ORIGINAL RESEARCH

Efficacy of Hematoxylin and Eosin, Periodic Acid Schiff and Gomori's Methenamine Silver stain technique in Demonstration of basement membrane in oral squamous cell carcinoma – A Comparative Histochemical Study.

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Abstract

Background: Basement Membrane is located between epithelium and connective tissue. It plays a crucial role in maintaining the tissue architecture and acts as a mechanical barrier preventing malignant cells from invading the deeper tissues. It is imperative to investigate and establish an efficient stain for demonstration of basement membrane that requires the use of reliable histochemical staining techniques that offer high quality visualization with clarity and contrast.

Aim And Objectives: The aim of the present study is to compare the efficacy of Hematoxylin and Eosin (H&E), Periodic Acid Schiff (PAS) and Gomori's Methenamine Silver (GMS) stain techniques in demonstration of basement membrane.

Materials And Methods: A total number of 40 archival slides including 10 normal oral mucosa and 30 Oral Squamous Cell Carcinoma(OSCC) cases were included in the study.

Results : Though all the three stains showed favorable features at different levels, The p-values for all three parameters—continuity ($p = 0.021$), contrast ($p = 0.009$), and intensity of colour ($p = 0.007$)—were found to be statistically significant indicating that GMS was better than the other stains in demonstrating BM continuity, contrast and intensity.

Conclusion: GMS exhibited superior efficacy in demonstrating the continuity and contrast of BM followed by PAS and H&E stain, which helps in diagnostic accuracy, staging and therapeutic planning for OSCC.

Keywords : Oral Squamous Cell Carcinoma, Gomori's Methenamine Silver, Basement membrane, Periodic Acid Schiff

Introduction

Oral Squamous Cell Carcinoma (OSCC) is a malignant epithelial tumor of the oral cavity, characterized by the proliferation of atypical squamous cells, which can invade the surrounding connective tissue and metastasize to regional lymph nodes.[1,2] The etiology of OSCC includes tobacco use, alcohol consumption, Human Papillomavirus (HPV) infection, poor oral hygiene and chronic infections, nutritional deficiencies, Genetic and Epigenetic Changes, chronic mechanical trauma[3].

The basement membrane (BM) is a specialized extracellular matrix structure that lies between epithelial or endothelial cells and the underlying connective tissue. It provides structural support, regulates cell polarity, and acts as a selective barrier controlling the exchange of molecules. A defining characteristic of cancer is invasion, which is marked by disruption of the basement membrane - a key diagnostic feature in OSCC.[2]

Immunohistochemistry (IHC) provides accurate localization and detection of basement membrane components such as laminin, collagen type IV, and fibronectin. Despite its precision, IHC techniques are often expensive and time-consuming, requiring specific antibodies and specialized laboratory facilities. Therefore, histochemical staining remains a valuable alternative for routine diagnostic applications[4,5].

For nearly a century, pathologists have relied on special

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histochemical stains to complement conventional hematoxylin and eosin (H&E) staining. While H&E serves as the cornerstone of histopathological examination due to its ability to demonstrate general tissue architecture and cellular morphology, it has limited capacity to identify specific structural or molecular components. In such cases, special stains—defined as any staining method other than H&E—play a crucial role[5].

These stains provide enhanced contrast and selectively highlight particular tissue elements, microorganisms, or molecular constituents, enabling more detailed identification of pathological alterations. In evaluating the basement membrane, special stains such as Periodic Acid-Schiff (PAS) and silver-based techniques can effectively delineate its continuity and integrity, offering a practical and cost-effective alternative to IHC in diagnostic settings.[4]

Materials and Methods

Archives of 30 OSCC samples were involved in the study (N =30). Hematoxylin and Eosin (H&E) stained sections were reviewed and suitable slides were selected. A total of 10 control samples – normal tissue samples were included. Institutional ethical approval was obtained prior to the study.

TABLE 1 : COMPOSITION OF H & E STAIN.

