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Alterations in the immunoexpression of claudin-1 between different grades of oral epithelial dysplasias

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ABSTRACT

Claudins are transmembrane proteins that play a role in cell proliferation and adhesion and tumourigenesis. This study evaluated the immunoexpression of claudin-1 in the oral epithelial dysplasia (OED) (19 mild, 26 moderate, 3 severe). Diffuse staining predominated in mild (89.4%) and moderate (80.8%) OEDs. Immunoexpression in the middle and upper third was observed in all mild cases, whereas in moderate/severe dysplasias staining was observed in the upper third in 41.4% of cases, in the upper and middle third in 41.4%, and in the upper, middle and lower third in 17.2% ($P < 0.05$). All mild OEDs and 73.1% of moderate cases presented only membrane staining, whereas membrane/cytoplasmic staining was observed in severe cases. Staining intensity was weak in 60% of parakeratinized OEDs and moderate/strong in orthokeratinized OEDs (60.8%) ($P > 0.05$). The differences in the immunoexpression of claudin-1 between different grades of OEDs suggest the involvement of this protein in the progression of dysplasias.

1. Introduction

The intercellular junctional complexes are important structures in the architecture and physiological function of tissues of multicellular organisms. There are three main types of intercellular junctions: tight junctions, adherent junctions, and gap junctions.[1–3] Tight junctions are proteins located in the apical portion of the junctional complex, which form a belt around the cell.[3,4] These junctions are found in various epithelial tissues, including stratified squamous epithelium.[5] Tight junctions are involved in mechanisms that regulate transcription, proliferation and cell polarity, in addition to forming a diffusion barrier.[1,6,7]

Tight junctions consist of peripheral and transmembrane proteins.[8] The transmembrane proteins can be divided into three types: occludins, junctional adhesion molecules, and claudins.[4,9] Claudins are considered to be the main tight junction-forming proteins.[8,10] These proteins comprise a family of 24 members and claudin-1 has been shown to be essential for the function of tight junctions.[2,6]

Claudins have been associated with the pathogenesis of neoplastic processes, since alterations in these structures may lead to increased nutrient diffusion and influence other factors that promote the development of tumours such as human carcinomas.[10,11] Biochemical and molecular changes in cells precede the establishment of cancer, and the dysregulation of different proteins might be involved in this process. Amongst these proteins there are claudins, which influence diverse cell functions.

Immunoreactivity for claudins can be seen in different potentially malignant lesions and carcinomas. There are, however, tissue type-specific differences in their expression. Investigators have showed the overexpression of these proteins in epithelial dysplasias,[5,12] cervical intraepithelial

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neoplasia,[13,14] oral squamous cell carcinoma,[5,15,16] while others showed low expression in skin carcinoma,[18] oesophageal carcinoma,[17–21] colon carcinoma,[19,22,23] breast cancer,[19,24,25] gastric carcinoma,[26] and prostate cancer.[27] Besides, cytoplasmatic and nuclear mislocalization were observed in oesophageal[21] and colon[23] carcinomas, respectively.

A potential mechanism by which alterations in the expression of claudins may contribute to oral carcinogenesis is through destabilization of tight junctions, resulting in loss of adhesion properties, known to be involved in early steps of invasion and metastasis.[5] Few studies have investigated the expression of claudins in oral potentially malignant lesions, especially oral epithelial dysplasias (OEDs). Therefore, the objective of the present study was to evaluate the immunohistochemical expression of claudin-1 in different grades of OED in order to determine whether a relationship exists between the expression of this protein and oral epithelial alterations that occur during the development of dysplasia.

