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ORIGINAL PAPER

Significance of anti-endothelial cell antibodies in paediatric kidney transplant recipients

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ABSTRACT

Introduction: Vascular endothelium, which expresses antigens, could be targeted by various antibodies, and it is the first barrier between the immune system and the allograft in kidney transplant recipients (KTRs). We aimed to outline the clinical significance of anti-endothelial cell antibodies (AECA) in paediatric KTRs.
Material and methods: Serum AECA IgG titres were measured pre and post renal transplantation in 46 paediatric kidney transplant recipients and in 12 age- and gender-matched healthy controls by ELISA technique.
Results: In KTRs, AECA titres were significantly increased after transplantation compared to both pre-transplantation (1.66 ±0.90 vs. 0.76 ±0.58 ng/ml, $p = 0.002$) and healthy controls (1.66 ±0.90 vs. 0.6 ±0.2 ng/ml, $p = 0.004$). In KTRs, AECA titres were significantly increased in living unrelated compared to living related renal grafts (3.3 ±3.9 vs. 1.09 ±0.87 ng/ml, $p = 0.003$) and were significantly affected by the type of induction therapy (in anti-thymocyte globulin, $n = 30$), basiliximab ($n = 9$) and no antibody induction ($n = 7$) groups; (1.32 ±1.18, 2.5 ±4.37 and 2.01 ±2.27 ng/ml respectively, $p = 0.0372$). Anti-endothelial cell antibodies titre was detected positive (≥ 1.2 ng/ml) in 21% (3 patients) of KTRs with acute rejection (AR) ($n = 14$) and in 28% (2 patients) of KTRs with chronic graft dysfunction ($n = 7$).
Conclusions: In KTRs, AECA titre is increased after kidney transplantation without a significant correlation with AR. Anti-endothelial cell antibodies titre is influenced by donor relations and antibody induction.

KEY WORDS:

anti-endothelial cell antibodies, acute and chronic rejection, children, immunological role, kidney transplantation.

INTRODUCTION

Kidney transplantation (KTX) is believed to be the ideal therapy for end-stage kidney disease (ESKD) in children [1]. Kidney transplantation is clearly superior to various dialysis techniques because the long-term outcomes are associated with a better growth, quality of life, productivity, and longer survival of ESKD children [2].

Recent advancements in immunosuppressive (IS) medications, surgical techniques, peri/postoperative care, early/pre-emptive KTX and long-term follow-up have improved the outcomes of KTX in children. Nevertheless, infection, rejection, and adverse drug reactions continue to cause transplant failure and death [3]. In children, acute rejection (AR) is responsible for 13–21% of graft failures. Within the first 6 months after transplantation, the number, the severity, and corticosteroid response of AR episodes are major determinants of long-term function and survival of the graft [2].

Non-HLA antigens are antigens that are expressed on endothelial, epithelial, parenchymal, and circulating immune cells. Non-HLA antibodies are directed against

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auto- or allo-antigens and can be pre-existing or newly developed after transplantation [4].

Anti-endothelial cell antibodies (AECA) are a diverse collection of non-HLA antibodies that target antigenic determinants expressed on endothelial cells [5]. The first barrier between the immune system of the recipient and the allograft in KTX is the vascular endothelium, which plays a significant role in the pathophysiology of antibody-mediated rejection (AMR). It expresses several antigens that can be targeted by various antibodies [6].

There is growing evidence that AECA plays a role in the host's immunological response to the allograft [4]. Anti-endothelial cell antibodies have been implicated in hyperacute rejection [7] and have been suggested to correlate with both AR and chronic graft dysfunction (CGD) [5]. However, the precise role and clinical application of AECAs are not yet well clarified. Moreover, results of published studies that define the importance of pre-existing AECA before KTX in predicting rejection after KTX are conflicting [8]. Patients with de novo AECAs are at increased risk for multiple AR, particularly in the early post-transplant period [9].

