Egyptian Journal of Medical Microbiology Volume 29 / No.2 / April 2020 159-167 Online ISSN: 2537-0979

ORIGINAL ARTICLE

Role of circulating $CD4^+CD25^+FOXP\text{-}3^+$T-regulatory cells and FOXP-3 rs3761548 gene polymorphism in acute liver allograft rejection

[1]Enas M. Ghoneim, [2]Mabrouk M. Ghonaim, [2]Amal F. Makled, [3]Mohamed A. Altabbakh, [4]Mohamed Helwa, [1]Radwa H. Salem, [1]Samah M. Awad

[1]Department of Clinical Microbiology and Immunology, National Liver Institute, Menoufia University, Egypt
[2]Department of Medical Microbiology and Immunology, Faculty of Medicine, Menoufia University, Egypt
[3]Department of Hepatology, National Liver Institute, Menoufia University, Egypt
[4]Department of Clinical Pathology, Faculty of Medicine, Menoufia University, Egypt

Key words:
FOXP-3 polymorphism, Treg cells, graft rejection, liver transplantation

Corresponding Author:
Radwa Hussien Salem , Department of Clinical Microbiology and Immunology, National Liver Institute,
Menoufia University, Egypt.
Tel.: 01026654792
salem_radwa@yahoo.com

ABSTRACT

Background: *FOXP3 gene, is a major regulator of T-regulatory (Treg) cells that have a critical role in immune responses to alloantigens and acute cellular rejection (ACR) after liver transplantation (LT).* ***Objectives:*** *To investigate the role of FOXP-3 rs3761548 gene polymorphism on ACR after LT and to determine the relation between the circulating $CD4^+CD25^+FOXP\text{-}3^+$Treg cells, and infection on the severity of ACR.* ***Methodology:*** *Blood samples were obtained from 60 allograft liver recipients and 20 healthy volunteers. FOXP-3 (rs3761548) polymorphism was studied using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). Flowcytometry was used to enumerate circulating $CD4^+CD25^+FOXP\text{-}3^+$Treg cells in peripheral blood of liver transplant recipients.* ***Results:*** *Bacterial and candida infections were more significantly detected in liver transplant recipients with ACR. A significant predominance of the AA genotype and the A allele of FOXP3 gene (rs3761548) was found in liver transplant recipients with compared to those without ACR. Highly significant reduction in percentage and count of $CD4^+$and Treg cells were demonstrated in the liver transplant recipients with ACR. A significant negative correlation was detected between degree of ACR and percentage and count of Treg cells.* ***Conclusion:*** *Genetic polymorphism of FOXP-3 (rs3761548) and decreased percentage and count of Treg cells were associated with ACR. Carriers of the AA genotype and the A allele were more liable to severe degree of ACR after LT.*

INTRODUCTION

Liver transplantation (LT) is a standard therapy for patients with end-stage liver diseases such as acute liver failure, chronic liver disease with advanced cirrhosis, hepatocellular carcinoma, and liver-based metabolic defects[1]. Acute cellular rejection (ACR) occurs in 25-50% of all liver transplant recipients within the first year after transplantation and the first sign is usually elevated liver function test results[2]. Rejection is accurately diagnosed by fine needle liver biopsy to determine the degree of rejection based on Banff schema[3]. Infectious complications are major causes of morbidity and mortality after LT; bacterial infections are the most frequent predominating during the first two months post transplantation and affect patient and graft survival[4].

T-regulatory (Treg) cells, which express the transcription factor forkheadbox P3 (FOXP3), express specific markers $CD4^+$,$CD25^+$ and $FOXP\text{-}3^+$[5]. In transplantation, these cells play a role in the suppression of donor-activated effector T-cells, in tolerance induction and preferential accumulation of Treg cells in liver allografts[5]. Development and function of Treg cells are controlled by *FOXP3* gene, which acts on certain genes to regulate expression of cell surface molecules on Treg cells and inhibit transcription of inflammatory cytokines[6].

