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Immunoexpression of PI3K, PTEN, and S6K1 in oral potentially malignant disorders

Imunoexpressão de PI3K, PTEN e S6K1 em desordens orais potencialmente malignas

Inmunoexpresión de PI3K, PTEN y S6K1 en trastornos orales potencialmente malignos

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ABSTRACT

Objective: To evaluate the immunoexpression of PI3K, PTEN, and S6K1 proteins in oral epithelial dysplasias (OED). **Methods:** Twenty cases of OED with different histological grades and twenty cases of healthy oral mucosa (HOM) were subjected to immunohistochemical reaction using anti-PI3K, anti-PTEN, and anti-S6K1 antibodies, which were analyzed semiquantitatively. Statistical analysis was carried out using Fisher's exact test or Pearson's chi-squared test for categorical variables, and the Mann-Whitney test or the Kruskal-Wallis test for quantitative variables. **Results:** A higher frequency of positive immunostaining for PI3K and S6K1 was observed in OED, together with decreased nuclear and cytoplasmic PTEN in OED compared to HOM. Furthermore, a higher mean cytoplasmic number of cells expressing PTEN was observed in low-risk OED compared to high-risk ones. **Conclusion:** Increased expression of PI3K and S6K1, along with reduced PTEN expression, may be linked to the development and progression of OED. Consequently, these markers may have potential diagnostic value, aiding in risk stratification and potentially contributing to prognosis assessment.

Keywords: Oral leukoplakia, Immunohistochemistry, PTEN phosphohydrolase, Phosphatidylinositol 3-kinase, Ribosomal protein S6 kinase.

RESUMO

Objetivo: Avaliar a imunoexpressão das proteínas PI3K, PTEN e S6K1 em displasias epiteliais orais (DEO). **Métodos:** 20 casos de DEO com diferentes graus histológicos e 20 casos de mucosa oral saudável (MOS) foram avaliados por reação de imunoistoquímica utilizando anticorpos anti-PI3K, anti-PTEN e anti-S6K1, os quais foram analisados semiquantitativamente. A análise estatística foi realizada utilizando o teste exato de Fisher ou o teste do qui-quadrado de Pearson para as variáveis categóricas, e teste de Mann-Whitney ou o teste de Kruskal-Wallis para as variáveis quantitativas. **Resultados:** Foi observada uma maior frequência de imunomarcação positiva para PI3K e S6K1 em DEO, juntamente com diminuição do PTEN nuclear e citoplasmático em DEO em comparação com MOS. Além disso, foi observado um número médio citoplasmático maior de células expressando PTEN em DEO de baixo risco em comparação com as displasias de alto risco. **Conclusão:** O aumento da expressão de PI3K e S6K1, associado a uma diminuição da

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expressão de PTEN, pode estar associada ao desenvolvimento e à progressão de DEO. Por conseguinte, tais marcadores podem apresentar potencial valor diagnóstico, auxiliando na estratificação de risco e podendo contribuir para a definição do prognóstico.

Palavras-chave: Leucoplasia oral, Imuno-histoquímica, PTEN fosfohidrolase, Fosfatidilinositol 3-quinase, Proteína S6 quinase ribossômica.

RESUMEN

Objetivo: Evaluar la inmunoexpresión de las proteínas PI3K, PTEN y S6K1 en displasias epiteliales orales (DEO). **Métodos:** 20 casos de DEO con diferentes grados histológicos y 20 casos de mucosa oral sana (MOS) fueron sometidos a reacción inmunohistoquímica utilizando anticuerpos anti-PI3K, anti-PTEN y anti-S6K1, los cuales fueron analizados semicuantitativamente. El análisis estadístico se llevó a cabo mediante la prueba exacta de Fisher o la prueba ji al cuadrado de Pearson para las variables categóricas, y la prueba de Mann-Whitney o la prueba de Kruskal-Wallis para las variables cuantitativas. **Resultados:** Se observó una mayor frecuencia de inmunotinción positiva para PI3K y S6K1 en DEO, junto con una disminución de PTEN nuclear y citoplasmático en DEO en comparación con MOS. Además, se observó un mayor número citoplasmático medio de células que expresaban PTEN en los DEO de bajo riesgo en comparación con los de alto riesgo. **Conclusión:** El aumento de la expresión de PI3K y S6K1, asociado a la disminución de la expresión de PTEN, puede estar relacionado con el desarrollo y la progresión del DEO. En consecuencia, estos marcadores pueden tener valor diagnóstico, ayudar en la estratificación del riesgo y contribuir a la determinación del pronóstico.

