

Immunohistochemical Evaluation of Renal Allograft Biopsies from a Sample of Iraqi Renal Transplant Recipients

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Abstract

Context: Renal transplantation is the best treatment for end-stage renal disease, although some complication may arise after transplantation including transplant rejection and infection, each of the two can occur oppositely to the other since the insufficiently suppressed immune system can still attack the graft while excessively suppressed immune system may allow latent virus to reactivate. The best way to diagnose both of the conditions is by immunohistochemical staining using monoclonal antibodies specific for BK virus and C4d. **Aims:** This study aims to investigate the frequency of antibody-mediated rejection (AMR) and BK virus nephropathy (BKVN) in renal allograft biopsies in Iraq. **Settings and Design:** The study encompassed 53 renal transplant recipients who developed signs of renal dysfunction. **Subjects and Methods:** Renal allograft biopsies were collected from centre of nephrology and kidney transplantation and AL-Yarmouk teaching hospital in Baghdad from January to September 2017, biopsies were embedded, sectioned, fixed on slides and stained with hematoxylin and eosin and monoclonal antibodies specific for C4d and BK virus. **Statistical Analysis Used:** Data were tested using Chi-square test; the analysis was conducted using the available SPSS-24 Package. **Results:** Samples were obtained from 38 males and 15 females in whom their mean age was 36.0 ± 13.8 years, the average time between transplantation and biopsy was 33.5 ± 22.5 months, and there was 31 donation from related donor and 22 from unrelated donor. The frequency of BKVN was 1.8% and the frequency of AMR was 52%. **Conclusions:** Using immunostaining of renal allograft biopsies using anti-BKV antibodies and anti-C4d help in identifying the causes of renal impairment after transplantation that otherwise remain hidden.

Keywords: Biopsy, BK virus, rejection, renal, transplant

INTRODUCTION

Renal transplantation

Renal transplantation is considered the treatment of choice for patients with end-stage renal disease; it is superior to hemodialysis because it lowers mortality rates and reduces the cost of treatment;^[1] however, there are a number of complications that can arise after transplantation jeopardizing the survival of the graft, like transplant rejection, in the past decades; there were considerable advances in immunosuppression protocols and compatibility testing which reduced the incidence of rejection to only 10%–15% in the 1st year of transplantation;^[2,3] however, these advances did not come without a cost since restraining the normal immune response leads to the reactivation of latent viruses.^[4] Renal transplantation in Iraq begun in 1973, and the first transplant was conducted at Al-Rasheed military hospital; later in 1985, the transplantation team was transferred to Baghdad Medical

City in Baghdad and established a renal transplantation unit; in 1990, the transplantation unit became a specialized center.^[5]

Rejection

Renal transplant rejection is a serious complication that can arise after renal transplantation, it is an immunological reaction toward the allograft tissue, and both innate and adaptive arms of the immune system participate in this reaction leading to tissue injury that impairs renal functions;^[6] in the present, the major cause of graft loss is antibody-mediated rejection (AMR);^[7] AMR is classified into hyperacute, acute, and chronic AMR. Hyperacute

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AMR may be caused by ABO-incompatible transplantation and may take place directly after transplantation.^[8] Acute AMR commonly occur within an extended period of time and usually becomes chronic; first, antidonor antibodies in the recipient serum bind to graft tissue leading to complement fixation; then, glomerular and peritubular capillaries undergo an acute injury, sequentially leading to transplant glomerulopathy and disruption of the tubulointerstitium.^[9] Chronic AMR is manifested by a specific morphological changes that are caused by tissue injury, and the word “chronic” is not used to describe the period of time required to develop rejection.^[10]

C4d staining

The binding of antidonor that is generated by activated B cells to the renal endothelium activates the classical pathway of complement system; phagocytic cells that possess receptors for complement components bind C3b and C4b on target cells which leads to the destruction of these targets; in addition, free components such as C3a, C4a, and C5a recruit macrophages to the site of complement activation and late components C6, 7, 8, and 9 join together to make membrane attack complex MAC which penetrates cell membrane and causes the cell components to leak out leading to cell death. C4b is further degraded in some cases into smaller fragments like C4d.^[11]

