RESEARCH ARTICLE

Periodic acid-schiff stain and p53 marker: reducing interobserver variability in microinvasive oral squamous cell carcinoma diagnosis

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Abstract

Objective: To assess the effectiveness of periodic acid-Schiff stain and p53 immunohistochemical marker in reducing interobserver variability for diagnosing microinvasive oral squamous cell carcinoma cases.

Method: The cross-sectional study was conducted at a tertiary care diagnostic hospital in Rawalpindi, Pakistan, from March 31 to July 31, 2023, and comprised diagnostically challenging biopsy specimens. The specimens were subjected first to haematoxylin and eosin stain, and then with periodic acid-Schiff stain and tumour protein p53 immunohistochemistry simultaneously. A preliminary diagnosis on routine staining alone and a final diagnosis with the two adjuncts were reported by two observers who were both blinded to the prior diagnosis. Data was analysed using SPSS 25.

Results: Of the 30 specimens diagnosed, 21 (70%) belonged to males and 9 (30%) to females. The mean age of the patients was 60.47±11.78 years. Periodic acid-Schiff staining and tumour protein p53 immunohistochemistry demonstrated a significant decrease in interobserver variability in the diagnosis of microinvasive oral squamous cell carcinoma, exhibiting enhanced visualisation in basement membrane breach and identifying the invading cells within the lamina propria that were masked on routine staining ($p<0.05$).

Conclusion: Periodic acid-Schiff stain and tumour protein p53 immunohistochemistry could assist in reducing interobserver variability in the diagnosis of microinvasive oral squamous cell carcinoma.

KeyWords: Interobserver variability, Periodic acid-Schiff reaction, Oral squamous cell carcinoma, Immunohistochemistry.

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Introduction

Oral squamous cell carcinoma (OSCC) is the most prevalent malignancy in the head and neck region.[1] The region of Southeast Asia, particularly high-risk countries like Pakistan, has the highest incidence rates of OSCC due to the extensive use of carcinogenic areca nut, and tobacco smoking habits.[2-4] Despite the high incidence of OSCC, an entity known as microinvasive OSCC (MIOSCC) remains a grey area in the literature. MIOSCC is cancer in its initial stages that has not infiltrated the underlying deeper tissue.[5,6] However, there is no definitive consensus regarding its diagnostic criteria, resulting in greater interobserver variability on routine histopathological examination. It can be misdiagnosed as oral epithelial dysplasia (OED) or early invasive OSCC (EIOSCC) in questionable cases, both having drastically different treatment options.[7,8] Detection of this early-stage tumour is important as it can lead to timely treatment, and improve morbidity and mortality of the patients.[9]

While staining with haematoxylin and eosin (H&E) is the gold standard in histological examinations, using it alone for diagnosing microinvasion in OSCC can be difficult due to the masking of basement membrane breach by dense inflammatory infiltrates.[8,10] A few studies have proposed that special stains and immunohistochemistry (IHC) can be used as adjuncts in the diagnosis of MIOSCC.[8,11,12] Periodic acid-Schiff (PAS) stain can identify breaches in the basement membrane, and tumour protein p53 IHC highlights the microinvasive foci, both of which are useful in such suspicious cases.[11,13]

The current study was planned to evaluate the use of PAS stain and p53 IHC in better evaluating OSCC, and to see how helpful they are in minimising interobserver variability in OSCC diagnosis in doubtful histopathological cases.

Materials and Methods

The cross-sectional study was conducted at a tertiary care diagnostic hospital in Rawalpindi, Pakistan, from March 31 to July 31, 2023. After approval form the institutional

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ethics review board, the sample size was calculated using the World Health Organisation (WHO) calculator with 5.2% prevalence of MIOSCC, and 8% margin of error.[14,15] Formalin-fixed, paraffin wax-embedded tissue blocks were obtained from the archives of the Histopathology Department. The selected cases were difficult to diagnose on routine H&E staining. Histopathologically diagnosed cases of OED, MIOSCC and EIOSCC were included. No discrimination was made on basis of age and gender. Specimens with inadequate fixation, having very scanty tissue biopsies, and those with extensive tissue necrosis, lesions with ulcerated epithelium and patients not consenting to the study were excluded. Informed consent from those included was taken on a data proforma after contacting them via a phone call.

The prepared blocks and slides were retrieved using non-probability convenience sampling technique.