Palabras clave: Leucoplasia oral, Inmunohistoquímica, PTEN fosfohidrolasa, Fosfatidilinositol 3-quinasa, Proteína quinasa ribosomal S6.

INTRODUCTION

Among all oral cancers, more than 90% are represented by oral squamous cell carcinoma (OSCC) (BADWELAN M, et al., 2023; LEONEL ACLS, et al., 2019; RADMAN M, et al., 2018). It is known that a proportion of these originates from oral potentially malignant disorders (OPMD), with leukoplakia being the most common (PAGIN O, et al., 2014; WARNAKULASURIYA S, et al., 2021). The World Health Organization (WHO) defines these lesions as morphologically altered tissue where cancer is more likely to occur compared to its seemingly normal counterpart (VERED M e WRIGHT JM, 2022). These disorders may sometimes exhibit varying degrees of oral epithelial dysplasia (OED), characterized by architectural changes associated with cellular atypia (LEONEL ACLS, et al., 2019; PAGIN O, et al., 2014; RADMAN M, et al., 2018).

The presence of OED in OPMD can predict malignant transformation into OSCC, although other factors, such as loss of heterozygosity of tumor suppressor genes, also influence progression to malignancy (CHAVES FN, et al., 2020; PAGIN O, et al., 2014). Different grades of OED can be histopathologically manifested, and various grading systems have been proposed to classify these grades, including the WHO system (VERED M e WRIGHT JM, 2022) and the binary grading system (KUJAN O, et al., 2006). However, clinical and histopathological characteristics alone are not effective predictors of stability, regression, or progression from OED to OSCC (MARTINS F, et al., 2016).

For this reason, many studies have investigated molecular alterations in OED, aiming to identify predictive biomarkers for malignant transformation (IDRIS A, et al., 2016; MARTINS F, et al., 2016; MIYAHARA LAN, et al., 2018; WANG H, et al., 2017). Several molecular markers have been recognized as important in the study of OED and carcinogenesis (GIUDICE FS e SQUARIZE CH, 2013). These markers are proteins involved in cell cycle signaling, tumor suppression, genomic stability, apoptosis, angiogenesis, and cell proliferation, among other cellular functions (WU J, et al., 2020). One prominent pathway studied in this context is the PI3K pathway, which has been explored for the expression of proteins involved in it and their functions to establish potential targeted therapeutic strategies for the treatment of various tumors (GIUDICE FS e SQUARIZE CH, 2013; WU J, et al., 2020).

The PI3K signaling pathway is related to various cellular functions, controlling protein synthesis, cell growth, apoptosis, proliferation, and angiogenesis, and is frequently altered in various malignancies (GIUDICE FS e SQUARIZE CH, 2013; WU J, et al., 2020). This pathway depends on three main proteins: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (PIK3CA), protein kinase B (AKT), and mammalian target of rapamycin (mTOR) (SQUARIZE CH, 2013). Activated PI3K phosphorylates the second messengers, namely phosphatidylinositol, PIP2, and PIP3, and activates AKT effectors and mTOR complex 2 (mTORC2). The activation of the PI3K-AKT-mTOR pathway includes loss of tumor suppressor phosphatase and tensin homolog gene (PTEN), amplification of or mutations in PI3KCA, AKT, and MTOR genes, and activation of growth factor receptors (WU J, et al., 2020). Although several studies indicate an important role of proteins in this pathway in the development of OSCC, there are no reports of research investigating the role of PI3K, PTEN, and S6K1 in OED, and the mechanism of malignant transformation into OSCC is not yet fully elucidated (GIUDICE FS e SQUARIZE CH, 2013; IDRIS A, et al., 2016; MARTINS F, et al., 2016; MIYAHARA LAN, et al., 2018; WANG H, et al., 2017; WU J, et al., 2020). Thus, the present study aimed to assess the immunoexpression of PI3K, PTEN, and S6K1 proteins in OED and in healthy oral mucosa (HOM) to understand how they are expressed in the process of OPMD progression.

