ASSESSMENT OF THE RISK OF MALIGNANT TRANSFORMATION IN OSMF CASES USING CUPRIC CHLORIDE- AN OBSERVATIONAL CROSS-SECTIONAL STUDY

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ABSTRACT

INTRODUCTION:-Oral cancer is a devastating disease prevalent worldwide, often preceded by precancerous disorders like oral submucous fibrosis (OSMF). The variable clinical presentation of OSMF in patients, coupled with non-standardized diagnostic methods and the inherent risk of malignant transformation, underscores the urgent need for accurate diagnostic methods and effective preventive strategies to combat this debilitating disease. AIM:-To investigate the crystallization patterns in oral squamous cell carcinoma (OSCC), OSMF, and healthy individuals using a minimally invasive and economical crystallization test. METHODOLGY:- A cross-sectional serological study was conducted on 114 subjects, divided into three groups of 38 each. A drop of blood was used to perform the test, and the results were statistically analyzed according to the arrangement of crystallization patterns using Chi- square and ANOVA tests. RESULTS:- The eccentric patterns were predominantly found in healthy individuals, while leaf-like and hollow glans patterns were more common in OSMF cases. Transverse forms were more frequently observed in OSCC cases. A highly significant difference was found between the study groups. The test was found to be highly sensitive. CONCLUSION:- Crystallization test is a rapid, economical, and minimally invasive laboratory method that can be used as an adjuvant to biopsy for identifying/predicting underlying malignancy in oral premalignant conditions.

Keywords:- Oral submucous fibrosis, blood crystallization, cupric chloride, malignant transformation

INTRODUCTION –

In India, oral cancers are frequently diagnosed at advanced stages primarily due to delayed diagnosis and inadequate disease management leading to a mortality rate of approximately 40-50% within five years of diagnosis.[1]OSMF is a chronic, insidious disease which is a potentially malignant disorder (OPMD) that is primarily linked to areca nut consumption and is characterized by progressive fibrosis of the oral submucosal layer.[2]The variation in expression of OSMF in patients leads to lack of unanimous acceptance of the clinical and histological grading of the disease. Also, visual inspection is the preferred method over routine biopsy procedures which often fails to detect early precancerous changes accurately further leading to neoplastic transformation of the disease. Thus, early screening for assessing the underlying malignancy becomes crucial as consequent diagnostic delays may lead to poorer prognosis.[3]

In this context, the crystallization test emerges as a valuable diagnostic tool for identifying persistent oral malignancies. Introduced by Pfeiffer and Steiner in 1938, this blood-based qualitative method leverages the phenomenon of morphogenetic forces.[4] The biocrystallization concept relies on oxidative stress induced by internal metabolic conditions. Cupric chloride salt is used to assess the quality of the substance, giving rise to organic and inorganic salts. The resulting crystallization patterns, including "Leaf pattern," "Transverse bar Formations," and "Hollow Glans Formations," are characteristic of premalignancy and malignancy.[5]

This study aims to investigate the differences in crystallization patterns between oral squamous cell carcinoma, oral submucous fibrosis, and healthy individuals. By detecting underlying severity in OSMF patients before clinical manifestation of OSCC, this research seeks to contribute to improved disease management and patient outcomes.

METHODOLOGY—

A total of 114 patients were categorized into three distinct study groups for this research. GROUP-I (n = 38): Healthy individuals with normal oral mucosa, serving as controls. GROUP-II (n = 38): Patients diagnosed with oral submucous fibrosis. GROUP-III (n = 38): Patients diagnosed with oral squamous cell carcinoma. Blood samples were collected from all participants after obtaining written informed consent at the Outpatient Department of Oral and Maxillofacial Pathology. Selective inclusion and exclusive criteria were followed.

