Ki-67 Proliferation Index in Renal Biopsy Samples of Patients With Systemic Lupus Erythematosus and Its Correlation with Clinical Findings

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Introduction. Systemic lupus erythematosus is an autoimmune disease that may affect almost all organ systems. Renal involvement is the most significant prognostic factor. Renal biopsy findings play an important role in treatment decision. Ki-67 is a monoclonal antibody that is only found in proliferative cells. This study aimed to investigate the proliferative activity in renal biopsy specimens of patients with lupus nephritis using the Ki-67 monoclonal antibody, and to compare the proliferative index between different subgroups of patients.

Materials and Methods. Renal biopsy specimens of 29 patients with systemic lupus erythematosus were retrospectively evaluated. Type of lupus nephritis and activity and chronicity indexes were determined. Ki-67 immunostaining was performed. For each patient, 1000 cells were counted and the number of Ki-67 positive cells was determined. The Ki-67 activity index was compared between different subgroups of lupus nephritis and correlated with systemic lupus erythematosus disease activity index, serum creatinine, proteinuria, anticardiolipin antibodies, and complement levels.

Results. A positive correlation between Ki-67 proliferation index, serum creatinine levels, and systemic lupus erythematosus disease activity index were found. Although conventional activity indexes were low, in 3 of 9 patients with class II lupus nephritis, Ki-67 proliferation indexes were high, indicating proliferation.

Conclusions. Ki-67 can be used as a proliferation marker in renal biopsy specimens for patients diagnosed with systemic lupus erythematosus.

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease affecting several organ systems including mainly the skin, joints, serous membranes, and kidneys. Renal involvement in SLE, also known as *lupus nephritis*, constitutes one of the most significant prognostic factors of the disease. Renal biopsy findings can especially play a determinative role in diagnosis and treatment planning. The assessment of renal biopsy specimens by immunofluorescence techniques in addition to light microscopy is extremely helpful to clinicians. Detection of the presence of glomerular and tubular cell proliferation in biopsy materials may significantly contribute to the selection of immunosuppressive treatment protocols.

Markers of cellular proliferation can be of help in renal biopsy examination of patients with SLE. Ki-67 is a monoclonal antibody that is found only in proliferating cells and reacts with an unknown epitope on human nuclear antigens.[1,2] Ki-67 is present in all active phases of the cell cycle (gap 1, synthesis, gap 2, and mitosis), but absent in silent (resting) cells.[3]

As Ki-67 is considered a proliferation marker, it has been especially investigated in the oncology field. Using Ki-67, proliferative tumor activity and its clinical correlation have been investigated in various solid organ tumors and hematological malignancies.[4-10] Although frequently used in oncology, Ki-67 use in non-neoplastic pathologies is limited. However, it has been demonstrated that cellular proliferation in renal biopsy specimens can be detected by Ki-67.[11,12]

This study aimed to investigate the proliferative activity in renal biopsy specimens of patients with lupus nephritis using the Ki-67 monoclonal antibody, and to compare the proliferative index between different subgroups of patients diagnosed with lupus nephritis. The relationship of these findings were also compared with several clinical and laboratory findings.

MATERIALS AND METHODS

Patients

A total of 29 SLE patients (26 women and 3 men), followed up in the Departments of Rheumatology and Nephrology in Uludag University Faculty of Medicine, were included in the study. The mean age was 28 ± 10 years. Renal biopsy findings were classified according to the 2004 lupus nephritis classification system[13]: class I, normal glomeruli (by light microscopy, immunofluorescence, and electron microscopy); class II, purely mesangial disease by (a) normocellular mesangium by light microscopy, but mesangial deposits by immunofluorescence or electron microscopy, and (b) mesangial hypercellularity with mesangial deposits by immunofluorescence or electron microscopy; Class III, focal proliferative glomerulonephritis (50%); class IV: diffuse proliferative glomerulonephritis (50%); and class V, membranous glomerulonephritis.

Measurements

Clinical and laboratory data and SLE disease activity index (SLEDAI) were determined. The antinuclear antibody, anti-double-stranded DNA, anticardiolipin immunoglobulin G, immunoglobulin M, complement C3, and complement C4 levels measured before renal biopsy were recorded. Anticardiolipin immunoglobulin G and immunoglobulin M antibody levels were interpreted as moderately positive at levels from 20 U/mL to 80 U/mL and strongly positive at levels above 80 U/mL. Renal biopsy specimens were retrospectively evaluated and activity and chronicity indexes were determined.[14] Renal biopsy activity and chronicity indexes are summarized in Table 1. Ki-67 immunostaining was performed in all biopsy materials. Patients who received immunosuppressive treatment at the time of renal biopsy were not included in the study.

