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Demonstration of Utility of Multiplex Immunohistochemistry Technique in Oral Pathology

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Abstract:

At present, researchers are continually attempting to identify different tissue markers to detect and identify target molecules involved in global malice such as Oral Squamous Cell Carcinoma to understand the carcinogenetic process and possibly develop therapeutic targets. However, the majority of research in the field of Oral Pathology involves the use of conventional immunohistochemistry techniques. The tissue specimens received by oral incisional biopsies are not always generously available, thus, making consecutive sectioning of the specimen to observe multiple biomarkers infeasible. The multiplex immunohistochemistry technique allows to simultaneously observe multiple markers within a single section, thereby having an advantage of conserving biological and economical resources. Even so, it is seldom used in the field of Oral and Maxillofacial Pathology possibly because of relatively scant and scattered literature. In this context, the following report demonstrates the utility of the multiplex immunohistochemistry technique in illustrating the concomitant expression of E-Cadherin and Podoplanin in Oral Squamous Cell Carcinoma. The results of this technique demonstrated obvious benefits of the multiplex immunohistochemistry technique in which it was possible to simultaneously visualize the changes in expression of target molecules occurring during the pathologic process, thus, providing a better insight into their possible role and correlation with each other within the tumor microenvironment. It could be inferred that the multiplex immunohistochemistry technique can be utilized in the field of Oral Pathology for efficiently obtaining a more elucidative microscopic picture in other oral lesions involving complex molecular interactions such as odontogenic tumors.

Keywords: dual immunostaining; oral cancer; cancer microenvironment; Oral Squamous Cell Carcinoma

Introduction

The immunohistochemistry (IHC) technique helps in assessing the distribution of various biomolecules involved in different pathological processes through their origin, localization, and intensity. It is commonly employed in for obtaining a precise and confirmatory diagnosis, which would otherwise not be possible by routine Hematoxylin and Eosin-stained sections. [1] A significant portion of cancers usually occurs in the head and neck region, particularly, the oral cavity, most of which are

subjected to incisional biopsies for diagnosis and treatment planning. The majority of the oral malignant lesions comprise Oral Squamous Cell Carcinoma (OSCC). [2] The relatively small and closed environment of the oral cavity is unable to confer much spatial freedom to oral surgeons and thus, the biopsies obtained from oral lesions tend to be of much smaller dimensions. [3] Consequently, an Oral Pathologist is unable to generously obtain serial sections of the specimen to observe various biomarkers separately which may lead to loss of tissue quantity and its subsequent exhaustion. As indeed, numerous molecular interactions are involved in the complex process of tumorigenesis, however, conventional IHC allows identification of only a single biomarker. It would only be logical to visualize the spatial relationship between different molecules involved in the pathogenesis of a lesion to better understand the disease process and correlate them for a relatively more accurate diagnostic and prognostic determination. This necessitates visualization of multiple antigenic epitopes simultaneously within a single microscopic picture that can be achieved by the multiplex immunohistochemistry (mIHC) technique. [4] Generally, dual immunostaining could be considered as a subset of the mIHC technique wherein the expression of two different antigenic epitopes is identified concomitantly. [5]

In this context, mIHC would prove to be advantageous in several ways. For example, it would help preserve the important biological resource i.e. the tissue specimen, as well as make the process more economically feasible by cutting short the time as well as expenditure required for multiple IHC procedures. This would especially hold for tissue specimens generally yielded by an incisional biopsy of an oral lesion. Despite numerous advantages, the mIHC technique has seldom been used in the field of Oral Pathology wherein the practitioners yet continue to expend multiple sections of the tissue for diverse immunohistochemical panels.

In the present research, we aimed to demonstrate the utility of dual immunostaining in illustrating the pathogenesis of a disease process, particularly, in the field of Oral Pathology. E-Cadherin is a cell adhesion molecule generally present in desmosomal attachments of squamous epithelial cells. Its downregulation has been associated with the loss of cohesion between the cells which further plays a key role in the pathogenesis of OSCC. [6] Podoplanin brings about changes in the actin cytoskeleton of the squamous epithelial cells that increase their motility consequently facilitating their migration and invasion. [7] Concurrent immunohistochemical expression of E-cadherin and Podoplanin was assessed in tissue specimens of OSCC to better understand the qualitative and spatial relationship between the two biomolecules. Yet another objective of the study was to correlate their expression for prognostic determination of the lesion.

