).

The IE of cathepsin B did not significantly differ as per the study variables (smoking, alcohol consumption, and signs/symptoms of laryngopharyngeal reflux) individually analyzed among patients in both Group A and Group B ($P > 0.05$). However, the analysis of the variable "local recurrence" separately in Group A and Group B showed a statistically significant difference in the IE of cathepsin B between Group B patients with and without recurrence ($P < 0.05$) (Table 1a).

TABLE 1A.
Distribution According to the IE of the Marker Cathepsin B Between Patients With and Without Local Recurrence Within Each Group

Variables	Local recurrence			
	Group A		Group B	
	Absent	Present	Absent	Present
n	11	5	3	13
Mean	105.51	106.11	76.9	122.42
Median	84.9	121.8	82.3	131.4
Standard deviation	52.69	43.92	18.6	40.14
Minimum	27.2	54.23	56.2	25
Maximum	179.6	160.6	92.2	189.8

Group A: $P > 0.999$; Group B: $P = 0.039$. IE, Index of expression. n = Absolute Frequency.

The comparison of the E-cadherin expression index between patients who relapsed and those who did not, separately in groups A and B. The results show that there was no statistically significant difference between patients with and without recurrence, neither for patients in Group A nor for patients in Group B ($P > 0.05$) (Table 1b).

The analysis of the correlation between the IE of E-cadherin and the study variables (smoking, alcohol consumption, signs/symptoms of reflux, and local recurrence) within each group demonstrated that there was no statistically significant difference among patients in both Group A and Group B ($P > 0.05$).

The comparison of the IE of cathepsin B and E-cadherin between smokers and non-smokers showed a statistically significant difference only for the IE of cathepsin B (Table 2).

The comparison of the IE of both markers between alcohol drinkers and non-drinkers showed no statistically significant difference for either marker.

TABLE 1B.
Distribution of the Expression Index of the E-Cadherin Marker Between Patients With and Without Relapse, Separately for Each Group

	Group			
	A		B	
	relapse		relapse	
	no	*yes*	*no*	*yes*
no	11	5	3	13
average	66.4	98.57	80.92	99.02
average	61.66	100	66.1	101.8
standard deviation	41.67	49.92	42.55	25.78
Minimum	11.29	22.5	47.77	59.1
maximum	126	148	128.9	146.2

Group A: $P = 0.267$. Group B: $P = 0.364$.

TABLE 2.
Distribution According to the IE of Markers Cathepsin B and E-Cadherin Between Smokers and Non-Smokers

Index of expression	Variables	Smoker		*P*-value
		Yes	No	
Cathepsin B	n	12	20	0.049
	Mean	89.99	121.67	
	Median	84.1	131.5	
	Standard deviation	39.34	43.86	
	Minimum	25	27.2	
	Maximum	160.2	189.8	
E-cadherin	n	12	20	0.423
	Mean	78.84	90.36	
	Median	79.09	93.8	
	Standard deviation	40.45	37.86	
	Minimum	19.88	11.29	
	Maximum	148	146.2	

IE, Index of expression. n = Absolute Frequency.

The results of the IE comparison of the markers between patients with and without signs and symptoms of laryngopharyngeal reflux showed a statistically significant difference only in the IE of cathepsin B (Table 3).

In terms of recurrence of leukoplakia among all patients in the sample, there was a statistically significant difference only in the IE of E-cadherin (Table 4).

DISCUSSION

Leukoplakia is considered a premalignant lesion, because its epithelium exhibits histomorphological alterations that lead to a higher risk of transformation into an invasive carcinoma than that of adjacent healthy tissues.[7–9,22]

In this study, there was a predominance of VFL among men (9.6 men: 1 woman). The high percentage of alcohol consumers (71.9%) and smokers (62.5%) may have contributed to the development of laryngopharyngeal reflux in the study sample (65.6%), which acts as an additional factor for larynx irritation, contributing to the development of dysplasia in the epithelium of the vocal fold.[2,4,6,10,30–33]

Cheng et al proposed the hypothesis that episodes of laryngopharyngeal reflux play an important role in *Helicobacter pylori* migration to the larynx and that this may be an independent risk factor for VFL. Smoking reduces the strength of the lower esophageal sphincter, thereby allowing additional reflux of gastric juice and bile salts to the esophagus, which may cause direct damage to the mucosa of the hypopharynx, larynx, and oropharynx.[30]

TABLE 3.
Distribution According to the IE of Markers Cathepsin B and E-Cadherin Between Patients With and Without Signs of Laryngopharyngeal Reflux Syndrome

Index of expression	Variables	Laryngopharyngeal reflux syndrome		*P*-value
		Absent	Present	
Cathepsin B	n	11	21	0.035
	Mean	132.38	97.96	
	Median	146.9	92.2	
	Standard deviation	41.32	42.11	
	Minimum	54.23	25	
	Maximum	189.8	160.6	
E-cadherin	n	11	21	0.349
	Mean	77.06	90.74	
	Median	81.92	100	
	Standard deviation	40.04	37.97	
	Minimum	22.5	11.29	
	Maximum	146.2	148	

IE, Index of expression. n = Absolute Frequency.