FOXP3 gene –3279C/A polymorphism may cause Treg dysfunction[5]. In addition, it may have a critical role in immune responses to alloantigens and may be associated with severity of alloimmune responses after organ transplantation[7].

The aim of this study is to determine the relation between Treg cells, *FOXP3 gene –3279C/A rs3761548* polymorphism; and severity of ACR in liver transplant recipients.

METHODOLOGY

Study participants:

The present study was carried out in the period from November 2017 to December 2019 in the Microbiology and Immunology Departments, Faculty of Medicine and National Liver Institute (NLI) in collaboration with Liver Transplantation Unit, NLI, Menoufia University. It included 60 liver transplant recipients who were classified into two groups: group I; 30 patients with ACR and group II; 30 patients without ACR. In addition, 20 age- and gender-matched apparently healthy volunteers served as a control group (group III). All participants in this study were subjected to full history taking and laboratory investigations. All transplant recipients received immunosuppressive drugs based on the NLI policy [combinations of tacrolimus or cyclosporine, steroids and mycophenolate mofetil (MMF)][8]. Classification of ACR regarding its severity was performed according to Banff Schema[3]. The study protocol was approved by Ethical Committee of Faculty of Medicine. Informed consents were obtained from all participants.

Microbial isolation and identification:

Clinical samples; blood (10 ml), urine, stool, ascitic fluid (10 ml), sputum and wound swabs were collected from the transplant recipients under aseptic technique during the first month after surgery. For blood and ascitic fluid samples; BacT/ALERT 3D Microbial Detection System was used (bioMérieux, Durham, N.C.). The growing microorganisms were identified by the standard microbiological methods and by VITEK2-Compact system (bioMerieux, France)[9,10].

Counting of the circulating $CD4^+CD25^+FOXP\text{-}3^+$T-regulatory cells by Flowcytometry:

For each sample (2 ml of EDTA-treated peripheral blood), two tubes were prepared; the first one for detection of $CD4^+CD25^+FOXP\text{-}3^+$Treg cells using anti-CD4-PerCP, anti-CD25-FITC and anti-FOXP3-PE (MACS, Miltenyi Biotec, USA). The second tube (autocontrol) was used to exclude the cell auto-fluorescence. Two ul of each monoclonal antibody were added to 100 ul of blood (with TLC adjusted to 5-10 x 10^3⁄mm³). Cells were washed by adding 1–2 mL of buffer and centrifuged at 300×g for 10 minutes, then cells were stained with anti-CD4-PerCP, anti-CD25-FITC according to the Manufacturer's recommendations. About 1–2 mL of buffer was added followed by centrifugation at 300×g for 10 minutes. Then, 100 µL of cold, freshly prepared fixation/permeabilization solution was added. After mixing, incubation was done in the dark at 2−8°C for 30 minutes. Then, 100 ul of the lysing solution and anti-FOXP3-PE were added and incubated for 10 minutes at room temperature followed by washing by 2 ml phosphate buffer saline azide. Cells were resuspended in 100 µL of sheath fluid solution and subjected to analysis by Flowcytometry (Becton Dickinson, San Jose, CA)[11]. (Fig. 1).