2. Materials and methods

Forty-eight OED specimens fixed in 10% formaldehyde and embedded in paraffin were obtained from the archives of the Discipline of Oral Pathology, Department of Dentistry, Federal University of Rio Grande do Norte. Haematoxylin-eosin-stained sections (5 μm) were used for morphological analysis. The classification criteria of the World Health Organization were used for the definition of the different grades of OED (Table 1).[28]

Histological sections (3 μm thick) were submitted to immunohistochemistry by the streptavidin-biotin method. Antigen retrieval was performed with citrate, pH 6.0, in a microwave for 10 min. The specimens were incubated with anti-claudin-1 (clone JAY.8, ZYMED, South San Francisco, CA), diluted 1:50, as primary antibody for 60 min.

The immunohistochemical expression of claudin-1 in the different grades of OED was analyzed considering distribution, epithelial localization, cellular localization and intensity of staining. The staining distribution was classified into focal (≤30% of the epithelium) and diffuse (>30% of the epithelium), adapted from Sheehan et al.[27] Epithelial localization, i.e., the predominant site of immunohistochemical expression in the epithelium, was divided into upper, middle and lower third according to the criteria of Usami.[20] Cellular localization, corresponding to the site of expression in the cell, was classified as membrane or membrane/cytoplasmic staining, adapted from Sobel et al.[13] Staining intensity, corresponding to the degree of immunohistochemical expression of claudin-1 in the specimens selected, was evaluated subjectively on a qualitative scale and was defined as weak, moderate or strong.[27] For statistical analysis, moderate staining was analyzed together with strong staining when staining intensity was correlated with the keratinization type of the epithelium and grade of OED. Similarly, moderate and severe OEDs were analyzed together when the grade of OED was correlated with epithelial localization and staining intensity. Epithelium of normal oral mucosa, obtained from cosmetic surgery, was used as positive control. All assessments were independently performed by two previously calibrated observers and were then compared until a consensus was reached.

Descriptive statistics and Pearson's Chi-square test were used for analysis of the results using the Statistical Package for the Social Sciences, version 17.0 (SPSS, Chicago, IL). A P value <0.05 was considered to indicate statistical significance.

The study was approved by the Ethics Committee of the Federal University of Rio Grande do Norte, Brazil (protocol 036/2009).

3. Results

The sample consisted of 48 cases of OED, including 19 (39.6%) mild cases, 26 (54.2%) moderate cases, and 3 (6.3%) severe cases, defined according to the 2005 classification criteria of the World Health Organization.[28]

With respect to distribution, staining was focal in 10.5% of mild dysplasia cases and diffuse in 89.4%. In moderate dysplasias, focal staining was observed in 19.2% of cases and diffuse staining in 80.8%. Focal staining predominated in severe dysplasias (66.7%), whereas diffuse staining was observed in 33.3% of these cases.

Table 1 – Criteria used for diagnosing grades of oral epithelial dysplasias (WHO, 2005).

Grades of OED	Criteria of grades of OED
Mild dysplasia	Architectural disturbance limited to the lower third of the epithelium accompanied by minimal cytological atypia.
Moderate dysplasia	Architectural disturbance extending into the middle third of the epithelium accompanied by cytological atypia.
Severe dysplasia	Architectural disturbance with greater than two thirds of the epithelium showing architectural disturbance with associated cytological atypia. However, architectural disturbance extending into the middle third of the epithelium with sufficient cytological atypia is upgraded from moderate to severe dysplasia.

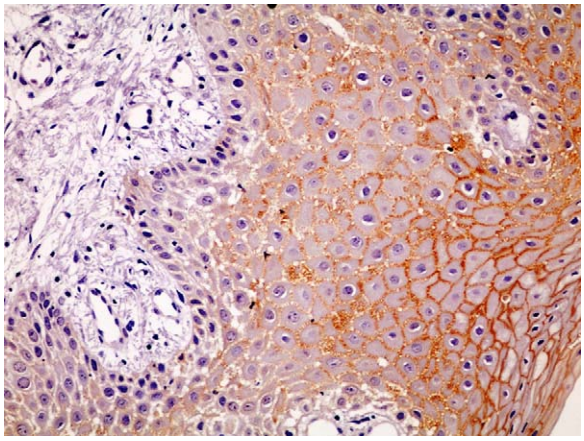

Fig. 1 – Immunohistochemical expression claudin-1 in the middle and upper third in mild OED (SABC, 400×).