C4d is a split complement component that attaches to endothelial cells of peritubular capillaries, and its binding is evident of interaction between complement-fixing antibodies and vascular endothelium. The detection of C4d expression in renal biopsy by methods like immunohistochemistry IHC or immunofluorescence IF is used since 1993 to diagnose AMR;^[12,13] though in the present, AMR is diagnosed in C4d negative as long as there is evidence of microvascular injury and circulating donor-specific antibodies DSA.^[14]

BK virus-associated nephropathy

BK virus is a member of Polyomavirus family; it is a small double-stranded DNA virus that can cause a syndrome called BK virus nephropathy (BKVN).^[15] BK virus infection occurs as early as 3–4 years of age;^[16] after infection, the virus remains latent in the tubular epithelial cells of the kidney;^[17] when renal transplant recipients receive immunosuppression, BK virus resumes replication and causes lysis of tubular endothelium.^[18]

Despite BK virus infection being mostly asymptomatic in immunocompetent individuals, it is found that up to 5% of healthy population and 60% of immunocompromised patients shed the virus in their urine.^[19-21]

The replicative cycle of BK virus starts as the virions adsorb to target cell surface by an N-linked glycoprotein which acts as a receptor;^[22] BK virions enter the target cell by caveolae-mediated endocytosis;^[23] the virions are then carried to the nucleus by the cytoskeletal transport machinery; once in the nucleus virions are uncoated and their genomes are transcribed, replicated, and encapsidated to form the new progeny;^[24] the reactivation of BK virus in the renal epithelial cells cause shedding of Decoy cells and virions in the urine.^[25]

This study was conducted to investigate the frequency of BKVN and AMR in a sample of renal transplant recipients and to show the role of immunohistochemistry in diagnosing posttransplant complications.

SUBJECTS AND METHODS

We prospectively studied 53 cases of renal allograft biopsies collected from center of nephrology and kidney transplantation and AL-Yarmouk teaching hospital in Baghdad (ethical permission was granted by these institutions) from January to September 2017; baseline characteristics of the patients (age, gender, time between transplantation and biopsy, donor–recipient relation and immunosuppression regimen) were obtained, and consents were signed by all the participants in this research.

Morphologic evaluation of the biopsies was performed using hematoxylin and eosin according to Banff 13 classification^[26] in consistence with clinical features of the patients.

Immunohistochemistry procedure

Processing of specimen biopsies was held in Al-Yarmouk teaching hospital laboratories. Paraffin blocks were cut into 4 μ , and sections were placed on positive charged slides, de-paraffinization of slides was done in xylene then the slides were rehydrated in alcohol. For antigen retrieval, slides were immersed in 10 mM sodium citrate buffer (PH = 7.4) and heated to 95°C for 30 min in microwave and to reduce endogenous peroxidase activity; three drops of peroxidase block were added to each section and incubated for 5 min; then, the slides were washed with phosphate buffer saline (PBS) for 2 min. Three drops of serum block were added to each section and incubated for 2 min; the excess serum was removed. Tissue sections were covered with diluted primary antibody specific for BK virus purchased from Santa Cruz, USA and for C4d purchased from Bio SB, USA, and incubated for 2 h; then, the slides were washed with PBS for 2 min. Each tissue section was covered with biotinylated secondary antibody for 30 min and then washed with PBS for 2 min. Sections were incubated with horseradish peroxidase HRP-streptavidin complex for 30 min then washed with PBS for 2 min. Slides were developed using three drops of HRP substrate. Sections were counterstained by hematoxylin for 5–10 s, then immediately rinsed in distilled water. Sections were mounted in DPX medium and covered with coverslips and examined by light microscope. Slides were processed using EnVision staining system purchased from Dako, USA. Data analysis was performed using the available statistical package of Statistical Packages for Social Sciences 24 (SPSS-24) (IBM Corp, Released 2016. IBM SPSS version 24.0, Armonk, NY, Chicago, USA).

