

Interleukin-17 Gene Expression and Serum Levels in Acute Rejected and non-Rejected Liver Transplant Patients

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ABSTRACT

Background: Interleukin-17 (IL-17), as a potent proinflammatory cytokine, has a critical role in post liver transplant outcomes. However, there is not much information about the effects of IL-17 cytokine on acute liver rejection. **Objective:** To evaluate the role of IL-17 in post-liver transplant acute rejection. **Methods:** Ninety seven adult liver transplant patients who enrolled in this cross sectional study were divided into Non-Acute Rejected (Non-AR) and Acute Rejected (AR) patient groups. Three blood samples were collected from each patient in days 1, 4 and 7 post liver transplantation. The IL-17 mRNA levels were evaluated using an in-house real time PCR protocol. IL-17 protein levels were also analyzed in Non-AR, AR and also control groups using ELISA method. **Results:** The IL-17 mRNA expression level continuously increased in AR patients in all days of follow-up post liver transplantation. IL-17 expression was, however, down regulated after day 4 post-transplant follow-up in Non-AR patients. Both IL-17 mRNA expression and protein levels were also significantly increased in AR patients compared with Non-AR ones. **Conclusion:** Based on these findings, significant increase of IL-17 mRNA and protein levels in AR patients highlights the important role of IL-17 in acute liver rejection.

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INTRODUCTION

Liver transplantation is the final therapeutic procedure for complicated end-stage liver diseases. Different problems complicate the outcome of liver transplantation (1,2). Acute rejection of the liver can lead to early graft loss and a risk factor threatening the recipient long-term survival after transplantation (3). Therefore, understanding the mechanisms of acute rejection especially immune related pathways helps to increase the success of graft expectancy (4).

Allograft rejection is a complex phenomenon arising from multiple cell type interactions and some other immune factors (5). In this process, T cells play a basic role in alloantigen recognition by interaction of MHC molecule with donor organ antigens. Under different cytokine milieu, naive T cells differentiate to multiple subtypes. Th1 cells produce IFN- γ while Th2 cells secrete IL-4, IL-5 and IL-13 (5). Transplantation models show the association of Th1 cytokine profile with allograft rejection, whereas Th2 profile tends to the acquisition of tolerance and stable graft survival (6). Based on previous studies, Th17 cells are represented to be a novel subset of Th cells characterized by producing IL-17 (7). IL-17 participates in orchestrating a specific kind of inflammatory responses (2).

Th17 cells are not only related to many immune/autoimmune diseases (8), but also allograft rejection (9), so that recent studies have demonstrated the prominent role of Th17 cells in the development of acute renal allograft rejection. It has been shown that IL-17 can be offered as an early diagnostic marker of acute renal allograft rejection in mice (10). Elevated IL-17 levels have been shown to be associated with renal and lung graft rejection in humans (11). Using IL-17 antagonism may have the potential for anti-rejection therapy, either alone or in combination with other immunosuppressive agents exhibiting complementary modes of action (12).

In addition, in human renal grafts a correlation was observed between shorter graft survival and the presence of intragraft Th17 cells producing IL-17 and IL-21 (13). Dramatic increases in IL-17⁺ cells are detected in the blood of kidney transplant patients who are experiencing delayed graft function. Th1, and Th17 are increased in the blood of these patients, suggesting that all mediate acute graft rejection (3,14-16).

Less information exists about the effects of Th17 and IL-17 in liver transplantation. It has been shown that stimulation with IL-17 in primary hepatocytes induces the expression of several inflammation-associated genes, including chemokine and C-reactive protein. IL-17 also activates other cells in the liver to produce proinflammatory cytokines (17). Activated liver-infiltrating Th17 cells are also responsible for neutrophil recruitment into the liver. Furthermore, serum IL-17 levels are increased and serve as a marker of the severity of acute hepatic injury (18).

Fabrega *et al.* described a possible correlation between acute liver rejections with higher proliferation of Th17 cells. This group also showed an overall increase in serum IL-17/IL-23 after transplantation compared to healthy controls. Even more elevated IL-17/IL-23 response has been found in acute liver graft rejection (19).

Considering the importance of Th17 and its related cytokines in the liver transplant outcome, design the aim of the present study to evaluate the association of IL-17 gene expression and its protein serum level with acute allograft rejection and graft surveillance in liver transplant patients.