Table 2 – Correlation between the grades of OED and epithelial localization of the immunohistochemical expression of claudin-1.

Grades of OED	Epithelial localization			
	Upper third *n* (%)	Upper and middle third *n* (%)	Upper, middle and lower third *n* (%)	Total *n* (%)
Mild *n* (%)	0 (0)	19 (100.0)	0 (0)	19 (100.0)
Moderate/severe *n* (%)	12 (41.4)	12 (41.4)	5 (17.2)	29 (100.0)
Total *n* (%)	12 (25.0)	31 (64.6)	5 (10.4)	48 (100.0)

Chi-square test ($P < 0.05$).

Table 3 – Correlation between the grades of OED and cellular localization of the immunohistochemical expression of claudin-1.

Grades of OED	Cellular localization		
	Membrane *n* (%)	Membrane/cytoplasm *n* (%)	Total *n* (%)
Mild *n* (%)	19 (100.0)	0 (0)	19 (100.0)
Moderate *n* (%)	19 (73.1)	10 (26.9)	26 (100.0)
Severe *n* (%)	0 (0)	3 (100.0)	3 (100.0)
Total *n* (%)	38 (79.1%)	10 (20.8%)	48 (100.0)

Chi-square test ($P < 0.05$).

Analysis of epithelial localization showed immunoexpression of claudin-1 in the middle and upper third in all mild OED cases (100%) (Fig. 1). In contrast, in moderate/severe dysplasias staining was only observed in the upper third in 41.4% of cases, in the upper and middle third in 41.4%, and in the upper, middle and lower third in 17.2% ($P < 0.05$) (Table 2).

All mild OED cases (100%) and 73.1% of moderate cases presented only membrane staining, whereas membrane/cytoplasmic staining was observed in all severe OED cases (100%) (Table 3; Fig. 2).

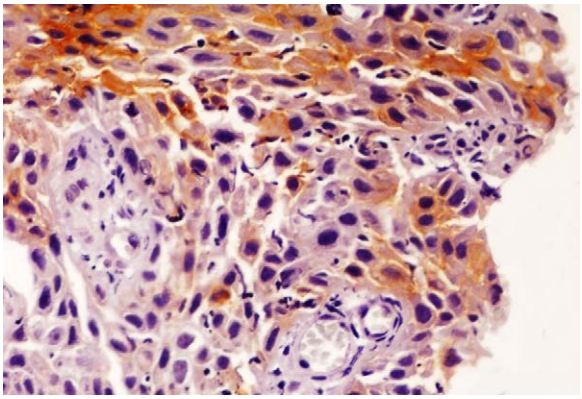

Fig. 2 – Membrane and cytoplasmic staining of claudin-1 in severe OED (SABC, 400×).

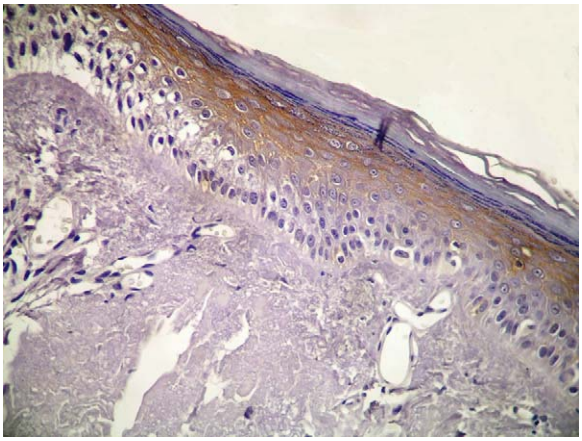

Fig. 3 – Differentiates in the intensity of the expression of claudin-1 in parakeratinized and orthokeratinized epithelium (SABC, 200×).