RESULTS

Of 53 individuals, 38 were males forming 71.6% while 15 were females (28.4%); the mean age of the individuals is 36.0 ± 13.8 years. Thirty-one individuals received organs from relatives while 22 received organs from unrelated

donors. The average time between transplantation and biopsy is 33.5 ± 22.5 months. Histopathological and immunohistochemical findings of the biopsies were as follows: acute AMR 10 cases, nine of them were C4d positive, chronic AMR 18 cases, 17 of them were C4d positive, acute tubular necrosis 13 cases all C4d negative, T-cell-mediated rejection TCMR five cases all C4d negative, calcineurin inhibitor nephrotoxicity six cases all C4d negative and BKVN one case which was also C4d negative [Figure 1]. There is a significant difference ($P = 0.0001$) between C4d expression and histological diagnosis as shown in Table 1. There was no significant association between C4d staining and recipient's age ($P = 0.651$), gender ($P = 0.772$), serum creatinine at the time of biopsy ($P = 0.771$), time after transplantation ($P = 0.27$), source of organ donation ($P = 0.718$), and immunosuppressive

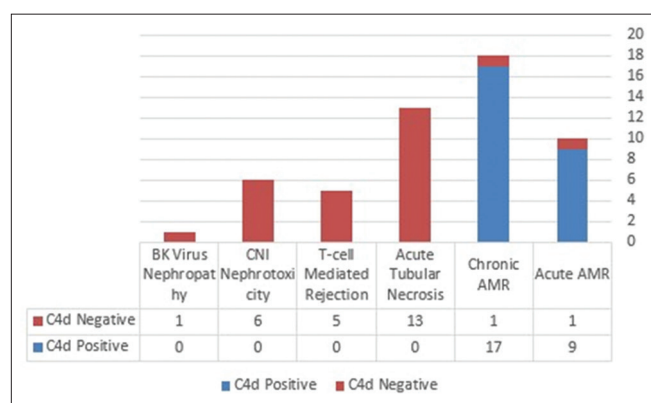


Figure 1: The association of histological diagnosis with C4d expression

Table 1: The correlation between histopathological diagnosis C4d expressions in the studied cases

Histologic diagnosis	n (%)	C4d positive	C4d negative	P
Acute AMR	10 (18.8)	9	1	0.0001
Chronic AMR	18 (33.9)	17	1	
CNI nephrotoxicity	6 (11.3)	0	6	
T-Cell mediated rejection	5 (9.6)	0	5	
Acute tubular necrosis	13 (24.6)	0	13	
BKVN	1 (1.8)	0	1	

AMR: Antibody-mediated rejection, CNI: Calcineurin inhibitor, BKVN: BK virus nephropathy

Table 2: The association of epidemiological and medical characteristics of the recipients with the histopathological and immunohistochemical findings

Variables	Acute AMR	Chronic AMR	CNI nephrotoxicity	TCMR	BKVN	ATN	P
Mean age (year)	34.9	35.6	34.8	41.2	27	32.3	0.651
Male/female	7/3	13/5	4/2	4/1	1/0	9/4	0.772
Mean serum creatinine ($\mu\text{mol/L}$)	230	283	221	256	150	229	0.771
Mean time after transplantation (month)	53.7	46.9	30.8	31.6	6	36.4	0.27
Related donor/unrelated donor	6/4	10/8	2/4	4/1	1/0	8/5	0.718
Cyclosporine/tacrolimus	7/3	9/9	5/1	4/1	1/0	7/6	0.392

AMR: Antibody-mediated rejection, CNI: Calcineurin inhibitor, TCMR: T-Cell-mediated rejection, BKVN: BK virus nephropathy, ATN: Acute tubular necrosis

regimen ($P = 0.392$) as shown in Table 2. Polyoma BK virus was detected in only one patient of 53 [Figure 2]; the patient's characteristics is shown in Table 3. The detection method was validated by staining BKV positive control slides using the same antibody and detection kit following the same procedure; the stained slides were positive [Figure 3]. In this study, anti-C4d antibodies were used to investigate the expression of C4d in the renal allograft tissue; to the best of our knowledge, this is also the first study in the country that addresses this issue. Twenty-six of 53 biopsies showed deposition of C4d in the renal tissue [Figure 4]; the expression patterns varied among peritubular, glomerular, and vascular expression.

