Original Article

Reproducibility of Clinico-histopathological Findings by Direct Immunofluorescence of Fixed, Paraffin Embedded Tissue Specimens for Diagnosis of Lichen Planus

AR. Ghannadan [1], M. Seyedmajidi [2], S. Mehrabi [3], A. Abdolabadi [4], P. Aminishakib [5], A. Bijani [6].

[1] Associate Professor, Department of Pathology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[2] Associate Professor, Dental Material Research Center, Babol University of Medical Sciences, Babol, Iran
[3]Assistant Professor, Department of Oral and Maxillofacial Pathology, School of Dentistry, Zanjan University of Medical Sciences, Zanjan, Iran
[4] Student, School of Dentistry, Babol University of Medical Sciences, Babol, Iran
[5]Assistant Professor, Department of Oral and Maxillofacial Pathology, School of Dentistry, Tehran University of Medical Sciences, Tehran, Iran AND Assistant Professor, Dental Material Research Center, Babol University of Medical Sciences, Babol, Iran
[6] Researcher, Non-Communicable Pediatric Disease Research Center, Amirkola Children's Hospital, Babol University of Medical Sciences, Babol, Iran

Abstract

Background and Aim: Lichen planus (LP) is a chronic mucocutaneous disorder mediatd by the impaired immunity, in which the association of clinical and histopathological findings is necessary for a definite diagnosis. In case of discrepancy between the clinical and histopathological findings, use of adjunct diagnostic methods such as direct immunofluorescence (DIF) is recommended. This study sought to assess the reproducibility of clinico-histopathological findings byDIF of fixed, paraffin embedded tissue specimens for the diagnosis of LP.

Materials and Methods: In this retrospective descriptive study, 49 oral (OLP) and cutaneous LP (CLP) specimens were subjected to DIF examination using fibrinogen, C3 and IgM. The intensity of staining was graded as 0, 1 or 2. Findings were compared in each group and between OLP and CLP groups using statistical tests.

Results: Statistical analyses revealed a correlation between C3 and IgM expression (Spearman's rho: 0.697, P<0.001); this correlation was more prominent in CLP (Spear man's rho: 0.746, P<0.001). A correlation was observed between fibrinogen and IgM expression in OLP (Spearman's rho: 0.769, P=0.02).

Statistical analysis with the Mann Whitney U test found no significant difference between OLP and CLP for the expression of C3 (P=0.3) or fibrinogen (P=0.5). But, a significant difference was noted between OLP and CLP for the expression of IgM (P=0.04).

Conclusion: It seems that DIF examination of formalin-fixed, paraffin embedded tissue specimens using C3, fibrinogen and IgM does cannot adequately reproduce clinico-histopathological findings; although, combined use of C3 and IgM in CLP and IgM and fibrinogen in OLP specimens yielded higher reproducibility.

Key Words: Lichen Planus, Fluorescent Antibody Technique, Direct, Diagnosis

Corresponding author:
P. Aminishakib, Assistant Professor, Department of Oral and Maxillofacial Pathology, School of Dentistry, Tehran University of Medical Sciences, Tehran, Iran

aminishakib@tums.ac.ir

Received: 27 Feb 2015
Accepted: 12 June 2015

Introduction

Lichen planus is a relatively common chronic mucocutaneous disorder mediated by the impaired immunity [1]. Oral mucosa is often involved in absence of cutaneous lesions [2] resulting in considerable pain and discomfort for the patients [1]. The glans penis, the vulvar mucosa, and the nails are also affected in some cases of mucosal LP [1, 2]. Cutaneous LP has a prevalence of 1%; the prevalence of OLP has reported to be 0.5-2.2% [1]. It most commonly involves patients in the age range of 30-60 years [2] and is slightly more common in females than in males [1]. The etiology of LP has yet to be fully understood [1,3,4]. Oral LP may have variable clinical manifestations ranging from asymptomatic white reticular or plaque-like patches mainly involving the buccal mucosa bilaterally to erythematous and painful lesions commonly appearing on the gingiva [2] with possible risk of malignancy, which is highly debated [1,5]. Clinical manifestations, especially bilateral presence of lesions or presence of classic Wickham lines at the margins, are necessary for clinical diagnosis. In case of absence of these symptoms, a biopsy may be required. Biopsy can enhance clinical diagnosis in cases where the typical clinical manifestation of LP does not exist or in those suspected for dysplasia or malignancy [2].