The pathologic role of AECA in inducing damage of the cell wall was investigated in several studies in non-transplanted populations. In Kawasaki syndrome, for example, AECA induces a complement-dependent endothelial cytotoxicity on cytokine-activated (interferon [INF]-γ, interleukin [IL]-1 α or β, and tumour necrosis factor [TNF]-α) but not on resting endothelial cells. Non-activated endothelial cells are not affected by antibodies, and the cytotoxic effect of AECA can occur when the activated endothelial cells bind with IL-1β. In KTX, ischaemia-reperfusion injury and inflammatory mediators (IL-1, TNF-, and INF-) can easily activate endothelial cells [8]. Thus, identification and characterization of AECAs could improve our understanding of their role in the pathogenesis of graft rejection, and it would enable the development of new non-invasive tools for monitoring of immune response and early diagnosis of rejection in kidney transplant recipients (KTRs) [10].

This study aims to outline the clinical significance of AECA by measuring titres before and after KTX and in different clinical subgroups of paediatric KTRs.

MATERIAL AND METHODS

STUDY POPULATION

This is a case-control study that included 46 paediatric KTRs recruited from the Kidney Transplantation Outpatient Clinic (CUCH) of Cairo University Children's Hospital and 12 healthy controls without any clinical signs or family history of renal disease recruited from the centre of excellence of the National Research Centre (NRC). All patients (KTRs) were recipients of a living donor (related or unrelated) kidney transplant. The study was conducted between 2016 and 2019. Kidney transplant recipients showing signs of infection induced fever, ureteral obstruction, arterial or venous thrombosis, or renal artery stenosis of the graft were excluded from the study.

Peripheral blood samples were withdrawn before KTX and at within the first 6 months of KTX for all included KTRs.

Quantitative assessment of AECA IgG concentration (ng/ml) in serum was performed using MyBioSource ELISA kit Cat. No. 263578. This assay implies a double-sandwich ELISA technique in which the wells are precoated with monoclonal antibody (human AECA), and the detecting antibody is a polyclonal antibody labelled with biotin. The avidin-peroxidase conjugate reacts with the TMB, yielding a colour. The positive development of the colour intensity correlates with the AECA concentration in the samples.

We used the mean +3SD of the AECA titre of healthy controls as the cut-off value for the AECA titre value. In our study, the value ≥ 1.2 (ng/ml) was considered positive for the AECA titre [7].

ETHICAL CONCERNS

The study was approved by NRC Ethics Committee, and the Paediatric Nephrology Unit Ethics committee of CUCH, Egypt. in compliance with the Declaration of Helsinki, blood samples from patients and controls were taken after written informed consent.

IMMUNOSUPPRESSIVE PROTOCOLS

Induction therapy was used in 39 (85%) KTRs (antithymocyte globulin – ATG in 30 and basiliximab in 9 patients), with no antibody induction used in 7 (15%) patients. All KTRs received perioperative pulse methylprednisolone therapy then oral steroids by the second postoperative week, which was tapered gradually to 2.5–7.5 mg/day by the end of the first transplantation year.

Maintenance IS medications consisted of steroids – calcineurin inhibitors (CNI) (cyclosporine or tacrolimus). Mycophenolate was administrated as an adjuvant therapy. In a CNI-based classic triple IS protocol (n = 44) (steroids, CNI, and mycophenolate), the initial cyclosporine A dose was 8–10 mg/kg per day by oral route with target trough level ranging between 150 and 200 ng/ml during the first 3 months in 14 patients, while the initial tacrolimus dose was 0.14–0.16 mg/kg per day by oral route with target trough level around 10 ng/ml in the first 3 months after KTX in 32 patients. In CNI minimization IS protocol (steroids – low-dose CNI and m-TORI) (n = 4) tacrolimus was used with a targeting trough level around 6 ng/ml, and everolimus was added instead of mycophenolate after the first 3 months post transplantation. Mycophenolate was initiated at a dose of 800–1000 mg/m^2 and was then

modified based on patient tolerance (appearance of adverse effects such as diarrhoea or leukopaenia).

