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RESEARCH ARTICLE

Assessment of Cytokines as Potential Biomarkers to Predict Allograft Rejection in Kidney Transplant Recipients

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Abstract

Objectives: Allograft rejection is the most common event that occurs post-transplant and the current diagnostic tools like the measurement of serum creatinine or core kidney biopsy could not serve as a predictive tool and have certain drawbacks. Thus, our target was to find potential, non-invasive, and predictive biomarkers which could identify recipients at the risk of post-transplant allograft rejection. **Methods:** It is a prospective longitudinal cohort study comprising of 40 live-related kidney transplant recipients whose peripheral blood was collected at three time points viz pre, one and three-months post-transplant to assess the plasma cytokine levels of FOXP3, IL-6, IL-17 and TGF-β using Enzyme linked immunosorbent assay (ELISA). The patients were clinically followed for period of two years to determine their post-transplant outcome. **Findings:** 13 of our recipients had biopsy proven allograft rejection at the end of follow-up period. The pre-transplant concentration of FOXP3 were less in the rejection group whereas the concentration of IL-6, IL-17 and TGF- β were more in the rejection group ($p<0.05$). The classification accuracy of the markers FOXP3, TGF- β, IL-17 and IL-6 were assessed and they had an AUC of 1.0, 1.0, 0.96 and 0.84 respectively with high sensitivity, specificity, and statistical significance. **Novelty:** Pre-transplant levels of plasma cytokines were able to predict recipients at the risk of allograft rejection. Thus, our study confirms the predictive power of our cytokine biomarkers FOXP3, IL-6, IL-17 and TGF-β and further paves way for personalized immunosuppressive regimen.

Keywords: Predictive Biomarkers; Allograft Rejection; FOXP3; IL6; IL17; TGFβ

1 Introduction

Kidney transplantation has become the best treatment of choice for people suffering from End-Stage Kidney Disease (ESKD), as it provides improved survival and better quality of life. Over the past few decades major technological improvements in surgical procedures, ancillary care and immunosuppressive regimen have significantly resulted in better short-term graft function but long-term graft function and survival remains as a major issue in kidney transplantation. One of the important factors that attributes to graft dysfunction is allograft rejection, which if diagnosed and monitored earlier could help in better sustenance of the allograft. Currently, allograft function is assessed through the serological measurements of creatinine and Donor- Specific Antibodies (DSA), urine analysis and histological evaluation which could miss early events and poses significant risks [(1)]. Hence the advent of omics technology has set directions using biomarkers to efficiently diagnose allograft rejection by overcoming the challenges and limitations of the conventional tool [(2)]. Our current study is one such approach under proteomics in which we focussed on the use of cytokines as predictive biomarkers to identify recipients at the risk of post-transplant allograft rejection. As cytokines are known to mediate the T- and B-cell activity, they have significant role in alloimmune response [(3)]. The cytokines FOXP3, IL-6, IL-17 and TGF-β are important mediators of inflammation and regulation of immune response and hence these were assessed in our study.

FOXP3 is a regulatory cytokine that acts as a key transcriptional factor in characterizing the lineage of thymically derived T-regulatory cells which plays an important role in immune homeostasis and suppression of inflammatory response to self-antigens [(4)]. IL-6 is a multifunctional pleiotropic cytokine that is involved in the regulation of immune responses, acute phase responses, haematopoiesis and inflammation thus having a very close association with allograft rejection [(5)]. IL-17 is a pro-inflammatory cytokine that exerts immunity against extracellular pathogens but a dysregulation of it leads to alloimmune response leading to graft rejection [(6)]. TGF-β is a multifunctional cytokine that is well documented for its immune suppression activity on various immune cells like T cells, B cells, macrophages and other cells and it also acts with other inhibitory molecules to maintain immune tolerance in peripheral tissues thus regulating immune response in kidney transplantation [(7)]. Hence all these important cytokines were assessed in our current work to ascertain their predictive ability for better management of the allograft.

