ORIGINAL ARTICLE

ROLE OF IMMUNOHISTOCHEMICAL EXPRESSION OF D2-40 MARKER IN HIGH-GRADE DYSPLASIA, MICROINVASIVE AND FRANKLY INVASIVE ORAL SQUAMOUS CELL CARCINOMA

Syeda Areeba Rehan, Wajeeha Rashid, Manahil Rahat

Department of Oral Pathology, Armed Forces Institute of Pathology, National University of Medical Sciences, Rawalpindi-Pakistan

Background: Oral squamous cell carcinoma (OSCC) is a prevalent malignancy that develops through a series of stages, ranging from high-grade dysplasia (HGD), microinvasive OSCC (MiOSCC) to frankly invasive OSCC. However, sometimes it is difficult to differentiate HGD from MiOSCC and invasive OSCC in histopathological morphology on routine staining. The aim of the study was to assess the expression patterns of D2-40 in these distinct stages of oral carcinogenesis, providing insights into its potential role as a biomarker for disease progression. **Methods:** This cross-sectional study was carried out in the Department of Histopathology, Armed Forces Institute of Pathology, Rawalpindi, Pakistan, from 1st Dec 2023 to 5th Feb 2024. It involved the application of a D2-40 immunohistochemical marker on a total of thirty tissue samples, ten patients diagnosed with High-grade dysplasia (HGD), ten with Microinvasive Oral squamous cell carcinoma (OSCC), and ten invasive OSCC cases. SPSS was used for data analysis and a p-value < 0.05 was considered significant. **Results:** The mean age of patients was 60.47±11.78 years, males were affected more (70%). D2-40 was expressed in different stages of oral carcinogenesis in increasing order as in 40% in HGD (4/10), and 90% in both microinvasive (9/10) and invasive OSCC (9/10) lesions. D2-40 IHC expression was associated with the patient's age and disease. **Conclusion:** Podoplanin (D2-40) has the potential to be a novel biomarker for the timely identification of microinvasion in early oral epithelial pathologies with diagnostic dilemmas.

Keywords: D2-40 monoclonal antibody; Dysplasia; Oral Squamous Cell Carcinoma; Podoplanin

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INTRODUCTION

In Asia, among malignancies in the head and neck region, oral squamous cell carcinoma accounts for two-thirds of the lesions.[1] The intricate progression from high-grade dysplasia to microinvasion and eventual transformation into frank invasion in oral squamous cell carcinoma is a complicated and pivotal process that warrants thorough investigation.[2] Incipient OSCC are asymptomatic lesions in contrast to advanced lesions, they do not affect deep tissues and tend to demonstrate discrete features,[3] resulting in diagnostic difficulties and misdiagnosis.[4] Severe dysplasia can be difficult to diagnose in cases of severe inflammation or tangential cuts, the histological features can overlap on routine staining with MiOSCC and can result in misdiagnosis in questionable cases.[5] Oral microinvasive OSCC (MiOSCC) has been defined as an early-stage lesion with foci of atypical neoplastic cells entering the lamina propria of underlying connective tissue, reflecting the first stage of invasion through the epithelium.[6,7] Obtaining accurate diagnosis in the histological assessment of high-grade dysplasia, microinvasion, and frank OSCC, on the other hand, poses various obstacles.[7,8]

The treatment modality for HGD and MiOSCC as well as OSCC can vary drastically, demanding more attention to accurate and timely diagnosis.[9,10] One key aspect of this exploration lies in understanding the immunohistochemical expression of the D2-40 marker.[11] In this research article, we delve into the nuanced patterns and significance of D2-40 expression within these stages of oral lesions, seeking to unravel the potential diagnostic and prognostic implications. This is, to the best of our knowledge, the first study to utilize the expression of D2-40 IHC in high-grade dysplasia, microinvasion and frank OSCC in Pakistan. This study was prompted by the belief that healthcare professionals can improve outcomes in initial detection by using D2-40 immunohistochemical staining in conjunction with routine staining in doubtful cases of early epithelial lesions, laying the groundwork for enhanced accuracy in diagnosis for histopathologists when evaluating such individuals in daily practice.