SOLUTIONS AND THEIR COMPONENTS	QUANTITY
HARRIS HEMATOXYLIN SOLUTION	100 ML
Hematoxylin (powder)	1 g
Absolute Ethanol	10 mL
Potassium or Ammonium Alum	20 g
Sodium Iodate *(oxidizer)*	0.2 g
Glacial Acetic Acid	1 mL (optional)
EOSIN Y (1% AQUEOUS OR ALCOHOLIC)	
Eosin Y (powder) Distilled Water or 95% Alcohol	1 g 100 mL
Glacial Acetic Acid	0.5–1 mL (optional, enhances staining)
ACID ALCOHOL (DIFFERENTIATOR)	
Concentrated HCl	1 mL
70% Ethanol	100 mL
BLUEING AGENT	
Lithium Carbonate	Saturated aqueous solution
Tap Water	With pH > 7
MOUNTING MEDIUM (DPX)	Ready-made or lab-made resin dissolved in xylene

Inclusion criteria:

- Tissue blocks of histopathologically confirmed cases of OSCC.
- Normal oral mucosa was obtained from the inflammation free gingival tissues from cases of periodontal flap surgery and surgicallyremoved impacted molars of healthy individuals.

Exclusion criteria;

- Insufficient tissue samples

Type of tissue fixation: Neutral buffered formalin -10%
Software used for statistics : SPSS software

From each case, three sections of 4 μm thickness each were prepared and stained using:

- Hematoxylin&Eosin (H&E)
- Periodic Acid–Schiff (PAS)
- Grocott's Methenamine Silver (GMS)

The slides were evaluated for parameters such as continuity of BM, contrast and intensity of staining. Slide evaluation was performed by two independent observers, and kappa statistics was calculated to assess inter- and intra-observer variation.

STAINING PROCEDURE:

Hematoxylin and Eosin (H&E) :

Principle :

Hematoxylin and eosin staining is a routine histological stain used to visualize the general architecture of tissues, including oral tissues. Hematoxylin is a basic dye that binds to acidic (basophilic) components like nuclei (DNA/RNA) and

TABLE 2 : COMPOSITION OF PAS STAIN.

SOLUTIONS AND THEIR COMPONENTS	QUANTITY
1. PERIODIC ACID	
Periodic acid	1 gm
Distilled water	100 ml
2. SCHIFF'S REAGENT	
Basic fuschin (is carcinogenic)	1 gm
Distilled water	100 ml
Sodium or potassium metabisulphite	2 gm
1 N hydrochloric acid	20 ml
Activated charcoal	0.3 gm
3. HARRI'S HEMATOXYLIN	
Hematoxylin	2.5 gm
Absolute alcohol	25 ml
Ammonium or potassium alum	50 gm
Distilled water	500 ml
Mercuric oxide (not recommended)	1.25 g
Sodium iodate (oxidizing agent)	0.5 gm
Glacial acetic acid	20 ml

stains them blue-purple. Eosin is an acidic dye that binds to basic (acidophilic) structures like cytoplasm, collagen, and muscle fibers, staining them pink to red. This differential staining allows for the clear distinction of cellular and tissue structures.[5] The composition with details of quantity is provided in Table 1.

Procedure :

Begin by deparaffinizing the tissue sections in xylene (2–3 changes), followed by rehydration through a series of graded alcohols: 100%, then 95%, and finally 70%. Rinse the slides in distilled water. Next, stain with hematoxylin for 5 to 10 minutes, and rinse thoroughly in tap water. If needed, perform differentiation using acid alcohol (1% HCl in 70% ethanol), then rinse again and proceed to blueing using either tap water or a lithium carbonate solution. After that, stain with eosin for 30 seconds to 2 minutes, followed by a brief rinse in tap water. Complete the process by dehydrating through graded alcohols (70%, 95%, and 100%), then clear in xylene, and finally mount the slides using DPX or another synthetic resin.