Table 4 – Correlation between the keratinization type and intensity of expression of the claudin-1.

Keratinization type	Intensity of expression		
	Weak *n* (%)	Moderate/strong *n* (%)	Total *n* (%)
Parakeratinized *n* (%)	15 (60.0)	10 (40.0)	25 (100.0)
Orthokeratinized *n* (%)	9 (39.2)	14 (60.8)	23 (100.0)
Total *n* (%)	24 (50.0)	24 (50.0)	48 (100.0)

Chi-square test ($P > 0.05$).

Table 5 – Correlation between the grades of OED and intensity of expression of the claudin-1.

Grades of OED	Intensity of expression		
	Weak *n* (%)	Moderate/strong *n* (%)	Total *n* (%)
Mild *n* (%)	10 (52.6)	9 (47.4)	19 (100.0)
Moderate/severe *n* (%)	14 (48.3)	15 (51.7)	29 (100.0)
Total *n* (%)	24 (50.0)	24 (50.0)	48 (100.0)

Chi-square test ($P > 0.05$).

Staining intensity was predominantly weak in OEDs with parakeratinized stratum corneum (60%), whereas moderate/strong staining was more frequent in orthokeratinized OEDs (60.8%) ($P > 0.05$) (Table 4; Fig. 3).

Overall analysis of staining intensity showed weak staining in half the cases (50%), moderate staining in 41.7%, and strong staining in 8.3%. No significant correlation was observed between staining intensity and OED grade, with staining being weak in 52.6% of mild OED cases and strong in 51.7% of moderate/severe cases ($P > 0.05$) (Table 5; Fig. 4).

4. Discussion

Cell–cell adhesion is generally altered in cancer, a fact resulting in cell dissemination, invasion of neighboring structures and metastasis.[5,8,13] The expression of claudins is dysregulated in a series of conditions such as potentially malignant lesions and malignant neoplasms as demonstrated by the overexpression, loss of expression and reduced expression of these proteins in different diseases.[5,29,30]

In the present study, moderate dysplasia was the predominant grade, followed by mild and severe dysplasia. Lee et al.[31] evaluating the prevalence of oral leukoplakia, identified OEDs in 45.6% of cases, including mild dysplasia in 42%, moderate dysplasia in 49% and severe dysplasia in 9%. In a study investigating the clinical presentation of epithelial dysplasias in 630 patients, Jaber et al.[32] observed a frequency of 41.7% of mild OED, 29% of moderate dysplasia and 23.8% of severe dysplasia. These results indicate a low prevalence of severe grade OEDs, in agreement with the present study, and alternation in the number of moderate and mild cases.

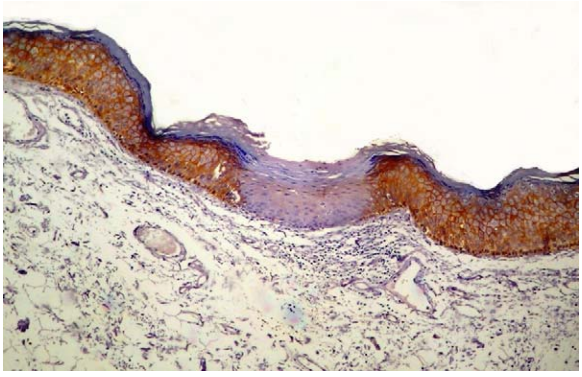

Fig. 4 – Strong immunohistochemical expression the claudin-1 in moderate OED (SABC, 400×).