2 Methodology

2.1 Study cohort

It is a prospective longitudinal cohort study from a single-centre wherein 40 kidney transplant recipients were recruited from 2018-2020 after obtaining Institute's human ethical committee approval (JIP/IEC/2017/0115 dated 27th May 2017). All our study participants consented to participate in this research study by giving a written informed consent. Recipients with deceased donor transplant and recipients who had malignancies were excluded from this study. Peripheral blood samples were collected from the participants at three time points namely pre-transplant (before the induction therapy) and at first- and third-month post-transplant. Two of our study participants could not cooperate with post-transplant sample collection and hence was excluded from the analysis. All of our study participants had a pre-transplant induction therapy of recombinant anti-thymocyte globin (R-ATG) 1.5 mg/kg body weight and 20 μg of Basiliximab followed by post-transplant maintenance therapy with Tacrolimus 0.1 mg/kg body weight, 1g of Mycophenolate mofetil and 20 mg prednisolone.

2.2 Estimation of Protein concentration

Two ml of Peripheral blood (n=114) was collected from the recipients before transplant and at first- and third-month post-transplant. Plasma was separated from the blood by centrifuging (Eppendorf 5430 R) at 3000 x g for 15 minutes and stored at -80^{o}C until processing. Plasma was thawed and used for the quantification of proteins through commercially available kits from Bioassay Laboratory bearing the catalogue numbers E0692Hu (FOXP3), E0090Hu (IL-6), E0142Hu (IL-17) and E3051Hu (TGF-β). 40μl of plasma was used for each protein assay using Sandwich ELISA technique and the OD value of each well was read in the microplate reader (iMark^{TM} from Bio Rad) set at 450nm as per the manufacturer's instructions. The concentration of the proteins was estimated using a standard curve generated using the standards provided with the kits.

2.3 Clinical data collection

All the study participants were clinically followed up for two years from the date of transplantation to measure the graft outcome that is estimated through serum creatinine. eGFR was calculated using CKD-EPI creatinine 2009 equation using National Kidney foundation application software. Other biochemical data like urea, albumin, and bilirubin as well as hematological

parameters like RBC, WBC, hemoglobin, neutrophils, eosinophils, basophils, lymphocytes, monocytes, and platelets were collected from hospital information system (HIS) which is a digital platform.

2.4 Statistical analysis

The data were statistically analysed through SPSS version 19.0 (Chicago: SPSS Inc.). The distribution of data was checked through Shapiro Wilk normality test and normally distributed data were shown as mean ± standard deviation whereas non-normally distributed data were shown as median and interquartile range. The pre-and post-transplant comparisons of variables were made through Wilcox signed rank test whereas Mann Whitney U test were used to compare between rejection and non-rejection groups. Post-hoc analysis with Wilcoxon signed rank test was conducted with a Bonferroni correction to estimate the time where the actual significant changes occurred. Logistic regression was used to find the best parameters to predict rejection and Receiver Operating Characteristic (ROC) curve was used to find the predictive potential of the parameters.

3 Results and Discussion

3.1 Patient characteristics

The study population consists of 40 kidney transplant recipients followed for a minimum duration of two years (3.39 ± 0.50 years) from the date of transplant. Of these 40 recipients, 13 of the recipients had allograft rejection at the end of our follow-up period. Out of these 13 recipients who experienced allograft rejection, 11 of them had only Antibody mediated rejection (ABMR), whereas the remaining two recipients with ABMR also had T-cell and borderline rejection during our follow-up period. Previous studies at various cohorts have reported an incidence of 1.1% - 21.5% of acute ABMR and 3% - 12% of incidence in the first-year post-transplant[8], but in our cohort the incidence of ABMR was on a higher side of 34.21% within a year of transplant. The characteristic difference between the rejection and non-rejection groups (Table 1) shows that cold ischemia time and donor age was more in the rejection group which could also be the reasons for higher incidence of ABMR. Our finding is similar to the report by Cruz et al. where a cold ischemia time of 1.9 hours and donor age of 39 years, negatively affected the allograft survival in a median time of 4.1 years[9]. Through our routine clinical follow-up, we also observed characteristic differences between rejection and non-rejection groups (Supplementary table 1) of which the pre-transplant levels of bilirubin and haemoglobin was significantly less in the rejection group. A study by Lee et al. also observed a similar finding in which it was exhibited that patients with lower serum bilirubin were at a higher risk of graft rejection[10] which further supports our findings.