MATERIALS AND METHODS

Patients. A total of 97 liver transplant recipients who underwent surgery at Nemazee hospital, Shiraz, Iran, were consecutively enrolled between years: 2011-2013. Three EDTA-treated blood samples were collected from each studied patient in days 1, 4 and 7 intervals post liver transplantation. Buffy coat and plasma were isolated from each EDTA-treated blood samples using Ficoll and preserved in -80°C till laboratory protocols were performed. Patients who expired before collection of all needed samples were rule out from study and increase the time of sample collection. The patients were divided in two groups based on experiencing acute rejection episodes or not: non-acute rejected (Non-AR) and acute rejected (AR) patients. Also, non-rejected transplant patients were considered as the control group. The AR patients consisted of 44 patients experiencing one or more acute rejection episodes. The Non-AR patients consisted of 53 patients not experiencing acute rejection. The study was approved by the Ethical Committee of Shiraz University of Medical Sciences (The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki). Acute rejection episodes were identified by an expert gastroenterologist team based on the accepted criteria such as increased serum levels of liver enzymes and total serum bilirubin level in the absence of biliary problems, histological findings after biopsy of the liver and clinical and biochemical response to high-dose steroids (20). The routine immunosuppression regimen consisted of tacrolimus or cyclosporine with mycophenolatemofetil and steroids. Drug dosage was adjusted to maintain target therapeutic blood levels of 200ng/ml for CsA (5 mg/kg/Day) or 10ng/ml for tacrolimus. Donors were selected on the basis of ABO blood group compatibility. HLA matching is not routinely done in Shiraz transplant center for liver transplant patients.

RNA Isolation and cDNA Synthesis. Total RNA was isolated from Buffy coats using RNX plus (Cinnagen, Iran). The purity and integrity of RNA were determined by measuring the optical density 260/280 and agarose gel (1%) electrophoresis. One microgram of each RNA samples was reversely transcribed to cDNA using reverse transcriptase and random hexamer (Vivantis, Malaysia). All the samples were treated with DNase (1u/μg of RNA) before synthesis of cDNA. Then cDNA synthesis was continued in two steps. First, RNA (10μg/10μl), dNTPs (1μ/10mM), and random hexamer (1μl/0.2μg) were mixed and incubated at 65°C for 7 minutes and then on ice for 2 minutes. Second, M-MLV Reverse transcriptase (RT) enzyme (1μl/200U), RT-buffer (2μl/10x), RNase inhibitor (1.3μl/ 60U), and nuclease free water were mixed and added to product of first step. Then final mix was incubated at 45°C for 90 minutes and 85°C for 5 minutes.

Real Time PCR. Real time PCR method was performed for the quantitative analysis of IL-17 mRNA expression profile in AR and non-AR liver transplant patients. The primer was originally designed for transcripts of IL-17 (NM_002190.2) and β-actin (NM_001101.3) as the internal control. After evaluation of the β-actin and GAPDH genes as internal controls, the β-actin gene was finally used as internal control for minor fluctuations. The primer sequences for amplification of IL-17 and β-actin transcripts were as follows: IL-17F: 5'-TCTGGGAGGCAAAGTGCCGC-3' and IL-17R: 5'-GGGCAGTGTGGAGGCTCCCT-3', β-actin F: 5'-GGCGGCACCACCATGTACCC-3' and β-actin R: 5'-GACGATGGAGGGGCCCGACT-3'. The PCR mix containing: SYBR green Premix (10 μl) by Ex taq (Takara, Japan), SYBR Green Dye (0.2 μl), forward and reverse primers (3 pM), and template cDNA (2 μl) were carried out. The

PCR thermocycling condition are included: One cycle 95 °C for 5 minutes, followed by 40 cycles of 95°C for 30 seconds and 65°C for 20 seconds using Step One Plus Real-Time instrument (ABI, Step One Plus, USA). The specificity of amplification reaction was confirmed by a melting-curve analysis. The results for the target genes were measured as fluorescent signal intensity and normalized to the internal standard gene β -actin.

Cytokine Level Measurement. The Plasma of EDTA-treated samples were collected from AR and Non-AR patients and also healthy controls and stored at -80°C till analysis of IL-17 protein level preformed using an IL-17 ELISA Kit (eBioscience, USA) according to the manufacturer's instruction.

