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Nuclear Factor of Activated T Cell-Regulated Cytokine Gene Expression for Tacrolimus Pharmacodynamics Assay among Live-Donor Kidney Transplant Recipients

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ABSTRACT

Background: The correlation between pharmacokinetic monitoring and clinical outcome is insufficient.

Objective: to evaluate the use of quantitative measurement of NFAT (nuclear factor of activated T-cells) regulated cytokine gene expression by real time PCR to profile kidney transplant recipients (KTR) on Tacrolimus (Tac) based immunosuppression in kidney transplant recpients.

Patients and Methods: In 45renal allograft recipients, samples were drawn at event of graft dysfunctionand stable condition. Tac concentrations were measured by immunoassay, while the expression of genes encoding NFATregulated cytokines [interleukin 2 (IL2), interferon gamma (IFNG), colony stimulating factor 2 (CSF2)] were determined by real-time polymerase chain reaction.

Results: In total, 45 kidney allograft recipients were enrolled. Patients in the first group showed a significant increase in NFAT-RE versus the second and thirdgroups (58.3 ± 15, 6.8 ± 1.7&30.9± 10respectively, P = 0.003). There was a significant stabilization of renal function in the normal group compared to theother group (P < 0.023).

Conclusions: Individualizing tacrolimus treatment with NFAT-RE as a translational immune monitoring tool demonstrated beneficial and safe, with the potential to lower rejection and infection risks while improving long-term renal allograft function.

Keywords: *kidney transplant, Gene expression, tacrolimus monitoring*

INTRODUCTION:

Immunosuppression is necessary for better transplant performance and patient survival after kidney transplantation ***(1).***

CNIs are critical-dose medications with a lot of pharmacokinetic heterogeneity between patients. Due to the small therapeutic window, therapeutic medication monitoring is required to balance effectiveness and safety. An individualised CNI treatment depending on sufficient monitoring techniques could be one step toward optimizing immunosuppression ***(2).***

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The cause of the lack of improvement in long-term graft function was unclear, and most late graft losses were related to chronic allograft nephropathy or death with functionning graft (causes of death include cardiovascular disease, infections and malignancies)***(3).***

So,thepurpose of this study to demonstrate that therapeutic monitoring of tacrolimus based on NFAT regulated gene expression in kidney transplants is a viable alternative to dose adjustment based on tacrolimus trough levels.

Subjects and methods:

A case control study was conducted at the urology and nephrology center,Mansoura University.

Ethical consideration: The study was approved by institutional review board (IRB) in Faculty of Medicine, Mansoura University (the Regional Committee for Medicaland Health Research Ethics). Prior to inclusion, written informed consent was obtained from all study participants and they were free to withdraw from the study.

Subjects:

The study population included stable KTR 18 years or older, who were maintained on triple immunosuppressive therapy with tacrolimus.

Three groups were included in this study:

First group: recipients with graft dysfunction resulting from biopsy proven acute rejection**(15 patients).Second group:**recipients with graft dysfunction resulting from suffering of infection **(15 patients).Third group:**recipients with normal renal function **(15 patients).**

All patients have tac trough level (FK0) within average level 4-8 ng/L

Tacrolimus monitoring was done via pharmacodynamics assay of the residual nuclear factor of activated T cell regulated cytokine gene expression (NFAT-RE) in all groups.

Immunosuppressive regimen:

All enrolled patients received an immunosuppressive regimen consisting of tacrolimus, mycophenolic acid, prednisone, and basiliximab induction. The initial oral daily dose of Tac was 0.1 mg/kg and administered twice daily.MPA was administered 1 g twice daily. Steroid treatment was initiated at day 0 with 500 mg of methylprednisolone, 250 mg on day 1, 125 mg on day 2, 80 mg on day 3, 60 mg on day 4, 40 mg on day 5, and progressively decreasing the dose to reach 20 mg on day 14, and from day 15 to day 29, 15 mg of prednisone. Patients received 10 mg of prednisone from day 30 to day 60 after transplantation. After the 60th day, 5 mg/d of prednisone was administered.

