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Research Article

Comparative Evaluation of Pfeiffer's Blood Crystallization Test for Early Detection of Epithelial Dysplasia in OSMF and Healthy Individuals: A Cross-Sectional Study

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Abstract

Background: Unlike other malignancies, oral cancer is preceded by Oral Potentially Malignant Disorders (OPMDs). Oral Submucous Fibrosis (OSMF) is one such OPMD with malignant potential of 7.6%. The purpose of present study was to determine the efficacy of Pfeiffer's blood Crystallization test for early detection of epithelia dysplasia in OSMF individuals.

Material and methods: The study participants included were involved 50 OSMF subjects and 50 healthy controls. The blood drop collected was subjected to crystallization test and the crystal patterns formed were analyzed after 20 hours. Incisional biopsy was performed in OSMF subjects to grade the degree of epithelial dysplasia. The data obtained was subjected to statistical analysis.

Results: The participant's age in the study groups ranged up to 56 years. Difference in the crystallization pattern between diseased group and non-diseased group was significant (p-value < 0.05%) with more transverse pattern in diseased group. The OSMF participants with no epithelial dysplasia showed normal crystallization pattern and maximum participants with dysplasia showed transverse pattern and the difference was statistically significant (p-value<0.05%). The sensitivity and specificity of crystallization test for OSMF was found to be 91.4% and 100.0% respectively.

Conclusion: Pfeiffer's Crystallization test can be considered as a less invasive and reliable screening aid for OSMF which helps clinicians to identify high-risk individuals for malignant transformation.

***Keywords:** Oral Submucosal Fibrosis, crystallization test, screening test, epithelial dysplasia.*

Introduction

In today's world, Cancer is one of the most dreadful health problem. There is enormous variation in the incidence of head and neck cancer all around the world. The Oral Squamous Cell Carcinoma (OSCC) is associated with significant morbidity and mortality. [1] Transformation of normal oral epithelial tissue to invasive oral cancer is a multistep process with an intermediate stage of oral lesions known as Oral Potentially Malignant Disorders (OPMD's). [2] OPMDs include Leukoplakia, Erythroplakia, Oral Lichen Planus, Discoid Lupus Erythematosus, Proliferative Verrucous Leukoplakia, Oral Submucous Fibrosis and Plummer-Vinson syndrome. [3]

Among the OPMD's, Oral Submucous Fibrosis (OSMF) is one of the most common oral potentially malignant disorder involving the oral cavity which has malignant transformation rate of 7.6% over a 17-year period. [4] Areca nut related habits (with or without tobacco) remain as an epicenter for etiological risk factors for the high incidence of these lesions in south-east Asian countries. In India, the prevalence of OSMF ranges from 0.2% to 4.6% and it is seen both in pediatric and adult population with are canut habit in any form.[5] Carcinogenic or mutagenic character of are canut which may result in alterations in DNA, cell senescence, tissue hypoxia, tumor cell proliferation etc and these are the mechanisms involved in its malignant transformation. OSCC developing from OSMF is highly invasive and despite the established risk factors and advances in treatment, there is only 5-year survival for oral squamous cell carcinoma.[6-10] Hence to improve the survival rate, early detection of malignant changes in OPMDs has to be done.[11] Many methods and techniques have been tried till date for early detection of oral cancer. This includes vital staining procedure (Toulidine Blue and Lugols iodine), Brush Biopsy (Oral CDx Brush), micronuclei anlaysis, DNA ploidy and light-based techniques. Chemiluminescence like ViziLite, ViziLite Plus, MicroLuxTM/DL and autofluorescent imaging like VELscope (Visual Enhanced Light scope) are promising light-based techniques for early detection of Oral Cancer. These methods available for early detection have certain clinical limitations or false positive results are more if the inflammatory component is present in OPMDs. [12-13] The need of the hour is a screening method that is simple, economical, reliable, less time consuming and less invasive.