METHODS

Ethical Aspects

This research was submitted to the Research Ethics Committee of the Federal University of Ceara and was duly approved under protocol number 3,728,390. CAAE: 22310819.6.0000.5054

Study Characterization

This study is an analytical, laboratory-based, retrospective, and observational study and consists of the morphological and immunohistochemical evaluation of OED and HOM cases.

Sample

The sample comprised 20 cases of OPMDs (presenting different OED degrees) obtained from incisional biopsies at the Stomatology Clinic of the Federal University of Ceara (UFC) campuses in Fortaleza and Sobral, and 20 cases of HOM from mucoceles excised at the same clinic. OEDs were classified by the WHO grading system as mild, moderate, and severe and by the binary system as low and high risk.

Inclusion and Exclusion Criteria for the Sample

OPMDs presenting different OED degrees and paraffin blocks with sufficient material for analysis were included in the study. Cases of actinic cheilitis, as well as duplicate lesions from the same patient, even if distinct, and cases with inadequately filled anatomopathological records were excluded from the sample.

Morphological Study

Histomorphological analyses were performed on a binocular light microscope (Leica DM500) at 400x magnification on 5µm thick sections, fixed and stained with Hematoxylin & Eosin (H&E) method. Each case's analysis was identified by the diagnosis with a histomorphological pattern individually and classified according to the WHO Dysplasia Grading System (VERED M e WRIGHT JM, 2022) and Binary System (KUJAN O, et al., 2006), as previously described.

Immunohistochemical Study

For the immunohistochemical study, 3 µm thick histological sections were made from paraffin blocks and prepared on pre-labeled slides. Antigen retrieval was performed by immersing the slides in a 10 mM citrate buffer solution (pH = 6.0) in a water bath at 98°C. Endogenous peroxidase activity was blocked for 30 minutes with 0.3% hydrogen peroxide, followed by a 1% protein block for 10 minutes. Sections were incubated with primary antibodies anti-PI3K (clone ab86714, Abcam®) at a dilution of 1:400, anti-PTEN (clone ab31392, Abcam®) at a dilution of 1:400, and anti-S6K1 (clone ab60948 Abcam®) at a dilution of 1:200, overnight, at 4°C. Samples were incubated with the LSAB Kit secondary antibody (DAKO®, Carpentaria, CA, USA) for 30

minutes at room temperature, and the reaction was revealed with a 3-3'-diaminobenzidine chromogenic solution (Sigma, St. Louis, MO, USA) for 5 minutes in a dark chamber. Harris hematoxylin was used for counterstaining. Finally, coverslips were placed on the samples on silanized slides, which were examined under a Leica DM 2000 optical microscope. A breast adenocarcinoma sample was used as a positive control for PTEN and S6K1 and muscular tissue for PI3K. The negative control was obtained by excluding the primary antibody.

Immunohistochemical Evaluation

The positivity parameter of immunostaining in all specimens included in the sample consisted of cells showing brown staining in membrane, cytoplasm, and/or nucleus regions, considering the intensity of cytoplasmic and nuclear immunoexpression as mild, moderate, or intense. PTEN, PI3K, and S6K1 expression levels were assessed based on the staining intensity (SI) and the percentage of positive cells (PP) in the entire tissue section. PTEN was analyzed counting the number of positive cells in each field and quantifying the intensity (no staining, weak, moderate or strong staining) and the cellular location (nucleus and/or cytoplasm), according to the study of Chaves FN, et al. (2020). The data gave normal variation and the positive cell average was determined in every case for possible statistical analysis. PI3K and S6K1 staining were evaluated by an H-score, calculated by multiplying PP by the corresponding SI (1=weak, 2=moderate, and 3=strong), with a maximum score of 300 (100% x 3), according to the study of Esteva FJ, et al. (2010). H-scores greater than 50 were considered positive. The analysis was performed by a trained observer, with immunopositive cells evaluated for the location of cellular immunostaining for PTEN (nucleus or cytoplasm), PI3K (membranous or cytoplasmic), and S6K1 (nuclear).