Ki-67 Immunohistochemistry

Six-micrometer sections were cut from paraffin blocks of the kidney specimen and were stored overnight in a laboratory oven at 550°C. The slides were immersed in xylol for 10 minutes, rinsed with absolute alcohol for 10 minutes, and exposed to ethanol (96°C) for 5 minutes (repeated 3 times). The sections were then rinsed under tap water, and hydrogen peroxide (3%) was applied over the sections for 5 minutes in order to inactivate endogenous peroxidases. The sections were thereafter washed with rinsing solution and were allowed to rest for 5 minutes. A blocking reagent was applied and the specimens were left to rest again for 5 minutes. The sections were then exposed

Table 1. Results of Activity and Chronicity Indexes in Lupus Nephritis

Activity Index	Score	Chronicity Index	Score
Cellular proliferation	0 to 3	Glomerular sclerosis	0 to 3
Cellular crescents	(0 to 3) x 2	Fibrous crescents	0 to 3
Fibrinoid necrosis	(0 to 3) x 2	Tubular atrophy	0 to 3
Hyaline thrombi	0 to 3	Fibrosis	0 to 3
Glomerular leukocyte infiltration	0 to 3	Interstitial mononuclear cell infiltration	0 to 3
Maximum score	24	Maximum score	12

to the anti-Ki-67 antibody (RB-081-A1, Neomarkers Fremont, CA, USA) for 10 minutes. After the sections were washed with a rinse solution, they were left to rest for 5 minutes and then were exposed to link solution for 10 minutes and streptavidin solution for 10 minutes. After the substrate solution was applied over the sections, they were left to rest for 5 to 10 minutes. Sections were stained by Mayer's hemotoxylin and mounted in Canada balsam after dehydration in the alcohol series.

Determination of Proliferative Index

Cell counts in immunohistochemically stained tissue sections were performed using a Carl Zeiss microscope (Carl Zeiss, Vienna, Austria) with A-plan objective under 40/0.65 × 10 magnification. Cell nuclei were counted starting from the regions with highest Ki-67 staining intensity. In all cases of lupus nephritis, cells within the glomerular capillary tuft were counted without discriminating between endocapillary and extracapillary cells, and tubular cells were not included in the calculation. Labeled nuclei were counted without considering staining intensity. In each slide, 1000 cells were counted and the number of Ki-67 positive cells was recorded. Examples of slides from a class II and a class IV lupus nephritis patient with Ki-67 positive staining are presented in Figures 1 and 2, respectively.

Statistical Analysis

Ki-67 proliferation indexes of patients with class II and class III and IV lupus nephritis (class IV was considered the proliferative nephritis group) were compared using the Mann-Whitney U test.

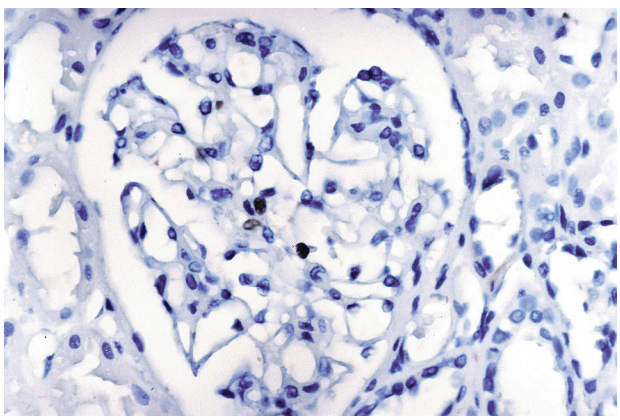

Figure 1. Brown-stained Ki-67 positive cells within the glomerular capillary tuft in class II lupus nephritis (Streptavidin-biotin peroxidase staining, × 400).

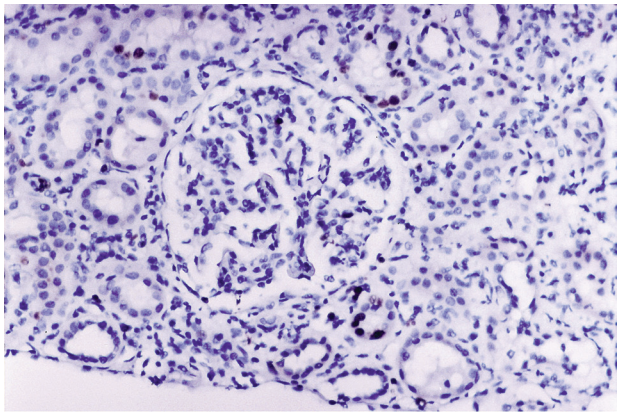

Figure 2. Ki-67 positive cells in class IV lupus nephritis (Streptavidin-peroxidase staining, × 200).

