Original Article

Characterization of anti-nuclear antibodies in patients with oral submucous fibrosis and its clinicopathologic implications – An immunofluorescence study

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Abstract

Background: Oral submucous fibrosis (OSF), widespread in the Indian subcontinent, is a chronic debilitating disease of the oral cavity having a high potential for malignancy. The etiology of OSF is debatable. However, recently, autoimmunity had been suggested to play a significant role in its etiology, yet unproven. Hence, this study was conducted to explore the presence of antinuclear antibodies (ANAs) in the serum of OSF patients which is one of the serum markers of autoimmune diseases and its possible clinicopathologic associations.

Materials and Methods: A total of 105 blood samples were collected from patients with OSF ($n = 45$), age- and sex-matched healthy controls with ($n = 30$) and without ($n = 30$) areca nut chewing habit. Serum positivity of ANA was determined by immunofluorescence and correlated with the oral habits and severity of the disease measured by maximum mouth opening (MMO) and site of involvement.

Results: Significantly higher incidence of ANA (35.6%) was found in 45 OSF patients than in the healthy group ($P = 0.001$). Prevalence of ANA positivity was found higher in females ($n = 11$; 68%) than males ($P < 0.001$). A significantly lower MMO ($P = 0.00$) was found in ANA positive patients (17 ± 6.21 mm) in contrast to MMO in ANA-negative patients (28.74 ± 6.58 mm). The mean duration of habit and frequency of habit between ANA positive and negative patients was not significant. A significantly more number of sites of involvement was seen in ANA positive cases ($P = 0.004$). Out of 16 ANA positive OSF cases, 10 cases showed + 2 and 6 cases showed + 3 fluorescence intensity. Speckled ($n = 8$), homogeneous ($n = 5$) and nucleolar pattern ($n = 3$) were the fluorescence patterns observed.

Conclusion: The presence of autoantibodies such as ANA, female predilection, alteration of humoral and cellular immunity justifies OSF as an autoimmune disease. This study provides broader prospective to adopt therapies that selectively target autoimmune pathways.

Keywords: Antinuclear antibodies, autoantibodies, autoimmune, immunofluorescence, oral submucous fibrosis

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Access this article online

Quick Response Code:

Website:
www.jomfp.in

DOI:
10.4103/jomfp.jomfp_376_20

INTRODUCTION

Oral submucous fibrosis (OSF) is a multifactorial, chronic, progressive, highly prevalent scaring disease of the oral cavity affecting the lamina propria and submucosa, resulting in loss of fibro elasticity.[1] Early signs are burning sensation and blanching of the oral mucosa. It is clinically characterized by leathery mucosal texture and palpable fibrous bands leading to limitation of mouth opening and sunken cheeks. Depending on the site of involvement, it shows wide variation in signs and symptoms. There is restricted movement of the tongue affecting speech and atrophic papillae. The palate if affected appears pale, with horizontal fibrous bands across the soft palate. Uvula may be shortened and deformed.[2] The clinical assessment of the severity of the disease is done by inter-incisal maximum mouth opening (MMO) and the number of sites of involvement.[3]

Literature has suggested chewing tobacco, chilies, high levels of copper in foodstuffs, vitamin deficiencies, malnutrition, resulting in low levels of serum proteins, anemia and genetic predisposition as possible etiological factors.[4] OSF also being termed as collagen disease shares some of the clinicopathological features including extracellular structural alteration of connective tissue with that of scleroderma and systemic lupus erythematosus (SLE), the latter being known as definable collagen diseases.[5] Autoimmune collagen diseases such as SLE, scleroderma and rheumatoid arthritis are characterized by the presence of high serum titer of autoantibodies known as antinuclear antibodies (ANAs). These are autoantibodies directed against nuclear constituents of cell.[6] Although extensive investigations have been performed to determine its etiopathogenesis, a definite etiology of OSF is still obscure.[1] Therefore, in the present study, we have attempted to explore the autoimmune etiology of OSF by indirect immunofluorescence (IIF), which is a gold standard method for the detection of autoantibodies. The presence of serum ANA in OSF was compared with healthy subjects with and without tobacco chewing habit. An effort has been made to investigate its relationship with the severity of the disease, frequency, and duration of the habit. Further, a note has been added on the observational finding on ANA pattern distribution.

