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Original Article

Immunohistochemical Evaluation of Matrix Metalloproteinase-9 Expression in Oral Epithelial Dysplasia and Oral Squamous Cell Carcinoma: A Clinicopathological Observational Study.

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Abstract

Background:
Oral squamous cell carcinoma (OSCC) is a major cause of cancer-related morbidity and mortality, particularly in developing countries. MMP-9 plays a critical role by degrading type IV collagen and facilitating tumor progression.

Objective:
This study aimed to evaluate the immunohistochemical expression of Matrix Metalloproteinase-9(MMP-9) in Oral Epithelial Dysplasia and Oral Squamous Cell Carcinoma, and to correlate these findings with histological grading.

Methods:
A prospective observational study was conducted on 100 formalin-fixed paraffin-embedded oral tissue samples, including benign, premalignant, and malignant lesions. Immunohistochemistry was performed using monoclonal MMP-9. Expression was graded based on staining intensity and proportion. Statistical significance was assessed using Chi-square tests ($p< 0.05$).

Result:
MMP-9 expression showed a significant association with lesion grade ($\chi^2 = 75.70$, $p < 0.001$). Low expression was observed in all mild OED cases (100%), whereas high expression was seen in all severe OED cases (100%). Among OSCC cases, low expression predominated in WDSCC (52.78%), moderate expression in MDSCC (50%), and high expression in PDSCC (80%). These findings demonstrate a progressive increase in MMP-9 expression with increasing severity of dysplasia and poorer differentiation of OSCC.

Conclusion:
MMP-9 expression increases with disease severity and tumour aggressiveness in oral epithelial lesions. Its evaluation may serve as a useful adjunctive biomarker for understanding tumour progression and improving prognostic assessment.

Recommendation:
Larger multicentric studies are recommended to validate its clinical utility.

Keywords: Matrix Metalloproteinase-9; Oral squamous cell carcinoma; Dysplasia; Immunohistochemistry
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Introduction
Although the adjacent normal tissue of oral carcinomas might be clinically normal, changes like molecular and chemical can reveal important information for predicting tumor behavior, recurrence, and metastatic potential.[1] Therefore, it is necessary to identify a better prognostic marker to categorize the patients for closer monitoring or more intensive treatment. The metastatic potential of OSCC depends upon its ability to digest the extracellular matrix (ECM), penetrate the basement membrane (BM), initiate tumor angiogenesis, and invade adjacent tissues and blood vessels.[2]

Most of these enzymes are involved in common physiological processes such as proliferation, differentiation, apoptosis, angiogenesis, and morphogenesis [3-5]. The breakdown of the basement membrane and

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interstitial connective tissue represents a key event in the process of tumor invasion. Tumor cells may accomplish this by secreting proteolytic enzymes or by inducing stromal cells to do so. Many different proteases are involved, mainly matrix metalloproteinases (MMPs), which regulate tumor invasion not only by remodeling the basement membrane and interstitial connective tissue, but also by releasing factors that contribute to the malignant behavior of cancers. MMP-9, a gelatinase, cleaves type IV collagen present within the epithelial and vascular basement membrane and thereby facilitates tumor invasion [6].

The Stimuli from transforming growth factor-β (TGF-β) and interleukin-8 (IL-8) are responsible for the synthesis of these enzymes. High levels of MMP-9 protein expression have been linked to tumor recurrence and the development of both nodal and distant metastases.[6]

The primary objective of the study is to compare MMP-9 expression among normal oral mucosa, dysplastic lesions, and malignant lesions, and to assess its association with the histopathological grades of OED and OSCC. Furthermore, the secondary objective is to determine whether increasing MMP-9 expression correlates with disease progression and tumor aggressiveness, thereby exploring its potential utility as a diagnostic and prognostic biomarker in oral epithelial carcinogenesis.

Methods

Study Design and Duration

An institution-based observational analytical study. The study period is from September 2023 to September 2024.

Study Setting

The study was conducted in the Department of Pathology at Baba Raghav Das Medical College, Gorakhpur, a tertiary care institution providing diagnostic and treatment services.

Sample Size Determination

A total of 100 formalin-fixed, paraffin-embedded tissue specimens were analyzed, including 90 cases of oral lesions and 10 samples of histologically normal oral mucosa.