Demographic and clinical data was collected. Fresh sections of 3-micron thickness were prepared and stained with H&E stain. The histopathologists independently interpreted the slides and gave a preliminary diagnosis. Then, the same tissue sections were stained with PAS and p53 IHC. The same observers, blinded to the previous diagnoses, viewed the slides again and gave a final diagnosis. The preliminary and final diagnoses were compared to assess interobserver variability before and after the PAS, p53 IHC adjuncts. Confounding factors were minimised by adhering to the inclusion and exclusion criteria.

For staining slides with PAS stain (Qaiser Scientific, Pakistan), deparaffinisation was carried out by heating the sections in an incubator for 1 hour at 68°C and treated with xylene for 3 minutes, followed by pure alcohol for 3 minutes, and methylated spirit for 2 minutes. They were then rinsed with tap water for 5 minutes, followed by 15 dips in distilled water, and were oxidised in 0.1% periodic acid for 15 minutes. After washing thoroughly for 5 minutes, they were treated with Schiff's reagent for 10 minutes, and kept dipped 3 times in 0.5% sodium metabisulphite for 2 minutes. Haematoxylin was stained as required, and the slides were dehydrated by submerging them in progressively higher alcohol concentrations. The slides were subjected to xylol solution and were mounted them with Canada balsam. The stained slides were viewed by the pathologists, and any breach in the basement membrane was recorded as positive.

For the detection of the p53 antigen, IHC was carried out using the indirect technique (Leica Bond 3 Microsystem equipment, Germany), that involved a primary antibody reacting with tissue antigen, followed by a secondary antibody reacting with the primary antibody. The sections were deparaffinised and hydrated using xylene and alcohol with decreasing grade, and washed in running water. The antigen unmasking solution (1500ml) was heated in a pressure cooker, and the sections were submerged in it for 1 minute. After unmasking, they were boiled in a stock solution of Tris and ethylenediaminetetraacetic acid (EDTA) for 20-25 minutes at 1000C. The sections were then placed in distilled water for 5 minutes, washed with phosphate buffer solution (PBS) for 6 minutes, peroxidase blocker was applied for 5 minutes, washed with PBS again for 6-9 minutes, incubated with primary monoclonal p53 antibody, and washed with PBS buffer for 2-3 minutes. The sections were then incubated with secondary antibodies for 15 minutes. The chromogen solution of 3,3' diaminobenzidine in the buffer was taken as antigen-antibody reactions. Haematoxylin was then used for final counterstaining. The sections were viewed under light microscopy by the two observers for final results.

For qualitative assessment of p53 interpretation, all the p53-stained sections were examined at low magnification. The scoring method for p53 immunoreactivity included labelling the OED, MIOSCC and EIOSCC specimen slides as negative and positive. The presence of a visible brown nuclear stain was the primary criterion for a positive stain. Staining confined solely to the basal layers was classified as a negative case. The proportion of stained nuclei was calculated by counting the number of p53 cells that were stained per 100 neoplastic epithelial cells in the best staining area, with a cut-off value of 10% nuclei stained with p53 immunohistochemically in the nests lying in the lamina propria.[16]

Data was analysed using SPSS 25. Frequencies and percentages were calculated for each categorical variable, and a Chi-square test was applied for statistical significance, where appropriate. Cohen Kappa test was used to analyse the interobserver variability before and after the use of adjuncts. $P<0.05$ was considered statistically significant.

Results

Of the 30 specimens diagnosed, 21 (70%) belonged to males and 9 (30%) to females. The mean age of the patients was 60.47±11.78 years. There were 10 (33.3%) OED, 8 (26.7%) MIOSCC, and 12 (40%) EIOSCC cases. The distribution of cases by site was as follows: 12 (40%) from the tongue, 10 (33.3%) from the buccal mucosa, 5 (16.7%) from the lower lip, 2 (6.7%) from the retromolar trigone,

Table-1: Periodic acid-Schiff (PAS) stain and tumour protein p53 immunohistochemistry (IHC) interpretation by histopathologists.