MATERIAL AND METHODS

This retrospective study was done from 1st Dec 2023 to 5th Feb 2024 in AFIP, Rawalpindi after approval from the Armed Forces Institute of Pathology's institutional ethical committee (MP-ORP22-

2/READ-IRB/23/2208) on 7th Nov 2023. After going through 105 tissue samples of early oral epithelial lesions, cases with diagnostic dilemmas on routine haematoxylin and eosin staining (H&E) were selected by an oral pathologist. Two histopathologists reviewed all cases' H&E-stained slides blindly. High-grade dysplasia cases were diagnosed according to the WHO classification of tumours. A carcinoma with no invasive foci larger than one mm was diagnosed as MiOSCC, whereas tumour foci larger than one mm were termed as frankly invasive OSCC.[12,13] Cases with inconclusive diagnoses weren't included in the study. The lab record forms were used to get patient information. A sample size of thirty was calculated using a confidence interval of 95%, a margin of error of 5%, and a population prevalence of 0.6%.[10] Out of the thirty cases, ten were of high-grade dysplasia, and 20 cases of oral squamous cell carcinoma (10 each of MiOSCC and frank invasive OSCC). D2-40 IHC was applied on fresh sections and interpreted. As patients were picked at random and most specimens were incisional biopsies, sample specifications based on OSCC grades could not be explored; detection of the precise grade of differentiation and their relationship to one another was not explored. Histopathologically diagnosed cases of HGD, MiOSCC and frank OSCC were included. However, patients undergoing treatment (surgery, chemotherapy, and radiotherapy), biopsy sample which was necrosed and patients with metastatic tumours in jaws from systemic malignancies were all excluded.

Immunohistochemical staining was carried out in accordance with previously published protocols.[14] For antigen retrieval, the dewaxed slides were rehydrated and submerged in a buffer containing ethylenediaminetetraacetic acid. The sections were subsequently exposed to three percent hydrogen peroxide and treated overnight with the principal antibody D2-40 (Leica Biosystems, Germany) at 4 °C.[14] Then, they were first stained with diaminobenzidine and then with haematoxylin. As a negative control, a PBS buffer was employed. The D2-40 IHC-stained slides were blindly examined by two histopathologists. The degree of the staining was graded as follows: 0 (no staining), 1+ (light yellow), 2+ (yellow-brown), and 3+ (strong brown). Samples with a score of 0 to 1+ were judged negative, while others were regarded positive.[14] In case of discrepancy between observers, a consensus was recorded by both observers using a multiheaded microscope.

Data analysis was done using SPSS 25. To establish the correlation between podoplanin (D2-40) positive and clinicopathologic features, a chi-square test was employed, where a *p*-value less than 0.05 was statistically significant.

RESULTS

The mean age of patients was 60.47±11.78 and males (70%) were affected more than their female (30%) counterparts. The tongue (40%) was the most involved region, followed by the buccal mucosa (33.3%), lower lip (16.7%), alveolar mucosa (6.7%), and retromolar trigone (3.3%). Appendix biopsy was used as a control for D2-40 (Figure-1A). The expression of D2-40 IHC was observed in the 30-tissue specimen as shown in Figure- 1 (B-D).

Our findings revealed that D2-40 was detectable in 40% of HGD cases but was lost in 60% of cases. The podoplanin protein was seen in a continuous linear pattern (60%) (Figure-2A) or a discontinuously linear pattern (40%) (Figure-2B) within the basal cell layer membrane in the cases with podoplanin signal. Aside from the non-specific staining of lymphatic vessels, only three patients (3/10) revealed D2-40 staining in the stroma.

MiOSCC arises from HGD, hence the pathogenic alterations of HGD were visible around the microinvasive focus (Figure-1C). To detect changes in D2-40 IHC expression throughout the transition of HGD to MiOSCC, we evaluated podoplanin (D2-40) expression discrepancies between small micro-invasive tumour cells and them around HGD regions. Our findings revealed that podoplanin expression was higher in MiOSCC patients (90%) than in HGD (60%), largely in a discontinuous manner. Aside from the lymphatic vessels, the majority of cases (9/10) displayed considerable staining in the stroma (Figure-3).

D2-40 immunohistochemistry stain was used in selected instances to examine podoplanin overexpression in frankly invasive OSCC (Figure- 1D). The majority of the cases displayed positive expression of D2-40 in a discontinuous pattern, according to our findings. Furthermore, half of the OSCC cases displayed staining within the stroma. We proceeded to look at the association between podoplanin IHC overexpression and the patients' clinicopathological characteristics. Thirty of the 105 cases with comprehensive pathology data were included. D2-40 positivity, not merely in neoplastic cells but also the stromal component, was found to be related to the patient's age and stage of oral cancer ($p<0.05$) (Table-1). The results of the D2-40 IHC scoring in tissue specimens revealed that scores 2 and 3 were the most common, however, they were not significant (Table-2).