A standardized protocol was employed to prepare blood samples for crystallization analysis. A single drop of blood was obtained from patients undergoing routine presurgical hematological procedures, for which 2 ml of intravenous blood was drawn under aseptic conditions. The blood sample was then diluted to a 6% hemolyzed solution by adding a single drop to 1 cc of double-distilled water in a test tube at room temperature. Subsequently, 0.1-0.2 cc of the hemolyzed blood solution was gently mixed with 10 cc of 20% cupric chloride solution in a calibrated test tube. The resulting admixture was carefully poured into prewarmed, flat-bottom petri dishes (assay petri dishes), specifically prepared for each participant. The petri dishes were then placed undisturbed in an incubator maintained at a temperature of 28°C-32°C and humidity of 35%-55% in an isolated room. Crystallization was allowed to occur over 18-19 hours. The patterns were studied using a hand lens and stereomicroscope. The crystallization patterns were observed in all subjects. Four distinct crystallization patterns were photographed and traced over an X-ray viewer. These patterns were analyzed to understand their significance in diagnosing OSMF and OSCC.

Statistical analysis

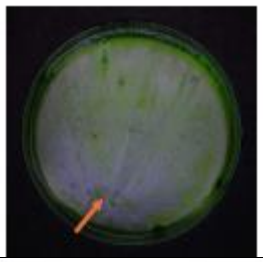
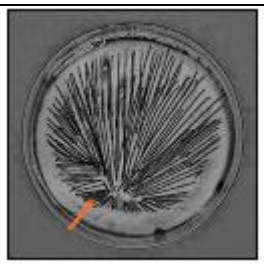
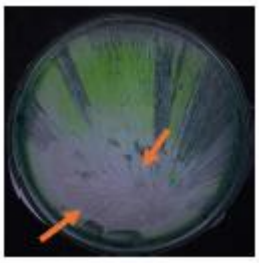
All statistical analyses were performed using the IBM SPSS 24 software (IBM Corporation, Armonk, New York, US). Descriptive data were presented as percentages. Two-sided tests were employed, with a significance level (alpha) set at 0.05. The chi-square test was used to evaluate categorical data among the groups. Cross-tabulation was performed to determine the frequency and association between groups. The sensitivity, specificity, positive predictive value, and negative predictive value of the crystallization test were calculated and tabulated. The mean transformation frequency (TF) was computed for each group. A p-value < 0.05 was considered statistically significant for all analyses.

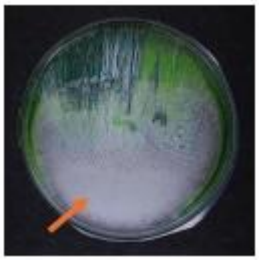
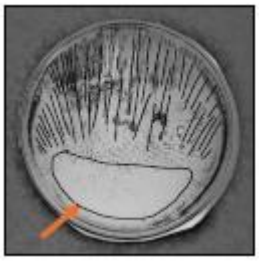
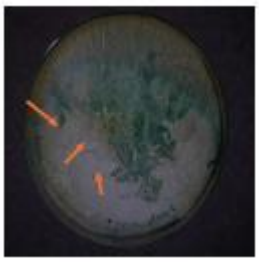
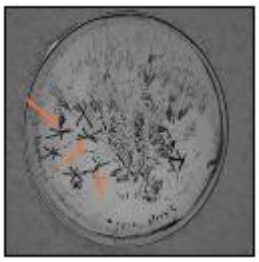
RESULTS—

Study observations:-

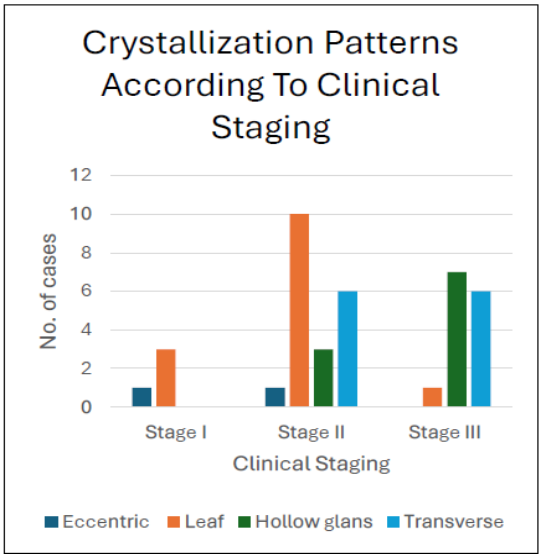
Our study of 114 subjects revealed that all four types of crystallization patterns (Eccentric, Leaf, Hollow Glans, and Transverse) were observed in Oral Submucous Fibrosis (OSMF) cases.**(Table 1)**. Eccentric pattern was predominant in healthy individuals. The Hollow Glans and Leaf patterns were most common in OSMF subjects. In OSMF cases, Leaf and Transverse patterns were equally prevalent, whereas Transverse pattern dominated in OSCC cases.**(Table 2)(Graph1)**The distribution of crystallization patterns varied significantly across OSMF, Oral Squamous Cell Carcinoma (OSCC), and healthy individuals (p=0.000).