PAS interpretation	Original Diagnosis			Total	p-value
Observer 1	**OED**	**MIOSCC**	**EIOSCC**		
Negative	5	1	0	6	0.006*
Positive	4	7	8	19	
Inconclusive	1	0	4	5	
Total	10	8	12	30	
Observer 2	**OED**	**MIOSCC**	**EIOSCC**	**Total**	**p-value**
Negative	4	1	0	5	0.026*
Positive	4	7	10	21	
Inconclusive	2	0	2	4	
Total	10	8	12	30	
p53 IHC interpretation	**Original Diagnosis**			**Total**	**p-value**
Observer 1	**OED**	**MIOSCC**	**EIOSCC**		
Negative	8	3	3	14	
Positive	2	5	8	15	0.073
Inconclusive	0	0	1	1	
Total	10	8	12	30	
Observer 2	**OED**	**MIOSCC**	**EIOSCC**	**Total**	
Negative	7	3	2	12	
Positive	2	5	8	15	0.057
Inconclusive	1	0	2	3	
Total	10	8	12	30	

*p-value is significant at level 0.05.

OED: Oral epithelial dysplasia, MIOSCC: Microinvasive oral squamous cell carcinoma, EIOSCC: Early invasive oral squamous cell carcinoma.

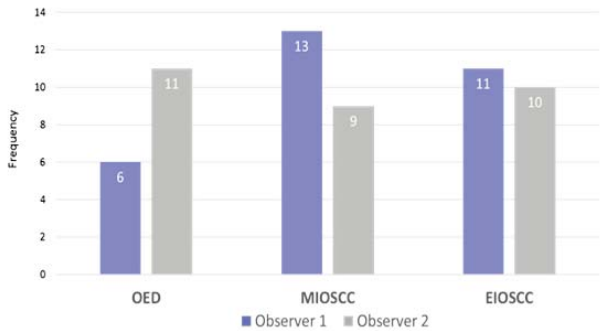

Figure-1: Preliminary diagnosis between two observers on haematoxylin and eosin (H&E) stain among the tissue specimens.

and 1 (3.3%) from the alveolar mucosa Preliminary diagnosis was recorded on H&E-stained slides, and showed significance (Figure 1). The interpretation of PAS and p53 adjuncts showed significant difference (Table 1).

Final diagnosis also showed significant differences (Figure- 2). Kappa value of preliminary diagnosis was 0.308 (p=0.001), showing fair agreement between the observers, while the value for the final diagnosis was 0.792 (p=0.001), indicating substantial agreement (Table 2).

Table-2: Interobserver variability.

Observations	Kappa	Interpretation	p-value
Preliminary diagnosis	0.308	Fair agreement	0.001*
Final diagnosis	0.792	Substantial agreement	0.000*

*p-value is significant at level 0.05.

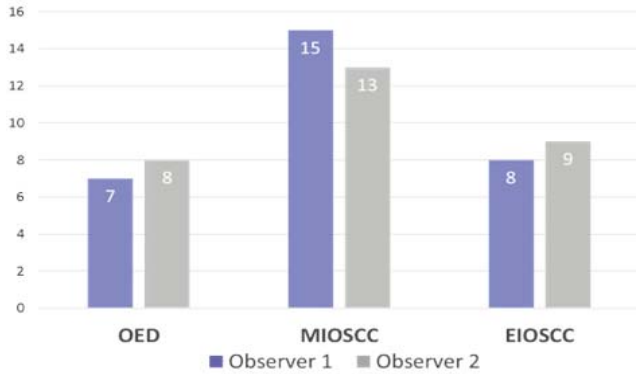

Figure-2: Final diagnosis between two observers after adjunct PAS stain and tumour protein p53 IHC.

PAS: Periodic acid-Schiff, IHC: Immunohistochemistry, OED: Oral epithelial dysplasia, MIOSCC: Microinvasive oral squamous cell carcinoma, EIOSCC: Early invasive oral squamous cell carcinoma.

Discussion

MIOSCC is an early-stage tumour with no deep tissue invasion.[6] In contrast to other anatomic regions, the American Joint Committee on Cancer (AJCC) does not currently define MIOSCC as a separate entity; in the breast, microinvasive carcinoma is defined as "an invasive carcinoma with no focus measuring >1mm" and it has even been stated through that "the clinical impact of multifocal microinvasive disease is not well understood at this time". Theoretically, this uncertainty might be extrapolated to the oral cavity.[6] Because there is no specified criterion for MIOSCC diagnosis, there may be interobserver variability on routine microscopic examination. The current study suggested that using PAS stain and p53 IHC adjuncts in addition to conventional microscopy could reduce interobserver variability in diagnosis. The gold standard is H&E staining in a normal histological examination and can clearly appreciate advanced stage in OSCC.[17] However, on H&E staining alone, it can be difficult to diagnose microinvasion in OSCC due to ulcerated epithelium, inflammatory processes, tangential cutting, relatively bland-looking invading areas, and squamous eddies.[10,13,18,19] As a result, a suitable differential stain or IHC marker that aids in differentiating epithelial elements from the mesenchymal components of connective tissue are required, which may serve as additional diagnostic aids. Several kinds of special stains for epithelial lesions have been introduced

