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ORIGINAL PAPER

Study the mRNA level of IL-27/IL-27R pathway molecules in kidney transplant rejection

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Summary ***Background: Renal transplantation stands as the sole remedy for individuals afflicted with end-stage renal diseases, and safeguarding them from transplant rejection represents a vital, life-preserving endeavor post-transplantation. In this context, the impact of cytokines, notably IL-27, assumes a critical role in managing immune responses aimed at countering rejection. Consequently, this investigation endeavors to explore the precise function of IL-27 and its associated cytokines in the context of kidney transplant rejection.***
Methods: The study involved the acquisition of blood samples from a cohort of participants, consisting of 61 individuals who had undergone kidney transplantation (comprising 32 non-rejected patients and 29 rejected patients), and 33 healthy controls. The expression levels of specific genes were examined using SYBR Green Real-time PCR. Additionally, the evaluation encompassed the estimation of the ROC curve, the assessment of the relationship between certain blood factors, and the construction of protein-protein interaction networks for the genes under investigation.
Results: Significant statistical differences in gene expression levels were observed between the rejected group and healthy controls, encompassing all the genes examined, except for TLR3 and TLR4 genes. Moreover, the analysis of the Area Under the Curve (AUC) revealed that IL-27, IL-27R, TNF-α, and TLR4 exhibited greater significance in discriminating between the two patient groups. These findings highlight the potential importance of IL-27, IL-27R, TNF-α, and TLR4 as key factors for distinguishing between individuals in the rejected group and those in the healthy control group.
Conclusions: In the context of kidney rejections occurring within the specific timeframe of 2 weeks to 2 months post-transplantation, it is crucial to emphasize the significance of cytokines mRNA level, including IL-27, IL-27R, TNF-α, and TLR4, in elucidating and discerning the diverse immune system responses. The comprehensive examination of these cytokines' mRNA level assumes considerable importance in understanding the intricate mechanisms underlying kidney rejection processes during this critical period.

KEY WORDS: *IL-27; Kidney; Transplantation; Rejection.*

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INTRODUCTION

End-stage kidney diseases (ESKD) afflict a significant number of patients, and regrettably, there is currently no available cure to restore their kidney function. As a result, kidney transplantation stands as the sole established treatment option (1). The preservation of transplanted kidneys is of paramount importance to ensure graft survival (2). Consequently, *kidney transplant rejection* (KTR) represents an irreparable detriment in this context. KTR is characterized by an inflammatory response accompanied by distinct pathological changes in the graft, triggered by the recognition of non-self-donor antigens present in the allograft by the recipient's immune system. Acute rejection, which can manifest within days to weeks after transplantation, can manifest in two forms: *antibody-mediated rejection* (ABMR) and acute *T-cell-mediated rejection* (TCMR). The interplay between the innate and adaptive immune systems plays a crucial role in the processes leading to transplant rejection. Nonetheless, T lymphocytes emerge as the primary cellular players in the development of rejection. Further investigations have revealed the critical involvement of numerous cytokines and costimulatory molecules in this intricate immune response (3).

Numerous investigations have been conducted with the aim of comprehensively unraveling the intricate dynamics underlying transplant rejection in KTRs, particularly in relation to the assessment of pro- and/or anti-inflammatory cytokines' involvement in this process. Among these cytokines, Interleukin-27 (IL-27) assumes a position of utmost significance. IL-27 exerts its influence on both the innate and adaptive branches of the immune system through various mechanisms that contribute to distinct immune responses (4, 5).

Previous studies have provided insights into the involvement of IL-27 in various transplantation contexts. Le Texier et al. proposed that transforming growth factor (TGF)β1 and IL-27 contribute to tolerance mechanisms in cardiac allograft transplantation (6). In our previous investigation, we demonstrated that IL-27 acts as an anti-inflammatory cytokine in the context of liver transplant rejection (7). Furthermore, an innovative role of IL-27 in lymphopenia-induced CD8+ T cell proliferation has been reported, suggesting that targeting B cell-derived cytokines could enhance the efficacy of lymph-ablation and improve transplant outcomes (8). Additionally, a separate study presented data indicating that IL-27 could serve as a potential immunological marker for identifying post-transplant neoplasia accurately (9).

Building upon these collective findings and drawing from previous studies, the present study aims to assess the importance of IL-27 cytokine mRNA level and its associated molecules, including IL-27R (also referred to as TCCR or WSX1), interferon (IFN)-γ, tumor necrosis factor (TNF)-α and its receptors (TNFRA and B), Toll-like receptor (TLR)3, TLR4, interferon regulator factor (IRF)3, and IRF7, in the context of kidney transplant rejection without the need to estimate their protein level.