From the histopathological point of view, LP lesions are diagnosed by two key features namely hydropic degeneration of keratinocytes in the basal layer of epithelium and band-like infiltration of lymphocytes [1,4,6]. Final diagnosis is made when the clinical features match the histopathological results. However, in case of discrepancy between the clinical features and histopathological results, several differential diagnoses are suggested and adjunct diagnostic methods may be used to make a diagnosis [2,7].

Direct immunofluorescence is among the most important adjunct diagnostic procedures. This histochemical technique is commonly used for detection and identification of antigens on fresh frozen tissue to diagnose three groups of diseases including vesiculobullous diseases, autoimmune diseases such as lupus erythematosus and leukocytoclastic vasculitis [6].

Direct immunofluorescence is often performed on fresh tissue samples, and special media are required for transfer of tissue samples, which may not be available in all clinics.

Since some previous studies reported that fixed paraffin blocks were usable for DIF, this study aimed to assess the reproducibility of clinic-histopathological findings by DIF of fixed paraffin embedded tissue specimens for diagnosis of LP.

Materials and Methods

This retrospective-analytic descriptive study was conducted on patient files in the archives of the Department of Pathology of School of Dentistry, Babol University of Medical Sciences from 2010-2013. Seventeen tissue blocks with definite histopathological diagnosis of OLP were selected. Six OLP and 26 CLP blocks were also retrieved from the archives of the Pathology Department of Tehran Razi Hospital from 2012-3013. In the next step, slides of the selected blocks stained with hematoxylin and eosin were evaluated to confirm the diagnosis of LP (23 cases of OLP and 26 cases of CLP). Slides were evaluated under a light microscope to confirm OLP according to the modified WHO criteria [8]. The following criteria were considered to confirm the diagnosis of CLP [9]:

1.Orthokeratinization
2.Wedge-shaped hypergranulosis
3.Irregular acanthosis
4.Vacuolization of the basal layer
5.Marginal lymphocytic infiltration in the superficial layer of dermis adjacent to the epidermis

In this study, DIF was performed on paraffin blocks. Three sections (5µm in thickness) were made of each block using a microtome, placed in a water bath and then fixed on slides and incubated at 150°C in a dry heat incubator. After deparaffinization in xylol in two steps (each for 15 minutes), rehydration was performed using graded ethanol (each for 15 minutes). Two rinses with distilled water were performed and then the sections were immersed in 0.1% sodium chloride and 0.1% trypsin at a pH of 7.8 (adjusted by sodium hydroxide) for trypsinization and incubated at room temperature (20-25°C) for four hours. The sections were then rinsed twice (each time for 10

minutes) in phosphate buffered saline (PBS). In the next step, the sections were stained with fluorescein-marked conjugated antibodies (Daco, Kyoto, Japan) including anti-fibrinogen, anti-C3 and anti-IgM with 1/30, 1/80 and 1/30 concentrations, respectively according to the manufacturer's instructions as well as small amount of Evans Blue dye to decrease background fluorescence. After 30 minutes of incubation in a humid chamber and twice rinse with PBS (each time for 10 minutes), mounting buffer was poured on the slides. Another slide was placed on top of it and the slides were evaluated under a Leitz Laborlux S fluorescent phase contrast microscope (Leitz GmbH & Co. KG, Oberkochen, Germany) with 50W HBO Illuminator and I 2/3 Blue filter.

The positivity of DIF reaction was based on linear, fibrillar or granular deposition of fibrinogen, C3 and IgM along the basal membrane zone or in the cytoid bodies in the epithelium or the superficial connective tissue. The severity of fluorescence staining in each sample was qualitatively scored as zero (when the intensity was lower than the background fluorescence), one (when the intensity was slightly higher than the background fluorescence) and two (when the intensity was much higher than the background fluorescence).

Descriptive findings were reported as frequency and percentage and the Pearson's correlation test and chi square test were used to compare the fluorescent antibodies and the OLP and CLP groups. The data were statistically analyzed using SPSS version 19 and $P<0.05$ was considered statistically significant.