Pharmacodynamic monitoring:

Sample preparation

Residual NFAT-RGE was assessed in whole blood samples. Heparinized peripheral blood was stimulated with 1 mL of complete RPMI 1640 containing 100 ng/mL PMA and 5 μg/mL ionomycin (Sigma-Aldrich Corp. St. Louis, MO, USA) for 3 hours at 37°C. After red cell lysis with ACK buffer (0.15 M NH4CI, 1.0 mM KHCO3), leucocytes were lysed with 400 μL of MagNA-Pure lysis buffer supplemented with an additional 1% (w/v) of dithiothreitol (RAS, Mannheim, Germany), and the sample was frozen at −70 °C. After thawing, mRNA was isolated with the MagNA-Pure-LC device using the mRNA standard protocol for cells. The elution volume was set to 50 μL. One aliquot of 8.2 μL RNA was reverse transcribed in a thermocycler using avian myeloblastosis virus reverse transcriptase and oligo (dT) as a primer (First Strand cDNA synthesis kit; Roche, Mannheim,

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Germany) according to the manufacturer's protocol. After the termination of the cDNA synthesis, the reaction mixture was diluted to a final volume of 200 μL and stored at −20°C until polymerase chain reaction (PCR) analysis.

Quantitative analysis of gene expression

The NFAT-regulated genes IL-2, IFNγ and granular-macrophage colony-stimulating factor (GM-CSF) 33 were selected for this assay from previous studies.4, 5 Gene expression was quantified using real-time (RT)-PCR with the LightCycler. Target sequences were amplified using commercially available LightCycler Primer Sets (Search-LC, Heidelberg, Germany) with the LightCyclerFastStart DNA Sybr Green I Kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. The transcript concentration for the measured genes was calculated from a virtual standard curve, obtained by plotting a known input concentration of a plasmid to the PCR cycle number at which the detected fluorescence intensity reaches a fixed value. mRNA input was normalized by a constant expression value of 2 housekeeping genes (β-actin and peptidyl propyl isomerase B).

- Mean RGE was calculated as mean RGE from the 3 measured NFAT-regulated genes: IL-2, IFNγ and GM-CSF.

Pharmacokinetic monitoring:

Blood samples were collected in EDTA-K3 tubes and processed immediately. Tac concentrations in whole blood were measured by liquid chromatography/tandem mass spectrometry.

Statistical Analysis:

Demographic data and the results of the prospective analysis were collected in a unified database. Statistical analysis was performed using SPSS software (version 26.0; Chicago, IL, USA). Data were presented as median ± standard deviation or percentages.. P-value < 0.05 was considered statistically significant.

RESULTS:

The characteristics of 45 recipients are shown in **Table 1.** There were no statistically significant differences between the two groups with respect to sex, age at transplantation, donor recipient relationship, pre-transplant immunological workup of the recipients and donors or pre-transplant blood transfusion.

Pre-transplant clinical characteristics for all groupsare displayed in **table 2**. There was shows statistical significant difference regarding the pre-transplantation hypertension P = 0.029 with no statistical significant difference regarding pre-transplant hepatitis serology, CMV status or pre-transplantation hemodialysis

Table 3showed the list of the post-transplant complications. Post-transplant complications showed insignificant statistical difference regarding post-transplant complication (diabetes mellitus, GIT complications, malignancy, surgical complications) with significant statistical difference as regard the post-transplant hypertension (P= 0.013)

Regarding thetype and categories of acute rejection episodes in the three groups**table 4** demonstrates no statistical difference.

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NFAT-RGE showed high interindividual variability. The pharmacodynamic marker NFAT-RGE increased in the first group58.3 ± 15% versus 6.8 ± 1.7&30.9± 10 in the second and third groupsrespectively, (P = 0.005).

Table (5) shows statistical significant difference regarding serum creatinine at last follow up (P= 0.023) with no statistical significant difference regarding the patient condition at last follow up.

DISCUSSION:

The main target would be to find a good balance between great alloresponse suppression and maximum immunocompetence against viral infections, reactivation, and cancer caused by viruses.

NFAT-RE could be used to guide CNI therapy and serve as a promising biomarker for the risk of rejection following transplantation. NFAT-RE has previously been recommended as a potential biomarker for predicting infection concerns in renal and hepatic transplant recipients ***(4).***

Tacrolimus is a medicine having a limited therapeutic range, as well as well-known adverse effects such nephrotoxicity, infections, and cancer ***(5).*** For this purpose, we had introduced this accurate assay to measure the functional effects of tacrolimus..

Our hypothesis wasthat the pharmacodynamics value of tacrolimus via assay the nuclear factor of activated T cell–dependent cytokines in kidney transplant recipients correlate with the immune status of the patients, to avoid its negative impacts while maintaining its immunosuppressive properties,to test this hypothesis, we designeda case control study performed in unselectedpopulation with end stage renal disease who were transplanted, these recipients underwentstandardized monitoring of tac trough level together withthe residual NFAT-RE.