Table 1 :- Characteristic Features Of Different Crystallization Patterns

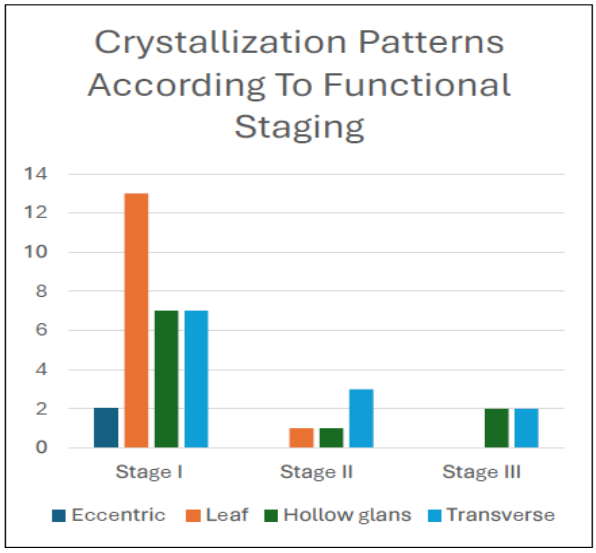
TYPE OF PATTERN	PATTERN CHARACTERISTIC	CRYSTALLIZATION PATTERN OBSERVED	PATTERN TRACING
Eccentric pattern	An orderly radiating arrangement of crystals from an eccentric center of gravity.		
Leaf pattern	A distinct pattern with a primary branch and numerous secondary branches, lacking transverse bars.		

Hollow Glans pattern	A combination of basal and tangential radiation, forming a rhomboidal hollow space		
Transverse Form	Presence of transverse needles withwing-like formations,showing horizontal bar, and unidirectional fanning patterns.		
	Presence of transverse needles with wing-like formations showing star shaped		

The crystallization patterns also varied across different clinical stages of OSMF, with Leaf pattern being most common in Stage II and Transverse pattern in Stage III (p=0.023).**(Graph 2)** However, no significant difference was found in crystallization patterns across different functional stages of OSMF.**(Graph 3)**



Graph 2: Bar graph depicting the distribution of crystallization patterns according to the Clinical staging in OSMF cases. Leaf pattern was more common in Stage I (75%), while hollow glans and transverse forms were more common in Stage III (50% and 43%, respectively.



Graph 3: Bar graph showing distribution of crystallization patterns according to the Functional staging in OSMF cases. Leaf pattern was more common in Stage I (45%), while transverse form was more common in Stage II (60%) and Stage III (50%).

Intergroup analysis:-

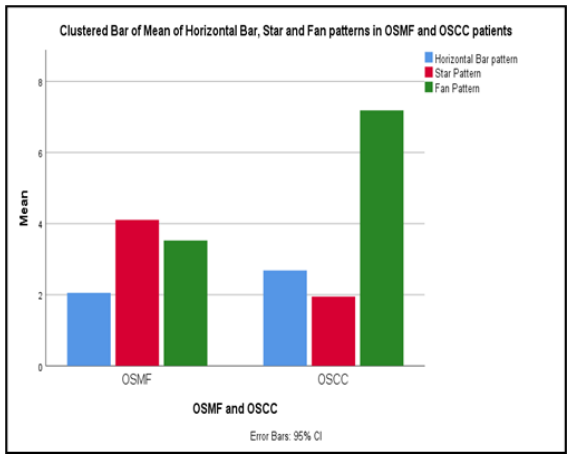
A comparative analysis of expressions of crystallization patterns between Oral Submucous Fibrosis (OSMF) and Oral Squamous Cell Carcinoma (OSCC) revealed statistically significant differences for distribution of Leaf pattern (p=0.043) and Hollow Glans pattern (p=0.003). Although the count of Transverse pattern showed no significant difference between the two groups (p=0.408), the number of Fanning subtype of Transverse pattern demonstrated a significant difference (p=0.008).**(Graph 4)**