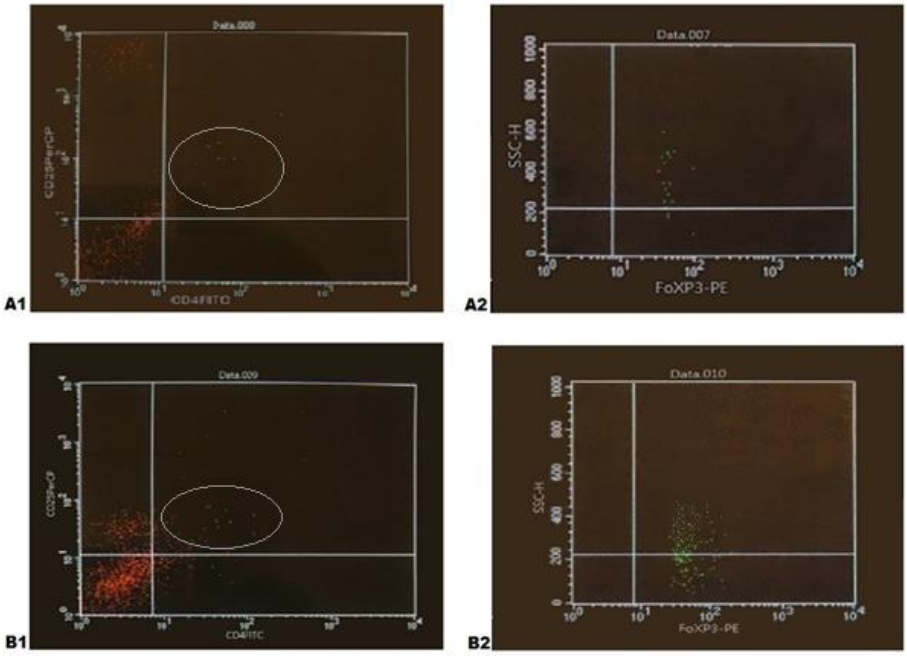

Fig.1: Flowcytometric analysis of $CD4^+CD25^+FOXP\text{-}3^+$Treg cells in liver transplant recipient with (A1, 2) and without (B1,2) ACR.

- CD4-FITC and CD25-PerCP double positive cells in the upper right quadrant (A1 and B1).
- Analysis of double positive cells for FOXP3-PE expression: the sum of upper and lower right quadrants (A2 and B2).

Detection of *FOXP-3 –3279 rs3761548 gene* polymorphism by PCR-RFLP:

Genomic DNA was extracted from 2 ml of EDTA-treated peripheral blood using QIAGEN DNA extraction kit according to the Manufacturer's instructions. PCR amplification was performed on thermal cycler (BioRad, USA Biometra) in a final volume of 25 µl containing: Master mix (12.5 µl) (MyTaq™ Red Mix (Bioline, UK), nuclease-free water (0.5 µl) and the extracted DNA (10 µl), and 1.0 µl (10 µmol/L) of: forward primer: 5'-GCCCTTGTCTACTCCACGCCTCT-3', reverse primer 5'-CAGCCTTCGCCATACAGAGCC-3'. The amplification steps were initial denaturation at 98˚C for 1 minute, followed by 35 cycles which consisted of; denaturation at 98˚C for 30 seconds, annealing at 67˚C for 30 seconds and extension at 72˚C for 1 minute, and then a final extension at 72˚C for 7 minutes. The amplification products were digested with a specific restriction enzyme (PstI) (Thermo Scientific™ Catalog number: ER0611) as follows: Nuclease-free water (6.5 µl), 2.5 µl 10X NEBuffer (New England BioLabs, Hitchin, UK), 1 µl PstI (10 U/µL) and 10 µl DNA amplification product were mixed and kept at 37˚C overnight. The 100 bp ladder and restriction products (10 µl) were loaded into appropriate wells. Electrophoresis on 2% agarose gel containing ethidium bromide was done. Bands were visualized under UV trans-illumination and photographed by a polaroid camera[5]. (Fig.2)

