Assessment of Selected Immunological Biomarkers in Active and Inactive SLE Patients in HUSM

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ABSTRACT: Lupus nephritis (LN) is characterized by renal deposition of immune complexes and it is a serious complication in Systemic Lupus Erythematosus (SLE). Normally, serum levels of anti-dsDNA antibodies and complement levels are used to identify patients with high disease activity and at risk for developing LN. The aim of the study was to assess the level of selected immunological biomarkers (anti-dsDNA, Complement C3 and Complement C4) in active and inactive LN patients. A total of 30 LN patients were enrolled in this study, 15 of which were diagnosed as having active LN, 15 with inactive LN and 15 healthy controls. The serums of each study groups were measured using indirect immunofluorescence assay (IIF) for anti-dsDNA and immunonephelometry for C3 and C4. Statistical analyses were performed using SPSS software, version 22.0 and values were expressed as mean ± standard deviation (SD). The results were analyzed by One-Way ANOVA, independent t-test and chi-square test. Anti-dsDNA antibodies were detected in less than one-third of patients in active and inactive LN patients (26.67%). Active LN patients presented a higher frequency of anti-dsDNA as compared to inactive LN patients (46.67% vs. 6.67%, p=0.013). However, no significant difference was found in C3 for both LN groups. Meanwhile, C4 demonstrated significant results (inactive LN; 0.24 g/l (0.11 g/l), active LN; 0.16 g/l (0.12 g/l), p=0.049). In conclusion, anti-dsDNA antibodies and C4 may have a great potential as diagnostic factors for active LN and as predictors for its disease activity.

Keywords: Systemic lupus erythematosus. Lupus nephritis. Anti-dsDNA. Complement 3. Complement 4.

Introduction

Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disease, of uncertain aetiology, with numerous patterns of clinical manifestations due to the production of autoantibody (Hussain *et al*., 2008). Some SLE patients have a high risk of developing lupus nephritis (De Zubiria & Herrcra-Diaz, 2012; Mavragani *et al.*, 2015). Lupus nephritis (LN) is characterized by renal deposition of immune complexes and it is a serious complication in Systemic Lupus Erythematosus (SLE) since it is the major forecaster of poor prognosis (Sterner *et al*, 2014; Wu *et al*., 2016). According to Birmingham *et al*. (2010), therapies thought to be effectively treat renal flares, but these therapies are themselves toxic. Regrettably, the pathogenesis of renal flare remains unclear in spite of the negative impact that renal flares have on SLE management. To date, there are no known predictors of an impending renal flare (Birmingham *et al*., 2010).

Based on Isenberg *et al.* (2007), the anti-double stranded DNA antibodies (anti-dsDNA) are considered as a specific marker for SLE. The presence of these autoantibodies might be practically diagnostic for SLE due to the high frequency ranging from 70% to 98%, sensitivity and specificity (57.3% and 97.4%, respectively) (Isenberg *et al*., 2007; Tsokos, 2011). Moreover, Arbuckle *et al*. (2003) stated that the identification of anti-dsDNA in SLE patients several years before disease onset proposes their involvement towards a clinically overt disease. Also, several lines of evidence reveal the pathogenic role of anti-dsDNA antibodies. Particularly, these autoantibodies have been associated with renal involvement in active lupus nephritis patients, as demonstrated by their deposition in several renal structures such as glomeruli, mesangium, basement membrane, subendothelial and subepithelial spaces, and also tubules (Foster *et al*., 1993).

Furthermore, complement is another immunological biomarker used in this study. Complement is a mediator of inflammation, complement deficiency predisposes to the development of SLE. Besides, complement activation might be involved in the tissue damage associated with SLE flare, predominantly reflecting nephritic activity (Rabbani *et al*., 2005; Bao & Guigg, 2007). The pathogenic roles of complement activation in human SLE were indicated from years of clinical observations. From clinical inspections, low total complement hemolytic activity (CH50) and decreased C3 and C4 levels have been found in about 75% of SLE patients with focal nephritis and 90% in patients with diffuse nephritis (Valentijn *et al*,

1985). Studies regarding the changes in plasma levels of complement C3 and C4 have been reported and showed a contradictory result. And yet, there is no single study measuring the complement levels at regular and recurrent intervals in order to assess the lupus flare up. Therefore, the role of complement is argued whether it is essential in the pathogenesis of SLE or it is just epiphenomenon (Hussain *et al*., 2008).

Hence, the aim of this study was to assess the level of anti-dsDNA antibodies, serum C3 as well as serum C4 in diagnosis of lupus nephritis.