Results

This study was conducted on tissue blocks present in the archives of the Department of Pathology of School of Dentistry, Babol University of Medical Sciences and Tehran Razi Hospital with definite diagnosis of LP. A total of 49 specimens were evaluated out of which, 26 were CLP and 23 were OLP. Of all specimens, 34 belonged to females and 15 belonged to males. The mean age was 55 years in males and 49 years in females. The overall mean age of patients was 52 years.

Of 23 cases of OLP, 13 (56%) were from the buccal mucosa, six (17%) were from the lip mucosa and four (17%) were from the tongue. OF 26 CLP, 18 (69%) were from the skull skin, four (15%) were from the foot skin and four (15%) were from the hand skin.

In CLP samples, C3 staining showed that the intensity of staining was zero in 21 specimens, one in three specimens and two in two specimens. In IgM staining, the intensity of staining was zero in 16 samples, one in four samples and two in six samples. In fibrinogen staining, the intensity of staining was zero in 17 samples, one in six samples and two in three samples (Tables 1-3).

In OLP samples the following results were obtained: In C3 staining, the intensity of staining of one and two were not seen in any sample. In IgM staining, intensity of staining of one was only seen in one sample. In fibrinogen staining, the intensity of staining was zero in 18 samples, one in three samples and two in two samples (Figures 1 and 2).

Statistical analyses showed that a direct correlation existed between the expression of IgM and C3 in OLP. The Spearman's correlation coefficient was 0.697 ($P<0.001$); this correlation was more prominent in CLP samples ($P<0.001$); The Spearman's correlation coefficient was 0.746. Also, a direct correlation existed between the expression of fibrinogen and IgM in OLP ($P=0.02$). The Spearman's correlation coefficient was 0.769.

Statistical analysis with the Mann Whitney U test found no significant difference between OLP and CLP for the expression of C3 ($P=0.3$) or fibrinogen ($P=0.5$). But, a significant difference was noted between OLP and CLP for the expression of IgM ($P=0.04$).

Discussion

Histopathological diagnosis of LP is challenging in some cases especially when mucosal lesions have an ulcerative clinical manifestation or are associated with severe inflammation [10]. The DIF technique was first used for diagnosis of skin conditions [11]. However, it was later confirmed that DIF was also valuable for diagnosis of bullous or ulcerative diseases of the oral mucosa [12].

On the other hand, immunofluorescence studies on OLP provide insight regarding the immunopathogenesis of this disease [10].

However, this method has some shortcomings as

Table 1. The frequency and percentage of the intensity of staining of fibrinogen in CLP and OLP samples

Lesion/Intensity of staining	0	1	2	P value
CLP (number/percentage)	17 65.4%	6 23.1%	3 11.5%	0.543
OLP (number/percentage)	18 78.3%	3 13%	2 8.7%	

Table 2. The frequency and percentage of the intensity of staining of IgM in CLP and OLP samples

Lesion/Intensity of staining	0	1	2	P value
CLP (number/percentage)	16 61.5%	4 15.4%	6 23.1%	0.046
OLP (number/percentage)	22 95.7%	1 4.3%	0 0.0%	

Table 3. The frequency and percentage of the intensity of staining of C3 in CLP and OLP samples

Lesion/Intensity of staining	0	1	2	P value
CLP (number/percentage)	21 80.8%	3 11.5%	2 7.7%	0.310
OLP (number/percentage)	23 100%	0 0.0%	0 0.0%	

well. For instance, it requires a specific transfer medium and takes more time for tissue processing, which delays diagnosis. Thus, assessment of the efficacy of this method for use on formalin fixed paraffin blocks can be helpful [12]. Therefore, this study aimed to assess the reproducibility of clinic-histopathological findings by DIF of fixed paraffin blocks for diagnosis of LP. Differential diagnosis by DIF is often performed based on four criteria:

1. The possibility of deposition of immune complexes
2. Type of deposited immunoglobulins
3. Number of deposited immune complexes
4. Deposition in areas adjacent to the target site [13]

In studies on LP using DIF, the greatest deposits belonged to fibrinogen and IgM, and deposition of IgA, IgG and C3 was less commonly seen [14]. Thus, in the current study, fibrinogen and IgM were chosen among markers with significant expression and C3 was chosen among markers with limited expression for further evaluation. In the current study, positive fibrillar deposition of fibrinogen was noted in the basal membrane of CLP and OLP specimens (Figures 1 and 2).