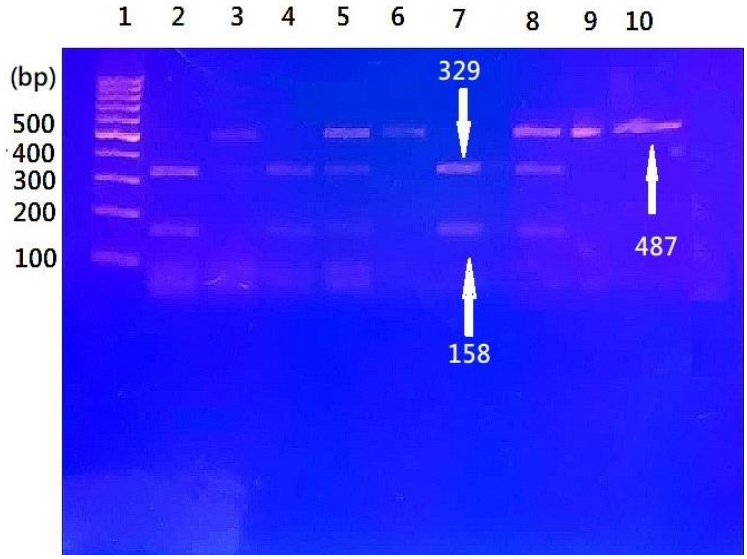

Fig.2: Agarose gel electrophoresis shows *FOXP3 gene –3279C/A rs3761548* polymorphism. Lane (1) 100 bp DNA ladder. Lanes (3, 6, 9 and 10) show AA homozygote genotype at (487 bp). Lanes (4 and 7) show CC homozygote genotype (158, 329 bp). Lanes (5 and 8) show A/C heterozygote genotype (158, 329 and 487 bp).

Statistical analysis:

Data were collected, tabulated, and statistically analyzed using an IBM personal computer with Statistical Package of Social Science (SPSS) version 22 (SPSS, Inc, Chicago, Illinois, USA) Chi-square, Student′s test, Kruskal-Wallis and correlation coefficient tests were done at 0.05% level of significance.

RESULTS

Clinical data and microbial infections in the studied patients:

Table (1); shows that the age of liver transplant recipients with severe ACR was younger than that of recipients with mild and moderate ACR. All liver transplant recipients with severe ACR and 92.9% with moderate ACR had severe symptoms of liver affection. However, there was no difference regarding other demographic data.

The types of infections are shown in table (2). Bacterial infections were detected in 76.7% of transplant recipients with ACR and in 46.7% of those without ACR. The commonest infection types were chest (30%) followed by blood stream (26.6%) and the predominant organism was *Klebsiella pneumoniae* (22.5%). Bacterial and fungal co-infections were present among 26.7% and 16.7% of transplant recipients with and without ACR respectively. However, there was no significant difference between the two groups.

Table 1: Degree of ACR in relation to demographic data among liver transplant recipients with ACR

Studied variables	Degree of ACR						Test of significance.	P value
	Mild (N=12)		Moderate (N=14)		Severe (N=4)			
	No.	%	No.	%	No.	%		
Age (years) **Mean ±SD** **Range**	52.1±6.91 38 – 60		46.4±7.37 35 - 58		28.7±7.13 23 - 39		*K= 11.6	**0.003*****
Sex							**X2= 0.742	0.690
Male	10	83.3	12	85.7	4	100		
Female	2	16.7	2	14.3	0	0.00		
Donor degree								
- First	4	33.3	4	28.6	3	75.0	X2= 4.06	0.397
- Second	4	33.3	7	50.0	1	25.0		
- Third	4	33.3	3	21.4	0	0.00		
Clinical presentation								
- No symptoms	0	0.00	0	0.00	0	0.00		
- Fatigue and itching	6	50.0	0	0.00	0	0.00	**X2= 13.1**	**0.007*****
- Fever and abdominal pain	3	25.0	1	7.10	0	0.00		
- Fever, jaundice, abdominal pain and ascitis	3	25.0	13	92.9	4	100		
Immunosuppressive regimen								
- Tacrolimus+ steroids	4	33.3	1	7.10	0	0.00		
- Cyclosporine+ steroids	4	33.3	6	42.9	1	25.0	**X2=**	0.511
- Tacrolimus+ steroids +MMF****	1	8.30	3	21.4	1	25.0	5.26	
- Cyclosporine+ steroids +MMF	3	25.0	4	28.9	2	50.0		

K:Kruskal-Wallis test** *****X2:Chi squared test *****P <0.01=highly significant** *******Mycophenolate mofetil***

Age was significantly younger in patients with severe compared to mild or moderate degrees of ACR.