Statistical Analysis

The data were tabulated in Microsoft Excel and exported to the Statistical Package for the Social Sciences (SPSS) version 20.0 for Windows, where analyses were performed with a 95% confidence level. Categorical data were expressed in absolute frequency and compared using Fisher's Exact Test or Pearson's Chi-square test, while continuous data were expressed as means ± SD of calculated indices and histoscores. These were analyzed using the Mann-Whitney test or Kruskal-Wallis test, followed by Dunn's post-test.

RESULTS

Clinicopathological findings

All the 20 cases of OPMD were clinically diagnosed as leukoplakia. According to clinical characteristics, a higher prevalence was observed in females (60%) in the age group of 60 years or older (average age of 64.24 years), with a slight predilection for the palate region (20%). Based on the WHO grading, mild dysplasia was prevalent in 48% of cases, moderate dysplasia in 32%, and severe dysplasia in 20% of cases. Regarding the binary dysplasia classification system, the majority of cases were high-risk (56%).

PI3K and S6K1 immunoexpression

The PI3K immunoexpression pattern was evaluated in the cytoplasm and cytoplasmic membrane of epithelial cells in OED and HOM cases. In all OED and HOM samples analyzed, there was no PI3K immunoexpression on the membrane. In the cytoplasm, 19 out of 20 DEO cases (95%) showed positivity for PI3K, while in HOM, this pattern was observed in 55% of samples (p=0.003). For S6K1, the nuclear immunoexpression pattern was analyzed, showing a statistical difference between OEDs (100% of positivity) and HOM (65%) (p=0.004) (**Table 1**).

Table 1 - Absolute numbers and percentages of PI3K and S6K1 immunoexpression in cases of HOM and OED.

	HOM		OED		P-value
PI3K (cytoplasm)	**Quantity**	**Percentage**	**Quantity**	**Percentage**	
Positive	11	55%	19	95%	**0.003**
Negative	9	45%	1	5%	
S6K1 (nucleus)	**Quantity**	**Percentage**	**Quantity**	**Percentage**	
Positive	13	65%	20	100%	**0.004**
Negative	7	35%	0	0%	

Source: Viana KF, et al., 2025.