The sample size was determined using **Cochran's formula**, considering a prevalence rate of 17%, an allowable error of 7.5%, and a 95% confidence interval, which yielded a total sample size of 100 cases.

Participants

Consecutive non-probability sampling was employed, wherein all eligible patients with oral premalignant and malignant lesions attending the Otorhinolaryngology Department and fulfilling the inclusion criteria during the study period were included until the required sample size was achieved.

Inclusion criteria

All oral biopsy specimens received from the Otorhinolaryngology department were obtained with documented informed consent.

Exclusion criteria

Specimens that were inadequate, autolyzed, or lacking informed consent were excluded from the study.

Data Collection

The specimens received in the Department of Pathology for histopathological examination during the study period constituted the source of data. Detailed clinical information, including age, sex, and relevant clinicopathological parameters, was

recorded for each case. Tissue samples were fixed in 10% neutral buffered formalin, dehydrated through graded alcohols, cleared in xylene, and embedded in paraffin wax. Sections measuring 2–3 µm in thickness were prepared and stained with Hematoxylin and Eosin (H&E) for microscopic examination and diagnostic confirmation.

Immunohistochemical evaluation was performed to assess the expression of MMP-9. Rabbit anti-MMP-9 antibody (Clone EP127), procured from Bio SB, was utilized for this purpose. Antigen retrieval was carried out using a Tris-EDTA buffer by a microwave-assisted technique. This was followed by blocking of endogenous activity, incubation with the respective primary antibodies, visualization using a polymer-based horseradish peroxidase (HRP) detection system, chromogen development with diaminobenzidine (DAB), and counterstaining with hematoxylin.

Immunoreactivity was evaluated based on both the proportion of positively stained cells and the intensity of staining. MMP-9 expression was graded following the scoring system proposed by **Jose and Mane et al. (2018) [7],** focusing on cytoplasmic staining. For each section, 1,000 cells were evaluated across ten high-power fields using a zigzag pattern to ensure representative sampling. Data were analyzed using appropriate statistical software. Results were presented in the form of tables. Descriptive statistics were expressed as percentages. The Chi-square test was used to assess the association between MMP-9 expression scores and histopathological grades. A p-value of less than 0.05 was considered statistically significant. In the present study, a statistically significant association was observed between MMP-9 expression and lesion grade ($\chi^2 = 75.70$, $p < 0.001$)

Bias

Selection bias was minimized by consecutive sampling. Observer bias was reduced by standardized diagnostic techniques.

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Statistical Analysis

All data were tabulated and subjected to statistical analysis using descriptive statistics and the Chi-square test to determine associations. A p-value of <0.05 was considered statistically significant.

Ethical Consideration

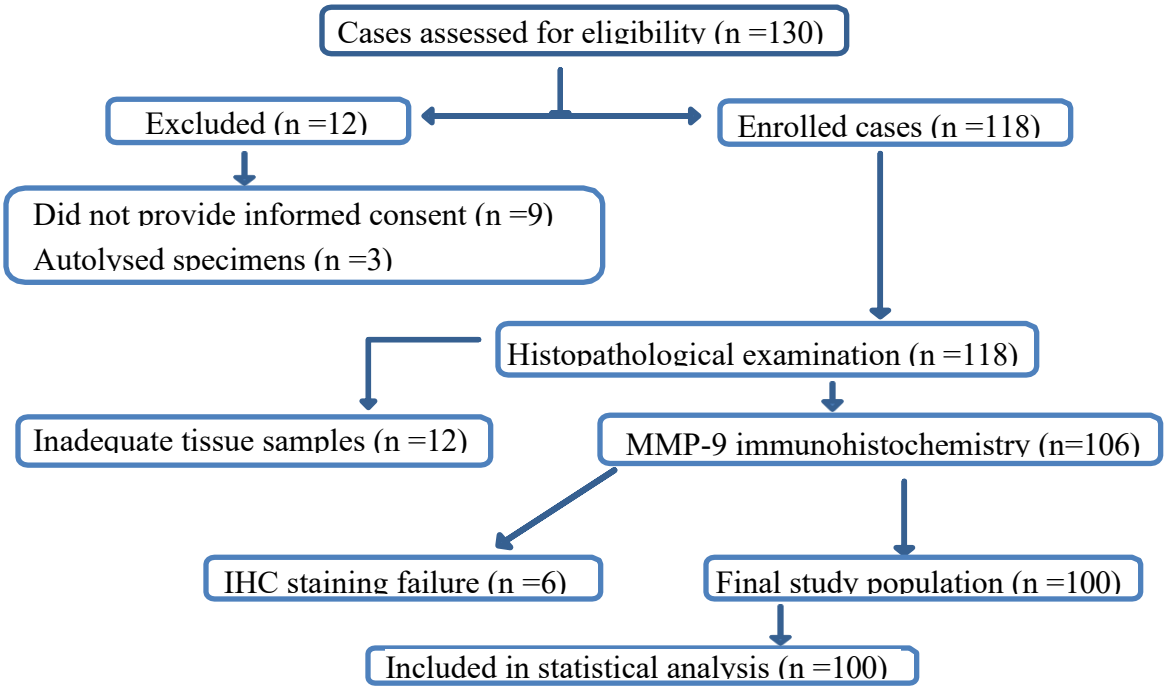
The study was conducted in accordance with ethical guidelines and received approval from the Institutional Ethics Committee of B.R.D. Medical College, Gorakhpur on 14 September 2024.

