

Evaluation of Ki-67, CD68 and Bcl-2 staining, dialysis and mortality in crescentic glomerulonephritis

Evaluación de tinción de Ki-67, CD68 y Bcl-2, diálisis y mortalidad en glomerulonefritis de crescética

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ABSTRACT

Introduction: Immunohistochemical staining of Ki-67, CD68 and Bcl-2 have been studied in glomerulonephritis. We aimed to assess these immunohistochemical staining features, hemodialysis initiation and 60 month mortality rates in crescentic glomerulonephritis. **Methods:** In this retrospective study, patients, with a previous diagnosis of crescentic glomerulonephritis were divided into two groups: Hemodialysis Initiated and Not Initiated groups. Kidney biopsy specimens' Ki-67, CD68 and Bcl-2 staining scores were defined as below 5% "0", 5-10% "+1", 11-20% "+2", over 20% "+3". Patients demographic, laboratory data, status of hemodialysis initiation, and mortality were obtained from medical records and immunohistochemical staining scores were compared between groups. Estimated glomerular filtration rates (eGFR) were assessed at 0, 6, and 12 months, except patients' ongoing hemodialysis. **Results:** A total of 56 patients were diagnosed as crescentic glomerulonephritis. Pauci-immune crescentic glomerulonephritis (58.9%) was the most common etiology. Hemodialysis was initiated in 36 patients. Mean age, baseline creatinine, urea, C-reactive protein levels were significantly higher and, hemoglobin and proteinuria levels were significantly lower in the Hemodialysis Initiated group. Immunohistochemical staining scores were not significantly different between groups. In Hemodialysis Initiated group, 8.33% of patients were recovered from hemodialysis.

Mortality rates were 44,4% and 10% in patients in the group of hemodialysis initiated and not initiated group respectively. When we combine the hemodialysis not initiated patients and patients recovered from hemodialysis; median eGFR at baseline, 6th and 12th month were 32.9, 43.9, and 58.0 mL/min/1.73m², respectively (p=0.016). **Conclusion:** Hemodialysis initiation was associated with high mortality. Degree of immunohistochemical staining was similar in both groups. Increment in eGFR was documented in first year in patients, other than the ones on still on hemodialysis.

KEYWORDS: crescentic glomerulonephritis; hemodialysis; dialysis; mortality; Ki-67

RESUMEN

Introducción: Se ha estudiado la tinción inmunohistoquímica de Ki-67, CD68 y Bcl-2 en glomerulonefritis. **Objetivo:** Evaluar estas características de tinción inmunohistoquímica, el inicio de la hemodiálisis y la tasa de mortalidad a los 60 meses en la glomerulonefritis crescética. **Material y métodos:** En este estudio retrospectivo, los pacientes, con diagnóstico previo de glomerulonefritis crescética se dividieron en dos grupos: Hemodiálisis iniciada y no iniciada. La puntuación de tinción Ki-67, CD68 y Bcl-2 de las muestras de biopsia de riñón se definió del siguiente modo: por debajo del 5% "0", 5-10% "+1", 11-20% "+2", más del 20% "+3". Se compararon los siguientes datos en los pacientes: demografía, resultados

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de laboratorio, de iniciación de la hemodiálisis y la mortalidad obtenida de los registros médicos y las puntuaciones de tinción inmunohistoquímica entre los grupos. La Tasa de filtrado glomerular estimada (TFGe) fue evaluada a los 0, 6 y 12 meses, excepto en los pacientes en hemodiálisis en curso. **Resultados:** Un total de 56 pacientes fueron diagnosticados con glomerulonefritis crescética. La glomerulonefritis crescética pauci inmune (58,9%) fue la etiología más común. Se inició hemodiálisis en 36 pacientes. La edad media, los niveles basales de creatinina, urea y proteína C reactiva fueron significativamente más altos, y los niveles de hemoglobina y proteinuria fueron significativamente más bajos en el grupo de Hemodiálisis Iniciada. Las puntuaciones de tinción inmunohistoquímica no fueron significativas entre los grupos. En el grupo de Hemodiálisis Iniciada 8,33% de los pacientes recuperó función renal y salió de diálisis. La tasa de mortalidad en el grupo de Hemodiálisis no Iniciada fue del 10,0% y en el grupo que inicio HD del 44%. Cuando combinamos los pacientes Hemodiálisis no Iniciada y los pacientes recuperados de hemodiálisis la mediana de TGFe en la línea de base, 6º y 12º mes fue 32,9, 43,9 y 58,0 mL/minuto/1,73m², respectivamente (p<0,016). **Conclusión:** El inicio de la hemodiálisis se asoció con una alta mortalidad. El grado de tinción inmunohistoquímica fue similar en ambos grupos. El incremento de la TFGe se documentó en el primer año en pacientes distintos de los que aún estaban en hemodiálisis.