Correlation between the indexes and other variables were examined by the Pearson correlation analysis.

RESULTS

Of 29 patients included in the study, 9 (31%) were diagnosed with class II, 5 (17%) with class III, 12 (41%) with class IV, and 3 (10%) with Class V lupus nephritis. The mean serum creatinine level for all cases was 1.0 ± 0.5 mg/dL and was significantly higher in class III and IV patients compared to class II patients (1.14 ± 0.60 mg/dL versus 0.70 ± 0.15 mg/dL, $P < .05$). Renal biopsy was performed for 5 patients (17%) who did not have proteinuria, of whom were diagnosed with class II lupus nephritis and 1 patient was diagnosed with class III and 1 with class IV lupus nephritis (Table 2).

A significant positive correlation was revealed between the Ki-67 proliferation index and serum creatinine. No correlation was noted between the Ki-67 proliferation index and proteinuria (Table 3).

Twenty patients had moderately or strongly positive anticardiolipin antibody levels. However, no correlation was found between anticardiolipin antibody positivity and the type of lupus nephritis, SLEDAI, or Ki-67 proliferation index. No correlation was found between serum complements C3 and C4 levels and the Ki-67 proliferation index, either.

The SLEDAI scores and the conventional renal biopsy activity index of patients diagnosed with class III and class IV lupus nephritis were found to be significantly higher compared to that of patients with class II nephritis ($P < .01$). Parallel to the SLEDAI and activity index, the Ki-67 proliferation index was also found to be significantly higher in patients diagnosed with class III and class IV

Table 2. Lupus nephritis type, systemic lupus erythematosus activity index (SLEDAI), Renal Biopsy Conventional Activity Index and Ki-67 Proliferation Index of the Patients

Patient	Lupus Nephritis Class	SLEDAI	Creatinine, mg/dL	Proteinuria, g/24 h	Activity Index	Ki-67 Proliferation Index
1	II	6	0.8	3	1	0
2	II	8	0.8	0	2	37
3	II	10	1.0	0.1	1	0
4	II	8	0.8	3	0	24
5	II	13	0.5	0.1	0	0
6	II	6	0.6	0	1	4
7	II	10	0.6	0.1	1	0
8	II	16	0.7	0	3	0
9	II	23	0.6	0.5	1	60
10	III	25	0.7	1.0	4	84
11	III	16	0.7	0.2	1	60
12	III	23	1.2	0	5	40
13	III	15	0.8	0.8	9	…
14	III	27	0.7	3.0	7	8
15	IV	12	0.5	0	8	45
16	IV	19	2.8	3.0	6	52
17	IV	16	1.2	2.0	15	36
18	IV	12	0.8	2.5	15	89
19	IV	13	0.9	0.1	16	96
20	IV	18	1.4	4.0	11	48
21	IV	24	2.4	3.5	13	96
22	IV	15	0.8	2.1	11	50
23	IV	20	1.7	2.8	14	140
24	IV	15	0.8	0.3	11	36
25	IV	15	1.4	3.0	9	50
26	IV	15	0.6	0.5	18	51
27	V	12	0.7	0.7	2	20
28	V	12	0.5	8.0	10	12
29	V	10	0.5	1.6	1	10

lupus nephritis.

Although conventional renal biopsy activity indexes were low in 3 of 9 patients with class II lupus nephritis (2, 0, and 1), Ki-67 proliferation indexes were high (37, 24, and 60, respectively), indicating proliferation. Lupus nephritis type, SLEDAI, activity index, and Ki-67 proliferation indexes are summarized in Table 2. Ki-67 staining was not detected in the samples for 1 patient diagnosed with class III lupus nephritis.

Table 3. Results of Correlation Analysis Between Ki-67 and Clinical and Laboratory Findings*

	Ki-67	
Parameter	Pearson Coefficient	*P*
Serum creatinine	0.457	< .05
Proteinuria	0.163	> .05
SLEDAI	0.467	< .05
Activity index	0.612	> .05

*SLEDAI indicates systemic lupus erythematosus activity index.