Table 2: Types and sites of infections among the liver transplant recipients

Studied variables	(GI) Recipients with ACR (N=30)		(GII) Recipients without ACR (N=30)		Test of significance **X2	P value
	No.	%	No.	%		
Infection						
Present	23	76.7	14	46.7	5.71	**0.016***
Absent	7	23.3	16	53.3		
Type of bacterial infection	**N=23**		**N=14**			
- Single	11	47.8	9	64.3	0.95	0.329
- Multiple	12	52.2	5	35.7		
Site of infection	**N=30**		**N=18**			
- Blood stream	8	26.7	5	27.8		
- Surgical site	6	20.0	4	22.2		
- Chest	9	30.0	5	27.8	2.38	0.665
- Intra-abdominal	3	10.0	0	0.00		
- Urinary tract	4	13.3	4	22.2		
Isolated organisms	**N=40**		**N=22**			
Gram-ve bacteria	**22**	**55.0**	**11**	**50.0**		
- *Klebsiella pneumoniae*	9	22.5	4	18.2		
- *E. coli*	5	12.5	2	9.10	0.20	0.904
- *Pseudomonas aeruginosa*	4	10.0	2	9.10		
- *Acinetobacter baumannii*	4	10.0	3	13.6		
Gram+ve bacteria	**9**	**22.5**	**6**	**27.3**		
- *Staph. aureus*	6	15.0	4	18.2		
- *Staph. epidermidis*	3	7.50	2	9.10		
Fungal infection (*candida albicans*)	**9**	**22.5**	5	**22.7**		
Bacterial and fungal coinfection						
Present	8	26.7	5	16.7	0.884	0.347
Absent	22	73.3	25	83.3		

P<0.05=significant** *****X2:Chi squared test

Number and site of infections exceed the actual number of cases because of multiple infection sites and co-infection with more than one organism in the same patient.

CD4$^+$cells and Treg cells among the studied groups:

The percentage and count of both CD4$^+$and Treg cells were significantly (P<0.001) lower in liver transplant recipients with compared to those without ACR. They were also significantly (P=0.001) lower in liver transplant recipients than healthy volunteers (table 3). Negative correlations were found between the degree of rejection and the count and percentage of CD4$^+$and Treg cells. (table 4).

Table 3: CD4$^+$and Treg cells among the studied groups.

Studied variables	(GI) Recipients with ACR (N=30)	(GII) Recipients without ACR (N=30)	(GIII) Normal controls (N=20)	Test of significance **K	Post hoc test
CD4$^+$cells					
-Percentage Mean ±SD	9.13±6.39	20.9±10.4	42.1±7.52	75.0 **P=0.001***	**P1:0.001*** **P2: 0.001*** **P3: 0.001***
-Count Mean ±SD	168.1±20.1	306.9±181.9	931.5±198.2	75.7 **P=0.001***	**P1: 0.001*** **P2: 0.001*** **P3: 0.001***
Treg cells					
-Percentage Mean ±SD	2.41±1.29	5.27±4.43	3.55±2.77	20.2 **P=0.001***	**P1:0.002*** **P2: 0.001*** **P3 0.001***
-Count Mean ±SD	37.1±30.1	72.4±55.9	48.9±25.2	30.5 **P=0.001***	**P1:0.008*** **P2: 0.001*** **P3:0.002***

P<0.01=Highly significant** ****K:Kruskal Wallis test

P1: Group I vs. group II, **P2:** Group I vs. group III, **P3**: Group II vs. group III.

- The normal range of CD4$^+$cells percentage and count are 25-60% and 600-1200/cmm respectively.
- The normal range of Treg cells percentage and count are 1-6% and 60-150/cmm respectively.

Significant differences were detected in the count and percentage of both CD4$^+$and Treg cells among the 3 studied groups.

Infections were more significantly (P<0.05) detected among patients with compared to those without ACR. However, there was no difference regarding the site or type of the detected organisms.

Table (4): Correlation between the degree of ACR and percentage and count of CD4$^+$and Treg cells

Studied variables	Degree of ACR (GI)	
	R	P value
CD4$^+$cells		
-Percentage	-0.499	**0.005****
- Count	-0.443	**0.014***
Treg cells		
-Percentage	-0.434	**0.017***
- Count	-0.408	**0.025***

***P<0.05=Significant** ****P<0.01=Highly significant**

There were significant negative correlations between the degree of ACR, and percentage and count of CD4$^+$and Treg cells.