Statistical Analysis. The analytical pattern of IL-17 expression level was calculated using $2^{-\Delta\Delta CT}$ (Livak method) between Non-AR and AR liver transplant patient groups. The parametric and non-parametric tests including: student's t-test, Mann-Whitney test, k independent test and χ square test were performed using SPSS, version 16 for Windows (SPSS, Chicago, IL, USA) to evaluate variations in IL-17 expression and serum levels between different studied groups. $p < 0.05$ was considered as statistically significant.

RESULTS

Patients' Profiles. The AR patients consisted of 44 patients experienced one or more acute rejection episodes. The age range in these patients was between: 2-69 years with a mean of 38.1 ± 14.96 years old. The AR group was composed of 31 (70.45%) males and 13 (29.25%) of them were females.

Table 1. Underlying diseases in Non-AR and AR liver transplant patients.

Underlying Diseases	Patients Group	
	Non-AR	AR
Hepatitis B Virus Infection	15 (28.3%)	7 (15.9%)
Auto Immune Hepatitis	9 (17%)	7 (15.9%)
Primary Sclerosing Colingitis	8 (15%)	11 (25%)
Cryptogenic Cyrrhosis	7 (13.4%)	9 (20.5%)
Wilson Disease	2 (3.7%)	3 (6.8%)
Biliary atresia	3 (5.7%)	0
Hypertyrosinemia	2 (3.7%)	0
Hepatitis C Virus Infection	2 (3.7%)	1 (2.3%)
others	5 (9.5%)	6 (13.6%)
Total	53 (100%)	44 (100%)

The Non-AR patients consisted of 53 patients' not experienced acute rejection. The age range was between: 1-74 with a mean of 34.33 ± 21.26 years old. The 36 of 53(68%) Non-AR patients were male and 17 of 53 (32%) of them were female.

The distribution frequency of the underlying disease in both Non-AR and AR liver transplant patients was presented in Table 1. The highest frequency of the underlying diseases was Hepatitis B Virus Infection and Primary Sclerosing Colingitis in Non-AR and AR transplant patients, respectively. The most frequent ABO blood group was O+ in both of AR and Non-AR transplant patients.

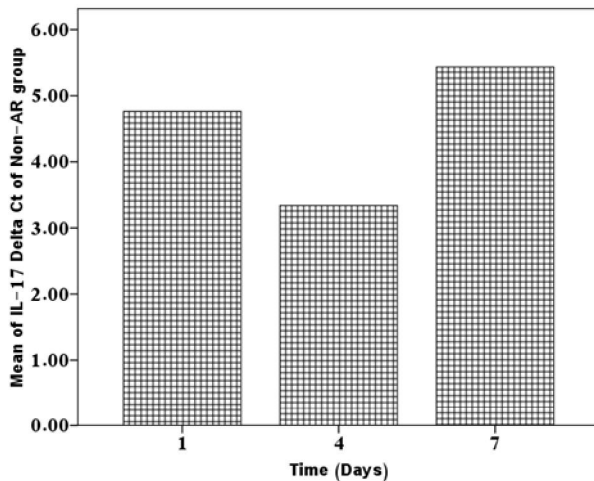


Figure 1. The pattern of IL-17 mRNA expression level in Non-AR liver transplant patients.

IL-17 Gene Expression in Non-AR and AR Liver Transplant Patients. The IL-17 expression level was down regulated in Non-AR patients after day 4 of post-transplant follow-up (Figure 1). But in AR patients, IL-17 expression level was significantly and steadily increased during all days of post-transplant follow up (Figure 2).

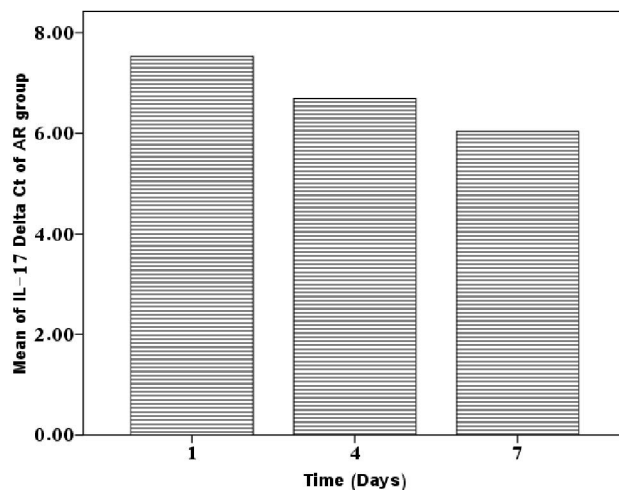


Figure 2. The pattern of IL-17 mRNA expression level in AR liver transplant patients.