PALABRAS CLAVE: glomerulonefritis crescética; hemodiálisis; diálisis renal; mortalidad; Ki-67

INTRODUCTION

Crescentic glomerulonephritis is a syndrome characterized by visceral epithelial cell proliferation and accumulation of leukocyte and blood components that partially or completely filling the Bowman's space due to glomerular basement membrane rupture. Crescentic glomerulonephritis may progress rapidly to end-stage renal disease within a few weeks or months, with signs of a nephritic syndrome.⁽¹⁾ Oliguria-anuria, serum creatinine level above 5.65 mg/dL at admission, need for hemodialysis, glomerular crescent ratio above 80%, presence of fibrinoid necrosis, Anti-glomerular basement membrane antibody staining, tubular atrophy, interstitial fibrosis are the poor

prognostics criteria.⁽²⁻³⁾

Ki-67 protein is a marker of cell proliferation. It is expressed in all phases of cell division except G0. Maximum expressions of Ki-67 in G2 and M phases, can be determined by immunohistochemical staining.⁽⁴⁻⁵⁾ Ki-67 expression is limited in non-neoplastic pathologies, while Ki-67 staining is widely used to determine activity in malignancies.⁽⁶⁻⁷⁾ It has been reported that Ki-67 proliferative index is related to treatment strategy and prognosis in some cancer types.⁽⁶⁻⁸⁾

CD68 is a glycoprotein highly expressed in macrophages and other mononuclear phagocytes. It is a valuable marker that can be used to identify macrophages and monocytes and has implications for the diagnosis of various disease conditions.⁽⁹⁾ In the long-term follow-up of proliferative lupus nephritis, kidney biopsy CD68 staining has been reported to be associated with poor prognosis.⁽¹⁰⁾ It has been reported that CD68 staining was higher in pauci-immune necrotizing glomerulonephritis compared to the control group and serum creatinine level is positively correlated with glomerular CD68.⁽¹¹⁾

Inadequate activation of apoptosis in glomerulonephritis can lead to loss of glomerular and tubular cells, which can lead to glomerulosclerosis and tubular atrophy. Anti-apoptotic and proapoptotic effects of Bcl-2 protein belonging to the Bcl-2 family have been described.⁽¹²⁾ In different types of glomerulonephritis, epithelial cells of the proximal, distal and collecting tubules and renal interstitial positive staining with Bcl-2 were detected. Bcl-2 staining intensity was negatively correlated with TGF-1 Beta staining, which activates interstitial fibroblasts.⁽¹³⁾

Migration and accumulation of immune cell to Bowman's space and visceral epithelial, and mesangial cell proliferations are the important histological findings in crescentic glomerulonephritis. Ki-67, CD68 and Bcl-2 could be a useful marker to evaluate migration, accumulation, and proliferation of cells in crescentic glomerulonephritis. In our study, we aimed to determine the dialysis initiation, mortality rate, and relationship between Ki-67, CD-68, Bcl-2 staining characteristics with disease prognosis, in crescentic glomerulonephritis.

MATERIALS AND METHODS

Patients aged 18 years and older who were diagnosed as crescentic glomerulonephritis by

kidney biopsy in our Nephrology Clinic between 2008 and 2015 were included in the study. Demographic and laboratory data of the patients were analyzed retrospectively. Patients age, gender, hemodialysis initiation, baseline urea, total protein, albumin, hemoglobin, platelets, proteinuria, erythrocyte sedimentation rate, C-reactive protein levels, and 0-6-12 months, serum creatinine, estimated glomerular filtration rate (eGFR) values were obtained from medical records. 60-month follow-up for mortality was evaluated. The eGFR was calculated by The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) Formula.⁽¹⁴⁾