FOXP3 gene polymorphism:

The *FOXP3 gene –3279C/A rs3761548* genotypes and gene alleles in the studied groups are presented in table (5) and Fig.(2). The frequency of the AA genotype and the A allele were significantly higher (P<0.05) in liver transplant recipients with ACR (66.7% and 71.7% respectively) than those without ACR, while the CC genotype and the C allele were more significantly predominant (P=0.001) in healthy volunteers (70% and 72.5% respectively). Similarly, the presence of the AA genotype and the A allele were more significantly (P<0.01) related to severity of ACR (table 5). Gene polymorphism was the only predictor for transplant rejection among the liver transplant recipients; presences of the AA genotype increased the risk for ACR 3.43 times more than the AC genotype and 10 times more than the CC genotype (P<0.05) (table 6).

Table 5: The *FOXP3 gene –3279C/A rs3761548* polymorphism and gene alleles among the studied groups and their relation to the degree of ACR.

Studied variables	(GI) Recipients with ACR (N=30)		(GII) Recipients without ACR (N=30)		(GIII) Normal controls (N=20)		Test of significance ***X2	Post hoc test
	No.	%	No.	%	No.	%		
***FOXP3* genotype**								
AA	20	66.7	9	30.0	5	25.0	8.11	**P1:0.017***
AC	3	10.0	7	23.3	1	5.00	10.5	**P2:0.004****
CC	7	23.3	14	46.7	14	70.0	0.93	P3:0.149
***FOXP3* alleles**								
A	43	71.7	25	41.7	11	27.5	11.0	**P1:0.001****
C	17	28.3	35	58.3	29	72.5	16.8	**P2:0.001****
							1.40	P3:0.148
	Degree of ACR (GI)						***X2	**Post hoc test**
	Mild (N=12)		**Moderate (N=14)**		**Severe (N=4)**			
	No.	**%**	**No.**	**%**	**No.**	**%**		
***FOXP3* genotype**								
AA	4	33.3	12	85.7	4	100	11.2	**P4:0.003****
AC	1	8.30	2	14.3	0	0.00	5.33	P5:0.069
CC	7	58.4	0	0.00	0	0.00	0.64	P6:0.422
***FOXP3* alleles**	**N=24**		**N=28**		**N=8**			
A	9	37.5	26	92.9	8	100	18.0	**P4:0.001****
C	15	62.5	2	7.10	0	0.00	9.41	**P5:0.002****
							0.61	P6:0.436

P<0.05=Significant** ****P<0.01=Highly significant** *****X2:Chi squared test

P1: Group I vs Group II, **P2:** Group I vs Group III, **P3:** Group II vs Group III, **P4:** mild vs moderate ACR**,**
P5: mild vs severe ACR, **P6:** moderate vs severe ACR.

Table 6: Predictable factors for ACR among liver transplant recipients.

Predictors	OR	Wald	P value	CI 95%
Age (years)	0.022	0.262	0.609	(0.093– 1.11)
Infection (bacterial and /or fungal)	0.300	0.182	0.670	(0.341 – 5.34)
$CD4^+$cells				
-Percentage	1.61	3.56	0.059	(0.982 – 2.64)
-Count	1.02	3.75	0.053	(1.00 – 1.04)
Treg cells				
-Percentage	2.33	0.875	0.350	(0.395 – 1.76)
-Count	0.929	0.671	0.413	(0.780 – 1.10)
***FOXP3 gene –3279C/A* rs3761548 genotype**				
(AA versus AC)	3.43	4.15	**0.041***	(1.04 – 11.2)
(AA versus CC)	10.0	6.62	**0.010****	(1.73 – 57.7)

***P<0.05=Significant** ****P<0.01=Highly significant**

Binary logistic regression analysis was used.