Materials and methods

Patients

This study included 61 adult kidney transplant recipients. Out of them, 32 had stable grafts, while 29 exhibited biopsy-proven rejection signs. The participants were selected from patients admitted to *Abu Ali Sina Hospital's Transplant* ward between 2018 and 2021, with an average of 350 kidney transplants per year. Protocol biopsies were conducted for all transplanted patients showing signs of graft rejection, with specialized pathologists using the Banff 10 classification to diagnose all rejected samples. The samples used in this study were chosen from the biopsy-proven ones, with 25% being TCMR and the remaining being ABMR.

Blood samples were collected from all participants in the study, including 61 kidney transplant patients and 33 healthy controls. The healthy controls chosen were age-matched normal individuals who had not experienced any infections or drug use for at least six months prior to sampling. Healthy controls were selected from hospital staff who volunteered to participate in our research. They were asked to answer related health questions, and a simple blood test was taken. The samples were treated with EDTA, and informed consent was obtained from all patients. The patients were then divided into two groups: a nonrejected group with 32 patients and a rejected group with 29 patients. The rejected samples were collected from kidney transplanted patients who referred to the hospital with graft rejection signs between 2 weeks and 2 months after transplantation. The blood samples included in the rejection group were taken before biopsy and after biopsy confirmation. All samples were selected from patients who had received their first transplant and were non-sensitized (*Luminex flow PRA negative*). Additionally, we have randomly selected from recipients who didn't show any signs of rejection between 2 weeks and 2 months after transplantation.

The *Ethics Committee of Shiraz University of Medical Sciences* approved the study, and all protocols adhered to the ethical guidelines of the Declaration of Helsinki. KTR patients underwent routine HLA and ABO blood matching tests, and all transplanted kidneys were from cadaver donors. Blood samples were screened for BK polyoma virus, *cytomegalovirus* (CMV), *hepatitis B* (HBV) and C (HCV), and *human immunodeficiency virus* (HIV) infection. Samples that tested positive for any of these infections were excluded from the study.

The study enrolled adult participants aged 18-74 years, excluding samples from younger participants. A questionnaire was created to collect demographic data and other relevant information, such as history of cancer, alcohol or tobacco use, and vasculitis renal disease. Individuals with these conditions were excluded from the study, as were those who experienced multiple episodes of rejection. Additionally, no mixed rejection samples were included in the study.

A routine immunosuppressive regimen was used for all patients consisted of tacrolimus or cyclosporine with mycophenolate mofetil and steroids. The blood level of 150-200 mg/mL was considered the therapeutic target for CsA (5 mg/kg/d) or for tacrolimus (8-10 mg/mL).

Molecular analyses

The buffy coat and plasma of all samples were separates using Ficol (*Nycomed, Zurich, Switzerland*) gradient for further analysis.

RNA extraction and cDNA synthesis

In order to extract the total RNA of each patient sample and controls buffy coats, *Trizol™* (*Invitrogen, Carlsbad, CA, USA*) was used according to manufacturer protocols. Furthermore, for evaluating the purity and concentration of extracted RNAs in each sample, their optimal density in 260/280 nm was calculated. 500 ng of total RNA used for cDNA synthesis by using Takara kit (*Dalian, Japan*) according to the manufacturer's instruction.

Quantitative Real-time PCR analysis (SYBR Green)

The expression level of different studied genes was analyzed using pre-designed primers (Table 1). Both GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) and β-actin genes were studied for internal control. Finally,

Table 1.
The sequences of the primer pairs used for gene amplification.

Gene names and mRNA IDs	Primer sequences (5'to 3')	Length (bp)	Annealing temperature (°C)
IL-27	Forward: GTGAACCTGTACCTCCTGCC		
NM_145659.3	Reverse: CGTGGTGGAGATGAAGCAGA	111	60
IL-27R	Forward: CGGAGCTGAAGACCATACCC		
NM_004843.4	Reverse: CGCCCGACAAATCCTCTTCT	114	59
IFN-γ	Forward: CAGCTCTGCATCGTTTTGGG		
NM_000619.3	Reverse: TCCGCTACATCTGAATGACCTG	110	58
TNF-α	Forward: CTTCTGCCTGCTGCACTTTG		
NM_001065.4	Reverse: CTACAGGCTTGTCACTCGGG	128	61
TNFARA	Forward: GAGAGGCCATAGCTGTCTGG		
NM_001065.4	Reverse: CTCTCACACTCCCTGCAGTC	124	60
TNFARB	Forward: CACATGCCGGCTCAGAGAAT		
NM_001066.3	Reverse: AGCTGGGTGTATGTGCTGTC	144	59
TLR3	Forward: GGGCAAGAACTCACAGGCCAGG		
NM_003265.3	Reverse: 5'-AAGGGCCACCCTTCGGAGCA	147	58
TLR4	Forward: 5'- TCAAGCCAGGATGAGGACTGGGT		
NM_003266.4	Reverse: 5'- CAGCAATGGCCACACCGGGA	118	59
IRF3	Forward: 5'- TTGGGGACTTTTCCCAGCC		
NM_001197122.2	Reverse: 5'- TCCAGAATGTCTTCCTGGGT	82	58
IRF7	Forward: 5'- GTGAGGGTGTGTCTTCCCTG		
NM_001572.5	Reverse: 5'- TCGTCATAGAGGCTGTTGGC	73	58
GAPDH	Forward: 5'-GGACTCATGACCACAGTCC		
NM_001357943.2	Reverse: 5'-CCAGTAGAGGCAGGGATGAT	119	58