Materials and Methods

Preparatory procedures

10 formalin-fixed paraffin-embedded tissue (FFPE) blocks of histopathologically confirmed cases of OSCC were obtained from the archives of the institutional Department of Oral Pathology and Microbiology. The specimens were graded according to Broder's criteria [8] and of the total number of OSCC tissues (n=10), 7 were diagnosed as Well-differentiated Oral Squamous Cell Carcinoma (WDOSCC) while the remaining 3 comprised of Moderately differentiated Oral Squamous Cell Carcinoma (MDOSCC). An equivalent number of FFPE blocks of normal tissue (n=10) mixed and matched for age and gender were used as controls. Sections of 4 μm thickness were obtained using a

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semi-automated microtome (Leica Biosystems, Germany), following which they were deparaffinized in xylene and rehydrated to water through a graded series of alcohol.

Dual Immunostaining technique

The stain-destain-restain technique was followed for the dual immunostaining procedure [9]. The tissue sections were incubated in 3% hydrogen peroxide buffer for 30 minutes. Antigens present in the tissue were retrieved in a 10mM citrate buffer for another 30 minutes (pH = 6.0). After a thorough wash with Tris-buffered Saline (TBS), the sections were incubated overnight in a cocktail solution comprising of appropriate concentrations of Monoclonal E-cadherin (Clone NCH-38, M3612, DAKO; Agilent Technologies, Inc., Santa Clara, CA, USA) and Podoplanin mouse monoclonal antibody (Clone 8.1.1 BioLegend, Inc, San Diego, CA, USA) at 4ºC. The following day, the tissue sections were incubated with secondary antibodies- anti-Mouse-Horseradish Peroxidase (HRP) conjugated with Diaminobenzidine (DAB) substrate and anti-Rabbit-Alkaline Phosphatase (AP) (MRCT525) conjugated with fast red chromogen for 60 minutes at room temperature. Subsequent staining was carried out by means of Biocare MACH2 Double Stain 2 Polymer Detection kit.

Mayer's Hematoxylin was utilized for nuclear counterstaining. The sections were then dehydrated in increasing grades of alcohol, cleared in xylene, and then stabilized by mounting with DPX. The overall procedure is delineated in **Figure 1**. Each batch was stained with phosphate-buffered saline (PBS) instead of the primary antibody as the negative control. Normal surface epithelial cells and lymphatic endothelial cells in the connective tissue stroma were stipulated as positive controls for E-Cadherin and Podoplanin respectively.

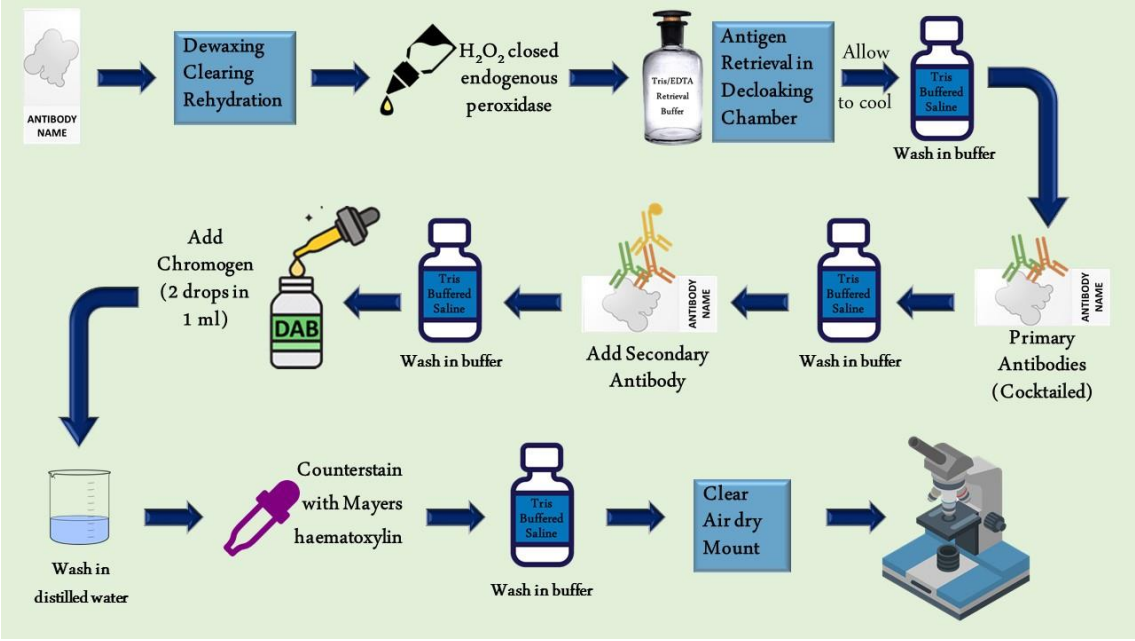

Figure 1: Delineates the steps involved in the dual immunostaining procedure Scoring of immune expression