CLINICAL PARAMETERS

Perioperative and follow-up clinical data were collected by reviewing records for all included KTRs. Graft function at time of AECA assessment was evaluated in terms of serum creatinine. Acute rejection was diagnosed when serum creatinine increased by 20–30% from baseline levels and this rise was associated by oliguria, fever, and graft tenderness [3].

Presumed AR presumed acute rejection (PRAR) was characterized as an episode of AR that was clinically recognized and treated with pulse methylprednisolone, without collecting a biopsy sample or there was no evidence of rejection in the biopsy sample according to the Banff-criteria [11]. Acute graft dysfunction with pathological evidence of rejection was defined as biopsy-proven acute rejection (BPAR).

Chronic graft dysfunction was clinically defined as a progressive reduction of graft function associated pathologically with interstitial fibrosis and tubular atrophy and ≥ 15% irreversible increase in creatinine level within 1 to 3 months as well as proteinuria ≥ 1 g/24 h [12].

LIMITATIONS

This study was limited by the small number of patients, the fact that it was a single-centre study, the frequency of AECA titre measurements (just twice before and after transplantation due to financial concerns), and the brief follow-up period.

STATISTICAL ANALYSIS

For data analysis, the Statistical Package for Social Science application version 16.0 was utilized. The data were presented as mean, standard deviation, range, or percentage. One-way analysis of variance or independent t-test were used to compare data between the experimental groups. Statistical significance was defined as a *p*-value of less than 0.05.

RESULTS

Our study included 46 paediatric KTRs and 12 healthy controls. Table 1 summarized the demographic and clinical characteristics, and laboratory data of transplanted patients and their controls. The mean age of KTRs was 10.36 ±3.84 years, pre-transplantation dialysis duration was 21.70 ±25.34 months, while the mean follow-up duration after KTX was 30.9 ±16.5 months. The male/female ratio was 31/15. All included patients received a living donor kidney transplant (related – $n = 34$, un-related – $n = 12$). All patients received their first renal transplant, except one patient with a previously failed graft due to venous thrombosis.

As regards to the donor relation, there was a significant difference in the levels of AECA titre in patients who received the graft from a living related donor (LRD) and patients who received it from a living un-related donor (LURD) (1.09 ±0.87 vs. 3.3 ±3.9 ng/ml, $p = 0.003$*) (Table 2).

As regards to the induction therapy, there was a significant difference in AECA titre when comparing the ATG, basiliximab, and no antibody induction groups (1.32 ±1.18 vs. 2.5 ±4.37 vs. 2.01 ±2.27 ng/ml, respectively, $p = 0.0372$).

By real-time polymerase chain reaction, cytomegalovirus was tested positive in 37 patients and negative in 9 patients, with no significant statistical difference as regards to AECA titre (1.77 ±2.53 vs. 1.22 ±0.64 ng/ml, respectively, $p = 0.0495$)

There was no statistically significant difference in AECA titre when comparing patients without previous PRAR episodes ($n = 16$) and patients with single previous PRAR episode ($n = 7$) or patients with ≥ 2 previous PRAR episodes ($n = 23$) (1.05 ±0.69 vs. 3.12 ±1.75 vs. 1.63 ±2.92 ng/ml, respectively, $p = 0.134$).

No significant difference was detected in AECA titre between patients with BPAR, ($n = 14$) and patients with no BPAR ($n = 32$), (1.27 ±0.8 vs. 1.83 ±2.69, respectively, ng/ml $p = 0.58$). Also, there was no statistically significant difference between the levels of AECA in patients who experienced CGD, ($n = 7$) and those with no CGD (1.11 ±0.67 vs. 1.76 ±2.46 ng/ml, respectively, $p = 0.853$).