GAPDH selected as internal control due to its less expression fluctuations in different samples.
The real-time mix for each primer pair was set up, using 10 µl of *SYBR Premix Ex TaqII kit* (*Takara, Shiga, Japan*), 0.2 µl of *ROX dye* (used for normalization), 0.8 µl of each forward and reverse primers (10pM) and 2 µl of synthesized cDNA. The total volume of each reaction reached to 20 µl adding sterile water. The amplification reaction was done in *Step One Plus Real Time instrument* (*ABI, Step One Plus, USA*). The cycling program used for amplification of each primer pair was 1 cycle of 95°C/2 mins, followed by 40 cycles of annealing temperature of each gene/20 secs and 72°C/30 secs. Previously the optimum annealing temperature of each gene was set up and used in real-time programming (for more information about the primer sequences refer to our previous report 11). At the end of each real-time, melting curves were generated by the instrument in order to verify the specificity of the amplification reaction. Finally, normalizing all data was executed using the result of GAPDH gene amplification.

PPI (protein-protein interaction) construction

For more investigation around the interactions between studied genes with each other at the protein level, the *Search Tool for the Retrieval of Interacting Genes* (*STRING, https://string-db.org/*) was used in order to produce the PPI network.

Statistical analysis

All data was collected in EPSPS ver. 22 (*SPSS, Chicago, IL, USA*). In order to calculate the mRNA expression level of studied genes, Livak (2-ΔΔCt) method was used. To analyze the variation in the gene expression levels in studied groups of patients, nonparametric tests were performed. Furthermore, two-sided Spearman correlation analysis was performed to estimate the variables' relationship (*GraphPad Software, Prism 6.01, CA, USA*). The *receiver operating characteristic* (ROC) curve analysis, sensitivity, and specificity of studied genes were determined using *MedCalc* (*MedCalc Software, Ostend, Belgium*) Statistical Software version 17.9. Finally, $p < 0.05$ was considered as statistically significant.

Results

Patients' demographic details

In this study, 61 kidney transplanted patients participated which were divided into two groups. These transplanted patients were composed of 41 (67%) men (mean age = 44.8 years) and 20 (33%) women (mean age = 36.7 years). The nonrejected group were composed of 32 patients and 21 (65.5%) of them were men (mean age of men = 45.7 years, mean age of women = 40.75 years). The rejected group were also composed of 29 patients consisting of 20 (69%) men (mean age of men = 42.9 years, mean age of women = 32.9 years).
The most abundant blood groups in rejected group were A+ (31%) and O+ (34.5%), nonrejected group were B+ (31.3%) and O+ (37.5%), and control group were A+ (34.8%) and B+ (31.5%). The underlying disease distribution is also categorized in Table 2. Some important blood factors that are listed in this table, were considered in both studied groups of patients. Statistical analysis between the two patients' groups shows the significant difference for all parameters.

Table 2.
The underlying disease, and blood factors' distribution among rejected and nonrejected patients.

		Study groups number (%)		p value
		Rejected	Nonrejected	
Underlying diseases	HTN	9 (31)	7 (21.9)	-
	DM	3 (10.3)	3 (9.4)	-
	ADPKD	3 (10.3)	7 (21.9)	-
	Others	14 (48.4)	15 (46.8)	-
Blood factors (mean; mg/DLit)	BUN	60.52	36.65	< 0.01
	Cr	4.08	1.24	< 0.01
	FBS	110	94.08	0.015
	Serum Ca	8.4	8.29	0.031
	Serum Na	134.84	136.82	0.015
	Serum K	5.2	4.6	0.02

Autosomal dominant polycystic kidney disease (ADPKD), Hypertension (HTN), Diabetes mellitus (DM), Blood Urea Nitrogen (BUN), Creatinine (Cr), fasting blood sugar (FBS), serum calcium (Serum Ca), serum sodium (Serum Na) and serum potassium (Serum K).

Table 3.
The AUC, p value, cut-off value, sensitivity and specificity of the studied genes are estimated and categorized.