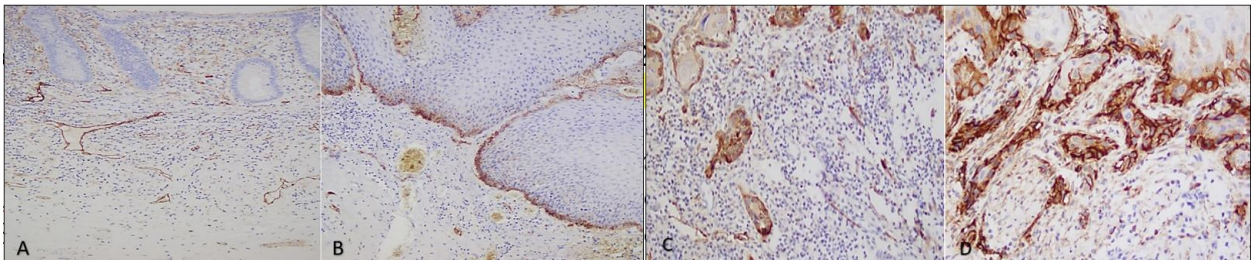

Figure- 1: Expression of D2-40 IHC in tissue specimen (X200 magnification) (A) Positive control (B) High-grade dysplasia (C) Microinvasive OSCC (Tumor Focus <1mm) (D) Frank OSCC (Tumor Foci >1mm)

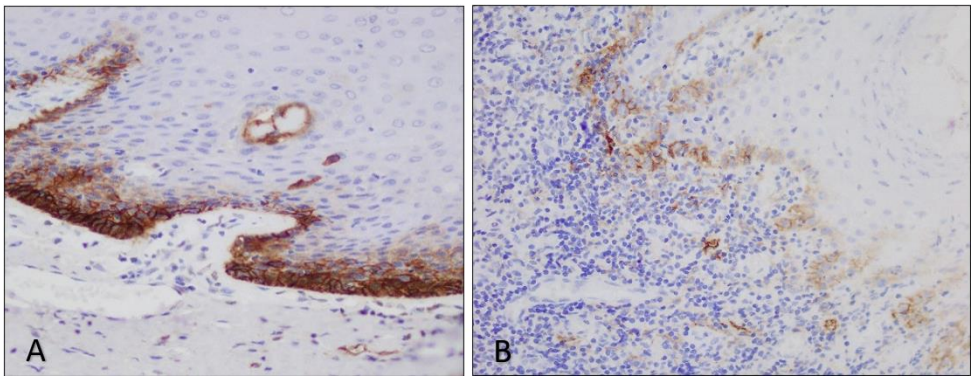

Figure-2: Pattern of D2-40 expression in High-grade dysplasia (X400 magnification) (A) Continuous linear pattern (B) Discontinuous pattern

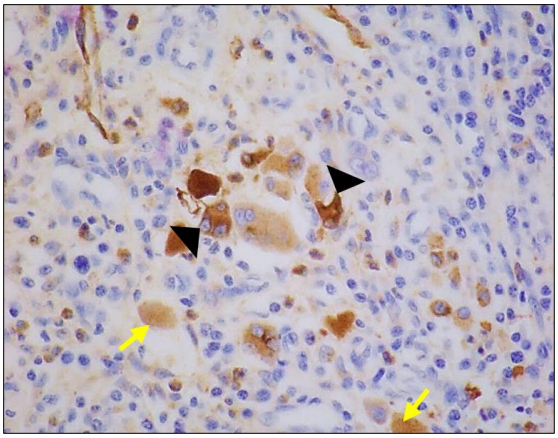

Figure-3: Expression of D2-40 in stroma (yellow arrow) and microinvasive tumour cells (black arrowheads) (X600 magnification)

Table-1: Correlation of clinicopathological characteristics with expression of D2-40 IHC

Parameter	D2-40 Positive	D2-40 Negative	*p*-value
Age, years			
≤50	3	4	0.037*
>50	19	4	
Gender			
Male	17	4	0.149
Female	5	4	
Diagnosis			
HGD	4	6	0.014*
MiOSCC	9	1	
OSCC	9	1	
**p-value is significant at 0.05 level.*			

Table-2: Scoring of D2-40 IHC in High-grade dysplasia, Microinvasive OSCC and Invasive OSCC[14]