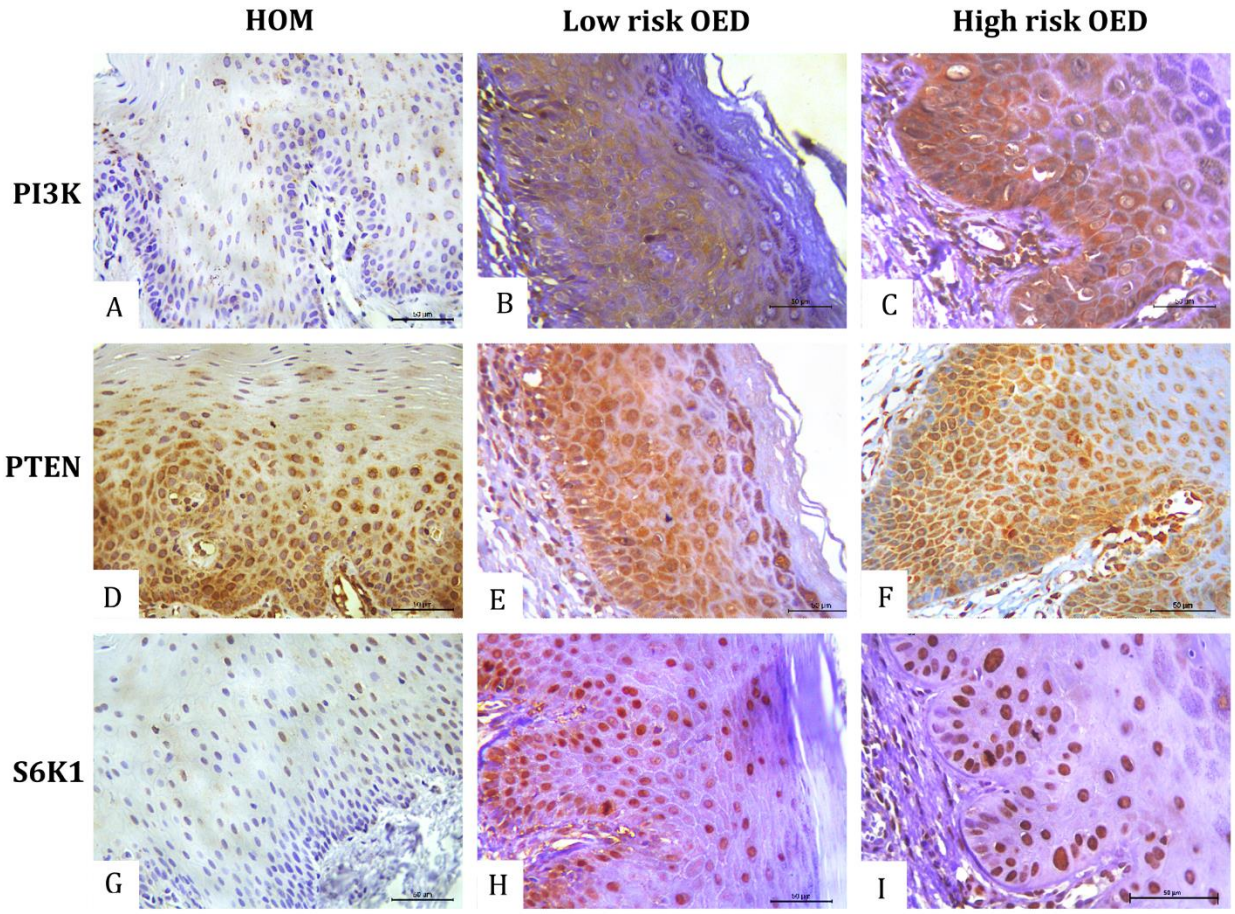
Using the WHO Grading System for OED, a higher percentage of PI3K immunoexpression was evident in moderate and severe OEDs (100%) compared to mild OEDs (88.9%). However, this data presented no statistical significance (**Table 2**). Applying the binary system evaluation, 100% of PI3K immunoexpression was observed in high-risk OEDs, compared to 87.5% in low-risk ones (p=0.209). For S6K1, in different grades of OEDs, no statistically significant difference was evident in the immunohistochemical pattern. **Figure 1** highlights the patterns observed in PI3K (**A**, **B**, **C**) and S6K1 (**G**, **H**, **I**).

Table 2 - Immunoexpression pattern of PI3K and S6K1 between the WHO and Binary grading systems expressed in absolute numbers and percentages.

	WHO grading system			
PI3K (cytoplasm)	**Mild**	**Moderate**	**Severe**	**P-value**
Positive	8 (88.9%)	6 (100%)	5 (100%)	0.526
Negative	1 (11.1%)	0 (0%)	0 (0%)	
S6K1 (nucleus)				
Positive	8 (100%)	8 (100%)	4 (100%)	1.000
Negative	0 (0%)	0 (0%)	0 (0%)	
	Binary grading system			
PI3K (cytoplasm)	**Low risk**	**High risk**		**P-value**
Positive	7 (87.5%)	12 (100%)		0.209
Negative	1 (12.5%)	0 (0%)		
S6K1 (nucleus)				
Positive	8 (100%)	12 (100%)		1.000
Negative	0 (0%)	0 (0%)		

Source: Viana KF, et al., 2025.

Figure 1 - A, B, and C: Photomicrographs showing the immunoexpression patterns of PI3K in HOM (A), low-risk OED (B), and high-risk OED (C). D, E, and F: Photomicrographs showing the immunoexpression patterns of PTEN in HOM (D), low-risk OED (E), and high-risk OED (F). G, H, and I: Photomicrographs showing the immunoexpression patterns of S6K1 in HOM (G), low-risk OED (H), and high-risk OED (I) (LSAB, 400x).



Source: Viana KF, et al., 2025.

