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Evaluation of PD-1 and TIM-3 Expression Levels of CD8+ T Cells in Renal Transplant Patients

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Abstract

Objective

After kidney transplantation, CD8+ T cells can infiltrate the kidney and cause necrosis, tubulitis, and even transplant rejection. For this reason, control of the T cell response is very important, and T cell immunoglobulin and mucin domain 3 (TIM-3) and programmed death 1 (PD-1) molecules play a role in regulating the T cell response. It is thought that the levels of TIM-3 and PD-1 expressions may be guiding in determining the clinical course after transplantation. This study aimed to determine the relationship between the mRNA levels of PD-1 and TIM-3 genes in peripheral blood samples taken from kidney transplant patients and the clinical conditions of the patients.

Material and Method

60 peripheral blood samples were collected from 30 kidney transplant patients, both pre-transplantation (pre-tx) and post-transplantation (post-tx). CD8+ T cells were separated from other lymphocytes by magnetic cell separation system (MACS) and their purity was determined by flow cytometry. Then, RNA was isolated and after cDNA conversion, the expressions of PD-1 and TIM-3 genes were determined by real-time polymerase chain reaction.

Results

While it was determined that the TIM-3 gene expression level increased in patients with acute tubular necrosis, antibody-mediated rejection and cell-mediated rejection findings ($p<0.001$), no correlation was found between PD-1 expression levels and the clinical findings of the patients.

Conclusion

It is thought that comparing TIM-3 mRNA levels before and after kidney transplantation may be a useful tool in evaluating the clinical status of patients.

Keywords: Immune checkpoint regulators, PD-1, renal transplantation, TIM-3.

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Introduction

Kidney transplantation is the best choice for patients with chronic kidney failure, which affects 13.4% of people worldwide, leading to millions of deaths. However, approximately 30% of patients undergo rejection episodes during the first year after transplantation (1,2). Rejection of the kidney is caused by a complex series of events that involves both adaptive and innate immune system responses, with T cells at the center of this process (3).

CD8+ T cells contribute to tissue necrosis and tubulitis in solid organ transplantation, despite the focus of most studies being on the CD4+ T cell subgroup (4). Moreover, CD8+ T cells secrete perforin and granzymes that can cause transplant rejection (5). It was shown that in acute and chronic allograft dysfunction, the number of CD8+ T cells increase in peripheral blood (6). It can be thought that immunomodulation of the CD8+ T cell population is important in long-term allograft survival and may bring a new perspective to increasing graft survival by targeting specific metabolic processes (7).

After maturation of T cells in thymus, naive T cells start to circulate between peripheral blood and lymph nodes to encounter MHC-peptide complex, which starts an immune response cascade (8). This MHC-peptide complexes are recognized by T cell receptors (TCR) and coreceptors, CD4 and CD8, stabilizes this synapsis between TCR-MHC-peptide complex. Then costimulatory CD28 and its ligand (CD80/86) interact, and cytokines start to secrete as a result of T cell activation (9). At the end of the T cell response these effector T cells started to express negative costimulatory molecules as CTLA-4, PD-1, TIM-3, LAG-3…etc. (10). PD-1 receptor can be expressed by both CD4+ and CD8+ T cells and has two ligand called PDL-1 and PDL-2 while the primary ligand is PDL-1 (11). It was shown that in different type of T cells, PD-1/PDL-1 interaction leads to the different results (12). For example, in follicular T helper cells (Tfh) binding of PD-1 to PDL-1 causes the localization of Tfh in germinal center. Otherwise, in CD8+ T cells PD-1 shortens the interaction between CD8+T cells and target cells which leads to the saving of the target cells from CD8+ T cell attack (13).

T cell immunoglobulin and mucin domain containing protein-3 (TIM-3) is a transmembrane protein that is encoded by HAVCR2 gene located on chromosome 5 (14). The best-known ligand of TIM-3 is Galectin-9 that is a S type lectin and expressed by several cells as T cells, B cells, mast cells and nonimmune cells. TIM-3/Galectin-9 interaction plays role on negative T cell costimulatory pathway (15). Recent studies have shown that TIM-3 can modulate the alloimmune response in allograft rejection. In heart transplants, it has been stated that both PD-1 and TIM-3 expression are increased in tolerant recipients compared to people who have undergone organ rejection. The idea that these molecules are very important in transplant tolerance remains important (16).