TABLE 3 : COMPOSITION OF GMS STAIN

COMPONENTS	QUANTITY
Stock methenamine silver solution	
3 % aqueous methenamine	400 ml
Silver nitrate, 5% aqueous	20 ml
Working methenamine silver solution	
Stock methenamine silver	25 ml
Distilled water	25 ml
5% borax (sodium borate) solution	
Sodium borate	5 g
Distilled water	100 ml
1% periodic acid solution	
Periodic acid	5 g
Distilled water	500 ml
0.2% gold chloride solution	
1% gold chloride	1 ml
Distilled water	49 ml
Stock light green solution	
Light green SF (yellowish)	1 g
Distilled water	500 ml
Glacial acetic acid	1 ml
Working light green solution	
Light green stock solution	10 ml
Distilled water	50 ml
Stock light green	10 ml
Distilled water	50 ml

Periodic Acid–Schiff (PAS):

Principle :

The PAS (Periodic Acid – Schiff) technique is based on the oxidation of certain tissue carbohydrates by periodic acid. It specifically targets 1:2 glycol groups or adjacent hydroxyl and amine groups, breaking the bond between neighboring carbon atoms to form aldehydes. These aldehydes then react with Schiff reagent to produce a bright red or magenta color. For a positive PAS reaction, the substance must have appropriate glycol or amino groupings, produce a non diffusible oxidation product, remain fixed in the tissue, and be present in a detectable amount.[5] The composition with details of quantity is provided in Table 2.

Procedure :

For the PAS staining procedure, 4–5 μm thick paraffin sections were first dewaxed in xylene and rehydrated through graded alcohols to distilled water. The sections were then oxidized with 0.5–1% aqueous periodic acid for 5–10 minutes, followed by thorough washing in distilled water. Schiff reagent was applied for 15 minutes, and the slides were washed in running tap water for 5–10 minutes. Nuclei were counterstained with Harris's hematoxylin for 1 minute, differentiated in 1% acid alcohol, and blued in tap water. Finally, the sections were dehydrated, cleared with xylene, and mounted using synthetic resin.[5]

Gomori'sMethenamine Silver (GMS)

Principle :

This method suppresses weak background reactions by breaking down aldehyde groups in collagen and basement membranes, ensuring only substances rich in polysaccharides—like fungal cell walls, glycogen, and mucins—react. Aldehyde groups reduce methenamine silver to metallic silver. Methenamine provides the necessary alkaline pH, sodium borate buffers the solution, gold chloride tones the stain, and sodium thiosulfate removes unreduced silver. It is the most effective technique for detecting fungi in tissue, especially when they are sparse.[6] The composition with details of quantity is provided in Table 3.

Procedure :

To perform the staining procedure, tissue sections are first deparaffinized, rehydrated, and brought to distilled water. They are then oxidized in 4% chromic acid for 30 minutes, briefly washed, and dipped in 1% sodium bisulfite, followed by thorough rinsing. Sections are placed in a preheated methenamine silver solution (56–60°C) for 15–20 minutes, checking after 15 minutes for the desired "paper bag brown" color—elastin should not appear black. After rinsing, sections are toned in 0.1% gold chloride for 5 seconds, treated with 5% sodium thiosulfate to remove unreacted silver, and washed again. A light green or H&E counterstain is applied, and finally, the slides are dehydrated, cleared, and mounted.[7]

Results

The study aimed to evaluate and compare the efficacy of three histochemical staining techniques- H&E, PAS, and Go-

TABLE 4 :

Groups		Continuity			Contrast			Intensity of color		
	Continu-ous	Frag-mented	Total	Distinct	Indis-tinct	Total	Weak	Strong	Total	
H&E		12	18	30	5	25	30	20	10	30
% total		13.3 %	20.0%	33.3%	5.6%	27.8%	33.3%	22.2%	11.1%	33.3%
PAS		19	11	30	17	13	30	12	18	30
% total		21.1%	12.2%	33.3%	18.9%	14.4%	33.3%	13.3%	20.0%	33.3%
GMS		25	5	30	21	9	30	6	24	30
% total		27.8%	5.6%	33.3%	23.3%	10.0%	33.3%	6.7%	26.7%	33.3%
Degree of freedom		2	2		2	2		2	2	
CHI- SQUARE		12.0			18.5			13.4		
P VALUE		0.02*			0.00*			0.001*		
KAPPA VALUE		0.806			0.763			0.792		
		*statistically significant								