Gene Name	Gene ID	AUC (95% CI)	p value	Cut off value	Sensitivity (95% CI)	Specificity (95% CI)
IL-27	246778	0.870 (0.759-0.942)	< 0.001	> 115.36	75.86 (56.5-89.7)	90.62 (75.0-98.0)
IL-27R (WSX-1)	9466	0.765 (0.639-0.864)	< 0.001	> 10.7	68.97 (49.2-84.7)	87.50 71.0-96.5
IFN-γ	3458	0.522 (0.390-0.652)	0.771	> 2.25	93.10 (77.2-99.2)	28.12 (13.7-46.7)
TNF-α	7124	0.818 (0.698-0.905)	< 0.001	> 630.35	48.28 (29.4-67.5)	100.00 (89.1-100.0)
TNFR1	7132	0.516 (0.384-0.646)	0.836	≤ 75.58	79.31 (60.3-92.0)	34.38 (18.6-53.2)
TNFR2	71323	0.581 (0.448-0.706)	0.277	> 2.38	86.21 (68.3-96.1)	34.38 (18.6-53.2)
IRF3	3661	0.583 (0.432-0.691)	0.268	> 0	96.55 (82.2-99.9)	25.00 (11.5-43.4)
IRF7	3665	0.629 (0.496-0.749)	0.077	≤ 11.55	86.21 (68.3-96.1)	40.63 (23.7-59.4)
TLR3	7098	0.650 (0.494-0.748)	0.037	≤ 1.27	58.62 (38.9-76.5)	68.75 (50.0-83.9)
TLR4	7099	0.704 (0.573-0.814)	0.004	> 0.23	86.21 (68.3-96.1)	59.38 (40.6-76.3)