PTEN immunoexpression

The immunohistochemical evaluation of PTEN investigated the expression pattern of this protein in the nucleus and cytoplasm locations, demonstrating a higher average number of cells with PTEN nuclear immunostaining in HOM cases (86.32±9.18) compared with OED cases (51.08±21.31) (p=0.003) (**Table 3**). In the cytoplasm, the immunoexpression pattern also presented a higher average number of cells with PTEN immunostaining in HOM (90.07±4.85), compared with OED cases (54.65±19.94) (p=0.001) (**Table 3**). Regarding the WHO grading system, a high average number of cells with both PTEN nuclear and cytoplasmatic immunostaining was found in mild OED, when compared with moderate and severe OED cases (p=0.005). In the binary grading system, there was a higher average number of cells with PTEN nuclear and cytoplasmatic immunostaining in low-risk OED compared to high-risk ones (**Table 3**) (**Figure 1**, **D**, **E**, **F**).

Table 3 - Immunoexpression pattern of PTEN between the WHO and Binary grading systems expressed in mean and standard deviation.

Nuclear PTEN				
Group	**Strong**	**Moderate**	**Weak**	**Positive**
HOM	1.13±0.81	51.60±13.01	33.58±8.03	86.32±9.18
OED	1.46±1.82	19.16±17.79	30.46±9.23	51.08±21.31
p-value	0.773	**0.005**	0,618	**0.003**
WHO grading				
Mild	2.31±1.89	32.31±18.79	32.31±9.09	66.93±18.70
Moderate	0.23±0.34	7.41±5.22	28.65±11.72	36.29±15.13
Severe	1.48±2.19	9.33±5.62	29.29±6.28	40.10±9.59
p-value	0.099	**0.012**	0.898	**0.005**
Binary system				
Low risk	2.57±1.81	35.86±15.97	33.49±8.79	71.92±10.66
High risk	0.69±1.44	7.60±5.46	28.36±9.27	36.66±12.96
p-value	**0.012**	**0.000**	0.333	**<0.001**
Cytoplasmatic PTEN				
Group	**Strong**	**Moderate**	**Weak**	**Positive**
HOM	1.25±1.13	45.88±13.21	42.94±13.08	90.07±4.85
OED	0.92±1.75	19.15±14.85	34.58±14.08	54.65±19.94
p-value	0.170	**0.005**	0.170	**0.001**
WHO grading				
Mild	1.87±2.27	28.10±15.92	34.66±13.76	64.63±19.40
Moderate	0.18±0.41	10.31±8.80	33.67±18.78	44.16±21.85
Severe	0.05±0.11	13.63±9.80	35.69±9.14	49.36±6.89
p-value	0.222	0.065	0.773	**0.049**
Binary system				
Low risk	2.08±2.31	31.19±13.32	36.00±13.88	69.27±13.46
High risk	0.11±0.31	10.81±9.14	33.60±14.70	44.52±17.42
p-value	**0.040**	**0.003**	0.616	**0.002**

Source: Viana KF, et al., 2025.

DISCUSSION

This study aimed to better understand the expression of different proteins in the PI3K pathway in cases of OPMDs presenting OED and HOM. Various factors, including age, gender, smoking habits, location, genetics, as well as different grades of dysplasia, have been suggested as predictors of the risk of malignant transformation in oral epithelial dysplasias, indicating a multifactorial and patient-specific nature in this process (WILS LJ, et al., 2023).

Molecular mechanisms involved in the progression and malignant transformation of oral epithelial dysplasias are not well elucidated, although the involvement of different molecules and signaling pathways in the process of epithelial dysplastic alteration and, consequently, in oral carcinogenesis is currently established (GUIMARÃES LM, et al., 2021; WILS LJ, et al., 2023). The PI3K pathway is one of the most dysregulated pathways in many types of cancer (HAN EJ, et al., 2023). PTEN acts as a tumor suppressor gene, and it is the pathway regulator, opposing the function of PI3K by dephosphorylating PIP3 into PIP2 (KAUR J, et al., 2010; MOURA ACD, et al., 2021). Low PTEN regulation is associated with PIP3 accumulation and, consequently, increased PI3K activation (CHAVES FN, et al., 2020). The results of this study revealed a more prevalent cytoplasmic immunoexpression pattern of PI3K in OED than in HOM, suggesting that this protein may play an important role in the development of dysplastic changes in the epithelium of OPMDs. PI3K activation seems to be closely related to the oral malignancy process since the positive immunoexpression of this protein is identified in the early stages of carcinogenesis through increased immunoexpression in OED (GIUDICE FS e SQUARIZE CH, 2013; KAUR J, et al., 2010; MOURA ACD, et al., 2021; WU J, et al., 2020).