Informed Consent

Written informed consent was obtained from all participants before their inclusion in the study. The nature, objectives, methodology, potential benefits, and confidentiality aspects of the study were explained to each participant in a language they could understand. Participants were given an adequate opportunity to ask questions and seek clarification regarding the study. Participation was entirely voluntary, and participants were informed that they could withdraw from the study at any stage without providing any reason and without affecting their medical care. Confidentiality of personal information was strictly maintained, and all collected data were used solely for scientific and research purposes. Only those participants who provided written informed consent were included in the study.

Result Participant Flow

During the study period from September 2023 to September 2024, a total of 130 patients with clinically suspected oral lesions were assessed for eligibility. Of these, 12 cases were excluded, including 9 patients who did not provide informed consent and 3 cases with autolysed specimens. The remaining 118 cases were enrolled and underwent histopathological examination. Subsequently, 12 cases were excluded due to inadequate tissue samples, leaving 106 cases for immunohistochemical evaluation of MMP-9 expression. Of these, 6 cases were excluded because of immunohistochemical staining failure. Consequently, the final study population comprised 100 cases, all of which were included in the statistical analysis.



In the present study, premalignant lesions were more frequently observed in younger individuals (≤40 years), whereas malignant lesions were predominantly seen in older age groups (51–60 years); however, this age-related difference did not reach statistical significance (p = 0.064). A marked male predominance was noted in both premalignant and malignant cases, with male-to-female ratios of 4:1 and 5.8:1, respectively, though no statistically significant association with gender was observed (p = 0.897), as summarized in Table 1.

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Table 1. Clinico-demographic Profile of the Study Population (n = 100)

Parameters	No. Of cases (n=100)
Age (years)	
< 40	16
> 40	84
Sex	
Male	80
Female	20
Risk factors	
Present	96
Not Present	04
Site	
Buccal Mucosa	48
Tongue	44
Lips	04
Other	04
Symptoms	
Ulcero proliferative growth	59
Ulcer	20
Fungating growth	08
Swelling	08
Other	05
Histological type	
Normal	10
Pre-malignant	15
Malignant	75

A clear trend of increasing MMP-9 expression was observed with disease progression, with complete absence in benign lesions and progressively higher expression in potentially malignant and malignant cases. The predominance of moderate to strong staining in malignant lesions underscores the role of MMP-9 in invasion and tumor progression, with a statistically significant association ($p < 0.001$) (Table 2)

Table 2: Correlation of MMP-9 Expression (Proportion and Intensity Grades) with Histological Diagnosis.