The purpose of this study was to evaluate possible changes in PD-1 and TIM-3 expression in CD8+T cells of patients before and after kidney transplantation.

Material and Method

Patient Group

Ethical approval for this study was obtained from Health Science University Tepecik Training and Research Hospital Noninvasive Ethics Committee with the number 2022/05-31. 60 blood samples were collected from renal transplanted patients both before the operation time and between 1 and 3 months after transplantation. 30 ml heparinized blood samples were diluted by PBS (Merck, Germany) (v/v) and added carefully on 5 ml lymphocyte separating solution (Capricorn, Germany). Tubes were centrifuged at 2500 rpm for 20 minutes. Then, the lymphocyte layer was collected to a new tube and washed two times with PBS by centrifugating at 1800 and 1500 rpm for 5 min., respectively. The lymphocyte pellet was suspended in 2 ml PBS and counted on Thoma chamber and then CD8+ T cells were separated by using magnetic separation system (MACS, Miltenyi Biotech, USA).

CD8+ T Cell Separation

$1x10^7$ cells were loaded onto columns of magnetic cell separation system (MACS, Miltenyi Biotech). CD8+ T cells were separated according to the manufacturers' protocol. Purity of isolated CD8+ T cells were analyzed by flow cytometer according to the procedure explained in our preliminary study (17).

Isolation and Quantification of Total RNA and Converted CDNA

RNAs were isolated from CD8+ T cell pellets by using GeneJET RNA Purification Kit (ThermoScientific, USA) and cDNA was converted by "VitaScript™ FirstStrand cDNA Synthesis Kit" (Procomcure, Austria) according to the instruction manual.

Real Time PCR

PD-1 primers were designed according to NCBI reference sequence: NC_000002.12 and NC_000005.10 while TIM-3 and β -actin primers were

Table 1 Primer sequences

Gene	Primer Sequences	Amplicon length	References
PD-1	F: 5'-TTCCACATGAGCGTGGTCAG-3' R: 5'-CCGCAGGCTCTCTTTGATCT-3'	102 bp	NCBI, Primer blast program
TIM-3	F: 5'-CCTATCTGCCCTGCTTCTAC-3' R: 5'-CTGGTGGTAAGCATCCTTGG-3'	364 bp	(18)
β-Actin	F: 5'-CTTCCTGGGCATGGAGTCCTG-3' R: 5'-GGAGCAATGATCTTGATCTTC-3'	21 bp	(19)

designed according to the literature showed in Table 1. NCBI Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast), IDT Oligo analyzer (https://www.idtdna.com), UCSC In-Silico PCR (https://genome.ucsc.edu) programs were used to control all three primer sequences. β-actin gene was used for the internal control (NM 001101.3). Primer sequences and amplicon lengths were shown in Table 1.

Amplification was performed by PicoReal Real Time PCR System (ThermoScientific, USA) with a polymerase chain reaction (PCR) protocol: 95°C for 10 minutes, followed by 40 cycles of 95°C for 45 seconds, 58°C 45 seconds, 72°C 45 seconds and final extension 72°C 10 minutes. PCR reactions were performed in triplicate by using RealQ Plus 2x Master Mix Green (Ampliqon, Denmark), according to the protocol of 6.25 µl SYBR Green, 0.5 µl forward and reverse primers, 2 µl cDNA and 2.75 µl pure water to a final volume of 12 µl. Relative gene expression analysis were performed according to the 2−ΔΔCT method. The results were normalized with β-actin gene expression levels. 60-95°C temperature range was used for melting curve analysis.