The three groups were well-matched in several baseline characteristics to rule out any preexisting risk factor of rejection or infection.All groups were comparable with no statistical significant regarding the recipient age to rule out any preexisting risk factor of rejection or infection.This is in agreement with ***(6),***who reported thatthe recipient's age is associated to rejection. Noncompliance with immunosuppression is common among paediatric, teen, and young adult kidney transplant recipients, increasing the risk of acute rejection ***(7).***

A greater risk of transplant rejection and delayed graft function has been associated to advanced donor age. In our investigation, the age of the donors was found to be comparable across all groups. When kidneys from older adult donors were evaluated at the extremes of donor age, no statistically significant incidence of and on DGF and rejection risks were identified ***(8).***

In our study, NFAT-RE was monitored in addition to standard Tac trough level. The interindividual heterogeneity of NFAT-RGE was great in Tac receiving patients. Patients with a high NFAT-RGE and little suppression of IL-2, IFN, and GM-CSF gene expression could be detected. The standard assessment technique in transplantation is the Tac C0 level.

Patients with frequent episodes of BPAR showedsignificantly higher values of RGE (58.3%). Acute rejection events occurred more often and earlier following transplantation if NFAT-RE high compared to patients with more inhibition of NFAT-RGE (P value = 0.003). Our results have shown that patients without rejection and infection had a NFAT-RE-mean value of (30.9%).

The observation that NFAT-RGE was progressively increased before the rejection event demonstrates its potential application as a non-invasive predictor of rejection risk.

We observed that COVID-19 and post-transplant DM occur more in the first group who had high NFAT-RE value. These complications may be because they are exposed to high doses of antirejection drugs. However, these results show no insignificant statistical difference.

The main finding of this study was a significant relationship between residual NFAT-regulated gene expression and the risk of rejection complications, whiletac trough blood concentrations did not correlate with infectious complications.

We found that highNFAT-RE values were associated with rejection episodes, independent of tacrolimus trough levels which affect significantly the kidney function (P= 0.023). Previous study in kidney transplantation has revealed NFAT-RE cutoffs of around 30% to differentiate between high and low risk of complications ***(9).*** Similarly, we observed here that rejection was rare below an NFAT-RGE value of 30%.

While this technique has been thoroughly assessed in cross-sectional, prospective observational, and interventional investigations of adult recievedCsA and paediatric patients with kidney, liver, heart, or lung transplants, there have been few studies in patientsrecievingtac.

Conclusion and Recommendation:

In our opinion, in respect to personalizing calcineurin treatment and reducing rejection, NFAT-RGE assessment can increase and augment the clinical value of monitoring tacrolimus.

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Table 1: Demographic Characteristics of the Patients:

	Group I (n=15)	**Group II** (n=15)	**Group III** (n=15)	***P*-value**
Recipients' age (years) (mean ± SD)	28± 11.8	30 ± 11.2	28 ± 10.9	0.494
Donors' age (years) (mean ± SD)	41± 7	39 ± 5	39 ± 7	0.874
Donor-recipient relationship:				
Genetically related (n, %)	12 (80%)	11(73.34%)	12(80%)	0.089
Emotionally related (n, %)	3(20%)	4(26.66%)	3(20%)	
Pre-transplant blood transfusion:				
Pre-transplant blood transfusion (n, %)	6 (40%)	5(33.3%)	6 (40%)	0.888
Source of blood transfusion (n, %) Donor specific: Unknown:	3(20%) 3(20%)	3(20%) 2(13.3%)	4(26.67%) 2 (13.33%	0.988
Number of blood transfusion No: 1-3: ≥4:	9 (60%) 3 (20%) 3(20%)	10(66.67%) 3 (20%) 2 (13.33 %)	9 (60%) 2(13.33%) 4(26.67%)	0.316
Immunological workup				
Blood grouping :(n, %)				
Same	12 (80%)	11(73.34%)	9 (60%)	0.295
Different matched	3 (20%)	4(26.67%)	6 (40%)	

Table (2): Pre-transplant clinical characteristics for all groups:

	Group I (n=15)	**Group II** (n=15)	**Group III** (n=15)	**P-value**
Pretransplant hypertension	6 (40%)	4(26.67%)	9 (60%)	**0.029**
Pretransplant dialysis	14 (93.3%)	13 (86.7%)	15 (100%)	0.092
Pre-transplant hepatitis serology status: Hepatitis C positive PCR (n, %) Hepatitis C antibody positive but negative PCR (n, %) Hepatitis B positive (n, %) HIVpositive antigen\antibody (n, %)	 1 (6.67%) 1 (6.67%) 1 (6.67%) 0 (0%)	 4(26.67%) (13.33 %) 1 (6.67%) 0 (0%)	 4(26.67%) 1(6.67%) 0 (0%) 0 (0%)	 0.298 0.970 0.179 1

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CMV IgG positive	14 (93.3%)	10 (66.7%)	12 (80%)	0.076

Table (3)*Post-transplant complication in all groups*:

	Group I (n=15)	**Group II** (n=15)	**Group III** (n=15)	***P*-value**
Post-transplant hypertension (n, %)	8 (53.33%)	4(26.67%)	9(60%)	**0.013**
Post-transplant diabetes mellitus (n, %)	3 (20%)	1(6.67%)	2 (13.33%)	0.648
COVID-19 (n, %)	2 (13.33%)	1(6.67%)	1(6.67%)	0.489
GIT complications (n, %)	0 (0%)	0 (0%)	1(6.67%)	0.409
Post-transplant malignancy (n, %)	0 (0%)	0 (0%)	0 (0%)	1
Posttransplant surgical complications:				
• Lymphocele (n, %)	0 (0%)	0 (0%)	0 (0%)	1
• Hematoma (n, %)	0 (0%)	1(6.67%)	1(6.67%)	0.409
• Urine leak (n, %)	0 (0%)	0 (0%)	0 (0%)	1
• Graft vascular complications (n, %)	0 (0%)	1(6.67%)	1(6.67%)	0.409

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Table (4): graft biopsies result of all groups:

	Group I (n=15)	**Group II** (n=15)	**Group III** (n=15)	***P*-value**
First graft biopsy result (n, %)				
Normal	0 (0%)	1(6.67%)	0 (0%)	0.28
Borderline rejection	7(46.67%)	7 (46.67%)	3 (20%)	
Acute T-cell mediated rejection (grade 1A)	2 (6.67%)	0 (0%)	1(6.67%)	
Acute T-cell mediated rejection (grade 1B)	1(6.67%)	0 (0%)	0 (0%)	
Acute T-cell mediated rejection (grade 2A)	0 (0%)	1(6.67%)	0 (0%)	
Acute antibody mediated rejection	7(46.67%)	2 (13.33%)	1(6.67%)	
Acute tubular necrosis (C4D:-ve) (DSA: -ve)	2(13.33%)	1(6.67%)	1(6.67%)	
Interstitial fibrosis and tubular atrophy	2(13.33%)	1(6.67%)	1(6.67%)	
Acute pyelonephritis	0 (0%)	2(13.33%)		
Second graft biopsy result (n, %)				
Normal	1(6.67%)	0 (0%)	0 (0%)	0.74
Borderline rejection	7(46.67%)	3 (20%)	3 (20%)	
Acute tubular necrosis (C4D:-ve) (DSA: -ve)	2(13.33%)	1(6.67%)	2(13.33%)	
Interstitial fibrosis and tubular atrophy	2(13.33%)	2 (13.33%)	2(13.33%)	
Third graft biopsy result (n, %)				
Borderline rejection	4 (26.67%)	1(6.67%)	1(6.67%)	0.98
Acute T-cell mediated rejection (grade 1A)	1(6.67%)	0 (0%)	0 (0%)	
Acute tubular necrosis (C4D:-ve) (DSA: -ve)	1(6.67%)	0 (0%)	0 (0%)	

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Table (5): Serum creatinine and Condition of last follow up of all groups:

	Group I (n=15)	**Group II** (n=15)	**Group III** (n=15)	***P*-value**
	Serum creatinine at last follow-up:			
Serum creatinine (mg/dL) (mean ± SE)	1.58± 0.98	2.2 ± 2.18	1.08 ± 0.20	**0.023**
	Patient and graft condition of last follow up: (n, %)			
• **Living with functioning graft**	13(86.66%)	15(100%)	14 (93.33%)	
• **Died with functioning graft**	1(6.67%)	0(0%)	1(6.67%)	
• **Living on dialysis**	1(6.67%)	0 (0%)	0(0%)	
• **Died with graft failure**	0 (0%)	0 (0%)	0 (0%)	0.388