Materials and Methods

Patients and Data Collection

A total of 30 LN patients were enrolled in this study, 15 of which were diagnosed as having active LN, 15 with inactive LN and 15 healthy controls. All patients enrolled in the current study were classified with SLE regarding to the revised American College of Rheumatology (ACR) criteria (Hochberg, 1997). 6 ml of blood was withdrawn from subject's peripheral vein. The blood samples were allowed to clot for two hours at room temperature prior to centrifugation at 4500 rate per minute (rpm) at 25ºC for five minutes (Universal 32R, Hettich Zentrifugen). The serum obtained from centrifugation was aliquot into three microcentrifuge tubes which were labelled with the subject and the intended tests i.e. anti-dsDNA, serum C3 and C4. The serum samples were frozen in aliquots at -80ºC. The serums of each study groups were measured using indirect immunofluorescence assay (IIF) by using *Crithidia luciliae* as substrate for anti-dsDNA and immunonephelometry (BN-ProSpec, Siemens, USA) for C3 and C4. The study protocol was approved by the Medical Research and Human Research Ethics Committee USM.

SLE Disease Activity Index Assessment

The global disease activity was assessed by total SLEDAI (tSLEDAI). Patients with SLEDAI score ≥6 were defined as having active disease and inactive disease with SLEDAI score <6 (Pradhan *et al*., 2010; Petri *et al*., 2017). For renal involvement, renal SLEDAI (rSLEDAI) was used to assess kidney disease activity. The score consists of the four kidney-related parameters: hematuria, pyuria, proteinuria, and urinary casts. Scores for the renal SLEDAI

can range from 0 (inactive renal disease) to a maximum of 16. Active lupus nephritis was those with an rSLEDAI score of 4 or more (Pitashny *at al*., 2007, Mustafa, 2014). By referring Weening *et al*. (2004), they are six histological types of lupus nephritis based on International Society of Nephrology/Renal Pathology Society (ISN/RPS): minimal mesangial LN (class I), mesangial proliferative LN (class II), focal proliferative (class III), diffuse proliferative (class IV), and membranous LN (class V) and advanced sclerosis (class VI) (Weening *et al*., 2004).

Statistical Analysis

All data entry and statistical analyses were performed using IBM© SPSS© Statistics version 22. Values were expressed as mean ± standard deviation (SD). Differences in mean values between the two groups were analyzed by independent *t* test for numeric variables and chi-square test for categorical variables. One-Way ANOVA is used to analyse the difference of means between more than 2 groups of samples. In all the analyses, a two-tailed $p < 0.05$ was considered to be statistically significant.

Results

Demographical and Clinical Data

The demographical and clinical data of the 30 LN patients and 15 healthy controls illustrated in Table 1. The LN patients and healthy controls consisted of 42 females (93.33%) and 3 males (6.67 %) with a ratio of 14:1. The mean age of the active LN was 34.47 years, while the mean age of the inactive LN was 36 years respectively. For healthy controls, the mean age was 28.07 years. The majority of distribution of ethnic population was 93.33% of Malays. Among 30 LN patients, 19 (63.33 %) patients were married while another 11 (36.67 %) patients were unmarried for both LN groups. About 8 (17.78 %) of LN patients and healthy controls from secondary level of education meanwhile 37 (82.22%) from tertiary level of education.

In our study, unemployed candidates were 26 (57.78%) whereas employed candidates were 19 (42.22%) for both LN groups and healthy controls. Mean duration between the onset of symptoms and diagnosis in active LN was 111.4 (111.85) and inactive LN was 74 (73.23)

with significant difference $p<0.001$. Furthermore, there was a significant difference in SLEDAI. The SLEDAI score was significantly higher in active LN groups compared to inactive groups (active LN; 11.93 (6.11) versus inactive LN; 2.20 (1.86), $p<0.001$). rSLEDAI also exhibited significant results specifically in active lupus nephritis (LN) patients (6.93 (3.20), $p<0.001$).