Some researchers suggested that change in immunofluorescence of LP lesions is a phenomenon secondary to the destruction of the deep layers of the epithelium/epidermis and the basal membrane. Finding of immunofluorescent objects and deposition of fibrinogen in some other diseases show that these changes may be non-specific phenomena related to cell.

deposits were related to destructive inflammatory processes at the interface of connective-epithelial tissues [10,14]. However, in a study by Laskaris et al, the pattern of fibrinogen deposits was reported to be non-specific; but most specimens showed a band of fibrinogen deposits in the basal membrane [3].

Degeneration in the basal layer but with a different process. Thus, our findings were in line with the

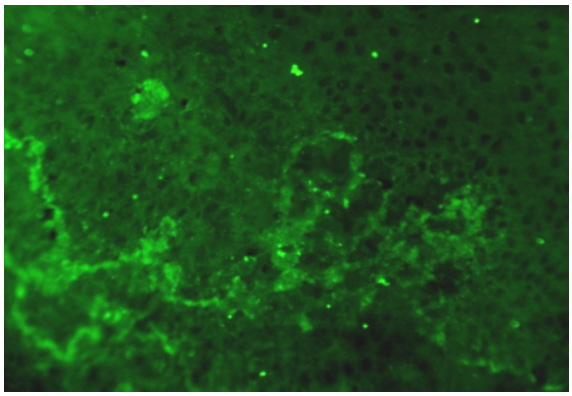

Figure 1. Expression of fibrinogen with 1+ intensity

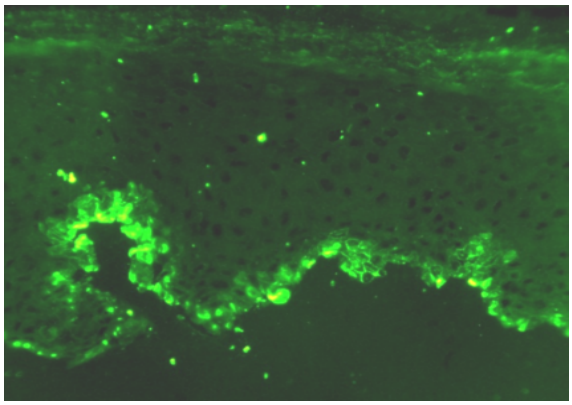

Figure 2. Expression of fibrinogen with 2+ intensity

results of previous studies, indicating that these Raghu et al. compared the efficacy of DIF for OLP and oral lichenoid reactions and reported uniform deposition of fibrinogen in the basal membrane of both lesions; the only difference was that the intensity of deposition was lower in oral lichenoid reactions [10].

Kulthanan et al. used this method on fresh-frozen specimens of patients with LP and reported shaggy deposition of fibrinogen at the interface of epidermis-connective tissue, which has been represented as the best indicative for lichen planus. IgM and C3 deposition in this study was like granular pattern or discontinuous region in basement membrane which was in agreement with the results of some previous studies [13].

Deposition of fibrinogen, C3 and IgM was also noted in cytoid bodies in the epithelium and superficial areas of lamina propria. However, it should be noted that in the current study, 65.4% of OLP and 78.3%of CLP specimens did not show the expression of fibrinogen. Also, negative specimens for C3 and IgM in OLP had a frequency of 100% and 95.7%, respectively; these values were 80.8% and 61.5%, respectively for CLP.

One factor affecting the measurement of the accuracy of DIF is proper preparation of tissue specimens, which is an important and technique-sensitive step. Buffered formalin is usually used to transfer biopsy samples to pathology laboratory. Immersion of specimens in formalin can cause cross-linking of tissue proteins such as antigens and makes their isolation by DIF difficult. However, Sano et al, and Arbesman et al. stated that immersion of specimens in formalin for a short period of time (10 minutes) did not compromise diagnosis [15,16].

Comparison of the positivity of DIF for C3 and IgM in the current study showed that a significant difference existed between CLP and OLP, and the positivity of these markers in CLP was higher than that in OLP. But, Kulthanan et al. found no significant difference in expression of these markers between OLP and CLP [13]. The difference between the resistance of skin and mucosa to formalin can explain such a difference in expression of antigens in the skin and mucosa.