DISCUSSION

Organ involvement and especially renal histopathological findings play a significant role in determining disease prognosis and treatment selection in SLE. Autopsy and biopsy results for patients diagnosed with SLE have demonstrated that renal involvement is quite common and can sometimes be present, independent from clinical manifestations.[15] Findings associated with lupus nephritis can be observed in renal biopsy specimens of patients with no clinical evidence of renal involvement.[16] Two of 5 patients with no evidence of proteinuria or active urinary sediment were diagnosed with class III and IV proliferative nephritis with renal biopsy. These patients had positive test results for anti-double-stranded DNA antibodies and had low serum complement levels. The Ki-67 proliferation indexes of these two patients were 40 and 45, respectively. Although it is not

common to perform renal biopsy in the absence of positive urinary findings, the conclusion for these two patients suggests that renal biopsy should be considered for patients who test positive for anti-double-stranded DNA antibodies and have low complement levels. Cell counts in the glomerular tuft also revealed significant proliferation, especially in the crescentic glomerulonephritis, vasculitic glomerulonephritis and Henoch-Schonlein purpura, and Ki-67 proliferation could not be demonstrated in diabetes mellitus and amyloidosis.

Howie and colleagues[11] were the first to perform Ki-67 staining in formalin fixed paraffin-embedded blocks of renal biopsy specimens. This method offers the opportunity for retrospective research on a large series of paraffin-embedded blocks that can be easily prepared. Groma and colleagues[17] investigated alpha smooth muscle actin (ASMA) antibody and Ki-67 expression in glomerulonephritis and demonstrated that the highest Ki-67 expression occurred in postinfectious endocapillary glomerulonephritis. The study reported that ongoing damage and diffuse activation were present even in normal-appearing glomerular mesangial cells in IgA nephropathies.

In this study, Ki-67 proliferation indexes were found to be as high as 37, 24 and 60 in three out of nine of patients diagnosed with class II lupus nephritis by light microscopy, for which we did not consider initiating a potent immunosuppressive treatment. Ki-67 staining was not detected in the remaining six patients diagnosed with class II lupus nephritis.

Although this study is limited by the low number of the biopsy samples, Ki-67 expression was found to be statistically higher in class III and IV lupus nephritis compared to class II lupus nephritis, there may be a potential need for more aggressive therapy in cases of class II nephritis showing proliferation evidence detected by Ki-67 staining. Long-term controlled studies investigating Ki-67 expression in class II nephritis are needed to support this theory.

Chung and Kim[18] investigated Ki-67 expression in poststreptococcal glomerulonephritis and failed to find a correlation with either creatinine clearance or proteinuria. Proliferation was assessed in a semi-quantitative fashion in that study, and cases were classified into 2 groups: mildly hypercellular with three to 5 cells, and hypercellular with more than 5 cells in one capillary tuft.

In contrast to Chung and Kim's study, this study did not perform cell counting per glomerulus or create groups according to cell counts. As it is much easier to detect nuclear staining by Ki-67, 1000 cells in each biopsy sample were counted and the number of stained cells was recorded. This study too, has failed to find a correlation between proteinuria and Ki-67 expression. However, it is important to note that the Ki-67 proliferation index was higher in cases of proliferative nephritis with high serum creatinine levels. This indicates the contribution of cell proliferation to glomerular hypercellularity.[19]

It has been shown that epidermal proliferation in SLE patients increases parallel to cutaneous Ki-67 and keratin-16 expression, especially in discoid lupus lesions.[20-22] However, studies investigating Ki-67 expression in lupus nephritis patients' biopsy samples are limited.

Jeruc and colleagues[23] studied activated complement C3 and Ki-67 expression in lupus nephritis and demonstrated that the apoptotic index correlated with the proliferative index. The study subsequently suggested that increased apoptosis lead to an increased inflammation.

Assuming that increased cellular proliferation in mesangial proliferative nephritis may also be due to changes in apoptosis, Uda and colleagues[24] investigated Bcl-2, Ki-67, and ASMA expression in a total of 55 renal biopsy specimens, out of which 6 were class IV lupus nephritis and found to have significantly higher levels of expression in cases of lupus nephritis and IgA nephropathies.

Cheah and colleagues[19] determined that Ki-67 proliferation increased in class IV lupus nephritis and concluded that this finding was an indicator of the contribution of cellular proliferation to glomerular hypercellularity.

CONCLUSIONS

Ki-67 can be used as a proliferation marker in lupus nephritis. The Ki-67 proliferation index is correlated with disease activity in SLE, class III and IV lupus nephritis, and renal biopsy activity index, and Ki-67 staining may be found to be high in some cases of class II lupus nephritis.

Studies relating to the morphologic evaluation of proliferation markers, aside from long-term follow-up studies evaluating functional and clinical variables will clarify the significance of this

activation marker for disease activity and prognosis. In particular, Ki-67-positive proliferative activity demonstration in some cases of class II lupus nephritis may also indicate subclinical damage. By demonstrating that a Ki-67 proliferation marker can be used for different types of lupus nephritis, this study provides a basic insight for future studies.

CONFLICT OF INTEREST

None declared.

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