Table 1: Socio demographic and clinical characteristics of studied groups (n=45)

Variables	Inactive lupus nephritis (n=15)	Active lupus nephritis (n=15)	Healthy control (n=15)	*p*-value
Gender				
Male, n (%)	1 (6.67)	1 (6.67)	1 (6.67)	0.309
Female, n (%)	14 (93.33)	14 (93.33)	14 (93.33)	
Age at Diagnosis				
Years; mean (SD)	36.00 (11.41)	34.47 (13.78)	28.07 (7.69)	0.135
Marital Status				
Married, n (%)	11 (73.33)	8 (53.33)	3 (20)	0.715
Unmarried, n (%)	4 (26.67)	7 (46.67)	12 (80)	
Race				
Malay, n (%)	12 (80)	15 (100)	15 (100)	0.068
Others, n (%)	3 (20)	0 (0)	0 (0)	
Level of Education				
Secondary, n (%)	3 (20)	4 (26.67)	1 (6.67)	0.666
Tertiary, n (%)	12 (80)	11 (73.33)	14 (93.33)	
Occupation				
Unemployed, n (%)	7 (46.67)	8 (53.33)	11 (73.33)	0.715
Employed, n (%)	8 (53.33)	7 (46.67)	4 (26.67)	
Disease duration				
Months; mean(SD)	74 (73.23)	111.47 (111.85)	0 (0)	<0.001
SLEDAI				
mean (SD)	2.20 (1.86)	11.93 (6.11)	0.00 (0.00)	<0.001
rSLEDAI				
mean (SD)	0.00 (0.00)	6.93 (3.20)	0.00(0.00)	<0.001

Renal Biopsy Classification

For renal biopsy classification, we found that the most common type of renal disease in lupus nephritis patients was mesangial proliferative lupus nephritis (class II) followed by diffuse proliferative glomerulonephritis (class IV), minimal mesangial LN (class I) and finally focal LN (class III) (Table 2).

Table 2: Histological grading in active and inactive lupus nephritis groups (n=30)

Renal biopsy in LN patients		Inactive LN n (%)	Active LN n (%)	Total percentage for both groups (%)
I	Minimal mesangial LN	3 (20)	0 (0)	10
II	Mesangial proliferative LN	7 (46.67)	9 (60)	53.33
III	Focal LN	1 (6.67)	1 (6.67)	6.67
IV	Diffuse LN	4 (26.67)	5 (33.33)	30
V	Membranous LN	0	0	0
VI	Advanced sclerosis	0	0	0

Immunological Biomarkers

Majority of patients were negative for anti-dsDNA antibodies (n=22; 73.33%), and if positive, the greatest frequency occurred at the highest serum dilution factor at 1:160 (n=7; 23.33%) and 1:80 (n=1; 3.33%). Anti-dsDNA antibodies were detected in less than one-third of patients in active and inactive LN patients (26.67%). Active LN patients presented a higher frequency of anti-dsDNA as compared to inactive LN patients (46.67% vs. 6.67%, p=0.013). However, no significant difference was found in C3 levels for both LN groups. Whereas, C4 demonstrated significant results (inactive LN; 0.24 g/l (0.11 g/l), active LN; 0.16 g/l (0.12 g/l), p=0.049) (Table 3).

Table 3: Immunological biomarkers of lupus nephritis patients and healthy controls (n=45)

Variables	Inactive lupus nephritis (n=15)	Active lupus nephritis (n=15)	Healthy control (n=15)	p -value
C3 mean (SD)	1.00 (0.22)	0.80 (0.55)	1.03 (0.16)	0.160
C4 mean (SD)	0.24 (0.11)	0.16 (0.12)	0.22 (0.05)	0.049
Anti-dsDNA antibody positive				
Titre: 1:10 – 1:160, n(%)	1(6.67)	7(46.67)	0(0)	0.013
Titre: No titre, n(%)	14(93.33)	8(53.33)	0(0)	

Discussion

In this study, we presented data with reference to the demographic, clinical as well as immunological biomarkers, among a local cohort of LN patients (n=30). As anticipated, the cohort LN patients were predominantly female. The prevalence of SLE in Asia-Pacific countries ranged from 7.7 to 68.4 per 100,000 populations in women while 0.8 to 7.0 per 100,000 populations in men. In our study, the female to male ratio for SLE was 14:1. SLE is predominant in women of child-bearing age. Its uncommon presentation in pre-pubertal and post-menopausal women suggests the role of endogenous sex hormones in SLE-pathogenesis. Many of the disease onset and flares are seen during period of rapid hormonal changes such as during pregnancy, post-partum period, ovulation induction and exogenous oestrogen administration.