No significant difference was found between healthy controls and pre-transplant levels of AECA titre (0.76 ±0.58 vs. 0.6 ±0.2 ng/ml, respectively, $p = 0.243$), while the titre was significantly increased after KTX (0.6 ±0.2 vs. 1.66 ±0.90 ng/ml, respectively, $p = 0.004$*). A significant difference was found in the pre-transplant levels of AECA titre as compared with the post-transplant levels of AECA (0.76 ±0.58 vs. 1 .66 ±0.90 ng/ml, respectively, $p = 0.002$*) (Figure 1).

Three out of 14 (21%) patients with BPAR had an AECA titre that was positive (≥ 1.2 ng/ml) (Figure 2), and 2/7 (28%) of the CGD patients had an AECA titre (≥ 1.2 ng/ml) (Figure 3). To identify the role of AECA in the occurrence of rejections, this must be further supported by larger investigations.

DISCUSSION

Anti-endothelial cell antibodies activate the vascular endothelium, promoting alloimmune responses as increasing expression of adhesion molecules and inflammatory cytokine production, which increase the degree of microvascular injury [14]. It was reported that AECA levels may rise as a result of pre-existing endothelial injury or viral infection [15].

TABLE 1. Demographic, clinical, and laboratory data of transplanted patients and controls and correlations of patients' data to anti-endothelial cell antibodies titre

Parameters	Patients ($n = 46$)	Controls ($n = 12$)	p-value	AECA p-value	AECA correlation coefficient
Age at KTX (years)	10.36 ±3.84	_	_	0.237	–0.165
Age at assessment (years)	12.94 ±4.23	10.7 ±4.51	0.132	0.213	–0.187
Sex (M/F)	31/15	8/4	0.123	0.233	–0.179
Duration of F/U after KTX (months)	30.94 ±16.51	_	_	0.233	–0.179
Dialysis duration (months)	21.70 ±25.34	_	_	0.418	–0.122
BMI at assess [kg/m^2]	22.63 ±7.88	23.60 ±8.44	0.7	0.501	–0.182
SBP [mm Hg]	109.40 ±10.50	95.54 ±9.70	0.0001*	0.590	–0.82
DBP [mm Hg]	70.40 ±8.91	61.55 ±10.10	0.003*	0.863	0.026
Donor age (years)	37.18 ±6.21	_	_	0.289	–0.160
Cold ischemia time (minutes)	52.45 ±12.30	_	_	0.259	–0.172
PRD dose at 1 month [mg/day]	19.02 ±5.44	_	_	0.403	0.133
PRD dose at 12 months [mg/day]	4.23 ±1.55	_	_	0.755	0.048
Trough CsA [ng/ml]	110.83 ±18.55	_	_	0.228	–0.580
Trough tacrolimus [ng/ml]	6.26 ±1.16	_	_	o.781	–0.082
eGFR [ml/min/1.73 m^2]**	76.20 ±22.10	86 ±18.8	0.16	0.452	–0.114
Hb [gm/dl]	10.84 ±1.17	14.32 ±1.50	< 0.0001	0.557	–0.104
HCT	32.14 ±4.20	38.88 ±3.62	0.0001*	0.903	0.022
TLC [$\times 10^3$/mm^{-3}]	7.83 ±2.61	3.57 ±1.42	< 0.0001*	0.224	0.214
G count [$\times 10^3$/mm^{-3}]	49.70 ±17.15	42.42 ±12.32	0.0181	0.312	0.191
L count [$\times 10^3$/mm^{-3}]	37.07 ±16.64	22.20 ±15.21	< 0.0001*	0.033	–0.390
PLT count [$\times 10^3$/mm^{-3}]	223.06 ±78.41	269.45 ±84.02	0.0057	0.046	0.345
CD4 (%)	34.32 ±9.58	34.78 ±10.01	0.882	0.070	–0.270