	Normal Mucosa		**Oral PreMalignant lesions**		**Malignant Lesions**		**Chi sq.= 89.35 p-Value**
	n	%	n	%	n	%	
MMP-9 proportion grade							
Grade 0	10	100.00	2	13.33	0	0.00	<0.001
Grade 1	0	0.00	3	20.00	25	33.33	
Grade 2	0	0.00	6	40.00	44	58.67	
Grade 3	0	0.00	4	26.67	6	8.00	
Total (n=100)	**10(10%)**		**15(15%)**		**75(75%)**		
MMP-9 intensity grade							
Grade 0	10	100.00	2	13.33	0	0.00	<0.001
Grade 1	0	0.00	6	40.00	24	32.00	
Grade 2	0	0.00	6	40.00	39	52.00	
Grade 3	0	0.00	1	6.67	12	16.00	
Total (n=100)	**10(10%)**		**15(15%)**		**75(75%)**		

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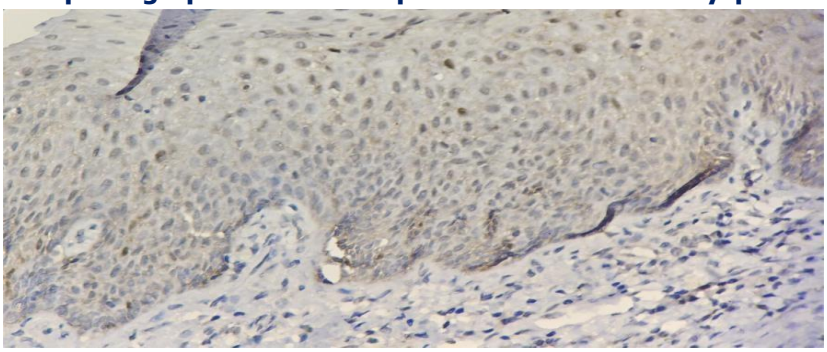
A progressive increase in MMP-9 expression was observed with increasing severity of oral epithelial dysplasia and poorer differentiation of oral squamous cell carcinoma. All cases of mild OED (100%) exhibited low MMP-9 expression, whereas severe OED cases (100%) showed high expression. Among OSCC cases, low MMP-9 expression was most common in WDSCC (52.78%), moderate expression predominated in MDSCC (50%), and high expression was observed in the majority of PDSCC cases (80%). These findings indicate a positive correlation between MMP-9 expression and disease progression (Table 3) (Microphotographs 1-4).

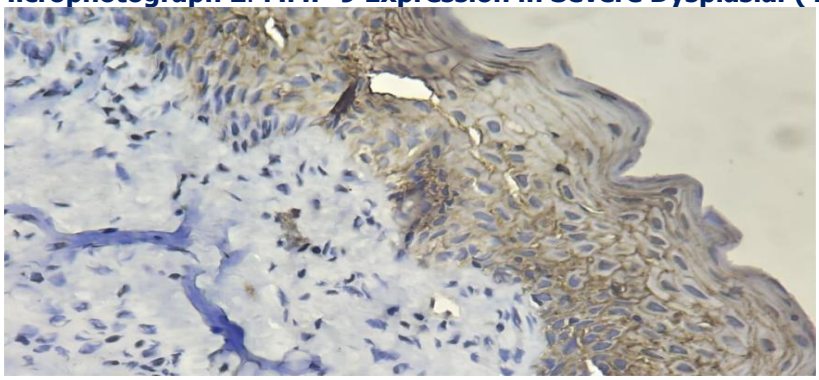
Table 3: Comparative Analysis of MMP-9 Expression Scores Across Different Grades of Oral Epithelial Dysplasia and Oral Squamous Cell Carcinoma

	Total No. cases (n)	MMP-9 Low (0-2)	MMP-9 Moderate (3-5)	MMP-9 High (6-9)
Benign/Non-neoplastic	n = 10	10(100%)	0(0%)	0(0%)
Mild OED	n = 8	8(100%)	0(0%)	0(0%)
Moderate OED	n = 5	1(20%)	2(40%)	2(40%)
Severe OED	n = 2	0(0%)	0(0%)	2(100%)
WDSCC	n = 36	19(52.78%)	11(30.55%)	6(16.67%)
MDSCC	n = 34	13(38.23%)	17(50%)	4(11.77%)
PDSCC	n = 5	0(0%)	1(20%)	4(80%)
Total	**n = 100**	**51(45.5%)**	**31(34.5%)**	**18(20%)**