The results of comparison between IL-17 gene expression level between Non-AR and AR liver transplant Patients was presented in Figure 3. IL-17 expression level significantly increased in AR patients compared with Non-ARs during day's 1 ($p=0.05$, 95%CI: 0.00-0.065) and 7 ($p=0.007$, 95%CI: 0.000-0.030) post-transplantation (Fig. 3, A and C). IL-17 expression level was also increased but not significant in AR patients during day's 4 ($p=0.173$, 95%CI: 0.126-0.287) post-transplantation (Fig. 3B). The IL-17 gene expression was increased more than 100 times in AR patients compared with Non-AR ones during post-transplant follow-up.

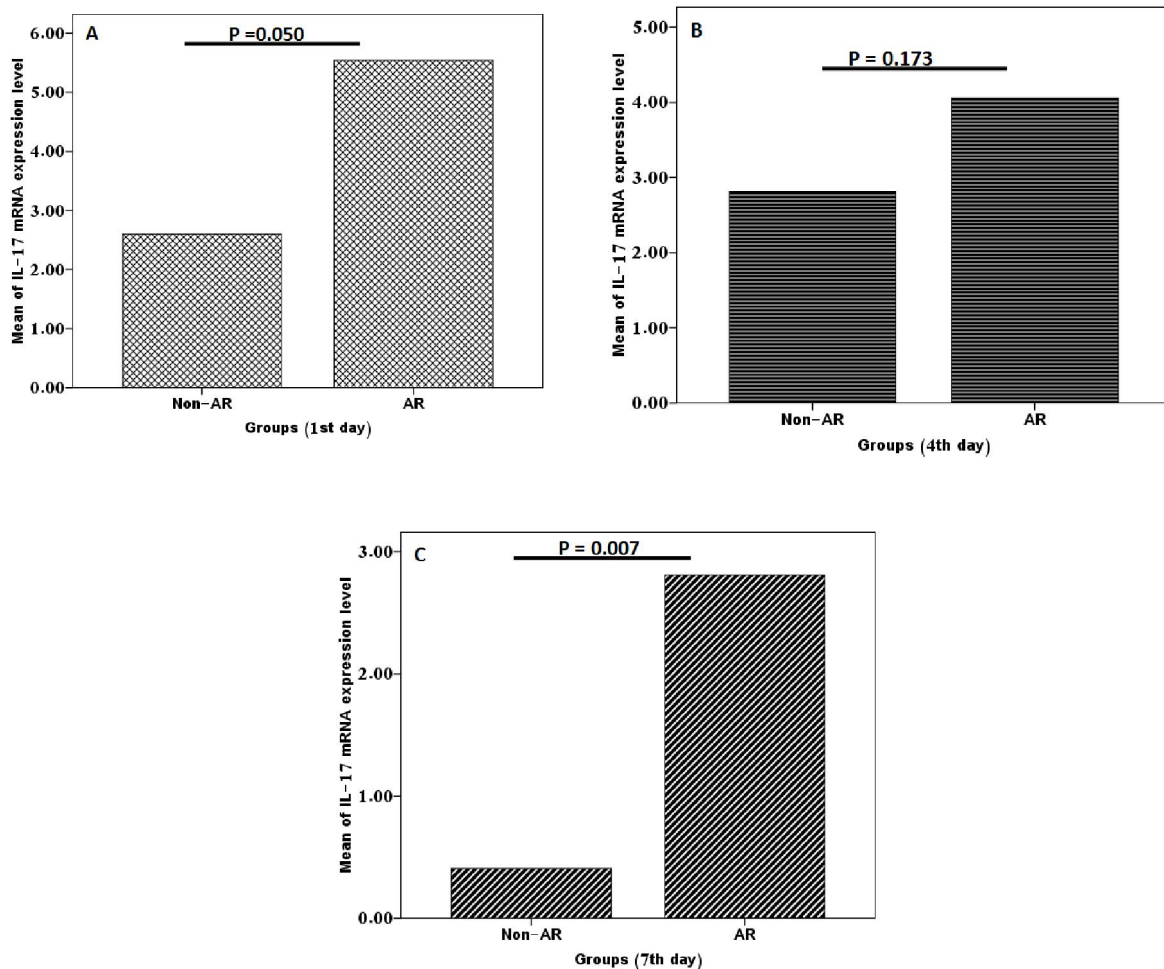


Figure 3. Comparing the IL-17 mRNA expression level* between Non-AR and AR patients. (A): IL-17 expression level in 1st day follow up, (B): IL-17 expression level in 4th day follow up, and (C): IL-17 expression level in 7th day follow up.