Infusion of cupric chloride ($CuCl_2$) to blood of a cancer patient leads to typical transverse type of crystallization. This crystallization test was developed in 1938 by Pfeiffer, a German scientist. It was suggested by Gulati et al, that the altered crystallization pattern is due to colloidal proteins that are present in extremely dilute solution in human blood act as impurities when mixed with $CuCl_2$ solution. Due to addition of $CuCl_2$ to blood, during evaporation process there is change in rates and amplitudes of molecular movements of organic and inorganic salts which leads to crystallization. Other researchers had an opinion that the protein degraded products such as polyamines and diamines will be increased in the blood of diseased is causing altered crystallization pattern. [14-15] The present study is planned to validate the blood crystallization test in OSMF individuals comparing it with the degree of epithelial dysplasia.

Material and Methods

The study was approved by Institutional Ethics Committee, with identification number D21039.

Sample Selection

Total 100 participants were included in the study, the participants were distributed in two groups having 50 (50%) participants were in Oral Submucous Fibrosis (OSMF) or diseased group and 50 (50%) were in Healthy Control (HC) or non-diseased group.

Inclusion criteria

Participants above 18 years, who were clinically and histo-pathologically diagnosed for having Oral Submucous Fibrosis associated with any type of areca-nut related habit (with or without tobacco) irrespective of duration of habit was included in study. Participants in the Control group were age and sex matched healthy individuals, with no history of past and present systemic disease and areca-nut or tobacco habit.

Exclusion criteria

The individuals with any type of systemic disease or who had undergone any type of treatment for Oral Sub mucous Fibrosis in past or present was excluded from the study. The participants with OSMF who had quit their areca-nut habit for at least for 6 months was excluded from the study.

Histo-pathological Analysis (for clinically diagnosed OSMF Individuals)

An Oral Biopsy was performed under local anesthesia (infiltration and/or nerve block) on patients who were clinically diagnosed with OSMF [16]. Either punch biopsy or incisional biopsy was taken and specimen was placed in a sterile pathology specimen jar, containing 10% buffered formalin for fixation. Histo-pathological diagnosis of Oral Epithelial Dysplasia was done as per classification given by WHO in 2017 (mild, moderate and severe dysplasia/carcinoma in situ). [17]

Pfeiffer's blood Crystallization test

A drop of blood was collected by pricking the ring finger/index finger by lancet under aseptic conditions. The blood drop was added to 1 cc of double distilled water, to achieve dilution of 6% hemolyzed blood and 0.1–0.2 cc of blood sample was then added to 10 cc of 20% Cupric chloride solution. Immediately, the mixture was poured in the pre-warmed petri dish of 10 cm size. The Petri dish was then placed in Biological Oxygen Demand (BOD) incubator (temperature: 28°C–32°C and humidity of 35% to 55%) in an isolated room for about 18-19 hours. Then the crystallization pattern was studied in proper day time and they were noted as crystals with eccentric centre of gravity with radiating needles pattern/Normal, Leaf like pattern and transverse pattern. [18] [Fig 1].