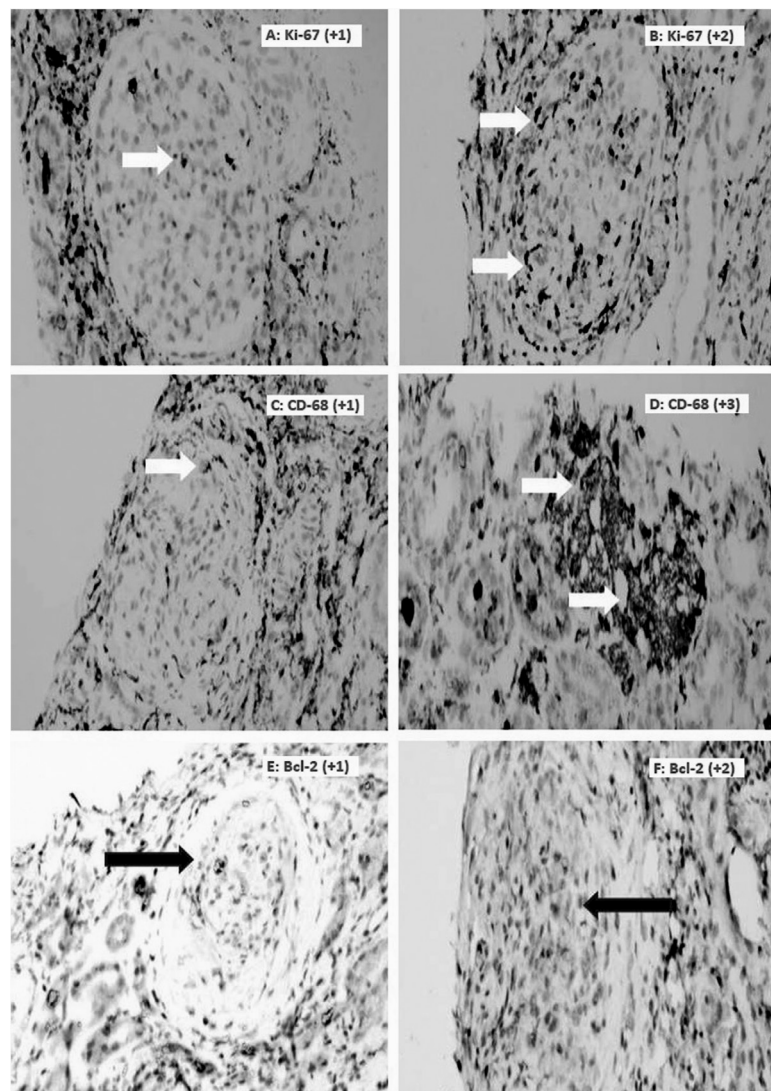
Preparation and evaluation of pathological specimens

Pathology blocks and tissue slides of the patients were obtained from the archive Ki-67,

CD68 and Bcl-2 immunohistochemical staining and evaluation were performed in paraffin blocks. Pathological examination; Tissue slide of 4µm thickness prepared from paraffin embedded blocks fixed with 10% neutral buffered formalin were examined by Hematoxylin and eosin stains. Immunohistochemical staining was performed using DAKO Autostainer Universal Staining System (Autostainer Link 48 DAKO, Glostrup, Denmark). After the staining stage, the sections were examined under the light microscope (Olympus BX51, Tokyo, Japan) at 40, 100, 200 and 400 magnifications by a single pathologist, blind to clinical information. At least 200 cells were counted in areas with the most intense cytoplasmic and nuclear staining (hot spots). It was scored as staining below 5%: "0", 5-10% staining: "+1", 11-20% staining: "+2", staining over 20%: "+3". (Figure 1)

Figure 1. Light microscope immunohistochemical staining in crescentic area.

- (A) Ki-67 immunostaining, score +1/+2 (200x).
 (B) CD68 immunostaining, score +1/+3 (200x).
 (C) Bcl-2 immunostaining, score +1/+2 (200x)



Study groups

The patients were grouped according to initiation of hemodialysis treatment as Group I: Hemodialysis not Initiated and Group II: Hemodialysis Initiated. In terms of disease prognosis, immunohistochemical staining characteristics were evaluated by classifying as +1 staining and \geq +2 staining.