Additionally, expressions of crystallization patterns in the groups were assessed in relation to the other clinico-demographic parameters like age, gender, habits, &tumor site, tumor size, histopathological grading and clinical staging in OSCC cases**.(Data mentioned in Table 3, Graph5, Graph 6, Graph 7, Graph 8, Graph 9, Graph 10)**

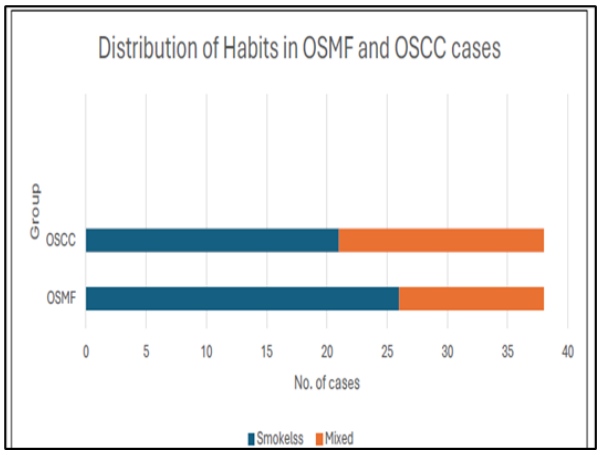
Table 3 :- Distribution Of Crystallization Patterns Among All Three Groups According To The Age And Gender

Distribution of patients according to the clinic-demographic parameters (age 15-70years)	Healthy individuals	OSMF	OSCC

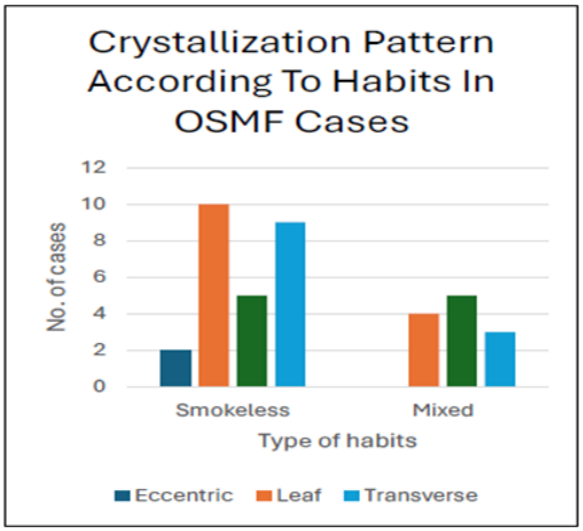
Age (≤40 and >40 years)	eccentric pattern was predominantly observed in both age groups (≤40 years: 87%, >40 years: 94%)	leaf-like and transverse patterns were more common in participants ≤40 years, while similar numbers of cases showed leaf, hollow glans, and transverse patterns in participants >40 years.	transverse form was predominant in both ≤40 years (84%) and >40 years (77%) age groups.
GENDER (Male/Female)	eccentric pattern was predominantly observed in genders (males: 81%, females: 100%).	male predominance (61%) with leaf pattern being more common in males (44%), while transverse form was predominant in females (40%) with statistical significance (p=0.054)	Gender-wise analysis showed male predominance (68%) with transverse form being predominant in both males (80%) and females (75%).



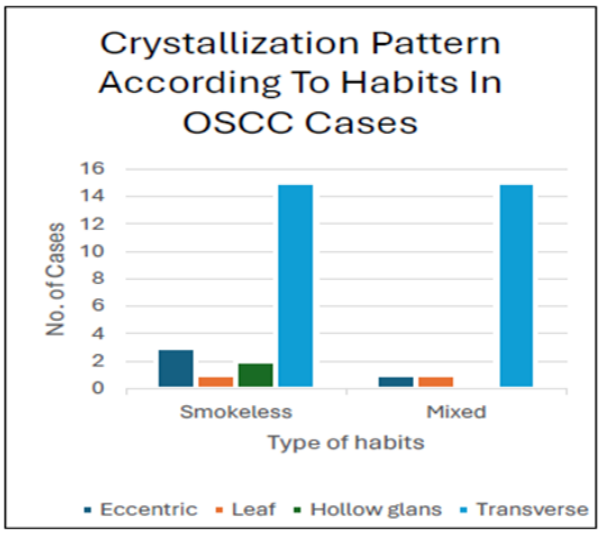
Graph 4 : Clustered bar showing mean of subtypes of transverse form in OSMF and OSCC patients.