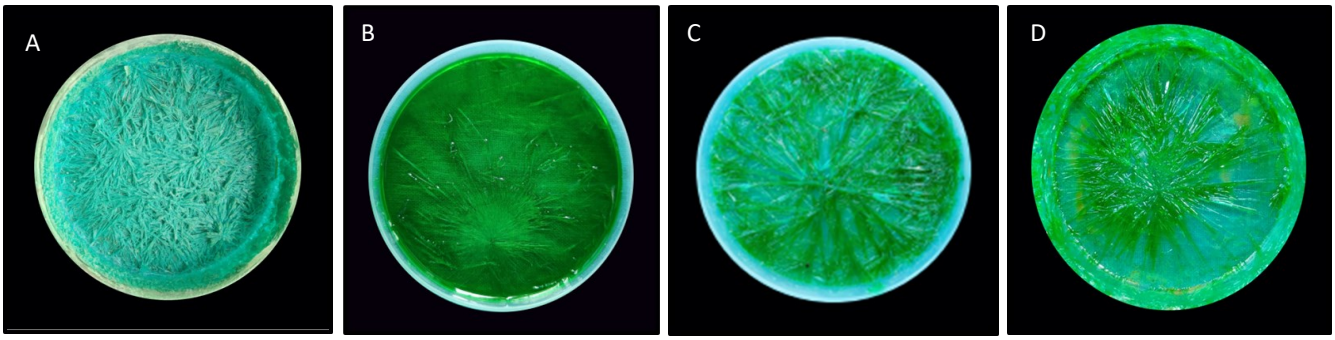

Figure 1 A-D: *Crystallization patterns of cupric chloride solution alone showing muddle formation (A), cupric chloride solution in healthy control group showing center of gravity and radiating crystals (B), Crystallization patterns of cupric chloride solution in OSMF individuals showing transverse pattern (C) and leaf like Pattern (D)*

Statistical Analysis

The data of each participant was be entered in Master chart prepared in Microsoft Excel Sheet. The data included the demographic details, clinical staging of the disease [16],histo-pathological grading of epithelial dysplasia.[17] and the crystallization pattern [18-20] The collected data then was be subjected to statistical analysis (Chi Square test).

Results & Observations

A total of 100(N) subjects were included in the study, 50 in each group. The study sample included the OSMF patients and healthy controls with age groups ranging from 19-56 years with the mean of 36.26+10.85 and 34.60+09.79 in Group I and Group II respectively. Chi-square test was used to compare age between groups and the p-value was 0.424. Out of 100 participants, 69 were males and 31 were females (Table 1) Maximum number participants were having Clinical Stage IV OSMF (n=22, 44.0%), M2 Functional Stage OSMF (n=17, 34.0%) and mild dysplasia (n=20, 40.0%)(Table 2). The comparison of distribution of OSMF clinical staging, functional staging and degree of epithelial dysplasia between different frequencies of areca nut chewing and different duration of are canut chewing using the chi-square test was statistically not significant. Crystallization pattern between diseased group and non-diseased group was significant (p-value < 0.05) with more transverse pattern in diseased group. (Table 3) The OSMF participants with no epithelial dysplasia showed normal crystallization pattern and maximum participants with dysplasia showed transverse pattern and the difference was statistically significant(p-value<0.05%)(Table 4). The sensitivity and specificity of crystallization test for OSMF was found to be 91.4% and 100.0% respectively (Table 5)

Table 1: Demographic characteristics of the included subjects in the study

S. No.	Group	N	Gender*			Age (Yrs.)			
			M	F	p-value (Chi Square test)	Min-Max Age	Mean	Std. D	p-value (Chi Square test)
	Group 1 (OSMF/ diseased)	50	41(82%)	9(18%)	0.065	19-56	36.26	10.85	0.424
	Group 2 (HC/ non-diseased)	50	28 (56%)	22 (44%)		21-56	34.60	9.79	

* M: Male, F: Female

Table 2: Clinical staging, Functional staging and epithelial dysplasia in Oral submucous Fibrosis patients.

Clinical staging*	N	Functional staging*	N	Epithelial Dysplasia**	N
I	1(2.0%)	M1	6(12.0%)	No dysplasia	15(30.00%)
II	7(14.0%)	M2	17(34.0%)	Mild dysplasia	20(40.00%)
III	20(40.0%)	M3	11(22.0%)	Moderate dysplasia	5(10.00%)
IV	22(44.0%)	M4	16(32.0%)	Severe dysplasia	10(20.00%)
Total	50(100%)	Total	50(100.0%)	Total	50(6100.0%)

* As per More et.al; **As per WHO classification of Epithelial Dysplasia

Table 3: Comparison of distribution of Pfeiffer's Crystallization pattern between both Group 1 and Group 2 (Chi Square test).

Pfeiffer's Crystallization pattern	Groups		Total
	Group 1	Group 2	
Eccentric centre of gravity with radiating needles pattern	18(36.0%)	48(96.0%)	66(66.0%)
Leaf like pattern	2(4.0%)	0(0.0%)	2(2.0%)
Transverse pattern	30(60.0%)	2(4.0%)	32(32.0%)
Total	50(100.0%)	50(100.0%)	100(100.0%)
χ^2 value = 43.765, p-value = 0.001			

Table 4: Comparison of distribution of Pfeiffer's Crystallization pattern between grading of epithelial dysplasia (Chi-square test).