Ethics committee approval

The present study protocol was reviewed and approved by the local Ethics Committee with protocol number 2014/414, dated 14.08.2014. Informed consent was not obtained due to the retrospective nature of the study. This study was conducted in accordance with the ethical standards of the World Medical Association Declaration of Helsinki.

Statistical analysis

The statistical analysis were performed with the Statistical Package for Windows version 18 [SPSS Inc; Chicago, IL, USA] packet program. The Kolmogorov-Smirnov test was used to assess the normal distribution of variables. Quantitative data were expressed as mean \pm standard deviation or median (25-75 percentile) and qualitative data as number and percentages (n, %). A T-test and Mann-Whitney U test were used to compare parametric and nonparametric independent groups, respectively. Chi-square test was used in comparisons of categorical groups. For mortality analysis Kaplan-Meier survival test was used. Friedman's one-way ANOVA was used for the repeated measure. A p

value <0.05 was considered statistically significant.

RESULTS

A total of 56 patients, aged 18 and above diagnosed with crescentic glomerulonephritis, of which 30% was focal and the rest of them was diffuse, were enrolled into the study. 37 of the patients were male (66%). Pauci-immune crescentic glomerulonephritis (n=33, 58.9%) was the most common etiology, and other pathologic diagnosis of patients were lupus nephritis (16.0%), and IgA nephropathy (14.2%). In four patients (7.1%), autoantibodies were negative on the other hand, immune florescence microscopy revealed focal mild Ig G, Ig M, C3 deposition along basal membrane, yielding the diagnosis of idiopathic proliferative glomerulonephritis. In one patient (1.7%), who had 10/10 crescent ratio, severe C3 staining was observed in mesangial region and along basal membrane, yielding the presumptive diagnosis of C3 glomerulopathy. One of the cases (1.7%) was considered as Goodpasture syndrome because of linear staining along basal membrane with Ig G, in spite of lacking Anti-GBM testing.

Hemodialysis treatment was initiated in 36 patients. The mean age in Hemodialysis Initiated and Hemodialysis not Initiated group was 57.72 ± 14.32 vs. 47.85 ± 15.26 respectively (p=0.019). Baseline creatinine, urea, C-reactive protein levels were significantly higher and eGFR, hemoglobin and proteinuria levels were significantly lower in the Hemodialysis Initiated group. The comparison of the demographic data of the two groups is shown in **Table 1**.

Table 1. Comparison of baseline values of the groups

| | Hemodialysis not initiated (n:20) | Hemodialysis Initiated (n:36) | P Değeri |
|------------------------------------|-----------------------------------|-------------------------------|------------------|
| ♀ / ♂ (n, %) | 8 (40%) / 12 (60%) | 11 (30.6%) / 25 (69.4%) | 0.474 |
| Age (year) | 47.85 ± 15.26 | 57.72 ± 14.32 | 0.019 |
| Creatinine (mg/dL) | 2.03 ± 1.06 | 5.76 ± 2.70 | <0.001 |
| e-GFR (ml/sec 1.73m ²) | 49.03 ± 38.58 | 12.04 ± 7.78 | <0.001 |
| Urea (mg/dL) | 85.95 ± 31.41 | 139.97 ± 69.22 | 0.002 |
| Total Protein (gr/dL) | 6.12 ± 0.97 | 6.30 ± 0.82 | 0.461 |
| Albumin (gr/dL) | 3.35 ± 0.61 | 3.26 ± 0.61 | 0.608 |
| CRP (mg/L) | $7.83 (2.63-46.94)$ | $26.35 (8.41-60.13)$ | 0.033 |
| Sedimentation (mm/h) | 68.60 ± 23.47 | 72.02 ± 29.45 | 0.658 |
| Hemoglobin (g/dL) | 10.87 ± 1.75 | 9.46 ± 1.25 | 0.001 |
| Platelet | 282.40 ± 91.52 | 250.27 ± 102.13 | 0.248 |
| Urine Volume (L/day) | 2.23 ± 0.61 | 1.42 ± 1.17 | 0.010 |
| Proteinuria (g/day) | $3.97 (1.63-6.24)$ | $1.26 (0.48-3.36)$ | 0.014 |

Considering the immunohistochemical staining with Ki-67, CD68 and Bcl-2, positive staining in different degrees were observed in all specimens. Immunohistochemical studies revealed similar staining pattern and distribution by all reactants in both groups. Frequency of +1 staining

was predominating in all groups; however, it was relatively lower in case of CD-68 staining. Staining scores of two groups were outlined in **Table 2**. No relationship was found in terms of Ki-67, CD-68 and Bcl-2 staining scores between the patients requiring hemodialysis or not.