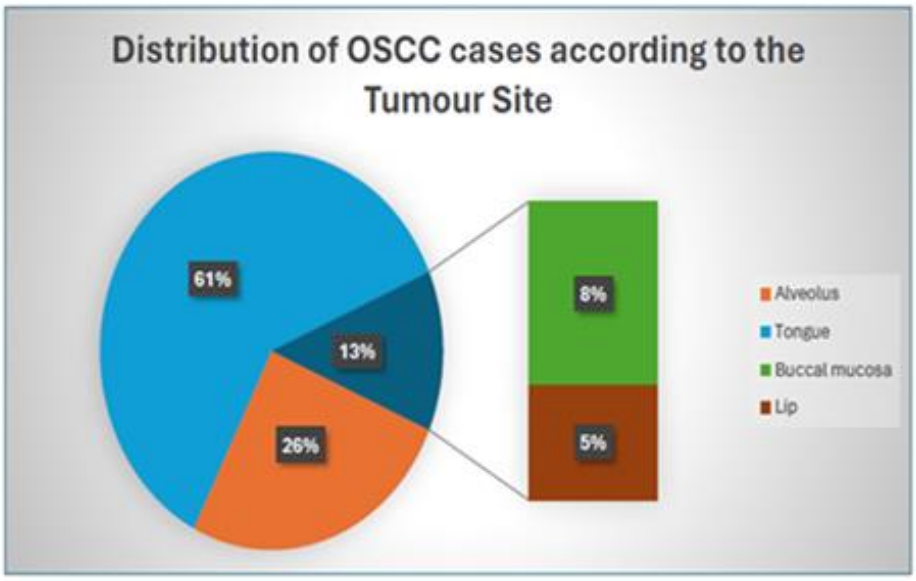
Graph 5:- Graph Showing Distribution Of Habits In OSMF And OSCC Cases



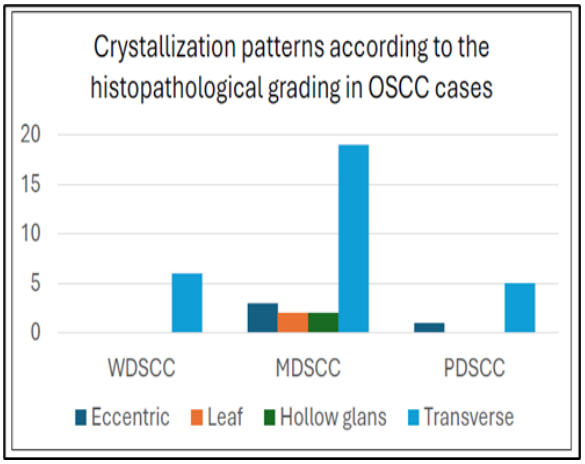
Graph 6 :- Bar graph showing habit wise analysis in OSMF cases. Leaf-like pattern (38%) and transverse form (35%) were more common in smokeless habit users, while hollow glans pattern (42%) was more common in mixed habit users.



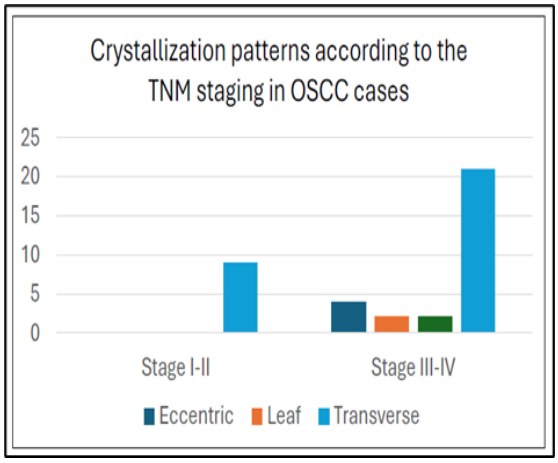
Graph 7:- Bar graph showing Habit-wise analysis in OSCC cases. Transverse form was predominant in both smokeless (71%) and mixed habit (88%) groups



Graph 8:- pie chart showing Tumor site-wise analysis. Transverse pattern was predominant in all sites, including alveolus (78%), tongue (80%), buccal mucosa (67%), and lip (100%). Statistical significance was observed for the difference in distribution of hollow glans pattern (p=0.028) and horizontal bar pattern (p=0.040).