Pfeiffer's Crystallization pattern	Staging of Epithelial dysplasia in OSMF*				Total
	No dysplasia	Mild	Moderate	Severe	
Eccentric centre of gravity with radiating needles pattern	15 (100%)	2 (10%)	1(20%)	0 (0%)	18(36%)
Leaf like pattern	0 (0%)	1(5%)	0 (0%)	1(10%)	2(4%)
Transverse pattern	0(0%)	17(85%)	4(80%)	9(90%)	30(60%)
Total	15 (100%)	20 (100%)	5 (100%)	10 (100%)	50(100%)
χ^2 value = 39.444, p-value = 0.001					

(*OSMF - Oral Submucous Fibrosis)

Table 5: Positive/negative crystallization test with statistical analysis in both the groups (Diseased and Non-diseased).

Crystallization Test Results	Diseased		Non-Diseased
	Dysplasia-Absent	Dysplasia-Present	
Positive	0	32	2
Negative	15	3	48
Sensitivity	**91.4%**		-
False negative	0	3	0
False Positive	0	0	2
Specificity	**100.0%**		-
Positive Predictive Value	**100.0%**		-
Negative Predictive Value	**83.3%**		-
Accuracy	**94.0%**		-

Discussion

Out of these OPMDs, OSMF is a condition demonstrates more morbidity as compared to oral leukoplakia and oral erythroplakia. [20] Screening tests with 100% accuracy is more valuable both for health care provider and the person who is suffering from the disease. [21] In a Taiwan's observational study conducted between 2010 to 2013, OSMF was found to be the 2nd most common OPMD next to oral verrucous hyperplasia to have malignant transformation potential. [22] As per estimation done in year 2018, over 177,000 deaths occur in a year due to Oral Squamous Cell Carcinoma (OSCC). [23] In another two previous follow-up studies done in Taiwan showed that individuals having OSMF with epithelial dysplasia developed OSCC more commonly when compared with OSMF with no epithelial dysplasia. [24,25] The overall malignant transformation rate of OSMF is found to be from 1.9 to 9%. [5] Hence, the main goal while screening OSMF cases should be to differentiate the cases with and without epithelial dysplasia. In many of the screening methods, authors recommend histopathological evaluation ultimately to confirm the dysplastic changes in epithelium. Hence there is a requirement of more apt and reliable and accurate technique for screening OSMF. This study was conducted with an aim to determine the efficacy of Pfeiffer's blood Crystallization test for early detection of epithelia dysplasia in OSMF individuals.

The present study, the study groups consisted of participants clinically and histo-pathologically diagnosed as a case of OSMF and healthy controls (HC) having 50 participants each. In OSMF group, the age of the participants ranged from 19 to 56 years with the mean age of 36.26± 10.85 years. When sex distribution was analyzed, male predominance (M: F=4.6:1) was noted indicating the consumption of areca nut products (with or without tobacco) which is responsible for OSMF disease process is more among males. The findings of our study are consistent with the studies conducted by Kumar A [26], Chole RH [27], Karemore T.V[28], Shah PH [29], Singh PK [30], Angadi PV [31] and Raina C [32]. Female predominance was noted in a study conducted by Arshad O [33]. In present study we found that the different stages of OSMF (clinical and functional) were independent of frequency and duration of areca nut related product consumption. In a study conducted by Hosein M et al. [34] and also in a study conducted by Jha VK et al. [35] authors found that the severity of OSMF increases with the increase in frequency of areca nut chewing per day, however authors found the duration of years of areca nut habit did not influence the severity of the disease. In a study conducted by Srivastava R et al., authors found that both higher frequency and higher duration of areca nut habit is associated with the severity of the OSMF. [36]