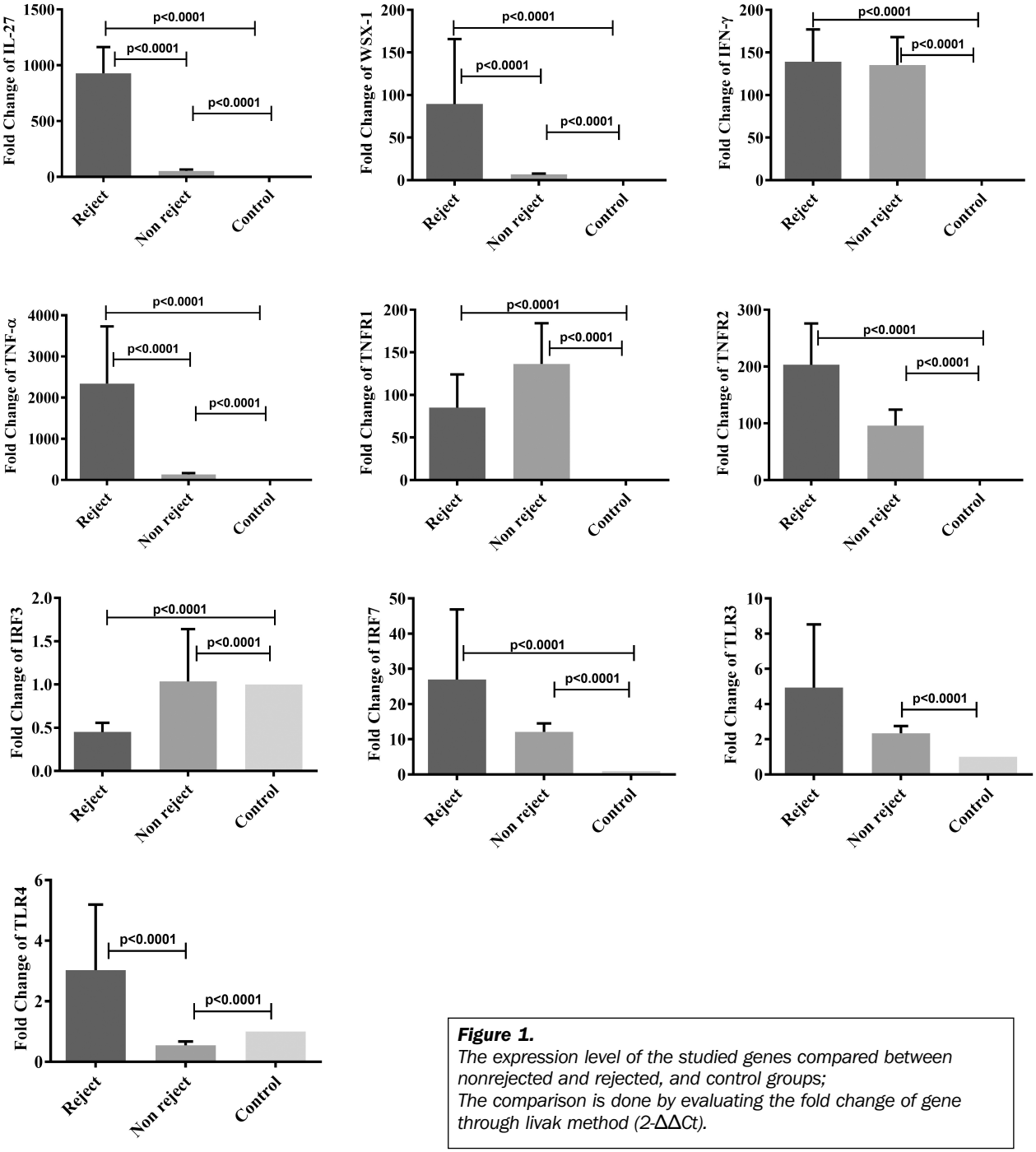


Figure 1.
The expression level of the studied genes compared between nonrejected and rejected, and control groups;
The comparison is done by evaluating the fold change of gene through livak method (2-ΔΔCt).

Autosomal dominant polycystic kidney disease (ADPKD), *Hypertension* (HTN), *Diabetes mellitus* (DM), *Blood Urea Nitrogen* (BUN), *Creatinine* (Cr), *fasting blood sugar* (FBS), *serum calcium* (Serum Ca), *serum sodium* (Serum Na) and *serum potassium* (Serum K).

Gene expression compared in nonrejected and rejected KTR

The mRNA expression level of all the studied genes were compared among patients' groups and healthy controls. This comparison is summarized in Figure 1 and statistical analysis showed that the difference in the expression level of genes between rejected group of patients and healthy control was statistically significant ($p < 0.0001$) in all studied genes except for TLR3 and TLR4 genes. The same comparison between nonrejected and healthy control group showed that in all genes this statistical comparison showed significant difference. Also, it is worth mention-