*: In figure 3, the increasing fold of IL-17 mRNA expression level is calculated using $2^{-\Delta\Delta Ct}$.

IL-17 Cytokine Level in Non-AR and AR Liver Transplant Patients. Significant increases were found in IL-17 serum level in AR patients compared with Non-AR and control ones during post-transplant follow-up [day's 1 ($p=0.001$), day's 4 ($p=0.001$), and day's 7 ($p=0.001$)] (Figure 4). Significant difference in cytokine levels of IL-17 in

controls compared with Non-AR and AR patients was presented as follows: in day1; 12 vs. 25 vs. 220 pg/ μ l, in day 4; 12 vs. 29 vs. 270 pg/ μ l and in day 7; 12 vs. 23 vs. 270 pg/ μ l, respectively. Significant associations were also found between the results of analysis of IL-17 mRNA and protein levels in both Non-AR and AR transplant patients, post-transplant follow-up.

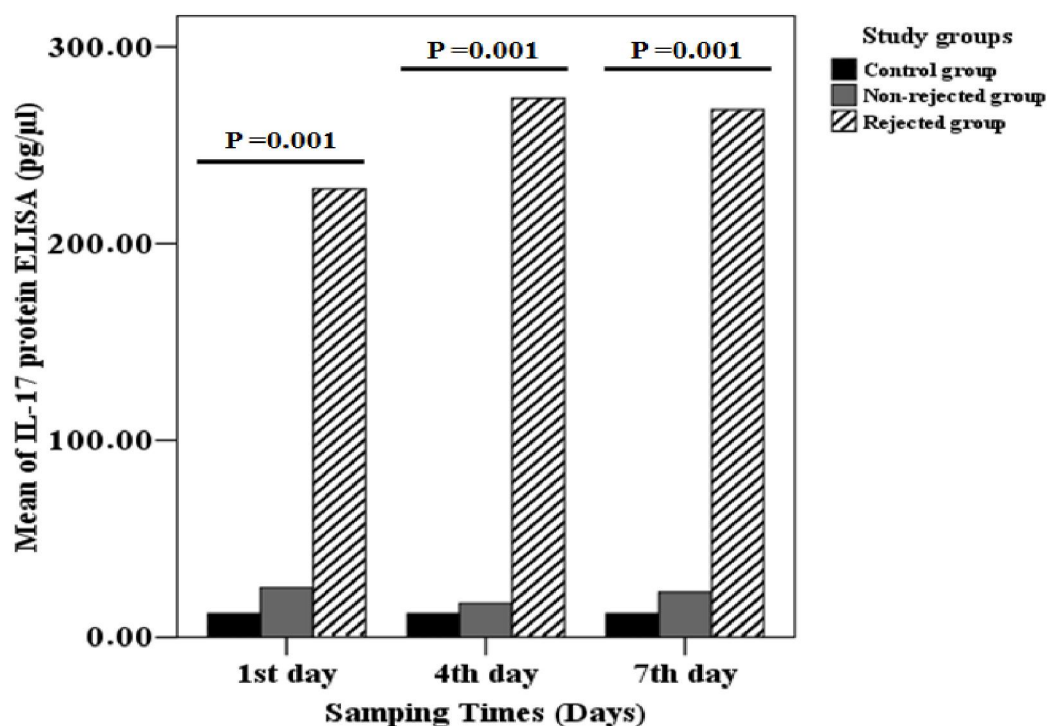


Figure 4. The comparison of IL-17 protein levels in AR and Non-AR patients and controls during post-liver transplant follow-ups.

IL-17 Gene Expression and Risk Factors. No significant association was found between risk factors including: age, gender, ABO blood grouping, and underlying diseases with the level of IL-17 gene expression in both Non-AR and AR liver transplant patients.

DISCUSSION

Acute rejection is still a common clinical complication early post-liver transplantation influenced by unsuccessful immunosuppressive therapy and inflammatory related disorders leading to infiltration of lymphocytes, macrophages and also eosinophils into the liver graft (4,9,21,22). Discovering Th17 which produces IL-17, open a new window in evaluation of how immune responses, host defenses, and pathogenesis of the inflammatory disorders are introduced and regulated in transplant patients (19).