Few studies have examined the risk of malignant transformation of different grades of OEDs. However, it has been suggested that the grade of dysplasia may guide the adequate treatment of these potentially malignant lesions.[33] A study involving Irish patients evaluated the potential of malignant transformation of oral mucosa white lesions and development of previously dysplastic lesions into malignant neoplasms was observed in 15% of cases.[34] Lee et al.[35] observed that moderate and severe dysplasias possess a 2.3 times higher risk of undergoing malignant transformation than mild dysplasias or hyperplasias. Another study conducted on patients with oral leukoplakia showed that cases of moderate or severe dysplasia presented a higher risk of transformation into carcinomas than mild dyplasias.[36]

In the present study, in cases of mild OED no immunoexpression of claudin-1 was detected in the lower third of the epithelium. This finding might be explained by the lack of constitutive expression of the protein in the basal cell layer of normal oral mucosa as demonstrated by Dos Reis et al.[5] In addition, several studies have shown that claudins present in tight junctions are mainly expressed in the upper layers of the epidermis.[6,37,38] Reduced expression of claudin-1 in the middle third was observed in moderate and severe cases of OED. These findings suggest that, in mild OEDs, the basal cell layer maintains characteristics similar to those of normal oral mucosa, and that more marked modifications, i.e., those producing molecular changes detected immunohistochemically, start to arise only in moderate and severe grade OEDs.

Studies have demonstrated the loss or reduction of claudin expression in both dysplasias and neoplasms. Arabzadeh et al.[17] induced tumourigenesis in the epidermis of rats and observed the loss of expression of claudin-1, -6, -11, -12 and -18 in the basal and suprabasal layer with the progression of carcinogenesis. Resnick et al.[22] showed the loss of claudin-1 expression in colon cancer, which was strongly correlated with recurrence and low patient survival. Kramer et al.[24] reported the loss of claudin-1 expression in primary breast tumours when compared to its expression in other human tissues, suggesting that this protein is involved in the development of breast cancer and, possibly, other epithelial tumours. Reduced expression of claudin-7 in ductal breast carcinoma was reported by Kominsky et al.[25], probably influencing cell dissemination and increasing the metastatic potential of the tumour. According to these authors, hypermethylation of promotor sequences in the claudin-7 gene is the main mechanism responsible for the reduced expression of this protein.

The loss or reduction of claudin-7 expression was also demonstrated in oesophageal squamous cell carcinoma and

was correlated with invasion and metastatic potential of the tumour.[20] Al Moustafa et al.[39] compared cDNA microarrays between normal epithelium and head and neck squamous cell carcinoma and found down-regulation of claudin-7 gene expression in neoplastic cells. Sheehan et al.[27] observed reduced expression of claudin-1 and -7 in prostate adenocarcinomas when compared to adjacent normal tissue areas, which was correlated with a more aggressive behaviour of the tumour. These findings agree with the view that the loss or reduction of expression of these proteins is involved in the mechanism of carcinogenesis. In the case of OEDs, this reduced expression in dysplastic areas might be associated with the onset of alterations within the cellular environment that result in the malignant transformation of epithelial tissue observed in some OEDs.

On the other hand, various studies have demonstrated the overexpression of claudins in different neoplasms and potentially malignant lesions. Dos Reis et al.[5] observed an increased expression of claudin-1 in oral squamous cell carcinomas, especially those presenting perineural and angiolymphatic invasion, and correlated this increase with the aggressive behaviour of the tumour. Strong staining for this protein in the lower third of the epithelium was also observed in adjacent dysplastic areas. Increased expression of claudin-1 and -7 was also found in squamous cell carcinomas of the tongue and was associated with reduced patient survival.[15] A recent study on cervical squamous cell carcinomas cited an increase in the expression of claudin-1 and -7 as one of the initial changes that occur during the progression and malignant transformation of normal cervical squamous cells.[14] In cervical intraepithelial neoplasia, high expression of claudin-1 was strongly associated with the initial phases of carcinogenesis and may serve as an important diagnostic marker for this type of tumour.[13] According to Resnick et al.[22] the increased expression of claudins suggests that their aberrant form may directly interfere with the structure and function of tight junctions, resulting in significant disorganization and increased cell permeability, and thus contribute to the development of cancer.