MATERIALS AND METHODS

Subjects and sample collection

The study group comprised 45 clinically diagnosed cases of OSF patients based on the criteria proposed by Sirsat and Pindborg (1967).[7] Sixty age- and gender-matched healthy subjects with ($n = 30$) and without ($n = 30$) areca nut chewing habit were selected as control groups. All OSF patients and healthy subjects were selected from the Department of Oral Pathology and Microbiology, SCB Dental College and Hospital, Cuttack. Patients with autoimmune disease, viral infection, malignancy and older people > 65 years of age, pregnant women and patients taking some specific drugs affecting ANAs were excluded from the study. A verbal explanation of the serum autoantibody test and risk was explained to all the patients and signed consent was obtained. History of the duration of habit and frequency of quid chewing per day was recorded. Determination of the severity of the disease in the study group was done by recording the number of oral mucosal sites of involvement and MMO of OSF patients.

The blood samples drawn from OSF patients and healthy control subjects were allowed to clot at room temperature and then centrifuged at 3000 rpm for 10 min to separate the serum. The samples were stored in a deep freezer at −20°C. The samples to be investigated within 48 h were stored at a temperature between + 2°C and + 8°C. Hemolysed, contaminated, lipemic and icteric samples were discarded.

ANA test method

The circulating ANA were detected by IIF using a diagnostic kit (HEp-20-10, EUROIMMUNE, Germany). Tissue sections of monkey liver and human epithelial cell lines were used as substrate and incubated with diluted patient serum. If there are specific antibodies in the serum they get attached to the antigens and the reaction becomes positive.

30 μl of the diluted sample (1:100 dilutions) was applied to each reaction field of the reagent tray, avoiding air bubbles using a polystyrene template. The reaction was initiated by fitting the biochip slides into the corresponding recesses of the reagent tray and was incubated for 30 min at room temperature (+18°C to + 25°C). It was then rinsed with a flush of PBS-Tween using a beaker and was immersed immediately afterward in a cuvette containing PBS Tween for at least 5 min and was shaken with a rotary shaker. 25 μl of fluorescein-labeled anti-human globulin was applied to each reaction field of a clean reagent tray. The slide was protected from direct sunlight and incubated for 30 min at room temperature (+18°C to +25°C). The Biochip Slide was rinsed with a flush of PBS-Tween using a beaker and was put into the cuvette filled with the new PBS-Tween for at least 5 min and was shaken with a rotary shaker. The biochip slide was dried with a paper towel and put facing downwards, onto the prepared cover glass. The presence of fluorescence,

its intensity and pattern was read using a fluorescence microscope with ×40 objective.

Statistical analysis

Data collected were subjected to statistical analysis using IBM corp. SPSS software version 22.0, Armonk, New York. Comparison for the presence of serum ANA among the study and the control group and comparison between the number of sites of involvement and ANA positivity was performed by Chi square test. An independent sample *t*-test was applied for comparison of age distribution among ANA positive and negative individuals. ANOVA test was applied for comparison of gender distribution among the study and the control groups. Mean MMO was compared between ANA positive and ANA negative groups using the Mann–Whitney test. Kolmogorov–Smirnov test was carried out to compare between frequency and duration of habit and ANA positivity. Evaluation of the results was within a confidence interval of 95% and a *P* value less than 0.05 was considered statistically significant.