Table 1. Characteristic difference between allograft rejection and non-rejection groups.The values are represented as mean ± SD or as median (Q1, Q3)

Characteristics	Allograft rejection (n=13)	Non allograft rejection (n=25)
Recipient's age (mean ± SD)	38.23 ± 9.85	35.28 ± 8.78
Recipient's sex (%)	Male 100%	Male 92%
	Female 0%	Female 8%
BMI Kg/m2 [median (Q1, Q3)]	20.10 (15.6, 21.46)	20.5 (18.4, 24)
Cold ischemia time, minutes [median (Q1, Q3)]	120 (67, 130)	86 (72.25, 102)
Warm Ischemia time, minutes [median (Q1, Q3)]	5.7 (5, 6.32)	5.08 (4.16, 8.07)
Donor's age (mean ± SD)	44.31 ± 12.00	40.30 ± 11.73
Donor's sex (%)	Male 38.46%	Male 16%
	Female 61.54%	Female 84%

3.2 Concentration of cytokines before and after transplant

The pre- and post-transplant levels of the cytokines were assessed (Table 2) and depicted in Figure 1. We found that the pre-transplant levels of FOXP3, IL-6 and TGF-β were high in comparison to the post-transplant levels, whereas IL-17 was less before transplant than after transplant. Post-hoc analysis (supplementary table 2) revealed that there was a statistically significant difference between the pre- and post-transplant levels of all the cytokines except IL-17 which was not statistically significant. A similar study by Ayato et al. have also assessed the plasma levels of 40 cytokines before and one year after transplant in 15 living donor kidney transplant recipients who did not have evidence of pathological and clinical rejection and recorded that

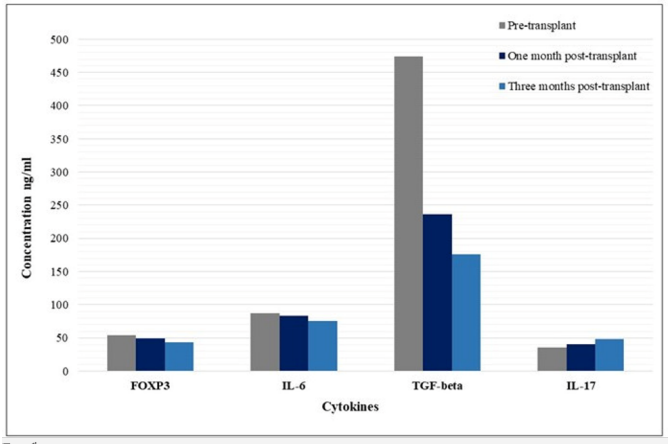

Fig 1. Plasma concentration of the cytokines before and after kidney transplantation

the levels of 22 cytokines remained stable over time whereas the levels of 18 cytokines decreased after transplant[11]. In our setting we found that there was a significant statistical difference in the levels of cytokines FOXP3, IL-6 and TGF-β before and after transplant but in IL-17 the difference in levels were not significant from a statistical point of view. From this we could understand that cytokines respond well to transplantation further pondering on its predictive or diagnostic utility.