AECA – anti-endothelial cell antibodies, BMI – body mass index, CsA – cyclosporine, DBP – diastolic blood pressure, eGFR – estimated glomerular filtration rate, FU – follow up, G – granulocyte count, HB – haemoglobin, HCT – haematocrit, KTX – kidney transplantation, L – lymphocyte count, MCH – mean corpuscular haemoglobin, MCHC – mean corpuscular haemoglobin concentration, MCV – mean corpuscular volume, PLT – platelet count, PRD – prednisolone, SBP – systolic blood pressure, TLC – total leucocyte count

** $p < 0.05$ was considered significant*

*** eGFR for children was calculated by using the revised Schwartz formula: ($k = 0.413 \times$ height/serum creatinine) [13]*

To study the effects of AECA in paediatric KTRs and to evaluate its role in diagnosing and monitoring graft rejection after KTX, serum AECA IgG titres were measured in 46 paediatric ESKD patients before and within the first 6 months after KTX and during the attack of PRAR.

In our study, no significant difference was found between pre-transplantation levels of AECA titre and healthy controls, while the titre was significantly increased after transplantation – we found a significant difference between the pre-transplantation and the post-transplantation levels of AECA titre.

Although the immune system has several checkpoints to maintain tolerance to self-antigens, such as central and peripheral tolerance, abnormalities in these checkpoints with constant presence of autoantigen lead to chronic inflammation [16]. Tissue damage induced by ischaemia-reperfusion injury can result in acute kidney injury and delayed graft function, which can impair graft survival [17].

As regards the type of antibody induction therapy, ATGs appear to be effective in limiting antibody formation. This is why they are used for induction protocols and, in some cases, for treatment of AR. The use of ATG with AR is discussed because of its side effects (for example, the higher risk of infection). The efficacy in reducing antibody formation is reflected in the results of this study, with a reduction in AECAs in patients with an induction with ATG. We found a statistically noticeable difference in the post-transplant levels of AECA titre between patients who received ATG, basiliximab, and no antibody induction therapy groups, with a low titre in the ATG treated group.

Early AR was reduced by induction IS drugs such as basiliximab, rabbit anti-thymocyte globulin (r-ATG), and interleukin-2 receptor monoclonal antibody (IL-2 RA) [18]. Rabbit anti-thymocyte globulin is known to have a higher IS effect than basiliximab. However, it also has a higher risk of enabling infection [19].

Anti-thymocyte globulin is a good choice for preventing and treating both acute T-cell-mediated rejection (TCMR) and AMR because of its ability to deplete T and B cells, inhibit B and T cell cooperation as well

TABLE 2. Comparisons of anti-endothelial cell antibodies titres in different subgroups of kidney transplant recipients ($n = 46$)

Parameters (n)	Mean ±SD	p-value
Donor relation		
LURD (12)	3.3 ±3.9	0.003
LRD (34)	1.09 ±0.87	
Antibody induction therapy*		
ATG (30)	1.32 ±1.18	0.0372
Basiliximab (9)	2.5 ±4.37	0.78
No antibody induction (7)	2.01 ±2.27	
CNI used		
CsA (14)	2.21 ±3.71	0.67
Tacrolimus (32)	1.42 ±1.27	
CMV status		
CMV RT-PCR –ve (37)	1.77 ±2.53	0.495
CMV RT-PCR +ve (9)	1.22 ±0.64	
Previous PRAR episodes		0.134
No PRAR (16)	1.05 ±0.69	
Lepisode PRAR (7)	3.12 ±1.75	
≥ 2 episodes PRAR (23)	1.63 ±2.92	
Previous BPAR episodes		0.58
Yes (14)	1.27 ±0.8	
No BPAR (32)	1.83 ±2.69	
CGD		
Yes (7)	1.11 ±0.67	0.85
No (39)	1.76 ±2.46	

ATG – anti-thymocyte globulin, BPAR – biopsy-proven acute rejection, CGD – chronic graft dysfunction, CMV – cytomegalovirus, CNI – calcineurin inhibitor, CsA – cyclosporine, LRD – living related donor, LURD – living un-related donor, PRAR – presumed acute rejection, RT-PCR – real time-polymerase chain reaction

as leucocyte adhesion, and induce certain 'tolerogenic' regulatory T cell and dendritic cell populations [20].