TABLE 4.
Distribution of the IE of Markers Cathepsin B and E-Cadherin Between Patients With and Without Local Recurrence

		Recurrence		*P*-value
		No	Yes	
IE (Cathepsin B)	n	14	18	0.248
	Mean	99.38	117.89	
	Median	84.1	123.7	
	Standard deviation	48.34	40.59	
	Minimum	27.2	25	
	Maximum	179.6	189.8	
IE (E-cadherin)	n	14	18	0.030
	Mean	69.51	98.9	
	Median	63.88	100.9	
	Standard deviation	40.65	32.49	
	Minimum	11.29	22.5	
	Maximum	128.9	148	

IE, Index of expression. n = Absolute Frequency.

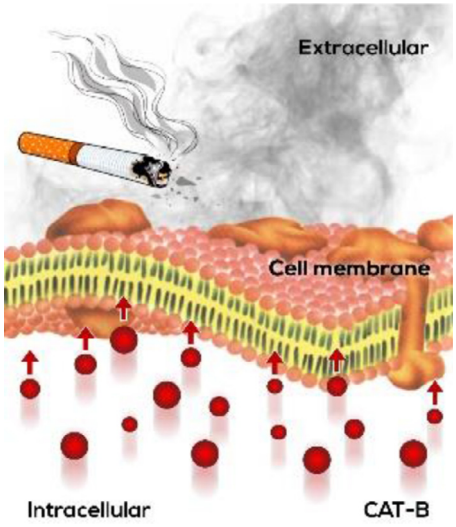

FIGURE 2. The intracellular activity and expression of cathepsin B, whose levels increase in cells close to or in contact with the extracellular matrix.

The correlation analysis between the IE of cathepsin B and E-cadherin and the variables in each group (Group A and Group B) separately showed a statistically significant difference only in the IE of cathepsin B of patients in Group B (high grade) with recurrence of leukoplakia, indicating that these patients have a higher IE of cathepsin B than those without recurrence (Table 1a).

Because multiple aspects of cell growth and differentiation were more altered in Group B (moderate-to-severe dysplasia and carcinoma *in situ*) than in Group A (low grade) in response to persistent stress caused by long-term chronic irritation because of tobacco and alcohol use and reflux, molecular changes are likely to have occurred, manifesting as an increased expression of cathepsin B specifically in the epithelium of the larynx, resulting in a more aggressive behavior of VFL, and making cells more prone to recurrence.

On comparing the IE of cathepsin B and E-cadherin with the study variables among all patients in the sample (n = 32), only the IE of cathepsin B showed a statistically significant difference between smokers and non-smokers, with the IE value being, on an average, higher among the former (Table 2).

Tobacco smoke is a potent inducer of cathepsin B activity; its presence in the laryngeal mucosa increases the expression of this proteolytic enzyme and the invasive phenotype of dysplastic cells. The mechanism of action of tobacco smoke in dysplastic cells is thought to comprise the exposure to carcinogens and co-carcinogens contained in the smoke and dose-dependent induction by them, as well as the tumorigenic effect of tobacco tar that is adsorbed on the fibers of cigarette filters. Note that these fibers are released and inhaled into the upper airways of smokers. In the larynx, particularly in the glottis, where the airway is naturally narrower, there is a higher concentration of both smoke and of cigarette filter fibers, which can penetrate the glottis tissue and become an additional risk factor for tumorigenesis.[34–36] These factors can deregulate the intracellular activity and expression of cathepsin B, whose levels increase in cells close to or in contact with the extracellular matrix (Figure 2).

Cathepsins play specific roles in the different stages of evolution to malignancy, and their presence on the cell surface can lead to the activation of a proteolytic cascade of events. On binding to other cell membrane proteins, Cathepsins act by degrading and destroying components of the extracellular matrix and cell membrane and participating in tumor angiogenesis. This proteolytic activity of Cathepsin B shows a relation with the transformation of normal cells into cancer cells, thereby increasing the migration and invasion of tumor cells, which can favor future metastases.[13-15,18,19,37–39]

Based on these results, we believe that higher levels of cathepsin B in vocal fold tissues (with moderate-to-severe dysplasia and carcinoma *in situ*) may be a sign of the beginning of its translocation to the cell surface and subsequent proteolytic activity in the cell membrane. This demonstrates that the elevation of this enzyme's levels is a potential marker of aggressiveness of these lesions, especially in patients who smoke.

When the variable laryngopharyngeal reflux was analyzed, a statistically significant difference was observed only in the IE of cathepsin B, with its values being, on an average, higher among patients without reflux than among those with reflux, regardless of the histological type of the lesion (Table 3).