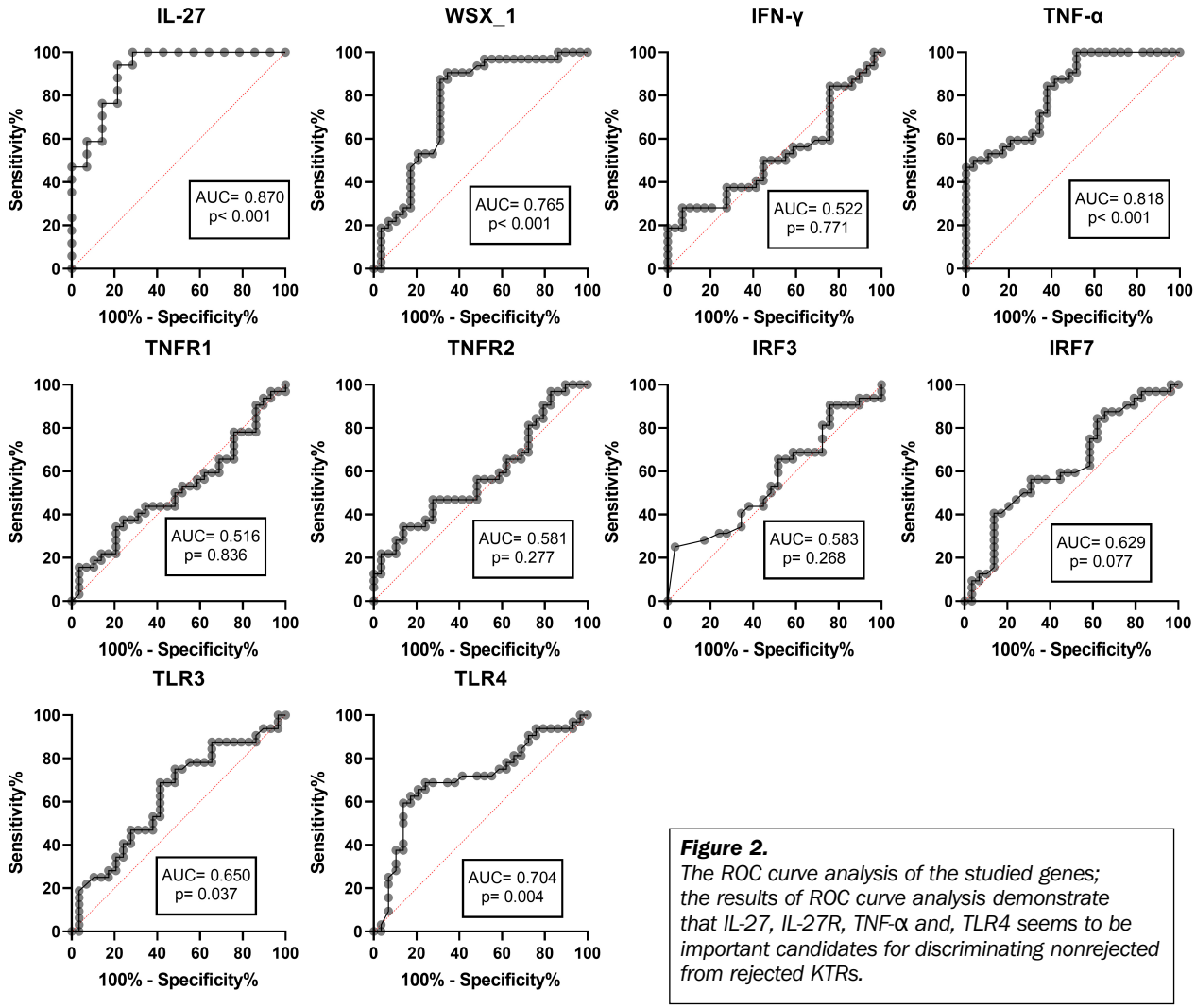


Figure 2.
The ROC curve analysis of the studied genes; the results of ROC curve analysis demonstrate that IL-27, IL-27R, TNF-α and, TLR4 seems to be important candidates for discriminating nonrejected from rejected KTRs.

ing that the statistical analysis between rejected and non-rejected groups showed significant difference in expression level of some of the studied genes (IL-27, IL-27R, TNF-α and TLR4). Finally, the expression level of TNFR1 and TLR3 was higher in nonrejected group versus the other two groups and the expression level of IRF3 in both of the patient groups was less than control group.

ROC curve analysis of expression level of genes between nonrejected and rejected KTRs

In order to evaluate the sensitivity and specificity of different studied genes between nonrejected and rejected groups of patients, ROC curve analysis used. The mentioned data is summarized in Figure 2 and Table 3. Also, the area under the ROC curve (AUC) for measuring the 2D (two-dimensional) area underneath the ROC curve was determined. These results show that some genes such as IL-27 ($p < 0.001$, AUC = 0.870), IL-27R ($p < 0.001$, AUC = 0.765), TNF-α ($p < 0.001$, AUC = 0.818) and TLR4 ($p = 0.004$, AUC = 0. 708) seems to be more important for discrimination between the two patients' groups.

Correlation study of selected genes in rejected KTRs

Studying the relation between the increased cytokines in rejected KTRs, in Figure 3 it is showed that only IL-27R with TNF-α ($r = 0.4574$; $p = 0.0126$) and TLR4 ($r = 0.5730$; $p = 0.0012$) had significant positive correlation.

Correlation between blood factors and studied genes in rejected KTRs

The relationship between studied lab indices with all the studied genes were analyzed. The analysis that was performed for finding the relation between studied gene expression levels and the blood factors in rejected KTRs showed that the expression level of IL-27R negatively and IFN-γ and TNFR2 positively correlate with serum Ca. As well, both TNFR2 and IRF7 positively correlate with serum Na and K, respectively in rejected KTRs (the figures are not shown).

PPI Network Construction for studied genes

In order to check the interactions of studied genes in the protein level and to confirm their relevance to each other, the PPI network was produced for all genes using the

Figure 3.
Correlation between increased cytokines in rejected KTRs which showed that IL-27R with TNF-α and TLR4 had positively significant correlation.