Earlier studies carried out before the year 1990 found that SLE was more frequent in Chinese (about 80%) (Frank, 1980). However, a more recent review in 2003 to 2005 revealed that Malay represented higher cases of SLE in Malaysia (63%) (Frank, 1980; Osio-Salido & Manapat-Reyes, 2010). In our present study, most of the population in Kelantan were Malay, followed by Chinese and Indian. The mean age of active LN patients was significantly lower than inactive LN patients in this study. Most of young SLE patients tend to be of newly diagnosed cases and the disease courses in this early stage were rather unstable. Hence, more flares were seen in the younger age group of the active LN patients. Besides, for both groups, most of the LN patients were already married and exhibited higher frequency of tertiary education. Most of our participants consist of students, teachers and businesswoman which were under tertiary educations.

SLEDAI is one of the scoring systems available to assess the SLE disease activity. In this study, SLE patients with SLEDAI score of more than six or equal to six were grouped as active SLE (Pradhan *et al*., 2010; Petri *et al*., 2017). In addition, renal SLEDAI (rSLEDAI) was used to assess kidney disease activity for renal involvement. There are four kidney-related parameters which are hematuria, pyuria, proteinuria, and urinary casts. Scores for the renal SLEDAI are range from 0 (inactive renal disease) to a maximum of 16. Active lupus nephritis was those with an rSLEDAI score of 4 or more (Pitashny *at al*., 2007, Mustafa, 2014). In the present study, the mean difference of SLEDAI score was statistically significant

between active LN patients and inactive LN patients. Similarly, rSLEDAI resulted significant difference among both groups. Hence, this result was justified the comparison of research interests between these two groups.

There is common agreement in various literatures that active class of biopsy proven LN are class III and IV, while class I, II, V, and VI are considered less active that requires limited immunosuppressive therapy (Segal & Johnson, 2009). In the present study, we found that more than half of the type of renal disease was from less active LN class (biopsy grade II). It was followed by more active LN class (biopsy grade IV), then followed with less active LN class (biopsy grade I) and lastly pursued by more active LN class (biopsy grade III). The cohort of our patients is consistent with other studies, in which majority of patients had proliferative nephritis (Gupta *et al*., 2016).

Immunological laboratory investigations widely used in clinical setting for SLE disease activity monitoring such as anti-dsDNA, serum C3 and serum C4 levels. Positive anti-dsDNA in SLE patients ranged from 36 to 69% (Julkunen *et al*., 2012). Negative anti-dsDNA levels could be due to non-active (stable) state of SLE even though it was documented that around 20% of SLE patients were persistently negative for anti-dsDNA (Min *et al*., 2002). In the present cohort, anti-dsDNA antibodies were detected in less than one-third of patients in active and inactive LN patients. Active LN patients presented a higher frequency of anti-dsDNA as compared to inactive LN patients. Evidence from previous studies revealed that the presence of anti-dsDNA antibodies was a factor associated with the presence of nephritis, suggesting a prevalent role of anti-dsDNA antibody in the disease profile regarding the renal involvement (Chan *et al*., 2002; Ourania *et al*., 2011). In contrast, Keiserman *et al.* (2013) showed that some LN patients with a positive anti-dsDNA do not present any renal abnormalities.

Generally, lupus nephritis patients have low C3 and C4 levels during disease flares because of the activation of the complement pathway by immune complexes (ICs). Often, that having inherited C4 deficiency confers a high risk to developing SLE. Meanwhile, C3 deficiency is rarely associated with SLE-like illness (Wallport *et al*., 1997). In the present study, there was no significant difference in C3 for both LN groups. Of note, decreased levels of C4 in active LN is consistent with study done by Hussain *et al*. (2008) who reported that C4 levels were decreased during disease flare and provides an essential protective role against the

development of LN. Correspondingly, Enjay *et al*. (2002) also stated that the development of severe SLE in the absence of both classical and alternative complement pathways suggests that it is the absence of C4, and not the presence of C3 that is critical in SLE pathogenesis. On the other hand, previous study found that C4 levels were more sensitive index of disease activity than C3 (Pradhan *et al*., 2010). This is because C3 was less sensitive parameter of disease activity and did not reflect accurate disease activity.

Limitation of this study is a small number of LN samples that may influence the impact of our findings. Therefore, this finding deserves validation in a larger population in future.

Conclusion

In conclusion, the present data suggests that anti-dsDNA antibodies and complement C4 may have a great potential as diagnostic factors for active LN and also predictors for its disease activity.

Acknowledgement

This study was funded by RUI Grant (1001/PPSP/812193) from School of Medical Sciences, Universiti Sains Malaysia and the MyBrain15 Ministry of Higher Education studentship awarded to Siti Khadijah Syed Mohammed Nazri. I want to extend my thankful to staffs of Klinik Pakar Perubatan and Immunology Department for their kind assistance.

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