Table 2. Pre- and Post-transplant levels of cytokines expressed as mean ± standard deviation

Cytokines	Pre-transplant	One - month post-transplant	Three - month post-transplant
FOXP3	54.36 ± 9.34	48.90 ± 27.88	43.44 ± 21.20
IL-6	87.75 ± 3.67	83.14 ± 33.58	75.19 ± 26.18
TGF-β	474.22 ± 525.14	236.31 ± 144.74	175.87 ± 65.50
IL-17	35.56 ± 4.10	40.16 ± 25.80	48.16 ± 32.49

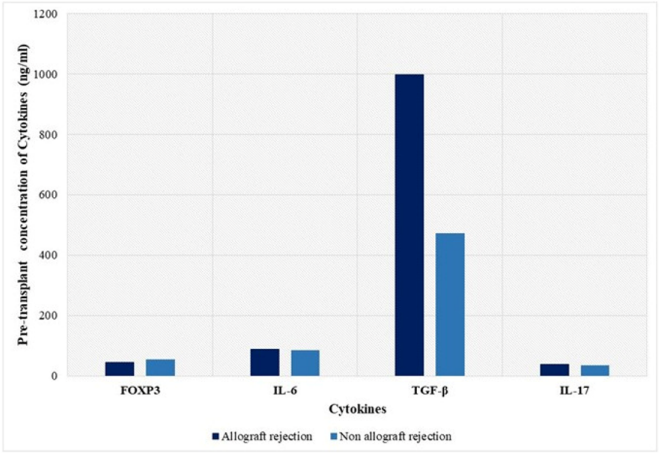

Fig 2. Pre transplant concentration of the cytokines between allograft rejection and non allograft rejection groups

3.3 Concentration of cytokines between allograft rejection and non-rejection groups

The concentration of cytokines FOXP3, IL-6, TGF-β and IL-17 was assessed between the rejection and non-rejection groups (Supplementary Figure 1). We found that the rejection group had a lower concentration of FOXP3 at all three time points which is on par with the recent reports by Shahroodi et al.[12] and Torabijahromi et al.[13] where a lower mRNA expression of FOXP3

was observed in recipients with acute rejection than those who had excellent long term graft function. We have also noted that the concentration of TGF- β to be high in the rejection group than the non-rejection group. This finding is supported by similar study by Salehi et al.[14] in which they found an increased mRNA expression of TGF- β in patients with ABMR but they could not find any difference at the level of protein, whereas we have found a significant increase at the protein level. In our study we have found an increased concentration of cytokines IL-6 in the rejection group before transplant, the levels of which decreased after transplant. This is in support to the randomized clinical trial conducted by a group of researchers in Austria where a blockage of IL-6 using Clazakizumab decreased late antibody mediated rejection in kidney transplant rejection[15] further supporting our result where we found a decrease in concentration after transplant which may be due to the immunosuppressive regimen that could modulate the cytokine milieu. A higher concentration of IL-17 in the rejection group was also documented in our study which is in concordance with an animal model study where an increased expression of IL-17 was associated with acute rejection[16]. All our findings were found to be statistically significant ($p<0.05$)

3.4 Association of pre-transplant cytokine levels with allograft rejection

Based on our findings in the difference of cytokines levels between rejection and non-rejection group, we could understand that the pre-transplant assessment between the groups would be more sensible to stratify recipients at the risk of post-transplant rejection, and hence the levels of cytokines FOXP3, IL-6, TGF-β and IL-17 before transplant was analysed. We found that the levels of FOXP3 was low, whereas the levels of IL-6, TGF-β and IL-17 were high in the rejection group in comparison to the non-rejection group (Figure 2). The results are tabulated in Table 3 and the differences were found to be statistically significant.