Therefore, hopes to find answers to questions in elucidating the exact role of IL-17 in liver transplant outcomes, especially rejections, led to analyze the role of IL-17 in acute rejected and non-rejected liver transplant patients.

A research group claimed that, Th17 cells could potentially contribute to allograft rejection but they were not sure whether the presence of Th17 cells is a prerequisite for allograft rejection or not (4,16). But documents showed that IL-17 as a pluripotent cytokine had multiple proinflammatory functions including transplant rejection in humans (15). Studies demonstrated that up-regulation of IL-17 expression is strongly associated with many autoimmune and inflammatory liver diseases (23-25). Studies also showed that in autoimmune diseases in which IL-17 blocking agents was used, alleviation in pathology was observed (24).

Arguments derived from both experimental models and clinical studies suggest that alloreactive Th17 lymphocytes can mediate the graft damage during renal, cardiac, and lung allograft rejection (26,27). Therefore, it was intended that the percentage of Th17 cells was increased during acute allograft rejection and it had a relationship with the grade of rejection (34). In this study, IL-17 expression level was steadily increased in AR patients during post-transplant follow-up which may resulted in induction of the inflammatory factors related to pathogenesis of acute liver rejection.

Also, studies in murine and humans models of acute renal rejection showed an elevation in IL-17 mRNA levels and protein early post-transplantation (11,16). IL-17 has been also detected in the urine and tissue samples from patients laboring early acute renal rejection (28,29). Similar observations have been made in heart transplant patients (12-30). Furthermore, in lung transplant patients, increased pre- and post-transplant IL-17 levels represent a risk factor for acute rejection (24,31).

As the IL-17 is a proinflammatory cytokine which is not elevated except in inflammatory conditions, and based on the earlier studies on the time of increase and decrease of this cytokine (11,12,28), in this study sampling time was chosen to be done on days 1, 4 and 7 post-transplantation. IL-17 production in Non-AR studied patients showed an increase in day 4 which may deal with ischemic reperfusion injury outcomes which is common early post transplantation and decrease on day 7 that may relate to remove of possible inflammatory inducing factors. Similarly, some studies showed the elevation of IL-17 protein as early as second day after renal mice transplantation model. In these studies the increased expression of IL-17 protein and its role on infiltrating mononuclear cells on day 2 after experimental renal transplantation were confirmed (12,28). In a rat renal allograft model, IL-17 mRNA expression also appeared on postoperative day 2, peaking at day 5, then declined, becoming undetectable by day 9 (11).

In our studied AR group, the steady increase of IL-17 mRNA expression level also is due to remaining of the inflammatory inducers of acute rejection. Also significant increase of IL-17 mRNA expression level in AR group in comparison with Non-AR ones, shows the important role of IL-17 in introducing acute rejection in liver transplant patients. In the line of these results, rare evidences exist about the role of Th17 in liver transplantation outcomes especially acute graft rejection like Fabrega *et al.*, who showed possible correlation of serum level of IL-17 with transplant rejection (19). Finally, simultaneous increase of the IL-17 mRNA and protein levels emphasize on the IL-17 functional role in acute liver rejection. Similarly, association between Th17 and IL-17 with acute rejection in non-liver solid organ transplantations was also reconfirmed by limited investigations in liver transplant allograft rejections. Other

researchers also found an association between hepatic ischemia reperfusion injuries with up-regulation of Th17 cells in liver transplant patients (32,33). In rat model, Xie *et al.* showed the participation of Th17 cells in post-liver transplant rejection (1). These findings can help to better understand the mechanism of acute rejection which may improve management of acute liver rejection (22). By evaluation of other interfering factors such as infections, underline diseases, genetic disorders, malfunction of immune system, and immunosuppressive drugs which not focused in this study, can better declare the importance of IL-17 in acute rejection.

On the other hand, some risk factors may have influence the post liver transplant outcome. HLA is one of this risk factors affect the surveillance and clinical outcomes in transplant patients. However, between transplanted organs liver is an immune privileged organ and HLA compatibility between recipients and donors seems to have minor importance in acceptability of graft livers, emphasized in earlier reports (35). In this study also no significant association was found between studied risk factors with the level of IL-17 gene expression in Non-AR and AR liver transplant patients.

In Conclusion, based on these findings, significant increase of IL-17 mRNA and protein levels in AR liver transplant patients compared with Non-AR ones, re-enforce on the important role of IL-17 in acute liver rejection needs to be confirmed in further studies.

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