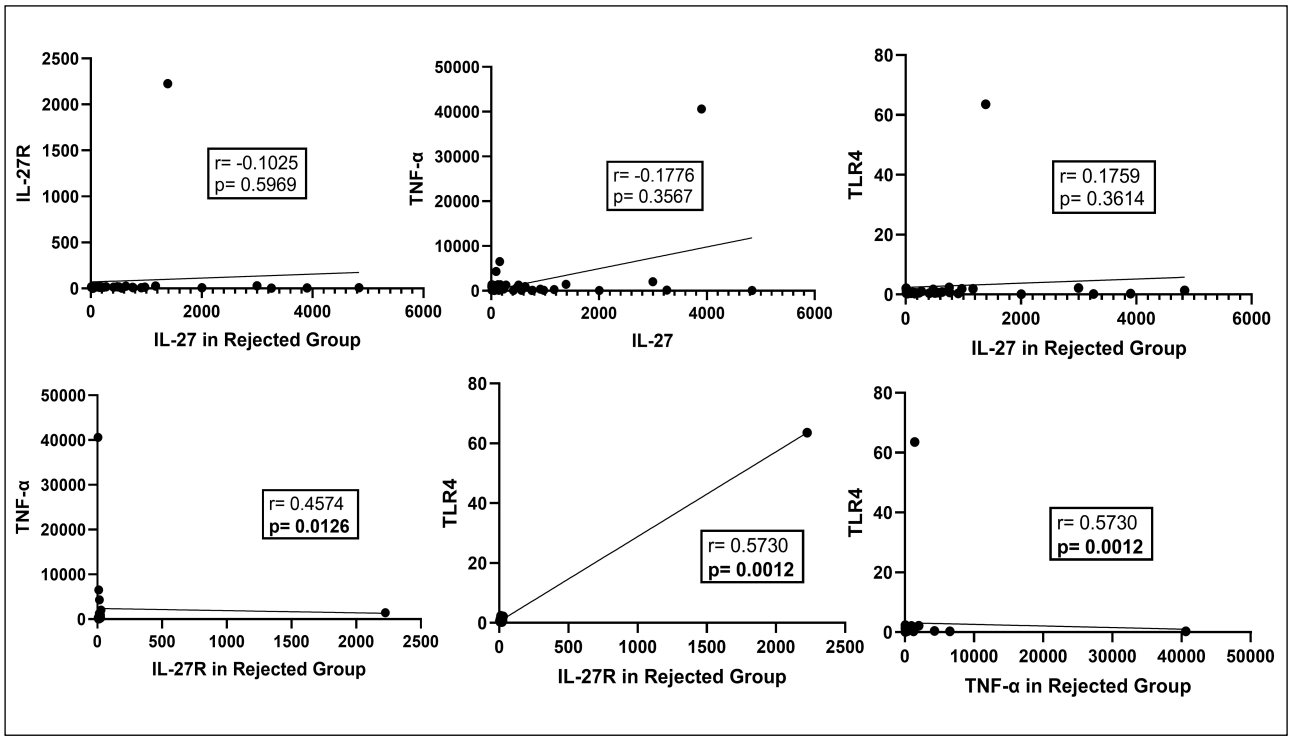
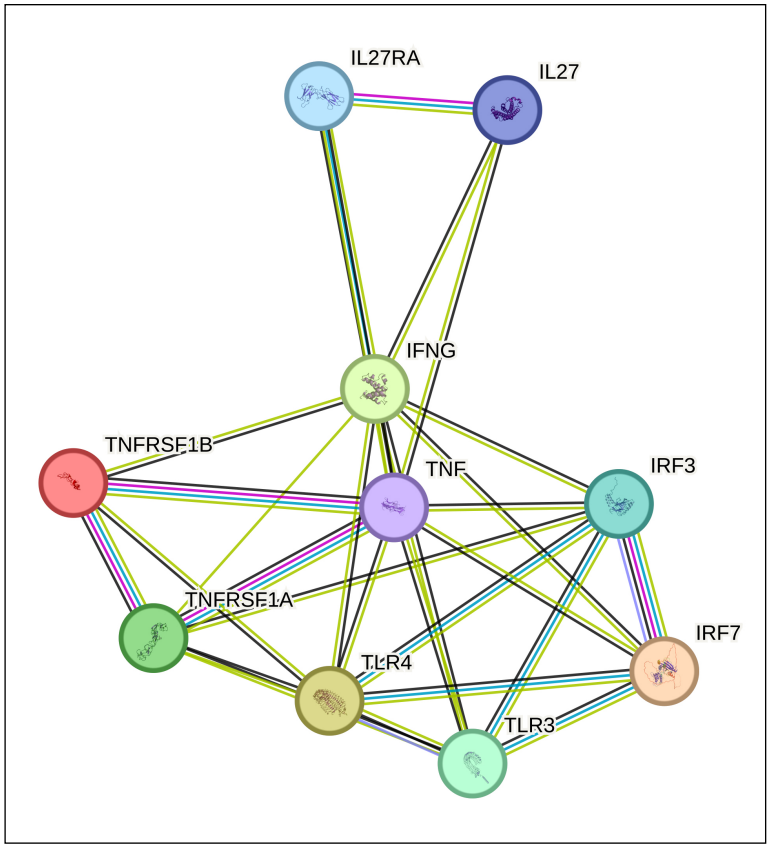


Figure 4.
The PPI network shows the association of all studied genes in protein level.



STRING database. The result of PPI network showed that all 10 studied biomarkers had several interactions with each other (Figure 4).

Discussion

Although the incidence of acute rejection in kidney transplant recipients has decreased, it remains a significant concern post-transplantation (12-15). A better understanding of this procedure can aid in predicting and preventing acute rejection in patients (16). Currently, serum creatinine is the gold standard for monitoring renal grafts (17). However, it is not specific or sensitive enough. Noninvasive and more robust methods are therefore needed. Cytokines and their receptors play a central role in allograft rejection. Therefore, in this study, we aimed to identify potential biomarkers such as IL-27 and its related cytokines and receptors (IL-27R, IFN-γ, TNF-α, IRF3 and 7, TLR3, and TLR4) in kidney transplant recipients.

Upon stimulation through TLR signaling, *antigen presenting cells* (APCs) rapidly initiate production of IL-27 4,18. IL-27 plays a critical role in initiating T cell responses by binding to its receptor, IL-27R (19). The main producers of IL-27R are activated T cells and natural killer (NK) cells (20). In vitro studies suggest that IL-27 is not essential for TH1 cell

differentiation in vivo, and its primary function appears to be as a negative regulator of the immune system (21). Two other studies have also identified the role of IL-27 in promoting tolerance in transplantation (6, 22). IL-27 has been shown to synergize with IL-12 in promoting proliferation of naive CD4+ T cells and production of IFN-γ from NK cells and CD4+ T cells (4)]. Based on these findings, this molecule is a potential candidate as a potent predictive and diagnostic marker.