RESULTS

Among 45 OSF patients, 35.6% of OSF patients had serum ANA compared to 6.7% (*n* = 60) in healthy group. Chi-square test revealed that the presence of serum ANA in OSF patients is higher than the other two healthy groups which is statistically significant (*P* = 0.001). However, no significant difference in presence of ANA was observed between the two healthy groups [Table 1]. No significant difference (*P* = 0.735) was found between the mean age of ANA positive subjects (30.53 ± 7.3) and ANA negative patients (31.77 ± 8.3). Prevalence of ANA positivity was found higher in females (*n*=11; 68%) than males.(*P* < 0.001)

A significantly lower MMO with *P* value of 0.00 was found in ANA positive patients (17 ± 6.21 mm) in contrast to MMO in ANA negative patients (28.74 ± 6.58 mm). The mean duration of habit was 7.63 ± 3.96 years in ANA positive patients and 9.48 ± 4.27 years in ANA negative patients and the difference was not statistically significant (*P* = 0.823). Mean frequency of habit in quids/day was comparatively higher in ANA negative patients (13.36 ± 7.01) than in ANA positive patients (11.44 ± 6.73), but the difference was nonsignificant (*P* = 0.701) [Table 2].

Intra-oral sites of involvement ranged from minimum of 2 to maximum of 7. Out of 45 OSF patients, 30 patients had ≥5 sites of involvement and 15 patients had <5 affected sites. Amongst patients with involvement of sites ≥5, 50% were ANA positive, whereas amongst patients with involvement of sites <5 only 6.70% were ANA positive. A significantly more number of sites of involvement was seen in ANA positive cases (*P* = 0.004) [Table 3].

The fluorescence intensity was assessed semi-quantitatively from 1 + to 4 + relative to the intensity of a negative (0) and a positive control (+4). The present study revealed that out of 16 ANA positive OSF cases, 10 cases showed + 2 intensity and 6 cases showed + 3 intensity. 3 different types of fluorescence patterns were observed in which 8 were speckled, 5 were homogeneous and 3 were of the nucleolar pattern [Table 4 and Figure 1].

DISCUSSION

OSF is a multifactorial disease characterized by progressive limitation of mouth opening and has a significant impact on the quality of life of affected individuals because of its high morbidity and malignant transformation rate.[2] Although there are several propositions suggested in the

Table 1: Comparison of presence of antinuclear antibodies among study groups

Study groups	Total	Presence of ANA	
		Negative, *n* (%)	Positive, *n* (%)
OSF patients with habits	45	29 (64.40)	16 (35.60)
Healthy group without habit	30	28 (93.30)	2 (6.70)
Healthy group with habit	30	28 (93.30)	2 (6.70)
Total	105	85 (81.00)	20 (19.00)

χ^2=13.918, *P*=0.001. ANA: Antinuclear antibodies, OSF: Oral submucous fibrosis

Table 2: Comparison of maximum mouth opening, duration and frequency of habit between antinuclear antibodies positive and antinuclear antibodies negative cases within the patient group

	Mean	95% CI for mean		SE	Significance, *P*
		Upper bound	Lower bound		
Maximum mouth opening					
ANA +ve	17.00±6.21	20.31	13.69	1.553	Mann-Whitney
ANA −ve	28.74±6.58	31.24	26.24	1.221	U=41.000, 0.000*
Duration of habit (years)					
ANA +ve	7.63±3.96	9.74	5.51	0.990	Kolmogorov-Smirnov
ANA −ve	9.48±4.27	11.11	7.86	0.793	*Z*=0.630, 0.823
Frequency of habit (numbers per day)					
ANA +ve	11.44±6.73	15.02	7.86	1.681	Kolmogorov-Smirnov
ANA −ve	13.36±7.01	16.03	10.70	1.301	*Z*=0.706, 0.701

*Significant. ANA: Antinuclear antibodies, CI: Confidence interval, SE: Standard error

literature to uncover the etiology of the disease, it still remains intriguing and complex to explore. Despite the fact of compelling evidence in favor of the areca nut as one of the etiologies for the disease, the persons developing OSF without chewing areca nut and every areca nut chewers not developing OSF makes the fact more obscure.[8] Adequate attention has not been paid to the genetic and immunologic aspects and therefore demands a reasonable insight. This study is aimed at exploring the autoimmune nature of the disease by determining the presence of serum ANA in OSF patients along with its pattern and to establish its correlation with the severity of the disease, frequency of habit and its duration.