Gene polymorphism (the AA genotype) was the only significant predictor factor for ACR.

DISCUSSION

Graft rejection is one of the major immunological complications following LT. It is elicited by genetic disparity between the donor and the recipient and is due to T-cell mediated or antibody-mediated immune response[12]. Treg cells play an important role in T-cell homeostasis and regulation of immune tolerance in LT through suppression of donor-activated effector T-cells[13]. *FOXP3 gene* is a major regulatory gene for development and function of Treg cells[13]. Polymorphism of this gene may have a role in ACR and post-transplant graft function [14].

In the present study, most of the transplant recipients (80%) were males, a finding which was previously reported [8, 15]. Men usually have higher risks of end-stage liver diseases which require LT. Moreover, 40.4 % of our liver transplant recipients with mild and moderate ACR received tacrolimus and steroids, while none of the recipients with severe rejection received tacrolimus and steroids. ACR was reported to be more common with cyclosporine as compared to tacrolimus[16]. Increased immune reactivity and metabolism of immunosuppressive drugs are more frequent in younger ages[17]. This study and that of others[12] found that younger recipients had higher risk for ACR. However, this finding was not reported by other investigators [5].

The NLI follows the Egyptian law which allows a living-related LT from a first-, second-or third-degree relatives[18]. There was no significant difference between these three donor types on ACR. Our patients with mild ACR had fever while patients with severe ACR had multiple clinical features (jaundice, abdominal swelling and tenderness, and ascites), a finding which was similarly reported[2]. However, no symptoms were associated with ACR in another study[16].

Although infection can occur at any time after LT, the highest incidence is during the first postoperative month due to mucocutaneous barrier alteration, use of invasive devices, immunosuppression and staying longer than 48 hours in ICU[19]. In this study, infection occurred among 76.7% and 46.7% of transplant recipients with and without ACR respectively during the first month after LT. Therefore, infection may affect the graft survival[4].

In the current study, *Klebsiella pneumoniae, Acinetobacter baumannii, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus and Staph. epidermidis* were isolated from 22.5%, 10%, 12.5%, 10%, 15% and 7.5% of transplant recipients with ACR respectively. However, lower isolation rates of *Klebsiella pneumoniae* and *Escherichia coli* (5.5% and 7% respectively)[20], and higher rates of *Klebsiella spp., Enterobacter spp.* and *E. coli* (42.6%, 30.8%, and 23.1% respectively) were reported[18]. In another study, the most common causative organism of post-operative infection was *E. coli* (77%), followed by *Pseudomonas* (9%), *Klebsiella* (8%), *Staphylococcus aureus* (4.5%) and *Enterobacter* (1.5%)[21]. The most frequent fungal infection encountered after LT is *Candida albicans*[22] which was also found in 22% of our patients.

This study showed that the percentage and count of $CD4^+$and Treg cells were significantly lower in the transplant recipients with ACR as compared to the other studied groups ($P<0.05$), a finding which was previously reported[5]. Moreover, there was a significant negative correlation between the degree of ACR, and percentage and count of $CD4^+$and Treg cells. Germani *et al.*,[23] showed that the frequency of the circulating $CD4^+CD25^+FOXP\text{-}3^+T$ cells was negatively correlated with rejection activity index (RAI) for grading severity of ACR. An interesting finding in this study is that the percentage and count of circulating Treg cells were significantly ($P<0.05$) higher in transplant recipients without ACR compared to patients with ACR and healthy volunteers. Wang *et al.*,[24]demonstrated an increased intragraft presence of Treg cells (histopathologically) during ACR, and they concluded that these cells were mobilized from the peripheral circulation to the immune activation site in order to regulate alloreactive responses during ACR.