The authors were divided into two groups each of which independently assessed the parameters about the nature of immunohistochemical staining. In case of gross discrepancy between the findings, the

slides were re-evaluated and results were decided based on a common consensus from both groups. The staining of both markers was assessed qualitatively based on their intensity and localization with subsequent scoring as follows:

E-Cadherin [10]

Based on Intensity:

0= Negative expression; 1= Mild intensity; 2= Moderate Intensity; 3= Intensive stain

Based on Localization

0 = No positivity, 1= Cell Membrane, 2= Cytoplasm, 3= Membranous + Cytoplasmic, 4= Nucleus

Podoplanin [11]

0= No expression is observed in any part of the epithelium;
1=expression is weaker than that in lymphatic vessels & observed in the basal layer of the epithelium;
2=expression is weaker than that in lymphatic vessels & observed in more than two layers of the epithelium;
3=equal to that in lymphatic vessels & observed in the basal layer of the epithelium;
4= equal to that in lymphatic vessels & observed in more than two layers of the epithelium.

Results

Immunohistochemical analysis revealed that E-Cadherin was intensely expressed in all the cases of WDOSCC (n=7) and normal tissues (n=10). A reduction in the intensity of E-cadherin expression was observed in MDOSCC. There was a demonstrable cytoplasmic shift in localization of its expression in 2 cases of WDOSCC and 2 cases of MDOSCC.

No expression of Podoplanin was detected in all the normal cases (n=10) and 1 case of WDOSCC. In the majority of OSCC cases, Podoplanin was expressed with a staining intensity equal to that in the lymphatic vessels and was observed in the basal layer of epithelium i.e Score 3. In only two cases of MDOSCC, more than two layers of neoplastic epithelium exhibited intense Podoplanin expression.

Overall, Podoplanin was expressed in the membrane of the cells present peripherally in the tumor nests and E-Cadherin was expressed in the membrane of the cells present centrally within the tumor nests **(Table 1)**. Although the sample size was not adequate to provide valid statistical results, a general trend of increase in qualitative expression of Podoplanin along concomittant with reduced expression of E-Cadherin in OSCC was demonstrable by means of mIHC technique **(Figure 2).**

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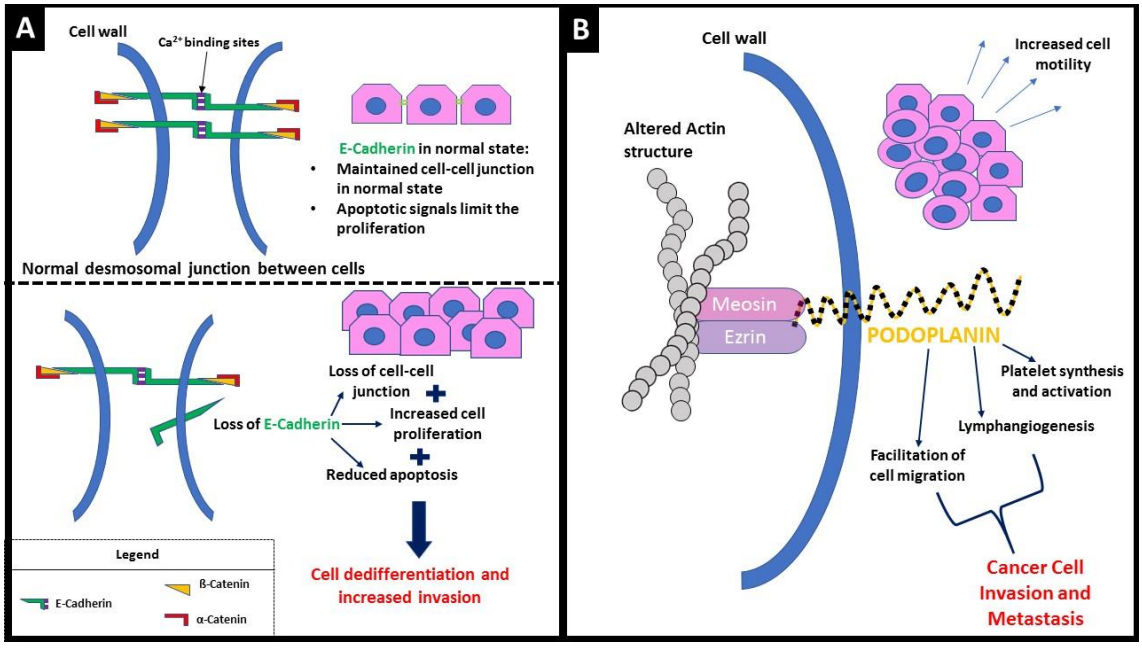