The present study showed a male predilection (64.4%) similar to studies by Chiang *et al*.,[9] Ranganathan *et al*.,[10] and Ahmad *et al*.[11] This observation of male preponderance for the disease was in contrast to the studies by VanWyk[12] and Aziz[13] wherein female predilection was seen. This could be explained by the fact that awareness and quid chewing habits in females of eastern countries is less than males,
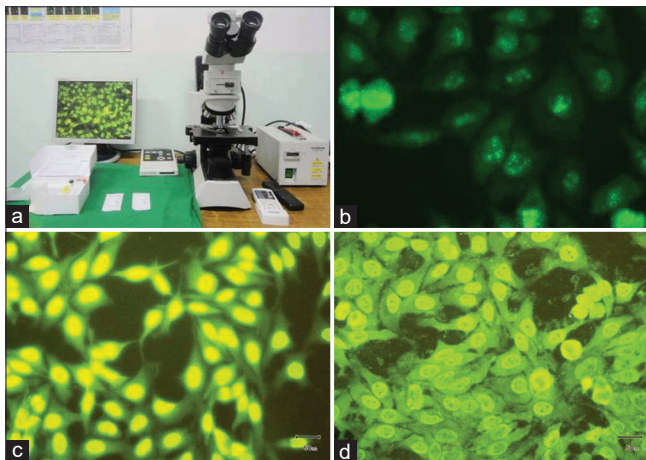

Figure 1: Fluorescence microscope and fluorescence patterns observed (a) Fluorescence Microscope with Monitor (b) speckled pattern (c) homogeneous pattern(d) nucleolar

Table 3: Study of association between intra-oral sites of involvement and presence of antinuclear antibodies in patient group

Number of sites involved	Presence of ANA	
	Negative, *n* (%)	Positive, *n* (%)
<5 (*n*=15)	14 (93.30)	1 (6.70)
≥5 (*n*=30)	15 (50.00)	15 (50.00)
Total (*n*=45)	29 (64.4)	16 (35.60)

*P=0.004, χ^2=8.195. ANA: Antinuclear antibodies

resulting in their low registration for the disease than the Western counterpart.

Many collagen connective tissue disorders of autoimmune nature can be detected by estimation of serum ANA.[14] OSF being considered as a collagen connective tissue disorder, it is reasonably presumed that such ANA might be present in OSF patients. This study showed significantly higher incidence of ANA (35.6%) in OSF patients than in healthy control groups [Table 1]. Similar result of significantly higher incidence of ANA in 23.9% of OSF patients was reported by Chiang *et al*.[14] The first study of ANA in OSF patients was done in 1986 by Cannif *et al*.[15] without the control group, and they showed the presence of ANA in 8% of the patients. Moreover, the prevalence of ANA positivity in the present study was found significantly higher in females (*n*=11; 68%) which corresponds to the autoimmune nature of the disease in western countries where quid chewing habit is low.

A close association of increased frequency of human leukocyte antigen (HLA) A24, DRB1-11, DRB3-0202/3, HLA-A10, B7, DR3 antigens and of haplotypic pairs A10/DR3, B8/DR3and A10/B8 in OSF patients suggest its genetic predisposition.[16] The HLA-DR antigens have been linked to susceptibility of autoimmune diseases owing to the location of immune response genes at or near the D locus of chromosome 6.[17] Different autoantigens need specific major histocompatibility complex (MHC) class II antigen on the antigen-presenting cell surface to induce autoantibody production. Canniff *et al*.[15] found that about 30% of OSF patients were associated with HLA-DR3 antigen. Moreover, only few of the normal population may possess a specific MHC class II antigen. This could explain why only 35.6% of our OSF patients had ANA in their sera. A significantly higher serum level of ANA than the control group can be explained by the presence of higher specific autoantibody production-related HLA-DR antigens in OSF patients. Presence of ANA in 6.7% of each healthy group with and without could be the effect of some subclinical changes. Further they might be considered at potential risk to develop an autoimmune disease or possibly OSF in future. This is also supported by the fact that the autoantibodies appear long before the disease becomes clinically evident.[18]