Graph 9- Bar graph showing Histopathological grading-wise analysis. Transverse pattern was predominant in all grades, including well-differentiated (100%), moderately differentiated (74%), and poorly differentiated (83%) squamous cell carcinoma.



Graph 10- Tumor staging-wise analysis revealed that transverse form was predominant in both Stage I-II (100%) and Stage III-IV (72%) groups.

Table 4: Cross -tabulation for determining sensitivity, specificity and predictive values with Leaf pattern in OSMF Vs OSCC

Cross tabulation for Leaf pattern in OSMF vs OSCC			Positive	Negative	Total
OSMF	Positive	Count	14	24	38
		% within OSMF	**36.8%**	63.2%	100.0%
		% within Leaf Pattern	**93.3%**	39.3%	50.0%
	Negative	Count	1	37	38
		% within OSMF	2.6%	**97.4%**	100.0%
		% within Leaf Pattern	6.7%	**60.7%**	50.0%
Total		Count	15	61	76
		% within OSMF	19.7%	80.3%	100.0%
		% within Leaf Pattern	100.0%	100.0%	100.0%

Table 5: Cross -tabulation for determining sensitivity, specificity and predictive values with Hollow-glans pattern in OSMF Vs OSCC

Cross tabulation for Hollow glans pattern in OSMF vs OSCC			Positive	Negative	Total
OSMF	Positive	Count	10	28	38
		% within OSMF	**26.3%**	73.7%	100.0%
		% within Hollow glans Pattern	**83.3%**	43.8%	50.0%
	Negative	Count	2	36	38
		% within OSMF	5.3%	**94.7%**	100.0%
		% within Hollow glans Pattern	16.7%	**56.3%**	50.0%

Total	**Count**	12	64	76
	% within OSMF	15.8%	84.2%	100.0%
	% within Hollow glans Pattern	100.0%	100.0%	100.0%

DISCUSSION

The crystallization test, developed by Pfeiffer (1938), utilizes cupric chloride to detect changes in protein structures in human blood. This test has been shown to differentiate between healthy and pathological conditions, including cancer. The formation of specific crystallization patterns, such as the "Transverse form," is characteristic of malignancy. The biochemical basis of pattern formation is attributed to the presence of cell surface components, enzymes, and intermediate products of protein metabolism, such as diamines and polyamines, in the blood.[6]These molecules influence the crystallization process, resulting in distinct patterns that reflect the underlying physiological or pathological state.[7]Research has demonstrated that the addition of blood to cupric chloride solution alters the crystal structure, leading to the formation of specific patterns.[8] The sensitivity of cupric chloride to proteins and other biomolecules enables the detection of subtle changes in protein structures, making this test a valuable tool for diagnosing and monitoring diseases, including cancer.[6]

Crystallization Patterns in Healthy Individuals

A comparative analysis of the crystallization patterns in healthy individuals from our study with existing literature revealed similarities with the findings of Sarode et al., Gulati et al., Mehrotra et al., Tarigoppula et al., and Rawat et al. [6, 9, 10, 11, 12]. Notably, our study observed an eccentric pattern in 87% and 94% of patients in the ≤40 years and >40 years age groups, respectively. Additionally, we found a leaf pattern in 8% of cases and a transverse pattern in 5% of cases in the control group.(Table 3)The variability in patterns may be attributed to the presence of impurities and the colloidal state of the substances.