DISCUSSION

At present, dialysis and symptomatic treatment are the major treatments for renal failure; however, renal transplantation is considered the best choice for patients in the end stage of the disease;^[27] transplant recipients in the present receive either too much or too little immunosuppression, which expose them to malignancies, drug toxicity and infection, or possibility of rejection;^[28,29] the restriction of the immune system made it possible for latent viruses to reactivate and resume their replication causing damage no less than that of rejection.^[30,31] One of these infections is caused by BK virus which is the etiological agent for BKVN, a syndrome characterized by necrosis of the tubular endothelium leading to impairment of tubular reabsorption; currently, there is no specific treatment for BKVN except for reducing immunosuppression, risking the reoccurrence of rejection.^[32] Hence, it is crucial for the survival of the kidney and the patient to precisely identify the cause of renal dysfunction.

The gold standard for the diagnosis of BKVN is the detection of BK virus antigens in renal biopsies.^[33,34] The characteristic features of BKVN are necrosis of tubular epithelial cells, sloughing of cells that bear inclusion from the basement of tubules and infiltrates of plasma cells and lymphocytes such making it resemble features of cellular rejection, so the correct diagnosis depends on immunohistochemical staining.^[35-37]

BKVN is prevalent in 1%–10% of renal transplant recipients;^[38] in a study done in China, only 9 positive cases were detected by immunofluorescence of 121 biopsy (7.4%),^[39] while in Germany, a research team examined

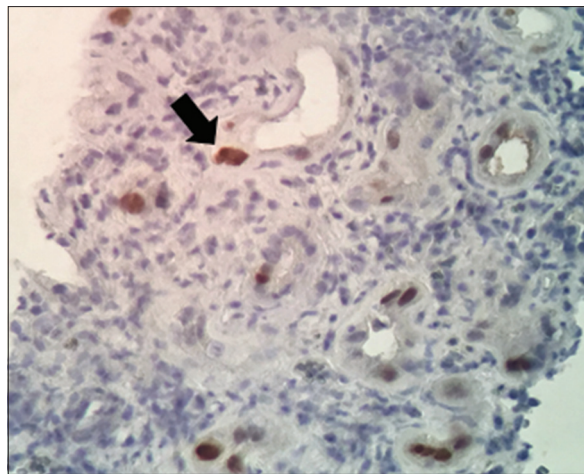


Figure 2: A renal allograft biopsy slide (×40) showing brown immunohistochemical positivity for BK virus in nucleus of renal tubule epithelial cells (arrowed)

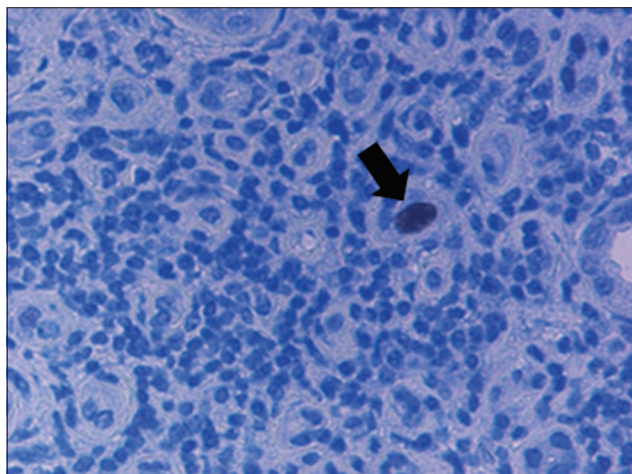


Figure 3: A positive control slide of renal biopsy (×40) showing brown immunohistochemical positivity for BK virus in nucleus of renal tubule epithelial cells (arrowed)

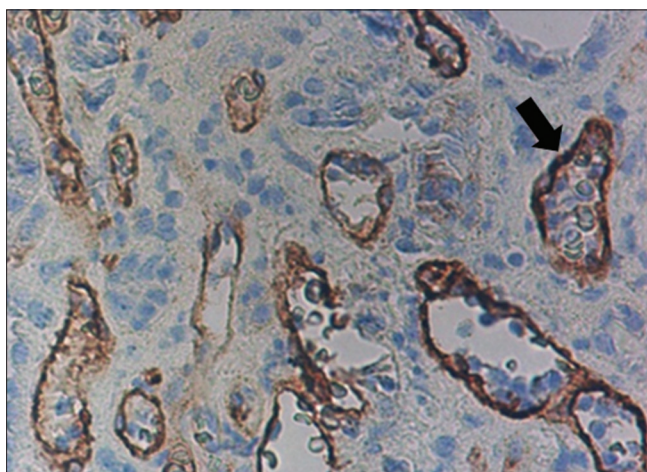


Figure 4: A renal allograft biopsy slide (×40) showing brown immunohistochemical positivity for C4d (Arrowed) in peritubular capillaries