Table 4: Trend of intensity and pattern of fluorescence in antinuclear antibodies positive patient group

Study groups	Total patients	Number of ANA positive	Intensity		Pattern		
			2+, *n* (%)	3+, *n* (%)	Homogeneous, *n* (%)	Speckled, *n* (%)	Nucleolar, *n* (%)
OSF patients	45	16	10 (62.50)	6 (37.50)	5 (31.25)	8 (50)	3 (18.75)

OSF: Oral submucous fibrosis, ANA: Antinuclear antibodies

In the present study, ANA positive and negative cases in the patient group did not show any significant difference in duration and frequency of the habit.[Table 2] Chiang *et al.*[14] documented no association with duration of habit and a borderline significant association with frequency of habit (*P* = 0.066). A combination of environmental factors in a genetically susceptible host may directly instigate, aid or aggravate the pathological immune process. Environmental chemicals may alter an autoantigen in a manner that makes it immunogenic. It has been postulated that to develop autoimmune disease the genetic contribution holds 30% whereas the contribution by environment is 70%.[19] All the OSF patients were areca nut chewers in our study. Therefore, it is possible that areca nut constituents damaged the lining epithelium of the gastrointestinal tract. The nuclear antigens released from the damaged cells might be modified by cytotoxic components of the areca nut. These autoantigens could be responsible for autoantibody production.

MMO and the number of sites of involvement in the oral cavity are indicative of the severity of the disease.[20] Hence, the analysis was done to find out the association of the presence of ANA with MMO and number of sites of involvement in OSF patients. Significantly lower mean MMO and the higher number of sites of involvement were seen in ANA-positive patients [Tables 2 and 3]. These findings suggest that the severity of disease was high in ANA positive OSF patients, which was contradicting the observations of Chiang *et al.*[14] who reported no statistically significant association of the presence of ANA with MMO and number of sites of involvement.

Different fluorescence patterns are found to be associated with a variety of autoimmune diseases.[21] The immunofluorescence patterns were more in the form of speckled (50%) followed by homogeneous (31.25%) and nucleolar (18.75%). The intensity of +2 and +3 was seen in 62.50% and 37.50%, respectively [Table 4 and Figure 1]. Similar homogeneous pattern is seen in SLE and mixed connective tissue diseases, whereas the speckled pattern is seen in SLE, Sjoegren's syndrome, scleroderma and polymyositis. The nucleolar pattern is seen in scleroderma and polymyositis.[22] With reference to such observed patterns the OSF could be considered as an idiopathic scleroderma of the mouth due to high incidence of speckled patterns and few nucleolar patterns. However, the homogeneous pattern remains undefined.

This study was limited to a sample size of 45 OSF cases and the study design being semi-qualitative analysis. Since literature pertaining to the topic was sparse with no satisfactorily conclusive results, we conducted this study to analyze the exploratory possibilities with small sample size and charted our findings on the fluorescence pattern and intensity. Further researches on a larger sample size can be done to quantitatively analyze and standardize these characteristic ANA patterns of IIF particularly to OSF.

CONCLUSION

This study found significantly higher positive rates of ANA in OSF patients than in healthy control subjects. The presence of serum ANA was associated with the severity of disease. Higher incidence of autoantibodies such as ANA, female predilection, alteration of humoral and cellular immunity concludes the autoimmune character of the disease. Further due to the chronic nature of the OSF disease the appearance of ANA in the form of serum autoantibodies long before the clinical symptoms can be considered as a good predictive marker for the potential to develop the disease. This study provides a broader prospective to adopt therapies that selectively target involved autoimmune pathways.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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