This is in line with what was reported by Brennan *et al.* [21]. They found that r-ATG at a total dose of 7.5 mg/kg can reduce AR but increase infection rates in high-risk KTRs. Similarly, Lee *et al.* [22] discovered that in low-immunologic KTRs who received mycophenolic acid tacrolimus and steroid therapy, the incidence of BPAR was significantly reduced with ATG induction and with borderline change as compared with basiliximab induction therapy.

Bertacchi *et al.* stated that even if the potential of ATG in AMR prevention is acquired and its use on AMR is recommended in the KDIGO guidelines for adults, evidence of its employment in AMR is scarce, and the literature on the use of ATG as adjuvant therapy in children is almost non-existent. Most centres do not use ATG as a standard treatment and reserve its use for AMR with a significant vascular component or concomitant TCMD. The main concern is the risk of long-term development of malig-

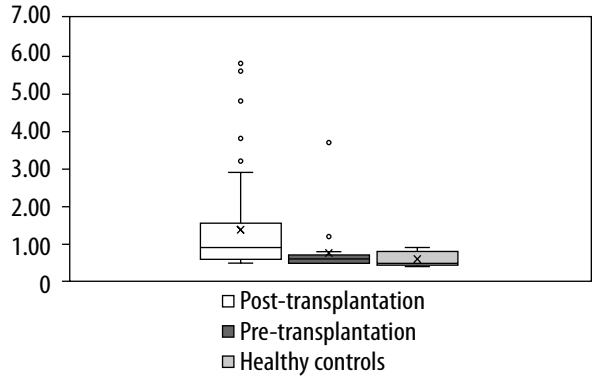

FIGURE 1. Anti-endothelial cell antibodies titres in healthy controls, pre-transplantation and post- transplantation

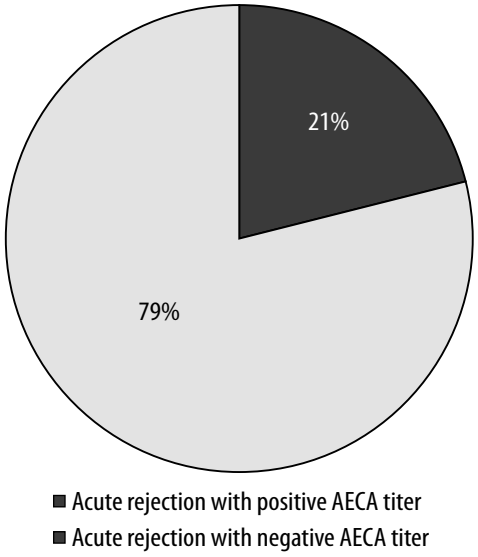

FIGURE 2. Frequency of anti-endothelial cell antibodies in biopsy--proven acute rejection patients

AECA – anti-endothelial cell antibodies
3/14 (21%) of patients with biopsyproven acute rejection had a positive anti-endothelial cell antibodies titer.

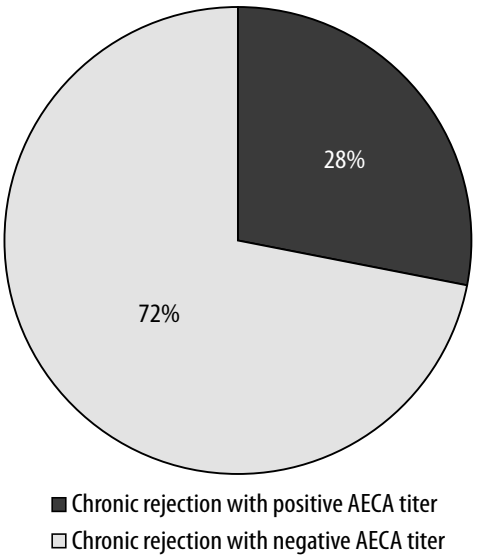

FIGURE 3. Frequency of chronic graft dysfunction patient with positive anti-endothelial cell antibodies titer