The reason for the discrepancy observed in the expression of claudins between different types of lesions is still unclear, but might be related to differences in the function of these proteins in each tissue or even to the specific characteristics of the tissue microenvironment.[10]

Membrane expression of claudin-1 was observed in all OED cases. However, additional cytoplasmic staining was found in all cases of severe OED and in about 1/3 of moderate cases. The cytoplasmic localization of claudins might be related to the loss of function of these proteins, resulting from different mechanisms such as protein phosphorylation.[17,40–42] Some studies associated the cytoplasmic localization of tight junctions with tumour progression. Investigating colon carcinoma, Dhawan et al.[23] observed an increased expression and translocation of claudin-1 to the cytoplasm in neoplastic cells. The degree of translocation was higher when expression was analyzed in metastatic neoplastic cells. Arabzadeh et al.[17] showed translocation of claudin-6, -11, -12 and -18 to the cytoplasm with the progression of carcinogenesis in the epidermis of rats. Lioni et al.[21] demonstrated that in the normal human oesophagus expression of claudin-4 and -7 is confined to the cell membrane of differentiated keratinocytes, whereas in oesophageal squamous cell carcinoma the expression of these proteins is translocated to the cytoplasm. This suggests that the presence of claudin in the cytoplasm has some function in intracellular signaling, which may play an important role in the progression of OEDs, since cytoplasmic expression was observed in more severe degrees. However, this mechanism is still unclear.

According to Ivanov et al.[41], translocation of tight junctions present in the cell membrane to the cytoplasm might also be related to the internalization (endocytosis) of these proteins and consequent loss or reduction of their function. However, studies investigating the involvement of tight junctions in this process are scarce. Several studies have focused on the mechanism of endocytosis of adherent junctions, such as E-cadherins, which seems to play an important role in the development and metastasis of different cancers.[43] Internalization of these proteins, as well as of tight junctions, has been shown to occur in response to physiological and pathological processes.[41] Pathological stimuli such as cytokines, growth factors, oxidative stress and bacterial and viral toxins have been indicated as factors that stimulate endocytosis.[41,44,45] On the basis of these studies, the translocation of claudin-1 to the cytoplasm observed in the present investigation may also be related to internalization of this protein in moderate and severe cases of OED, promoting the down-regulation of this protein.

Yuki et al.[6] and Yamamoto et al.[37] showed that tight junctions are mainly present in the granular layer of the epidermis, thus playing an important role as an epithelial barrier. Yoshida et al.[46] reported the expression of occludins only in the granular cell layer of the epidermis, and Furuse et al.[38] demonstrated that claudin-1 and -4 are also concentrated in this layer. These findings agree with the present results and support the view that claudin-1 plays a fundamental role in the upper portions of epithelial tissue, especially in the granular cell layer.

Since the recent discovery of claudins, numerous studies have demonstrated the loss, reduction or increase of expression of these proteins in various types of cancer, findings indicating their involvement in tumourigenesis. Thus, these proteins might be a promising target in studies investigating both the diagnosis and prognosis of cancer and cancer therapy.

Furthermore, the alterations in the immunoexpression of claudin-1 observed between different grades of OEDs suggest that this protein might be involved in the progression of dysplasias that may culminate in the malignant transformation of the epithelium. However, as the present work is not a prospective study, it cannot evaluate the potential for malignant transformation of OED. In addition, Smith et al.[47] report other significant potential sources of bias, such as the lack of analysis of the effect of environmental risk factors and method of biopsy (incisional vs. excisional), and results of retrospective, single centre, observational studies.

Thus, further long-term outcome studies, in which follow-up data were recorded, and a large number of cases, with multi-centre collaboration, are necessary to determine the specific mechanism underlying this event. Although our work does not overcome these problems, it can serve as guide for future prospective well-designed studies.

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Ethical approval

The Ethical Approval for our research was given by the Ethics Committee of the Federal University of Rio Grande do Norte, Brazil (protocol number: 036/2009).

Conflict of interest

We do not have any conflicts of interest to declare.

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