In general, it seems that for assessment of the efficacy of DIF on formalin-fixed paraffin blocks, specimens must be thoroughly inspected from the beginning of fixation to the time of evaluation because the process of fixation and timings can affect the tissue properties and its antigenic characteristics. However, accurate control of these factors is not possible in a retrospective study.

Conclusion

Based on the results of this study, it can be concluded that DIF of formalin-fixed paraffin blocks using C3, fibrinogen and IgM markers alone cannot suitably reproduce clinic histopathological findings. Although combination of IgM and C3 in CLP and combination of IgM and fibrinogen in OLP yielded higher reproducibility.

References

1. Neville BW, Damm DD, Allen CM, Bouquot J.

Oral & maxillofacial pathology. 3rd ed. philadelphia: Saunders Elsevier. 2009. P:782-8.
2. Al-hashimi I, Schifter M, Lockhart PB, Wray D, Brennan M, Migliorati CA, et al. Oral lichen planus and oral lichenoid lesions: diagnostic and therapeutic considerations. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2007 Mar; 103 Suppl:S25.e1-12.
3. Laskaris G, Sklavounou A, Angelopoulos A. Direct immunofluorescence in oral lichen planus. Oral Surg Oral Med Oral Pathol. 1982 May; 53 (5):483-7.
4. Pekiner FN, Demirel GY, Borahan MO, Ozbayrak S. Cytokine profiles in serum of patients with oral lichen planus. Cytokine J. 2012 Dec; 60 (3):701-6.
5. Canto AM, Muller H, Freitas RR, Santos PS. Oral lichen planus: clinical and complementary diagnosis. An Bras Dermatol. 2010 Sep-Oct; 85 (5):669-75.
6. Regezi JA, Sciubba JJ, Jordan RCK: Oral pathology , clinical pathologic correlations. 6th ed. Missori: Saunders Elsevier. 2012. P:97-102.
7. Ismail SB, Kumar SK, Zain RB. Oral lichen planus and oral lichenoid reactions: etiophatogenesis, diagnosis, management and transformation. J Oral Sci. 2007 Jun;49(2):89-106.
8. Hiremath S, Kale AD, Hallikerimath S. Clinico-Pathological Study to Evaluate Oral Lichen Planus for the Establishment of Clinical and Histopathological Diagnostic Criteria. Turk Patoloji Derg. 2015;31(1):24-9.
9. Elder DE, Elenitsas R, Johnson Jr BL, Murphy GF, X Xu. Lever's histopathology of the skin. 10th ed. Philadelphia: Lippincott Williams & wilkins: 2009.P:186.
10. Raghu AR, Nirmala NR, Sreekumaran N. Direct immunofluorescence in oral lichen planus and oral lichenoid reactions. Quintessence Int. 2002 Mar; 33(3):234-9.
11. Daniels TE, Quadra-White C. Direct immunofluorescence in oral mucosal disease: A diagnostic analysis of 130 cases. Oral surg Oral Med Oral Pathol. 1981 Jan;51(1):38-47.
12. Rinaggio J, Crossland DM, Zeid MY. A Determination of the Range of Oral Conditions Submitted for Microscopic and Direct Immunofluorescence Analysis. J Periodontol. 2007 Oct; 78(10):1904-10.
13. Kulthanan K, Jiamton S, Varothai S, Pinkaew S, Sutthipinittharm P. Direct immunofluorescence study in patients with lichen planus. Int J Dermatol. 2007 Dec;46(12):1237-41.
14. Mutasim DF, Adams BB. Immunofluorescence in dermatology. J Am Acad Dermatol. 2001 Dec; 45(6):803-22, quiz 822-4.
15. Sano SM, Quarracino MC, Aguas SC, Gonzalez EJ, Harada L, Krupitzki H, et al. Sensitivity of direct immunofluorescence in oral diseases. Study of 125 cases. Med Oral Patol Cir Bucal. 2008 May 1;13(5):E287-91.
16. Arbesman J, Grover R, Helm TN, Beutner EH. Can direct immunofluorescence testing still be accurate if performed on biopsy specimens after brief inadvertent immersion in formalin? J Am Acad Dermatol. 2011 Jul;65(1):106-11.