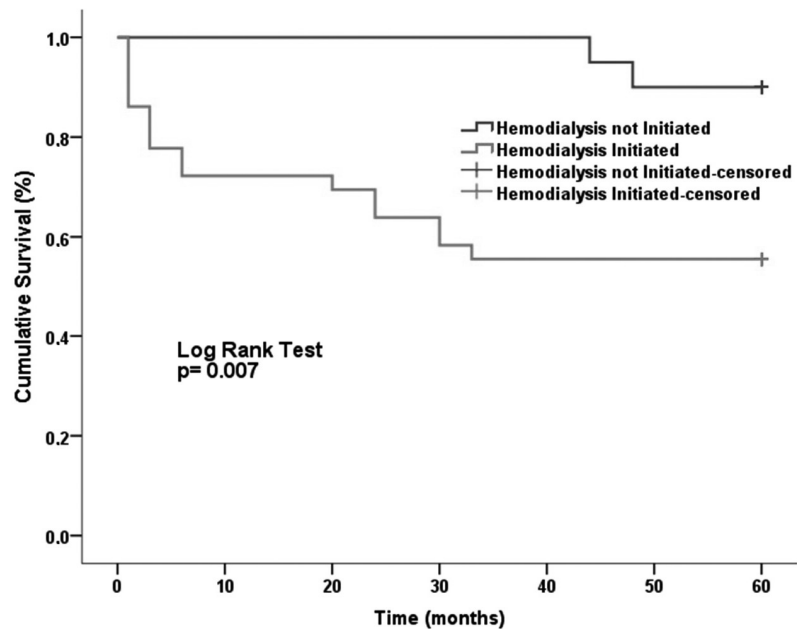
Table 2. Ki-67, CD-68 and Bcl-2 staining scores between the two groups

| | Group | Total (n:56) | Hemodialysis not Initiated (n:20) | Hemodialysis Initiated (n:36) | P |
|--------------|-------|--------------|-----------------------------------|-------------------------------|-------|
| Ki-67 (n, %) | +1 | 44 (78.5%) | 16 (80.0%) | 28 (77.8%) | 0.845 |
| | ≥ +2 | 12 (21.5%) | 4 (20.0%) | 8 (22.2%) | |
| CD 68 (n, %) | +1 | 35 (62.5%) | 13 (65.0%) | 22 (61.1%) | 0.773 |
| | ≥ +2 | 21 (37.5%) | 7 (35.0%) | 14 (38.9%) | |
| Bcl-2 (n, %) | +1 | 46 (82.1%) | 15 (75.0%) | 31 (86.1%) | 0.298 |
| | ≥ +2 | 10 (17.9%) | 5 (25.0%) | 5 (13.9%) | |

Only, in three patients (8.33%) of the Hemodialysis Initiated group renal recovery were observed and hemodialysis was suspended within three months. Patients were followed up to 60

months for mortality and 16 (44.4%) patients died in Hemodialysis Initiated Group. Only two (10%) patients died in Hemodialysis not Initiated Group (p=0.007). (**Figure 2**)

Figure 2. Kaplan–Meier survival curves of the groups



The Hemodialysis not Initiated group and hemodialysis suspended patients were analyzed together. Analysis showed that the eGFR was increased to 43.9 (32.4-74.7) in 6th month follow-up and 58.0 (29.9-71.5) in 12th month follow-up compared with baseline (0th month) 32.9 (17.6-

48.7) mL/min, 1.73 m² (**Figure 3**). The median eGFR was significantly higher in 6th and 12th month than the baseline (p= 0.016). The change of eGFR at 6th and 12th months were compared according to the Ki-67, CD68, Bcl-2 immunohistochemical staining intensity as +1 vs. ≥ +2 staining. The

patients with +1 staining pattern by all reactants disclosed significant increases in eGFR, on the other hand, even higher increments in eGFR were

observed in patients with +2 staining pattern, although it did not reach statistical significance level in one year follow up. (Table 3)