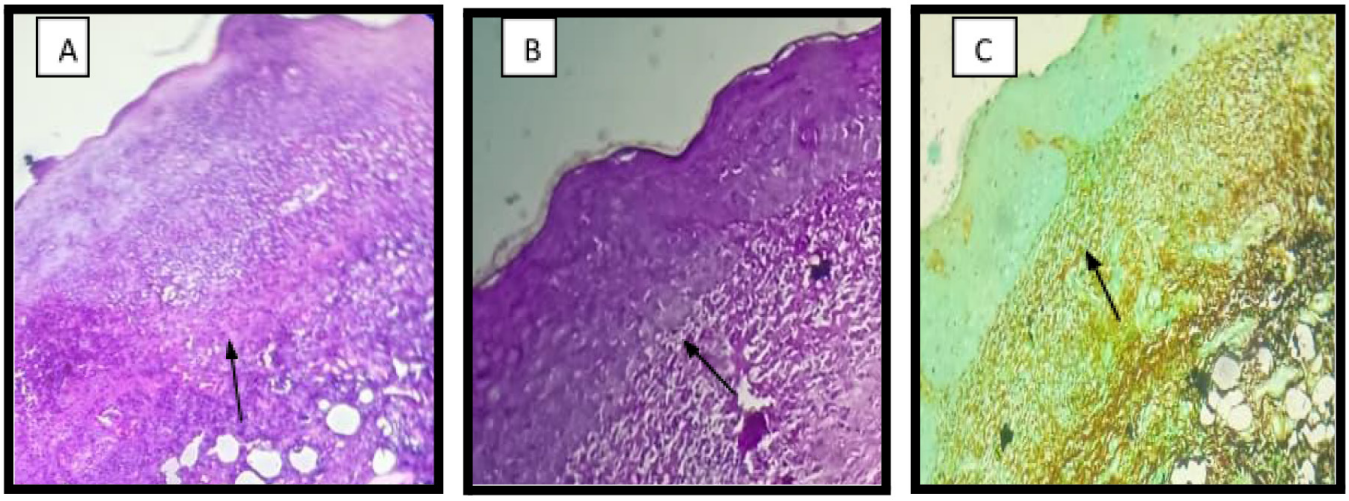

Fig. 1 (10X): Photomicrograph showing fragmented, indistinct, weak basement membrane in OSCC under light microscopy using **(a)** H&E stain, **(b)** Periodic Acid Schiff stain and **(c)** Gomoris Methenamine Silver Stain.

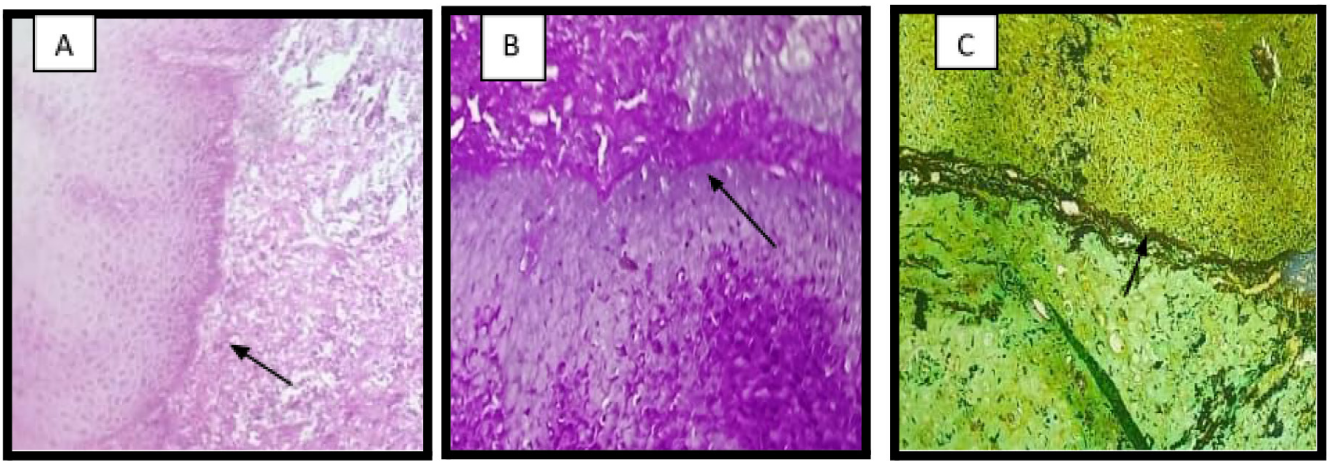

Fig 2 (10X): Photomicrograph showing continuous, distinct, strong basement membrane in OSCC under light microscopy using **(a)** H&E stain, **(b)** Periodic Acid Schiff stain and **(c)** Gomoris Methenamine Silver Stain .

mori's Methenamine Silver (GMS) - in the demonstration of the basement membrane in oral squamous cell carcinoma (OSCC) tissues. A total of 30 histopathologically confirmed OSCC cases were selected. For each case, serial sections of 4-micron thickness were stained with H&E, PAS, and GMS and assessed for three parameters: continuity of basement membrane, contrast, and intensity of stain colour.