Statistics

IBM SPSS Statistics 21 program was used for the statistical analysis. Student t test was used to compare continuous variables, while chi-square test was used for categoric variables. Box-plots allowed to visualize gene quantifications. Spearman correlation test was used to calculate correlations. Results were expressed as mean value ± standard deviation. $p<.05$ was considered as statistically significant.

Results

Study Group

Demographic characteristics of the patients are summarized in Table 2. Six recipients were diagnosed for C4d+, CD3+ and acute tubular necrosis. Among these patients one underwent nephrectomy while the other patients were cured with intravenous immunoglobulin (IVIG) and became stable between 1 to 3 months after transplantation.

mRNA Levels of PD-1 and TIM-3 Genes

The mRNA expression levels of PD-1 and TIM-3 before and after transplantation were evaluated and calculated by using β-actin expression levels to

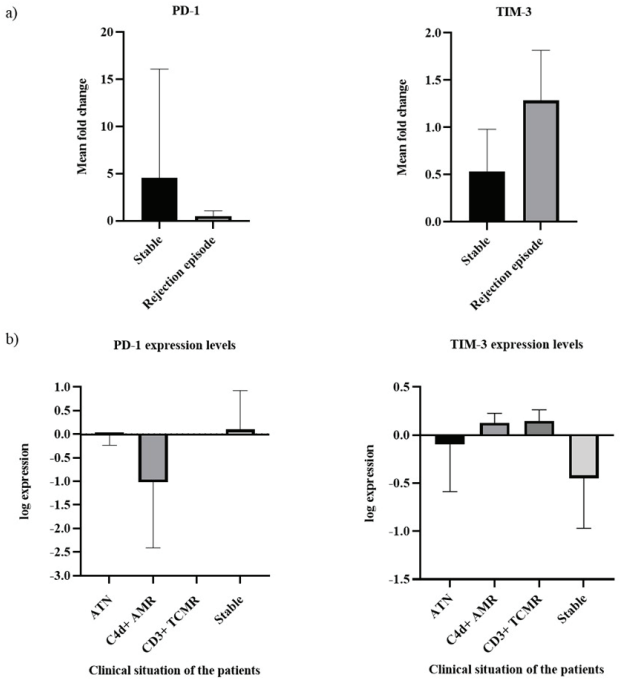

Figure 1

a) TIM-3 and PD-1 mean fold change according to the clinical status of the patients at the time of specimen collection. b) log expression values of TIM-3 and PD-1 according to the rejection types. ATN, acute tubular necrosis; AMR, antibody mediated rejection; TCMR, T cell mediated rejection.

Table 2 Patient demographics

	ATN*	C4d+	CD3+	Stable	p
No. of patients	2	2	2	24	>.05
Patients age (year, mean ± SD)	46,5±7,8	43±15,6	33±8,5	45,0±12,5	>.05
Gender (F/M)	0/2	2/0	1/1	7/17	>.05
GFR pre-Tx	6,4±0,8	6,7±1,5	13,4±10,7	8,5±3,1	>.05
GFR post-Tx**	61,5±23,3	49,2±8,8	35,5±40,3	56,1±12,7	>.05
Creatinin pre-Tx	3,5±0,6	7±2,3	4,3±2	6,9±3	>.05
Creatinin post Tx**	1 ±0,02	1,6±0,3	4,1±3,5	1,4±0,3	>.05
Cause of CKD (HT/DM/Others)	0/0/2	1/1/0	2/0/0	2/13/7	>.05
Donor (Cadaveric/Alive)	2/0	0/2	1/1	10/14	>.05
Donor age (year, mean ± SD)	34±7	47±11,3	55,5±7,7	43,8±12,2	>.05
HLA mismatches (A,B,DR,DQ; mean± SD)	1,5 ±0,5	2±1	2,5±1,5	2,4±1,4	>.05

ATN, Acute tubular necrosis; Stable, stable renal function; SD, standard deviation; F, female, M, male; GFR, glomerular filtration rate; pre-tx, before transplantation; post-tx, after transplantation; CKD, chronic kidney disease; HT, hypertension; DM, diabetes mellitus. *ATN was diagnosed at 0 time after transplantation by biopsy. **Post-tx GFR and creatinine levels were obtained 1 month after transplantation.