Changes in the pH of pharyngeal and laryngeal mucosa occur in patients with symptoms of laryngopharyngeal reflux. Therefore, the pH of the extracellular, and

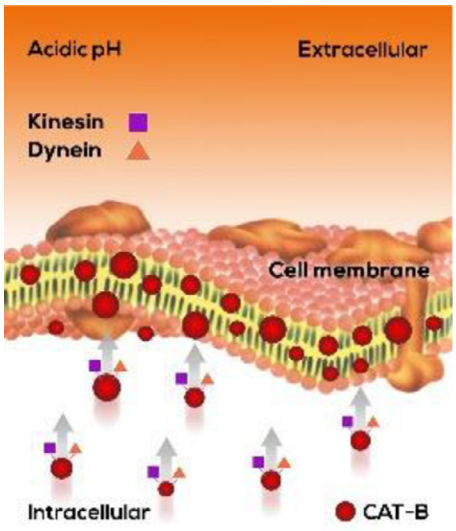

FIGURE 3. Cathepsin B in the cells of patients with symptoms of laryngopharyngeal reflux.

intracellular environments of these tissues is low. The focal dissolution of extracellular matrix proteins caused by cathepsin B occurs more rapidly at an acidic pH, in which lysosomal enzymes are more active. It is believed that in an acidic environment, cathepsin B is redistributed from the perinuclear region to the periphery of the cell, and this process is coordinated by the motor proteins dynein and kinesin.[11,14,37,40]

This would lead to a higher concentration of cathepsin B in the cell membrane and later in the extracellular matrix, followed by a decrease in cathepsin B concentration in the cytoplasm, which explains the lower IE of cathepsin B in the cells of patients with symptoms of laryngopharyngeal reflux (Figure 3).

The location of cathepsin B in the cytosome determines its main functions. In normal cells, cathepsin B is located in lysosomes close to the nucleus; however, in neoplastic cells, there is a higher concentration of this enzyme both in the perinuclear lysosomes, and at the cell's periphery.

When cathepsin B is in an acidic microenvironment, it may play an important role in the transition from a premalignant lesion into a malignant lesion by promoting a runaway proteolysis of the components of the extracellular matrix, both through direct degradation of extracellular matrix, and through the activation of other proteases that trigger the proteolytic cascade.[11,14,16,37,39–42]

When the IE of cathepsin B and E-cadherin were correlated with the variable local recurrence of leukoplakia in all patients, the IE of cathepsin B tended to be high, but a statistically significant difference was only reported in the IE of E-cadherin, with its value being higher in the group of patients with VFL recurrence than in the group of patients without recurrence (Table 4).

E-cadherin is linked to the establishment and integrity of the cell adhesion system (cadherin−catenin complex), and it plays an important role in the structure of stratified squamous epithelium.[20,22]

E-cadherin expression varies at different stages of carcinogenesis; this fluctuation probably depends on a longer duration and higher frequency of exposure to risk factors for premalignant and malignant lesions. E-cadherin expression is higher in tissues with hyperkeratosis or parakeratosis, followed by tissues with mild, and moderate dysplasia. Downregulated E-cadherin expression is more evident in infiltrative tumors than in superficial tumors, and this is related to reduced histological differentiation, potentially more aggressive lesions, and a poorer disease prognosis.[20,22,25,38,43,44]

There is a study that found different values in the expression of E-cadherin in leukoplakia samples with different degrees of dysplasia, which may be affected by the duration, and frequency of the patients' lifestyle. The authors concluded that the loss of E-cadherin can be used as a tumor marker to identify a greater susceptibility of normal or potentially malignant tissue to transform into a malignant neoplasm.[45]

The initially increased expression of E-cadherin in VFL is probably a mechanism for maintaining the integrity of the adhesion system in the context of cellular stress and chronic, cumulative aggression to the larynx from multiple risk factors (tobacco, alcohol, and reflux).[30] This defense mechanism may predispose the patient through a not yet understood pathway to the local recurrence of the disease and induce, at a later stage of leukoplakia progression, the reduction of E-cadherin function at the membrane, impairing the cadherin −catenin system and reducing cell adhesion, which would lead to lesions with a more aggressive behavior, ie, higher tumor invasiveness and metastasis.[20]

We believe that additional prospective studies with homogeneous cohorts of patients with VFL are necessary to draw definitive conclusions about these markers.

CONCLUSION

Patients with moderate to severe dysplasia and carcinoma *in situ* with a high IE of cathepsin B and a habit of smoking were more susceptible to local recurrence. Patients with signs of laryngopharyngeal reflux syndrome had a lower IE of cathepsin B, regardless of the histological type of the lesion. The IE of E-cadherin increased in patients with VFL who relapsed after the initial treatment.

The E-cadherin fluctuates during the various stages of carcinogenesis. The fluctuation instability of its expression would be conditioned to the presence of some other factors not yet known. Dysplasias- mild, moderate, severe - and carcinoma in situ, there were no genetic alterations of this protein. E-cadherin remains normal or slightly increased expression. In later stage there is a reduction of E-caherin expression, loss of the cadherin-catenin complex, and a decreased cell-cell adhesion.

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CONFLICT OF INTEREST

All the authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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