Quantitative assessment revealed notable differences among the three staining techniques. For the continuity of the basement membrane, GMS staining exhibited superior performance, with 25 of the 30 cases showing continuous BM, as compared to PAS (17 cases) and H&E (13 cases). Fragmented or indistinct BMs were more frequently observed with H&E staining (18 fragmented/indistinct), demonstrating its limitations in clearly visualizing the basement membrane.

In terms of contrast, GMS again outperformed the other techniques, with 21 cases demonstrating strong contrast of the basement membrane against the surrounding epithelium and connective tissue, followed by PAS with 17 cases, and H&E with only 11 cases showing strong contrast. The superior contrast with GMS is likely attributable to its ability to stain reticulin fibers black, providing a stark visual delineation of the basement membrane.

The parameter of intensity of color further reinforced the findings, with GMS achieving strong intensity in 23 cases, PAS in 18 cases, and H&E in 12 cases. GMS staining yielded a sharp, dark visual of the basement membrane, in contrast to the pink and magenta hues produced by H&E and PAS respectively, which may be more difficult to distinguish, especially in poorly differentiated or inflamed tissues.

Chi-square analysis was employed to assess the statistical significance of these findings. The p-values for all three parameters—continuity ($p = 0.021$), contrast ($p = 0.009$), and intensity of colour ($p = 0.007$)—were found to be statistically significant, indicating that the differences in BM visualization across the staining techniques were not due to chance.

Photomicrographs further substantiated these findings. H&E-stained sections (Figure 1a) often showed fragmented and indistinct basement membranes, while PAS-stained sections (Figure 1b) demonstrated improved, but still variable, visualization. In contrast, GMS-stained sections (Figure 1c) consistently highlighted the basement membrane with strong contrast and continuity, validating the quantitative assessments.

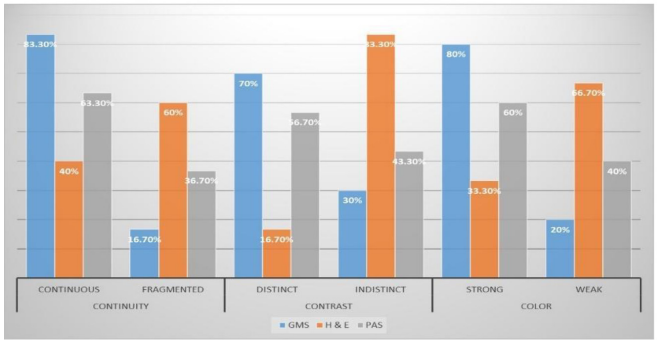

Fig. 3: Comparison of GMS, H&E, PAS staining based on continuity, contrast and color characteristics

Evaluation of consistency was ensured through independent observation by two examiners, and inter- and intra-observer reliability was assessed using kappa statistics. The use of two blinded observers added to the robustness of the findings, minimizing subjective bias.

Overall, both PAS and GMS were significantly more effective than H&E in demonstrating the basement membrane in OSCC tissues. Between the two, GMS was clearly superior across all evaluated parameters. This suggests that GMS staining provides the most reliable and high-contrast visualization of basement membranes in OSCC, which is crucial for assessing the degree of invasion - a key diagnostic and prognostic indicator.

Discussion

The basement membrane (BM) is a specialized structure of the extracellular matrix that lies at the interface between epithelial cells and the underlying connective tissue. It serves as a mechanical barrier and a platform for cellular attachment, proliferation, differentiation, and polarity. Structurally, the BM is composed of type IV collagen, laminin, nidogen, and heparan sulfate proteoglycans. It plays a crucial role in maintaining tissue integrity, compartmentalization, and selective permeability.[8]

In oral squamous cell carcinoma (OSCC), the invasive potential of malignant epithelial cells is characterized by their ability to breach the BM. BM disruption is, therefore, a defining histological hallmark of malignancy and an important diagnostic criterion that separates carcinoma in situ from invasive carcinoma.[9] The ability to clearly visualize the BM is essential for pathologists when determining the invasive front of tumors and predicting prognosis.