In a previous study, we examined the expression rate of IL-27 in liver transplant patients during the first week of acute rejection. However, no significant changes were observed between the rejected and non-rejected groups 7. Our team also evaluated the expression level of other related cytokines, such as IL-17, in this context (23). In our current research, we investigated the expression level of IL-27 in kidney transplant patients during the period between 2 weeks to 2 months post-transplantation. Our findings revealed a significant increase in IL-27 expression level among rejected patients compared to non-rejected patients. The same pattern was observed for the IL-27 receptor (IL-27R), with a statistically significant increase in the rejected group. The ROC curve analysis for both IL-27 and IL-27R were also significant.

In response to TLR4 signals, NF-kB is activated and binds to the IL-27p28 promoter (24). Additionally, IFN-γ exhibits synergistic effects in this system (25), working in cooperation with other cytokines such as TNF-α. Various studies have been conducted to evaluate the role of IFN-γ in transplant rejection (26-28), and our study shows an increase in IFN-γ expression levels even in the blood of stable renal graft recipients compared to healthy individuals ($p < 0.05$). Furthermore, our results demonstrate a significant increase in kidney transplant rejection patients compared to healthy individuals. The reason for the increase in IFN-γ production post-transplantation is related to modifications in the extent of methylation of its promoter (28). TNF-α plays a crucial role by binding to its receptors (TNFR1 and TNFR2) expressed on the surface of various target cells. Immunological responses are attributed to the signals produced through TNF-α binding to TNFR1, while T cells are affected by binding to TNFR2 (29). Studies have reported synchronized elevation of TNF-α and TNFR2 in rejected kidney transplant patients (30, 32). Our data supports this finding, showing a significant increase in both TNF-α and TNFR2 levels in rejected patients compared to healthy controls. Furthermore, TNF-α levels were significantly higher in rejected patients than in stable graft participants, as demonstrated by ROC curve studies with a p-value of < 0.05. Other studies have also reported elevated protein levels of TNF-α in serum (33, 24) and urine (34) samples from kidney transplant rejected patients.

The expression levels of the TLR4 gene and protein in solid organ transplants have been investigated in various studies (35-38). These studies have detected an increased expression level of the TLR4 gene in the liver (39) and kidney (40, 41) in blood and tissue biopsies of patients experiencing acute rejection episodes, respectively. Our results are consistent with these studies, as we also found a significant increase in TLR4 expression rates in patients with transplant rejection compared to other study groups (non-rejected and normal). Additionally, ROC curve analysis demonstrated that this gene can be considered a valuable prognostic factor for renal transplant rejection by distinguishing between rejected and non-rejected patients. However, there is insufficient evidence to support the importance and role of the TLR3 gene in graft rejection based on our study and others (42, 43).

The TLR4 pathway activates a mediator molecule called TRIF (ToAll/IL-1R-related domain containing adaptor inducing IFN), which then triggers the translocation of IRF3 and 7 to the nucleus via a MyD88-independent signaling pathway. Previous research has shown that the IRF3/IRF7 heterodimer plays a crucial role in viral infections, inflammatory diseases, and septic shock by regulating IFN production (44). These two molecules are known to be key regulators of IFN production induction. However, their role in kidney transplant rejection is not well understood. Our findings indicate that IRF3 expression levels are significantly lower in both rejected and non-rejected patient groups compared to healthy controls, while IRF7 expression levels are significantly higher in both patient groups. Additionally, our PPI network analysis revealed a strong correlation between the proteins of these genes as potential biomarkers for kidney transplant rejection.

This study has demonstrated the importance of increased gene expression levels, particularly in the rejected group. However, what is more crucial is the ability of these genes to differentiate between rejected and non-rejected patients. Among all the genes studied, IL-27, IL-27R, TNF-α and TLR4 were found to be significantly expressed in the rejected group of KTRs. These four genes also showed significant variations in ROC curve analysis ($p < 0.05$). Ultimately, this study aimed to identify a critical gene expression pattern related to transplant rejection that could potentially serve as biomarkers for predicting and diagnosing rejection independently of protein level expression.

Conclusions

In our current research, we propose that monitoring the mRNA expression patterns of certain cytokines, such as IL-27 and its receptor (IL-27R), TNF-α, and TLR4 genes in the blood of patients, could have non-invasive prognostic and diagnostic potential and free researchers from testing protein level. This could guide clinical decisions regarding the appropriate extent of immunosuppressive therapy for each patient and potentially improve outcomes following kidney transplantation.

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