Figure 3. Course of the estimated glomerular filtration rates of the patients hemodialysis not initiated and recovered from hemodialysis (median (25–75 percentile) ± min, max value; *: difference between 0th and 6th month, **: difference between 0th and 12th month, p=0.016)

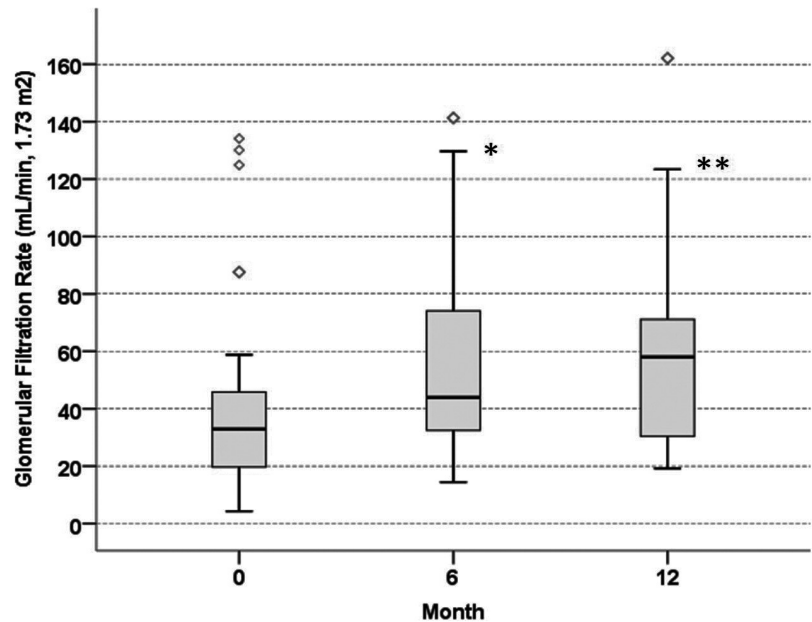


Table 3. One-year estimated glomerular filtration rate of the patients hemodialysis not initiated and recovered from hemodialysis according to immunohistochemical staining scores

| | Group | Patients Number (n, %) | eGFR (mL/min,1.73 m ²) | | | P |
|-------|-------|------------------------|------------------------------------|-------------------|-------------------|-------|
| | | | 0. Month | 6. Month | 12. Month | |
| Ki-67 | +1 | 19 (82.6%) | 27.7 (14.0-42.9) | 40.4 (31.5-68.7) | 55.3 (29.9-70.0) | 0.015 |
| | ≥+2 | 4 (17.4%) | 46.5 (31.7-108.3) | 83.1 (41.7-83.1) | 87.6 (32.2-118.3) | 0.779 |
| CD 68 | +1 | 16 (69.5%) | 30.3 (13.7-42.0) | 37.3 (29.8-66.9) | 48.2 (30.2-68.0) | 0.017 |
| | ≥+2 | 7 (30.5%) | 34.3 (21.8-124.9) | 68.6 (32.8-118.2) | 71.5 (22.9-123.2) | 0.565 |
| Bcl-2 | +1 | 18 (78.2%) | 33.6 (16.7-44.3) | 44.4 (30.9-71.3) | 56.6 (29.9-70.3) | 0.012 |
| | ≥+2 | 5 (21.8) | 24.7 (17.1-127.5) | 43.5 (34.9-123.9) | 70.7 (29.3-123.3) | 0.819 |

DISCUSSION

Crescentic glomerulonephritis is a rare group of diseases in society. Three subgroups have been described and the most common type is pauci immune crescentic glomerulonephritis.⁽¹⁾ Etiologies of crescentic glomerulonephritis differ between countries. Pauci immune crescentic glomerulonephritis was the most common type in Spain 67.2%,⁽¹⁵⁾ Japan 64%,⁽¹⁶⁾ and America 60%,⁽¹⁷⁾ while immunocomplex nephritis was reported to be the most common type in China 62.7%.⁽¹⁸⁾ Data on patient survival in crescentic glomerulonephritis are limited. In the short-term follow-up, 3 and 6 months patient survival was found to be 81.1% -

72.4%, respectively.⁽¹⁹⁻²⁰⁾ In another study, diffuse crescentic glomerulonephritis with mean follow-up period of 9.4±15 months, mortality rate was reported to be 4.5%.⁽²¹⁾ In our study 5-year patient survival rate was 67.9%. Mortality was 14.2% in the first 3 months and 17.8% in the six months.