Although Hematoxylin and Eosin (H&E) staining is the most commonly used method in histopathology, it has limited sensitivity in highlighting the BM. As a result, special histochemical stains like Periodic Acid-Schiff (PAS) and Gomori's Methenamine Silver (GMS) have been employed for better visualization.

Periodic Acid-Schiff (PAS) stain detects polysaccharides and glycoproteins by oxidizing them to aldehydes, which react with Schiff reagent to yield a magenta color. PAS highlights the BM due to its glycoprotein content and is used in the diagnosis of tumors, fungal infections, glycogen storage diseases, and mucin-secreting lesions.[10] In the context of OSCC, PAS is beneficial in delineating the extent of BM integrity, helping distinguish between pre-invasive and invasive lesions.[11]

Gomori's Methenamine Silver (GMS) stain, traditionally used for detecting fungi and reticulin fibers, is also effective in visualizing the BM. GMS reacts with aldehyde groups in carbohydrates, forming a black precipitate, thereby providing excellent contrast against the tissue background. It specifically stains type III collagen and reticulin fibers, both of which are present in the BM.[12] Due to its high contrast and sharp delineation, GMS is considered superior in detecting BM invasion in squamous malignancies and renal pathology.[13,14]

Our study compared the effectiveness of H&E, PAS, and GMS in demonstrating the BM in OSCC tissue sections. The

findings revealed that both PAS and GMS stains were significantly superior to H&E. Among the three, GMS demonstrated the highest level of clarity, contrast, and continuity of the basement membrane, making it the most reliable method for detecting BM disruption. These observations are consistent with those of Seifi et al. (2017), who reported that GMS stain provided clear and continuous visualization of BM, allowing for better discrimination of malignant invasion.

Statistical analysis: The data obtained were transferred into excel format and its analysis was done using IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp. Descriptive statistics including frequency and percentages were calculated for continuity, contrast and intensity of colour. Comparison between the various study groups was made using cross tabulations and statistical significance was assessed using Pearson's chi-square test. The statistical significance in the present study was kept at $p<0.05$.

Using the chi-square test, a significant difference among the staining methods in all three evaluated parameters: continuity, contrast, and staining intensity were observed. GMS achieved statistically significant superiority, confirming its diagnostic utility. Furthermore, kappa statistics for inter- and intra-observer agreement demonstrated high reproducibility of results with GMS staining, supporting its reliability in routine histopathological use.

These results align with previous literature, including findings by Pujar et al., that emphasized the limitations of routine H&E staining in basement membrane evaluation. The enhanced performance of GMS staining may be attributed to its ability to detect silver-binding reticulin fibers, which are integral components of the BM.

From a clinical perspective, the detection of BM integrity is crucial in guiding treatment decisions. For example, in early OSCC or borderline epithelial dysplasia, confirming the presence or absence of BM breach determines whether conservative management or aggressive surgical intervention is appropriate. Misinterpretation caused by suboptimal BM visualization, such as when using H&E alone, may lead to under- or over-treatment.

Immunohistochemistry (IHC) remains as a highly sensitive technique for demonstration of BM using markers such as Collagen IV, Laminin, Tenascin offering molecular-level specificity.15,16 However, GMS will be a cost-effective alternative to these IHC markers especially in a resource-constrained laboratories. Limitation of our current study is smaller sample and adherence to single method of fixation. However, future prospective research on a large sample size with different fixation methods are recommended to validate our findings.

Conclusion

This study demonstrates that GMS is the most effective histochemical stain for identifying BM in OSCC, offering superior clarity, contrast, and reproducibility compared to H&E and PAS. Incorporating GMS into routine diagnostic protocols could significantly enhance the diagnostic accuracy and staging of OSCC, facilitating appropriate therapeutic planning and better prognostication.

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CONFLICT OF INTEREST:

No potential conflict of interest relevant to this article was reported