Table 3. Pre-transplant assessment of cytokines and its ROC analysis

Cytokines	Allograft rejection	Non allograft rejection	AUC	Cut-off	Sensitivity	Specificity	p-value
FOXP3	45.31	55.31	1.0	49.51	100	100	<0.01
IL-6	89.22	85.76	0.84	88	80	70	<0.01
TGF-β	997.62	472.70	1.0	735	100	100	<0.01
IL-17	38.72	34.40	0.96	36.4	90	80	<0.01

3.5 Utility of pre-transplant serum proteins to predict allograft rejection

We further investigated the utility of the pre-transplant levels of the cytokines FOXP3, IL-6, TGF-β and IL-17 by constructing a ROC curve defining the sensitivity, specificity, and area under the curve (AUC), individually for each of these cytokines (Figure 3). We found that the pre-transplant levels of FOXP3 and TGF-β had a higher AUC of 1.0 with 100% sensitivity and specificity and a cut-off of 49.51 ng/ml and 735 ng/ml respectively to identify recipients at the risk of post-transplant rejection. The cytokines IL-17 and IL-6 were also good predictors of allograft rejection with an AUC of 0.96 (90% sensitivity and 80% specificity, cut-off of 36.4 ng/ml) and 0.84 (80% sensitivity and 70% specificity, cut-off of 88 ng/ml) respectively.

Even though our study has a minimum sample size to conclude our findings, we found significant conclusions which if implemented in a larger cohort could serve as a better tool aiding in the diagnosis in a cost-effective and timely manner.

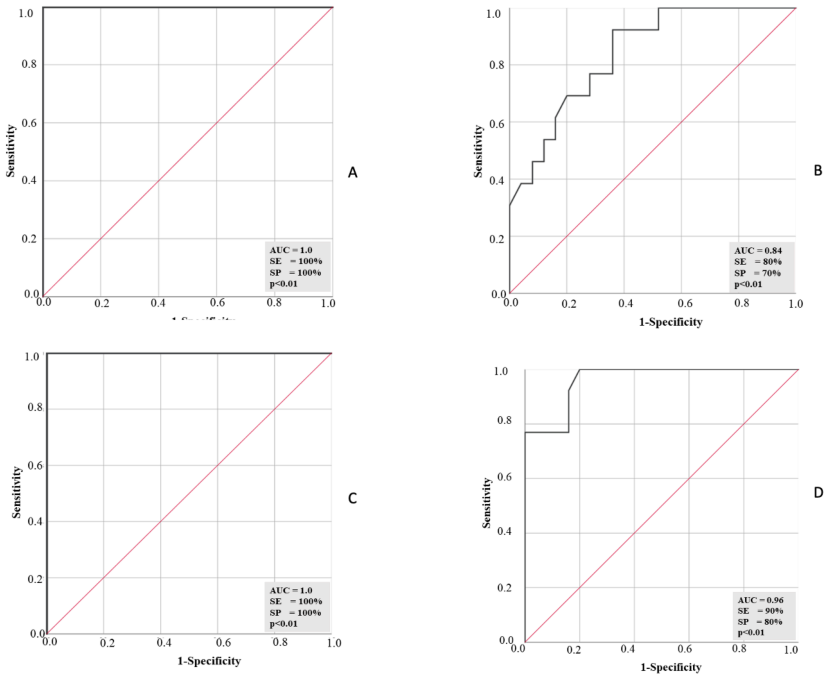

Fig 3. ROC curve to assess the potential of the cytokines to predict allograft rejection. a:ROC curve for FOXP3, b:ROC curve for IL-6, c:ROC curve for TGF-β, d:ROC curve for IL-17

4 Conclusion

Our current work has assessed the utility of plasma cytokines as a predictive biomarker to identify recipients at the risk of post-transplant allograft rejection. We have found that recipients with low pre-transplant levels of cytokine FOXP3 and high pre-transplant levels of cytokine FOXP3 and high levels of cytokines IL-6, TGF-β and IL-17 are more prone to post-transplant rejection and its diagnostic utility assessed through ROC curve has further strengthened the findings exhibiting an AUC of above 0.8 with high sensitivity and specificity. Thus, through this study, we were able to stratify recipients at the risk of allograft stratify recipients at the risk of allograft rejection which could further pave way to tailor the immune suppressive regimen for better management of the allograft thereby enhancing long-term graft function.

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