Ki67, which is a proliferation index, has provided to pathologists a new analytical tool for evaluating kidney biopsies. Ki-67 was evaluated in kidney biopsy specimens in terms of clinical course and disease prognosis in lupus nephritis, acute post streptococcal glomerulonephritis, membranoproliferative glomerulonephritis and non-inflammatory nephritis such as membranous

glomerulonephritis.⁽²²⁻²⁴⁾ Ki-67 expression was found to be high in active period of poststreptococcal glomerulonephritis, lupus and membranoproliferative glomerulonephritis.⁽²⁴⁻²⁶⁾ Also, a positive correlation between proliferation index and serum creatinine value and lupus activity index in adults and children have been reported.⁽²⁵⁻²⁶⁾

Glomerular and interstitial macrophage accumulation is associated with hematuria, proteinuria, and higher creatinine level. CD68 staining indicator of macrophage accumulation was detected in all types of crescentic glomerulonephritis.⁽²⁷⁻²⁸⁾ In crescentic glomerulonephritis, chemokines are mainly expressed by CD68 positive macrophages and parietal epithelial cells. Inflammatory process is more severe in chemokine expression and is associated with poor prognosis.⁽²⁸⁾ Chemokine expression in crescentic glomerulonephritis was found to be correlated with CD68 + immunohistochemical staining. In pauci-immune crescentic glomerulonephritis, CD68 + staining was detected predominantly in areas of fibrinoid necrosis, and serum creatinine level during biopsy was reported to correlate with glomerular CD68 score.⁽¹¹⁾ As a result, the intensity of macrophage infiltration is closely associated with poor prognosis, disease progression, hematuria, and disease activity.

Bcl-2 has anti and proapoptotic effects and apoptosis take part in remodeling in the kidney. Intensely Bcl-2 expression has been demonstrated in crescentic glomerulonephritis, and conflicting effect of Bcl-2 expression in glomerular diseases have been reported. Bcl-2 expression has a possible role in deregulation of apoptosis and the progression of glomerular scarring in cellular crescentic glomerulonephritis.⁽²⁹⁾ In mice, Venetoclax (ABT-199), a selective Bcl-2 oral inhibitor prevented the development of proteinuria and interstitial nephritis, and glomerular immune complexes were partially reduced.⁽³⁰⁾ In histological based studies, it has been shown that Bcl-2 expression was predominantly regulated in proximal tubule cells and T and B lymphocytes infiltrating the interstitium, and was not found in the glomerular capillary tuft.⁽²⁹⁻³⁰⁾ In contrast, over expression of Bcl-2 in podocytes was reduced the Bax / Bcl-2 ratio in IgA nephropathy and may have a protective effect on the overall development of glomerular lesions by restricting glomerular cell

apoptosis.⁽³¹⁾ This protective effect is thought to act by interacting with the mitochondrial voltage-dependent anion channel and increasing its activity, leading to membrane potential loss and cytochrome c release. In addition, renal tubular toxicity due to cyclosporine A, reversed by repaglinide, resulting in increased Bcl-2 expression in the tubular cell.⁽³²⁾

Ki-67 positivity in crescentic glomerulonephritis is often accompanied by Bcl-2 positivity.⁽²³⁾ In our study, in patients with +1 pattern by Ki-67, CD68 and Bcl-2, rate of median eGFR increase in one year was 99.6%, 59% and 68.4% respectively. However, the degree of eGFR increment in patients with $\geq +2$ staining pattern were 88.3%, 108% and 186% in Ki-67, CD68 and Bcl-2 stained groups respectively. The rate and magnitude of recovery seems to be superior in patients with $\geq +2$ staining pattern, however this increments were not statistically significant probably due to limited number of patients in those groups.

Our study has potential limitations. First our results are based on retrospective observational data. Second, relatively small number of crescentic glomerulonephritis subjects.

In conclusion hemodialysis initiation has worsened the prognosis and associated with high mortality. No relationship was found between degree of immunostaining with Ki-67, CD68 and Bcl-2 between groups in terms of requiring or not requiring hemodialysis.

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