The immunohistochemical analysis of this research also aimed to perform a detailed evaluation of the intracellular localization of PTEN since this protein may be present in both the cytoplasm and the nucleus, playing an important role in maintaining cellular morphology (when present in the cytoplasm) and in nuclear homeostasis regulation (when present in the nucleus) (CHAVES FN, et al., 2020). The immunohistochemical evaluation of PTEN revealed nuclear and cytoplasmic immunoexpression in both OED and the control group (HOM). The expression pattern presented either homogeneous or heterogeneous (positive and negative areas) in the OED and HOM samples.

In this study, the average number of cells with nuclear and cytoplasmic PTEN immunoexpression was significantly higher in HOM compared to OED cases, and it was lower between more advanced OED grading. These findings agree with those obtained in a study which investigated PTEN immunoexpression in HOM, OED, and OSCC (CHAVES FN, et al., 2020), observing that, in HOM, the presence of this protein was more prevalent than in OEDs. It is suggested that the difference in this event could be explained by possible genetic alterations of PTEN in the early stages of the PI3K pathway, as well as possible changes in protein translation after gene transcription. Thus, the nuclear and cytoplasmatic immunoexpression of PTEN in HOM and OED samples in this research point to an effective anticarcinogenic role of this protein.

Moreover, a lower average of PTEN stained cells in the cytoplasm was observed in high-risk OED cases compared to the low-risk group. This event may be related to an attempt by PTEN to suppress the phosphorylation of PIP2 into PIP3 in more advanced dysplasia grades, as a higher percentage of PI3K immunoexpression was evident in high-grade dysplasias. Despite the findings of this research presenting immunoexpression of both PI3K and PTEN in OED, in areas with a higher quantity of cellular atypia, there was a higher PI3K immunopositivity and lower PTEN immunoexpression, demonstrating the antagonistic role these two proteins play in the pathway.

Regarding S6K1 expression, the higher frequency of immunoexpression of this protein in OED compared to HOM samples corroborates the fact that this marker is associated with events such as increased protein synthesis and cell growth, which are related to dysplastic processes (DELGADO L, et al., 2022). The results of this research also showed higher expression of this protein in low-risk dysplasias. This result can be explained by the fact that this protein is one of the main nuclear targets of the PI3K pathway through the activation of mTORC1, resulting in the phosphorylation of S6K1. This may be associated with the initial development and progression of OPMD. Therefore, it can be suggested that S6K1 may be involved in mechanisms related to dysplastic changes in the development and progression of OED.

Although the results of this research are significant, this study has some limitations. The first of these concerns the sample size, considered small to accurately express the results obtained, thus requiring studies to be carried out with a more robust sample that corroborate such findings.Furthermore, the present study presents a case-control design, therefore, there is also a need to carry out cohort studies with the aim of validating the findings of this research, mainly in the context of the role of the main PI3K pathway proteins in the progression of OED into OSCC and in the prognosis of patients affected by this condition.

CONCLUSION

The present study demonstrated that increased expression of PI3K and S6K1, coupled with reduced nuclear and cytoplasmic expression of PTEN, may contribute to the development and progression of oral epithelial dysplasias. The elevated expression of PI3K and S6K1 in OED suggests activation of the PI3K pathway, whereas the decreased PTEN expression in OED underscores its suppressive role in oral carcinogenesis. These findings indicate the potential use of these proteins as biomarkers in the progression of OED to oral squamous cell carcinoma. However, further studies are required to evaluate their diagnostic, therapeutic, and prognostic roles.

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