Table 3 Correlation between gene expression levels and demographic and clinical properties of patients

		P.gend.	P.age	D.gend.	D.age	PreTx-Cre	Post-Tx-Cre	PreTx-GFR	PostTx-GFR	HLA-MM	Rej.
PD-1	R	0,130	-0,064	-0,225	-0,072	0,026	-0,006	0,091	-0,150	0,11	-,159
	p	0,246	0,369	0,116	0,353	0,445	0,488	0,317	0,215	0,281	,400
TIM-3	R	-,404*	0,268	,393*	-0,015	-,351*	0,270	0,091	-0,145	-0,107	,558**
	p	0,014	0,076	0,016	0,469	0,029	0,075	0,316	0,222	0,288	,001

*Correlation is significant at the 0.05 level (1-tailed).
**Correlation is significant at the 0.01 level (2-tailed). P, patient; gend, gender; D, donor; Cre, creatinine; GFR, glomerular filtration rate; MM, mismatch; Rej, rejection attack

normalize the results. The pre-transplantation mRNA levels of the TIM-3 and PD-1 genes were used as control. Clearly, the mean values of PD-1 expression values were determined to be higher in stable patients. However, TIM-3 mean fold changes were evaluated higher in patients with rejection episode (Figure 1a). When we divided patients due to the rejection episode reason, PD-1 expression levels could be variable. On the other hand, the evaluation of the TIM-3 expression levels showed that patients with AMR and TCMR have increased levels of expression, while one patient had increased, and the other one had decreased levels of TIM-3 expression in patients with acute tubular necrosis (Figure 1b).

Only one stable patient had higher TIM-3 expression levels than the other stable patients. When we observed clinical status of this patient there was no certain case. Patients that have the highest PD-1 gene expression levels (9.44; 16.51; 55.33, respectively) are evaluated as stable patients. On the other hand, patients with ATN, AMR and TCMR had similar PD-1

expression levels and lower than most of the stable patients.

According to the correlation analysis, PD-1 and TIM-3 expression levels were found as non-significantly (p=0.731) negative correlated (r=-.066). Moreover, when we evaluated the correlation between patients' characteristics and PD-1, TIM-3 expression levels, rejection episode was found statistically significant positive correlation with TIM-3 expression levels. Gender of both patient and donors, and pre-tx creatinine levels were also found statistically significant positive, negative, and positive correlation, respectively (Table 3). No significant correlation was found between PD-1 expression levels and any of the clinical properties of the patients.

Discussion

Invasive methods as core needle biopsy are still the most reliable and valuable method to detect antibody and cell mediated rejection episodes after kidney transplantation (20). However, any of the invasive methods have risks of complications both for the kidney and patient (21). Studies focused on noninvasive biomarkers to predict the rejection has been developed and at the present time associations between many molecules and rejection was evaluated (22, 23). mRNA profiles of the cytokines, cytotoxic molecules, and receptor/ligands on immune cells of the renal transplant patients showed to be used for the diagnosis of the rejection (24).

TIM-3 is one of the most important immune checkpoint regulator molecules (25). Although it was first identified on the surface of helper T cells, it was determined that cytotoxic CD8+ T cells, Th17, NK cells, monocytes, dendritic cells, mast cells and microglia cells can express this molecule on their surfaces. Moreover, soluble form of TIM-3 molecule can be detected in serum and urinary samples (26). In particular, the TIM-3 molecule is involved in the termination of the Th1 immune response and induction of tolerance (27). However, expression of the TIM-3 molecule by the other immune cells leads to the idea of having different roles in different cell types, environment and cellular signaling can affect the TIM-3 mediated response (28). Renesto et al showed the TIM-3 mRNA levels of the urinary samples could be a potential biomarker to detect acute rejection (29). Otherwise, Luo et al. reached the same results in their studies in which they used peripheral blood samples (30). Shahbaz et al. compared the pre-and post- transplantation TIM-3 expression levels by using both blood and urinary samples. According to their results TIM-3 levels were found higher in patients with graft disfunction (26). Ponciano et al. compared the patients with organ rejection episode and control group, and they reported increasing level of TIM-3 (31). Renesto et al. showed higher expression levels of TIM-3 in patient with acute rejection than stable patients (29). Manfro et al. detected increased levels of TIM-3 in tissue, blood, and urinary samples in patients with acute rejection when compared to patients with graft dysfunction. They also reported the significant correlation between gene expression levels in different tissue and organs (32).