AECA – anti-endothelial cell antibodies.
7 (28%) patients of chronic graft dysfunction had a positive AECA titer.

nancy and severe infections; for this reason, a precautional use of repeated doses of ATG is needed [23].

In the present study, we found that post-transplant levels of AECA titres were significantly higher in KTRs with LURD as compared to those with LRD. We could assume that the better immunological matching of both HLA and non-HLA antigens increases tolerance, reducing the formation of de novo AECAS.

Fuller *et al.* [24], who compared outcomes of LRD vs. LURD kidney transplants, stated that the total number of HLA mismatches as well as the number of HLA-DR mismatches were higher in LURD, and that poorer HLA matching in LURD may reflect the higher one-year rejection rates despite the use of potent immunosuppression.

In our study, pre-transplantation, 2 patients (4.4%) were positive for the AECA titre (≥ 1.2 ng/ml), while 16 patients (34.8%) became positive post transplantation, with 14 developing AECA de novo after transplantation.

In the present study we found no significant difference in AECA titre between patients with AR (n = 14) and those without AR (n = 32). Also, we did not find a significant difference between the patients with CGD (n = 7) and patients without CGD (n = 39). Among patients with AR, we found that 3 patients out of 14 (21%) were positive for the AECA titre (≥ 1.2). Also, we found that 2 out of 7 patients who were diagnosed as having CGD (29%), were positive for the AECA titre (≥ 1.2). So, the occurrence of AR was not significantly different in the AECA-positive group as compared with the AECA-negative group, and the occurrence of CGD was not significantly different in the AECA-positive group as compared with the AECA-negative group

Our results are in accordance with the findings of Ismail AM *et al.* [25]. They found that the occurrence of AR was not significantly different in the AECA-positive group as compared with the AECA-negative group; however, the number of rejections and severity of rejection seemed to be higher among patients with AECAs.

Although AECA was found to be a very important non-HLA antibody in KTX, the incidence of AECA-positive AR is very low [5]. Despite the generation of autoantibodies after KTX, most of patients did not exhibit rejection or graft dysfunction. This finding suggests that the autoantibody pathogenicity is dependent upon other factors such as ligand expression, ischaemic injury, and/or the state of inflammation within the microenvironment of the allograft.

The expression of autoantigens on endothelium can vary greatly depending on their anatomical location, vessel type, and inflammatory milieu, which might make it difficult to attribute its clinical relevance to non-HLA autoantibodies [26].

Acute rejection is a significant risk factor for CGD and thus a powerful predictor of long-term graft survival in both cadaveric and living donor kidney transplants, despite its steady decline in recent decades [27]. Our results were contrary to Shin *et al.*, who found that AECA IgG titres were increased in KTRs with AR but decreased following anti-rejection therapy. Furthermore, they discovered that recipients with AR had greater pre-transplant AECA titres than those without AR episodes [8].

CONCLUSIONS

The anti-endothelial cell antibodies titre increased significantly after renal transplantation, but there was no significant correlation with AR. The efficacy in reducing antibody formation is reflected in our results, with a reduction in AECAs in patients with an induction with ATG. The anti-endothelial cell antibodies titre was influenced by donor relations; it was significantly higher in KTRs with LURD as compared to those with LRD.

A large-scale prospective study and an animal model may be more helpful for detecting AECA titre as a non-invasive marker for diagnosing AR, and a longer follow-up period is required for predicting CGD in KTRs.

DISCLOSURE

The authors declare no conflict of interest.

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