In present study, histological evaluation was done in diseased group (OSMF) for evaluating epithelial dysplasia. When different epithelial dysplasia stages were compared with the clinical and functional staging of the disease, there was no correlation established. There was no significant correlation observed when duration and frequency of areca nut habit was compared with epithelial dysplasia. Previously, many authors have compared the histologic staging of OSMF with the clinical and or functional staging of OSMF and they have not found any correlation between the histological stages of the disease with the clinical stages of the disease. [36,37]

Few authors have found that the histological severity of disease is correlated with clinical severity [37, 38] of the disease and also with frequency and duration [39] of areca nut habit. But these authors have not compared the epithelial dysplasia in OSMF with the clinical staging of the disease or duration and frequency of areca nut habit. From present study we found that the epithelial dysplasia can be seen in any clinical stage of the disease and is independent to the frequency and duration of the habit. In a preliminary study conducted by Sarode SC et al [40], authors recommended that OSMF individuals showing dysplastic features like increased basal cell layer hyperplasia, hyperchromasia, increased nuclear-cytoplasmic ratio etc should be considered as high risk group for malignant transformation and should be kept on follow up for early detection of OSCC. In another study the authors have concluded that the degree of epithelial dysplasia increases with increased thickness of fibrosis. [41] The long term follow up studies have shown that the rate of malignant transformation is more in OSMF with epithelial dysplasia. [24,25]

In present study, the Pfeiffer's crystallization test was performed on individuals suffering from OSMF and the crystallization patterns were compared with different stages (clinical and functional) of the disease, degree of epithelial dysplasia associated with the disease and with the crystallization patterns of healthy individuals. In non-diseased group, out of 50 healthy controls 48 demonstrated the orderly arrangement of crystals which is typical to healthy persons i.e. single eccentrically situated center of gravity with radiating crystals coming from the center toward the margin. Two individuals demonstrated transverse patterns. Out of 50 study participants in diseased group, 18 participants had crystallization pattern of eccentric centre of gravity with radiating needles, 2 participants were having Leaf like crystallization pattern and 30 participants showed transverse crystallization pattern. When crystallization patterns of both the groups were compared the significant difference was noted suggesting this test be effective in screening OSMF for malignant transformation. The results of our study are similar to the studies conducted by Ingle V [42] and Tarigoppula RK [19]. Few authors [15,18,42,43] have studied crystallization pattern in other OMPDs like oral leukoplakia and they have found the similar results and hence this test can be effective tool in screening all OPMDs for malignant transformation. In present study the crystallization pattern in diseased group was compared with clinical and functional staging of the disease which was found to be significant. Also, when degree of epithelial dysplasia in diseased group was compared with crystallization pattern, the difference observed was statistically significant. In present study the crystallization pattern observed in cases with no dysplasia was normal pattern what we observe in healthy individual. Sarode SC et al. [30] did clinico-pathological correlation of OSCC with the crystallization pattern in which the authors counted the transverse patterns and compared it with the histo-pathological grades and clinical stages of OSCC. Sarode SC and his colleagues found that the mean transverse pattern increased as clinical stage and histological grade of the disease increased. [30] In present study also we found the positive (transverse or leaf like) crystallization pattern in mild, moderate and severe epithelial dysplasia.

In present study, sensitivity and specificity of Pfeiffer's crystallization test for OSMF was found to be 91.4% and 100.0% respectively. Makkar V et al. [15] found 80% sensitivity of crystallization test for oral leukoplakia. In Tarigoppula RK et al. [19] study, sensitivity and specificity of crystallization test for the OPMD group were found to be 83.33% and 86.84% respectively. Ingle VM et al. [42] found 85% sensitivity and 90% specificity of crystallization test for OPMDs. Rawat G et al. [18] found the sensitivity of crystallization test for OSCC to be 96% and for leukoplakia 92%.

In present study the quantification of transverse bar was not calculated. Obtaining the mean number of transverse crystals and comparing it with degree of epithelial dysplasia will give clearer picture when screening for oral cancer is conducted.

Conclusion

From present study, we can conclude that the Pfeiffer's Crystallization test can be considered as a less invasive and reliable screening aid for OSMF which helps clinicians to identify high-risk individuals for malignant transformation.

Conflict of Interest

The author declare no conflict of interest.

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