Table 3: Some epidemiological and medical characteristics of the patient diagnosed with BK virus nephropathy

Age	27 years
Gender	Male
Source of donation	Related donor
Time since transplantation	6 months
Immune suppression regimen	Tacrolimus, mycophenolic acid, and prednisolone
Serum creatinine	1.7 mg/dl

352 biopsies by immunohistochemical staining only to record 22 cases (6.2%),^[40] and even less number of cases were recorded by Mengel *et al.* using immunohistochemical staining in 1276; only 11 cases were positive for BK virus (>1%).^[41]

In Iraq, two studies have been conducted to investigate BK virus infection in renal transplant recipients; one of them detected Decoy cells in urine by pap smear method in 27.3% of study participants;^[42] the other detected BK viruria by PCR in 2.1% of study participants;^[43] to the best of our knowledge, there have not been an attempt to detect neither BK viremia nor BK proved by biopsy.

The rejection of transplanted organs is complicated process involving humoral and cellular pathways which cause a wide range of graft injuries if not managed eventually leading to graft loss,^[44] in Iraq; according to the most recent study that investigated survival of renal transplant recipients, incidence of rejection in the 1st year of transplantation was 16.3%, AMR formed 15% of this proportion^[5] however these cases are only diagnosed on bases of histological evidence of tissue injury.

Banff 2013 classification of renal allograft biopsies stated that three features must be present in renal allograft biopsy to be diagnosed as acute or chronic AMR:

1. Histologic evidence of tissue injury
2. Evidence of antibody interaction with vascular endothelium
3. Presence of circulating DSA in recipient serum.^[14]

C4d is an important marker of AMR that indicates an interaction of antibodies with vascular endothelium and such can be used as evidence of rejection.^[12]

Since 1973, hundreds of transplantations were performed in Iraq; however, to this date, the evaluation of renal allograft biopsies is done only on the basis of histopathological characteristics of the specimens due to unavailability of required immunohistochemical markers, and very few studies were conducted about BK polyomavirus infection and less about transplant rejection, and for the best of our knowledge, this is the first study in the country to discuss both of the individuals.

Donor-specific antibodies can produce a damage to the graft tissue by two pathways; indirect pathway by initiation of complement fixation leading to the deposition of C4d and direct

pathway by adsorbing to inflammatory cells like neutrophils natural killer cells and macrophages; this pathway, however, does not involve complement fixation so that no C4d will be deposited.^[45]

In this study, according to the results obtained by histological diagnosis, 28 of 53 biopsies were diagnosed as AMR; 26 of those were confirmed by C4d staining, and the remaining two lacked C4d expression; it is possible that the injury occurred in these two cases was mediated by inflammatory cells rather than complement fixation.

We aimed by conducting this study to evaluate a sample of renal allograft biopsies using immunohistochemical markers for AMR and BKVN; one limitation of the study is the small sample size; the other is the unavailability of DSA detection methods which could be useful in measuring C4d sensitivity.

Only one case of BKVN was diagnosed in this study making the frequency 1.8% which corresponds with the global frequency rates,^[46] the diagnosed case is a male who received Tacrolimus as an immunosuppressant; both are risk factors for BKVN; factors also include old age, episodes of rejection, and cold ischemia time, although the detected case is 27 years of age, with no history of rejection and received the kidney from a living donor.^[47,48]

We also found that 52% of the examined biopsies showed evidence of acute and chronic AMR based on histopathological features and C4d immunostaining; in the absence of DSA screening technology, the frequency could be higher.

CONCLUSIONS

The frequency of BKVN in the studied cases is 1.8% which corresponds with the global frequency. C4d helped in reaching diagnosis in approximately half the studied cases. AMR was diagnosed in more than half the studied cases, thereby the immunohistochemical staining of renal allograft biopsies using anti-BKV and anti-C4d Abs is important for the diagnosis of complications that arise after transplantation; in the absence of such method, the accurate diagnosis can be missed and posttransplant complications may be poorly managed.

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Conflicts of interest

There are no conflicts of interest.

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