Microphotograph 1: MMP-9 Expression in Moderate Dysplasia. (400X)



Microphotograph 2. MMP-9 Expression in Severe Dysplasia. (400X)



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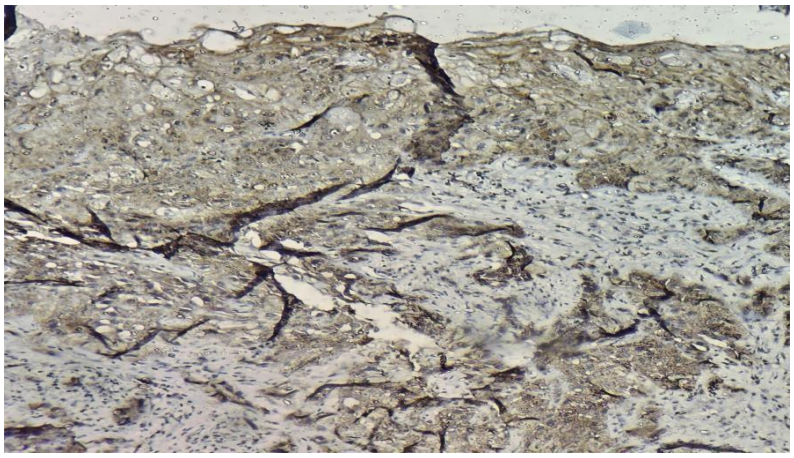
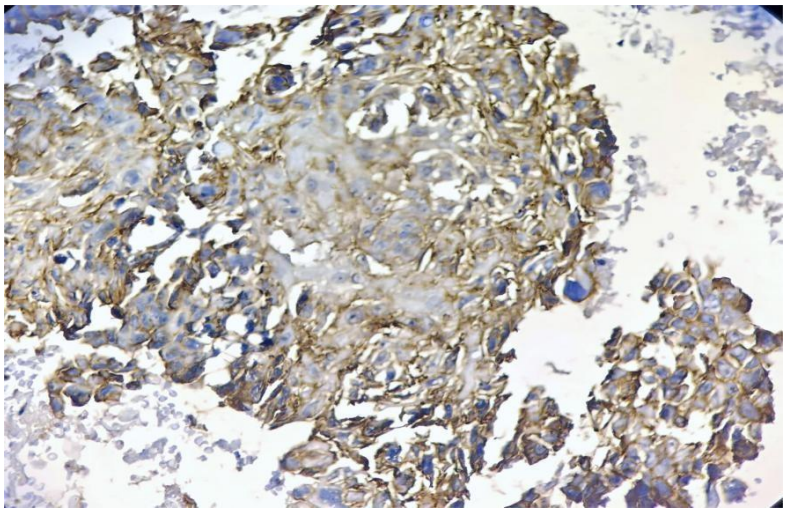
Microphotograph 3. Mmp-9 Expression in Moderately differentiated squamous cell carcinoma (Mdscc). (100X)



Microphotograph 4. Mmp-9 Expression in Poorly differentiated squamous cell carcinoma (Pd scc). (400X)



Discussion

Cancer is one of the most common causes of morbidity and mortality. The hallmark of cancer is unregulated cell growth that evades typical regulatory processes. Oral squamous cell carcinoma (OSCC) ranks as the 6th most prevalent epithelial malignancy globally and is a significant contributor to morbidity and death. In India, oral cancer ranks as the third most prevalent cancer, after breast and cervical cancers, constituting 30% of all cancer cases.

In the present study, immunohistochemical assessment of MMP-9 was carried out in 100 cases encompassing benign, premalignant, and malignant oral lesions.

Analysis of age distribution revealed a higher prevalence of premalignant lesions in individuals aged <40 years, whereas malignant lesions were most frequently observed in the 51–60 year age group. These findings align with studies by **Nayak et al. (2017) [8],** who reported a peak in OSCC cases in the sixth decade. Demographic observation in the present research revealed a significant male predominance in the OED and OSCC groups. This trend is consistent with **Balasundaram et al. (2014) [9**], who reported a higher incidence of OSCC in males. This gender disparity may be attributed to higher exposure to risk factors such as tobacco and alcohol among males, particularly in rural settings.