and have been said to be effective in differentiating microinvasive foci in the connective tissue.[8,20,21] A study used PAS stain to examine basement membrane breach and to comment on microinvasion into the lamina propria.[13] Another study suggested that Modified Cajal Trichome stain can be used instead of regular H&E staining in the diagnosis of MIOSCC.[8] It performed somewhat better than conventional microscopy in terms of measuring the depth of invasion (DOI) and distinguishing between epithelial and connective tissue components.[8,20] In the current study, suspected cases were easier to identify as MIOSCC after the application of PAS stain, but the DOI was not measured. Even though the majority of oral lesions with microinvasion have a favourable prognosis, microinvasive carcinoma has the ability to infiltrate lymphatic and vascular channels, making it critical to be diagnosed properly.[6] If it is misdiagnosed as OED, it might progress to aggressive OSCC, making cure difficult when it could have been treated earlier. However, if it is over-diagnosed as EIOSCC, extensive treatment may be given unnecessarily. Therefore, the clinician requires a careful diagnosis to arrange the treatment regimen appropriately. These treatments increase morbidity and mortality.[9] Because microinvasive OSCC is an uncommonly reported phenomenon, the current study focussed on re-evaluating questionable OED, MIOSCC and EIOSCC cases for microinvasion.[8,22] According to the findings, MIOSCC could have been accurately diagnosed, but it was missed due to extensive inflammation at the epithelial-connective tissue interface, obscuring invasive tumour cells in stromal tissue.[6,8] Similarly, IHC markers have been proposed in certain studies to assist in the diagnosis of early epithelial disease.[11,13] IHC distinguishes the invasive foci from the background inflammatory cells.[6,11] A study on MIOSCC cases found that assessing tumour DOI by p53 IHC can be used as a criterion for classifying MIOSCC and can even aid in defining MIOSCC treatment modalities, which can be less comprehensive than the traditional therapies.[7,23]

In the current study, p53 exhibited various reactions in tissue specimens. High-grade dysplasia was characterised by strong staining in the basal and suprabasal epithelium. Microinvasive OSCC, on the other hand, revealed nuclear staining in invasive foci in the lamina propria. The nuclear staining in EIOSCC was comparable, although the invasive foci were >1mm. The PAS stain and p53 IHC marker both aided in the diagnosis of doubtful cases and lowered interobserver variability between histopathologists in general. In regular microscopy, H&E staining is still the gold standard. In contrast, PAS and p53 IHC can be valuable adjuncts in diagnosing difficult cases of early OEDs. The correct diagnosis is crucial for a successful treatment plan and predicting prognosis, and this stain aids in the simple discovery of challenging cases of MIOSCC.[24] The PAS stain and p53 IHC aided in the early and rapid detection of OSCC, which is crucial for therapeutic interventions and survival rates.[8]

To the best of our knowledge, the current study is the first to determine the effectiveness of the PAS and p53 adjuncts in reducing interobserver variability in OSCC diagnosis in Asia. However, the study has its limitations, like the sample size which was kept to a minimum due to the rarity of the entity and financial constraints. Also, the findings may have limited generalisability due to the use of non-probability convenience sampling method. Further studies with larger sample sizes are required to predict the application of these special stains in various lesions of the oral cavity. Also, the PAS stain did not accurately demonstrate basement membrane in all the cases, which may be due to poor fixation or staining process. Similarly, p53 showed variations in staining in all the invasive cases. This may again have been due to variations in the expression of this gene in MIOSCC, implying that other surrogate immunomarkers should be investigated that may help reach a conclusive diagnosis. Also, the effectiveness of PAS stain and p53 IHC should be evaluated in MIOSCC cases. A better staining process and careful handling of specimens can help in achieving even more consistent results.

Conclusion

While H&E staining remains the conventional gold standard, using the PAS stain and p53 showed promising results in lowering interobserver variability among pathologists. PAS stain and p53 IHC could serve as adjuncts, contributing to improved diagnostic agreement and reliability in identifying MIOSCC by providing objective and standardised criteria.

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Authors' Contribution:
SAR, BP: Research proposal, data collection and interpreted results.
NA, HUD, MUR, SMM: Critical analysis and drafting.