Crystallization Patterns in Oral Submucous Fibrosis

Our study observed four distinct crystallization patterns in oral potentially malignant disorders (OPMDs). The transverse pattern was the most significant, with a sensitivity of 80.0%. We also found that the leaf pattern was prominent in clinical staging of oral submucous fibrosis (OSMF), with a statistical significance difference of p=0.048 and showed sensitivity of 93.3% and specificity of 60.7 %.(Table 4) The hollow glans pattern, observed in 18% of OSMF cases, showed a sensitivity of 83.3% and specificity of 56.3%.(Table 5)The transverse pattern was associated with stage II and III clinical staging of OSMF, with statistical significance (p=0.023). Our findings suggest that the crystallization test can be a useful tool in diagnosing and monitoring OPMDs, including OSMF as the results obtained were in accordance with the previous studies carried out for other OPMDs.[6,11,13] Thus, our study highlights the importance of close follow-up for OSMF cases with transverse patterns, as they may develop OSCC in the future.

Crystallization Patterns in Oral Squamous Cell Carcinoma

The transverse formation of crystallization patterns is a hallmark of malignancy, observable in both early and advanced cancer cases.[14] Our study found that 24 out of 38 oral squamous cell carcinoma (OSCC) cases showed positive results (63%), with 14 cases exhibiting negative results. The crystallization test's high sensitivity is based on physical phenomena, and

shortcomings in maintaining required environmental conditions may lead to negative results. We observed three subtypes of transformation frequencies (TFs): Horizontal bar, Star pattern, and Fanning. The Fanning pattern was most prominent in malignancy cases, followed by combinations of Horizontal bar and Fanning. Our study found that the transverse form was predominantly noted for all tumor sites, with statistical significance for horizontal bar ($p=0.040$). The variation in tumor site may be linked to tobacco intake duration, frequency, and type. We also found a statistical significance of $p=0.028$ for hollow glans patterns correlated with tumor site in OSCC patients. The comparison between TNM staging of OSCC and various TF patterns did not reach statistical significance. However, our study showed contrasting findings with slight variations in the occurrence of transverse form according to histopathological grades of OSCC patients.

Comparing And Correlating OSMF And Healthy Individuals

The eccentric crystallization pattern was predominantly observed in healthy individuals and oral submucous fibrosis (OSMF) cases. Notably, this pattern was least common in OSMF (Table 2). A statistically significant correlation was found between OSMF and healthy individuals($p< 0.05$), consistent with the findings of Tarigoppula et al., who reported a significant difference between oral potentially malignant disorders and normal oral mucosa.[9]

Comparing and Correlating OSCC and OSMF

The present study revealed a male predominance in oral submucous fibrosis (OSMF) cases, with 84% of males affected ($p=0.054$ for transverse form, $p=0.031$ for fanning). The high incidence of OSMF among males may be attributed to the higher prevalence of tobacco intake among males (68%) compared to females (32%).The study also found that the alveolus was the most commonly affected site in oral squamous cell carcinoma (OSCC) cases, followed by the tongue, buccal mucosa, and lip. This finding contrasts with previous studies, which reported the buccal mucosa as the most commonly affected site.[15]The crystallization test revealed significant correlations between OSMF and OSCC cases, with high significance observed for leaf pattern ($p=0.043$) and hollow glans crystallization pattern ($p=0.003$). The fanning pattern subtype of transverse form also demonstrated high significance ($p=0.008$) in OSMF and OSCC patients. The study's findings are consistent with previous research, which reported the high significant difference of expression of transverse crystallization patterns in oral potentially malignant disorders and oral malignancies.[9,16, 17] However, the study's small sample size and single-center design limit its ability to assess the underlying malignancy in OSMF cases.

CONCLUSION

The crystallization test could be used as a valuable diagnostic tool for identifying underlying oral malignancies, particularly in oral submucous fibrosis (OSMF). Our study demonstrated significant correlations between OSMF and OSCC cases, with high significance observed for leaf pattern, hollow glans crystallization pattern, and fanning pattern subtype of transverse form. However, further studies with larger sample sizes and multicenter designs are needed to validate the crystallization test's efficacy in detecting underlying malignancy in OSMF cases. Additionally, research should focus on standardizing the crystallization test protocol and exploring its potential applications in other oral and systemic diseases. The crystallization test

offers a simple, non-invasive, and cost-effective method for assessing the potential risk of malignant transformation in OSMF cases. Its incorporation into routine clinical practice may improve disease management and patient outcomes, particularly in resource-constrained settings.

FINANCIAL SUPPORT AND SPONSORSHIP—
Nil.

CONFLICTS OF INTEREST—

There are no conflicts of interest.

Reg. No: CTRI/2024/02/062193

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