According to the present study, the most common substance associated with oral malignant lesions (64%) was tobacco chewing. Only 4% of cases had no specific addiction

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described in oral premalignant and malignant lesions. These findings were similar to the study of **Akhilesh Krishna et al.[10]** Site distribution revealed buccal mucosa as the predominant site for the OED group and the gingivobuccal complex for OSCC. This observation is in concordance with **Moore and Catlin et al[11]**, where they have also stressed that gingival and buccal vestibule cancers generally originate in the area of placement of tobacco.

In this study, Analysis of MMP-9 expression showed a progressive increase in both proportion and intensity from benign through premalignant to malignant lesions. Similar findings have been reported by **Maurya et al. (2023)[12].** The variable expression of MMP-9 in WDSCC suggests differences in invasive potential among well-differentiated tumors. Although WDSCC is generally less aggressive, increased MMP-9 expression in some cases may indicate early invasive activity similar to that of **Jose and Mane et al. (2018) [7]** study, highlighting the potential role of MMP-9 in identifying tumors with more aggressive behavior. Collectively, these findings suggest that the evaluation of MMP-9 offers valuable insight into oral carcinogenesis and may improve diagnostic and prognostic assessment in routine clinical Pathology.

Generalizability

The findings apply to similar tertiary care settings in developing countries with comparable demographic and risk factor profiles.

Conclusion

The present study demonstrates that altered expression of MMP-9 plays a significant role in the progression of oral epithelial lesions. A concomitant increase in MMP-9 expression was observed from premalignant lesions to invasive oral squamous cell carcinoma, reflecting increased tumor aggressiveness and invasive potential. The forward relationship of this biomarker supports the involvement of epithelial–mesenchymal transition in oral carcinogenesis. Further large-scale, multicentric studies with long-term follow-up are warranted to validate the prognostic significance of MMP-9 expression. Integration of the biomarkers with molecular and clinical parameters may help refine risk stratification, guide targeted therapeutic strategies, and improve outcome prediction in patients with oral epithelial dysplasia and oral squamous cell carcinoma.

Limitations

One of the major limitations of the present study is the relatively small sample size, particularly within individual histopathological subgroups, which may limit the generalizability of the findings. In addition, the study was conducted at a single institution, thereby restricting population diversity. Although standardized criteria were used, some degree of interobserver variability in immunohistochemical scoring cannot be completely excluded. Furthermore, this was a cross-sectional study, and long-term clinical outcomes, including recurrence, metastasis, and survival, were not evaluated.

Recommendations

Based on the findings of the present study, MMP-9 may serve as a valuable biomarker for assessing the progression and aggressiveness of oral epithelial dysplasia and oralsquamous cell carcinoma. Larger multicentric studies with a greater sample size and longer follow-up periods are recommended to validate its diagnostic and prognostic utility. Further research exploring the molecular mechanisms of MMP-9 and its potential as a therapeutic target may enhance its clinical applicability in the management of oral premalignant and malignant lesions.

List of abbreviations

BM = Basement Membrane DAB = Diaminobenzidene
ECM = Extracellular matrix HRP = Horseradish peroxidase
IL-8 = interleukin-8
MDSCC = Moderately differentiated squamous cell carcinoma MMP-9 = Matrix metalloproteinase-9
OED = Oral epithelial dysplasia
OSCC = Oral squamous cell carcinoma
PDSCC = Poorly differentiated squamous cell carcinoma, TGFβ = Transforming growth factor β
WDSCC = Well-differentiated squamous cell carcinoma

Source of Funding

The study had no funding.

Conflict Of Interest Statement

The authors declare no conflicts of interest.

Data Availability

Data available on request.

Author contributions

AB-Conceptualization, study supervision, methodology, validation of histopathological findings, critical review of data, interpretation of results, and manuscript revision. **KP-**Study design, data collection, histopathological and immunohistochemical evaluation, statistical analysis, interpretation of results, manuscript drafting, and literature review. **SS-**Statistical analysis, data interpretation, methodological support, and critical review of the manuscript. **RKR-**Study supervision, validation of histopathological findings, interpretation of results, and critical revision of the manuscript.**AB-**validation of histopathological findings, interpretation of results, and

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critical revision of the manuscript.

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