Diagnosis	D2-40 score				Total	*p*-value
	No staining	Light yellow	Yellow-brown	Strong brown		
High-grade dysplasia	1	5	1	3	10	0.192
Microinvasive OSCC	1	1	5	3	10	
Invasive OSCC	0	1	5	4	10	
Total	1	6	13	10	30	

DISCUSSION

Histopathologists are commonly requested to identify if the initial invasion is present during early epithelial tumors,[14] because the treatment options for individuals with severe dysplasia, microinvasive OSCC, and frankly invasive carcinoma are substantially different. However, in many situations, simply observing H&E sections does not result in a definitive diagnosis.[5] As a result, researchers have looked at several immunohistochemical markers used by pathologists that highlight basement membrane proteins such as collagen IV and laminin.[15,16] Indication of infiltration of the basement membrane by a particular marker is yet to be determined in the oral cavity.[2] As a result, continued efforts in this field are worthwhile. We investigated the expression of D2-40 IHC in HGD, MiOSCC, and invasive OSCC in this study.

The monoclonal antibody D2-40 detects podoplanin, a family of mucin-like transmembrane glycoproteins found on the endothelium of lymphatic arteries.[17] D2-40 reactivity increased gradually from normal epithelium to dysplastic epithelium and finally to SCC, as observed by Partu *et al*., suggesting that these immunomarkers may be implicated in the early phases of squamous cell carcinogenesis, with D2-40 acting as a surrogate marker for lymphatic endothelial response to neoplastic transformation.[18] Similar studies indicate that this may be an increasingly aggressive cancer phenotype and suggest that D2- 40 may play a role in predicting OSCC prognosis.[11,19–21] In our study, D2-40 expression increased gradually from high-grade dysplasia to higher levels in cases of microinvasion and frank OSCC.

We observed the expression of D2-40 IHC within the stroma of high-grade dysplasia as well as in MiOSCC and OSCC. Our outcomes contribute to the growing body of evidence supporting D2-40's usage as a microinvasion marker in early epithelial diseases. Microinvasion detection is crucial for selecting treatment methods and predicting patient outcomes.[22,23] D2-40 IHC's staining features ensured its use in improving the diagnosis of early oral lesions and better-determining microinvasion. Three out of ten cases of HGD cases showed strong expression in the stroma that surrounds neoplastic cells which was consistent with previous research done.[14,22]

Khan *et al*. carried out research to determine the importance of D2-40 as a risk indicator in oral leukoplakia. They also found a link between its expression and different degrees of OSCC. IHC was used to investigate podoplanin expression within 40 cases of leukoplakia and OSCC. In this investigation, podoplanin expression increased dramatically from low-grade dysplasia to high-grade dysplasia and from different grades of OSCC with decreasing differentiation, indicating that it can be used as a prognostic marker to detect early-stage oral cancer.[23,24] This information could be valuable in tailoring therapeutic approaches and stratifying patients based on their risk of disease progression. Our study added evidence to this research but did not observe expression in varying grades of dysplasia or OSCC due to the nature of certain biopsy specimens.

However, it is critical to recognize the limits of our research. The limited sample may create biases that impair the generalizability of our findings. Future prospective studies with bigger cohorts and continuous monitoring of such patients are also needed to establish D2-40's prognostic value in oral SCC.

Finally, our study of the immunohistochemistry expression of D2-40 in high-grade dysplasia, microinvasive OSCC, and frankly invasive OSCC, sheds light on the probable function of lymphangiogenesis in oral carcinogenesis. The higher the expression of D2-40 higher the chances more lymphovascular invasion leading to a poorer prognosis. The observed association between D2-40 expression and clinicopathological characteristics implies that it may be useful as a prognostic marker, albeit more research is needed to confirm these findings and demonstrate its generalizability. This study adds to the growing body of information in oral cancer research and establishes the groundwork for future research into tailored therapy options guided by molecular markers.

Conflict of interest: There are no conflicts to disclose.

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AUTHORS' CONTRIBUTION

SAR: Came up with the idea and did data collection, manuscript writing, and statistical analysis. WR, MR: Did data collection and manuscript writing.

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Address for Correspondence:

Dr. Syeda Areeba Rehan, Department of Oral Pathology, Armed Forces Institute of Pathology, National University of Medical Sciences, Rawalpindi-Pakistan

Cell: +92 334 266 4412

Email: dr.syedaareebarehan@gmail.com