According to the previous studies, it is obvious that TIM-3 gene expression levels were detected higher in graft dysfunction and acute rejection (29). In our study we hypothesized TIM-3 expression on CD8+T cells could also be a marker for the graft failure and rejection episodes after transplantation. Therefore, in this study TIM-3 was one of our target molecules that we compared expression levels before and after kidney transplantation. According to our results we were determined that there was a significant positive correlation between graft failure and the increase in TIM-3 gene expression level of CD8+ T cells before and after transplantation.

PD-1 is expressed on NK cells, dendritic cells, both B and T lymphocytes and its ligand PDL-1 is expressed by most of the cancer cells to escape from the immune response (33). Although the role in organ transplantation and allograft rejection cannot known well, it is thought that it can be an important biomarker. Therefore, several studies were focused on the role of PD-1 in organ transplantation. Kinch et al. determined PD-1, PD-L1 and PD-L2 gene expression levels in patients who developed lymphoproliferative disorder after solid organ transplantation (kidney, heart, liver, lung), and observed positive immunostaining results for all 3 molecules in 67% (34). Bishawi et al. discovered that lack of PD-1 causes the change in peripheral lymphocyte balance and because of this allograft rejection detected in heart transplanted patients (35). In our study it was detected that PD-1 expression levels were increased in 9 patients. Two patients had the highest PD-1 levels however, these patients were stable and had good kidney functions. When the patients with rejection episode were investigated, there was no PD-1 expression change in 50% and decreased level of PD-1 in 50% of the patients. Pike et al. compared the level of PD-1 expression in CD4 and CD8 T cell populations of patients and detected increased level of PD-1 expression in patients with rejection episode (36). However, it was reported that costimulatory mediated

tolerance after lung transplantation depended on the expression of the PD-1 molecules on CD8+ T cells and if PD-1 expression is absent, the interaction of CD8 + T cells with immune cells infiltrating the graft is prolonged and causes acute rejection (37). The meaning of this finding is that acute rejection develops in cases where PD-1 expression is decreased and therefore it is parallel to our findings.

In conclusion, this study suggests that detection of TIM-3 mRNA transcripts in CD8+ T cells can be used as a potential biomarker for kidney rejection. The best way to observe patients' clinical status may be the evaluation of the TIM-3 and PD-1 expression levels after the first, third, sixth and one year of transplantation and by comparing them with the results of pre-transplantation levels can be more sensitive and reliable. Further studies can be planned to observe the expression levels of other immune checkpoint regulatory molecules that play important role in immune response.

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Conflict of Interest Statement
The authors have no conflicts of interest to declare.

Ethical Approval
Ethical approval for this study was obtained from Health Science University Tepecik Training and Research Hospital Noninvasive Ethics Committee with the number 2022/05-31. This study was conducted in line with the principles of the "Helsinki Declaration".

Consent to Participate and Publish
Written informed consent to participate and publish was obtained from all individual participants included in the study.

Availability of Data and Materials
Data sharing is not applicable.

Authors Contributions
BÇA: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Writing-original draft.

MS: Conceptualization; Formal analysis; Investigation; Methodology; Project administration; Supervision; Writing-review & editing.

MP: Conceptualization; Formal analysis; Investigation; Methodology; Project administration; Supervision; Writing-review & editing.

TKA: Project administration; Supervision; Writing-review & editing.

ET: Data curation; Formal analysis

MT: Data curation; Formal analysis

HİKÇ: Investigation; Methodology

İP: Project administration; Supervision

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