Figure 2: Illustrates immunohistochemical expression of E-Cadherin (pink) and Podoplanin (brown) under the 40x objective of the light microscope. A) Intense membranous expression of E-Cadherin within the central epithelial cells and weak membranous expression of Podoplanin within the peripheral epithelial cells of the neoplastic island in WDOSCC; B) Reduction in intensity of membranous expression of E-Cadherin with the relatively intense membranous expression of Podoplanin in MDOSCC; C) and D) Cytoplasmic shift of E-Cadherin along with intense membranous expression of Podoplanin in more than two layers of peripheral neoplastic epithelial cells in MDOSCC

Discussion

E-cadherin is closely associated with trans-membranous catenins of the squamous epithelial cells in order and is responsible for maintaining the structural integrity of the intercellular bridges present between them. Its downregulation would, therefore, lead to loss of cellular cohesion which is an essential hallmark of invasion. [12] The cytoplasmic shift of E-Cadherin has been associated with invasion and poor prognosis in various carcinomas, including OSCC which was demonstrable by our findings. [10] Generally absent in the epithelial cells, Podoplanin gets accumulated peripherally in their cytoplasm in association with Ezrin and Meosin (molecules forming an integral part of the actin cytoskeleton). This association brings about changes in the actin cytoskeleton and increases the motility of the cells which in turn facilitates the invasion of epithelial cells into the connective tissue. [11] Thus, its upregulation would be an indicator of a more progressed malignancy as observed in our cases. The staining can be either single-color, two-color, and inverse two-color. [5] The staining intensity depends on some factors like, the type of enzyme conjugate, the type of chromogen that has been selected, the incubation time and the temperature maintained during the incubation. Optimal color contrast is obtained with two complementary colors, for example, blue and yellow. Even the slightest amount of antigen can be detected due to highly sensitive detection systems and vice versa. [13]

The technique facilitated a faster, conservative, and more precise visualization of multiple markers, thus, providing a better insight into their relationship in a single picture. Previous studies have also emphasized these advantages of the mIHC technique. Wang et al. had correlated the expression of STOML-1 and STOML-2 with tumor progression and established a positive association between both

the markers while highlighting the advantages of the mIHC technique. [14] The role of the mIHC technique in the detection of changes in the tumor microenvironment of OSCC has also been demonstrated in recent research [15]. Yet another research has demonstrated the utility of the mIHC technique in prognostication of OSCC by concomitantly evaluating immunoexpression of tumor-associated lymphocytes. [16] Cross-reactivity amongst antibodies, inability to analyze markers in a similar location (membranous/ cytoplasmic/ nuclear) or in a similar type of cells (epithelial/ mesenchymal/ neural, etc.) for quantitative analysis, and relative unavailability of directly conjugated antibodies are a few limitations of mIHC technique. Since both the markers were expressed in the cellular membranes in some cases, quantitative analysis (counting the percentage of positive cells) became inadmissible because of their co-localization. This fact reinforces the need of using markers that are exclusively located in different parts of a cell, (nucleus or only membranous or in different parts of a tissue (epithelium or connective tissue), or different cell types (endothelial cells and fibroblasts) if the quantitative analysis is required in a tissue stained for multiple antigen epitomes. The success of dual immunostaining depends largely on the prevention of cross-reactivity between the immunoreagents and the choice of good color contrast. [13] Since both, the markers have already been associated with the carcinogenetic process and also their expression overlaps in the cell membrane, the standard quantitative analysis of immunoexpression by positive cell count was not undertaken. Our report presents a qualitative demonstration of the utility of the mIHC technique in demonstrating the spatial relation between two biomarkers within the same lesional tissue whilst maintaining feasibility. mIHC technique can also be effectively employed in other benign and malignant oral lesions, especially in lesions with complex odontogenesis wherein numerous biomolecules are involved.

Conclusion

Dual immunostaining is being widely used currently in determining the diagnosis and prognosis of various malignancies in the body, due to its advantage of determining multiple antigens simultaneously. However, there are only a few studies conducted on its applicability in oral lesions. A microscopic slide ideally stained by the mIHC technique can assist the Oral Pathologist in better understanding the behavior and gaining an insight about the pathogenesis of the lesion, making an accurate diagnosis with greater efficiency and determining the prognosis with greater precision. For these reasons, the mIHC technique would be indispensable too in the field of Oral Pathology and should be more frequently employed.

Declaration of interest statement: The authors have no conflicts of interest to declare.

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Table 1: Represents summarized results after grading the study samples for qualitative assessment of E-Cadherin and Podoplanin

Cases		E-Cadherin									Podoplanin				
		Intensity				Localization									
		0	1	2	3	0	1	2	3	4	0	1	2	3	4
WDOSCC	7				7		5	2			1			6	
MDOSCC	3		1	2			1	1	1					1	2
Normal	10				10		10				10				
Total	20		1	2	17		16	3	1		11			7	2