The role of *FOXP3 gene –3279C/A* polymorphism in ACR after LT was analyzed in the current study. The AA genotype (66.7%) and the A allele (71.7%) were significantly more predominant in the transplant recipients with ACR. On the other hand, the CC genotype (70%) and the C allele (72.5%) were significantly more predominant in healthy volunteers as compared to transplant recipients with ACR (23.3% and 28.3% respectively). Thude *et al*,[25] reported that the frequency of the genotype −3279 AA was 41% and 25% in transplant recipients with and without ACR respectively. Similarly, the −3279 A allele was more frequent in the transplant recipients with (50%) than in those without ACR (35%). Khuja *et al.*,[13]revealed that ACR frequency was 17.6% and 40.9% in the transplant recipients with rs3761548 AC and AA genotypes respectively while rs3761548 CC was not detected in their study. Furthermore, it was found that the *FOXP3 rs3761548* AA genotype carriers were associated with a fourfold increased risk for ACR compared to those with the CC genotype[26].

The AA genotype and the A allele were the only significant predictors of ACR in our patients. This result may suggest that the A allele of *FOXP3 gene* may be implicated in decreased number of Treg cells and ACR. The −3279 A allele of rs3761548 was demonstrated to alter the consensus sequence "GGGCGG" for the zinc finger transcription factor specificity protein 1 (SP1) which is located in an upstream enhancer element of the *FOXP3 gene*. Binding of SP1 to the consensus sequence induces strong and stable transcription of the *FOXP3*

gene. This binding may be impaired by the −3279 A allele, reducing transcription and consequently expression of the FOXP3[26]**.** The A allele may alter the E47 and c-Myb transcription factor binding sites, leading to modifications in the gene expression[27]**.** However, another study revealed that *FOXP3 rs3761548* polymorphisms were not correlated with incidence of ACR and suggested that medications or other pathophysiological conditions may mask the effect of the *FOXP3 rs3761548* polymorphisms on ACR[28]**.**

The *FOXP3 gene rs3761548* AA genotype and the A allele in liver transplant recipients were important risk factors for ACR. Decreased circulating Treg cells and occurrence of infection in transplant recipients may have a role in ACR. However, other studies which include adequate numbers of patients are required to support our findings.

Funding: No funding sources.

Authors' Contributions: All authors contributed to conception, design, analysis of data and the manuscript writing.

Conflicts of interest: The authors declare that they have no financial or non-financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

REFERENCES

1. Yang JD, Larson JJ, Watt KD, Allen AM and Wiesner RH. Hepatocellular carcinoma is the most common indication for liver transplantation and placement on the waitlist in the United States. Clin Gastroenterol Hepatol (2017)15:767-775.
2. Panackel C, Ganjoo N, Saif R and Jacob M. Decompensated liver disease: liver transplantation is the best option. not the last option. IMA Kerala Med J (2016) 9 (1):17.
3. Demetris AJ, Bellamy C, Hübscher SG, O'Leary J and Randhawa PS. Comprehensive update of the Banff working group on liver allograft pathology: Introduction of antibody-mediated rejection. Am J Transplant (2016) 16(10):2816-2835.
4. Kim SI. Bacterial infection after liver transplantation. WJG 20th Anniversary Special Issues (7): Liver transplant. World J Gastroenterol (2014) 20(20): 6211-6220
5. Verma S, Tanaka Y, Shimizu S, Das L and Ohdan H. Significant association between *FOXP3 gene* polymorphism and steroid-resistant acute rejection in living donor liver transplantation. Hepatol Commun (2017) 1(5):406-420.
6. Li Z, Li D, Tsun A and Li B. FOXP3$^+$regulatory T cells and their functional regulation. Cell Mol Immunol (2015) 12(5):558–565.
7. Qiu XY, Jiao Z, Zhang M, and Chen JP. Genetic association of *FOXP3 gene* polymorphisms with allograft rejection in renal transplant patients. Nephrology (2012) 17(4):423-30.
8. Gad EH, Alsebaey A, Lotfy M, Eltabbakh M and Sherif AA. Complications and mortality after adult to adult living donor liver transplantation: A retrospective cohort study. Ann Med Surg (Lond) (2015) 4(2):162–171.
9. Tille MP. Baily and Scott's Diagnostic Microbiology. Gram –ve bacilli and coccobacilli (MacConkey positive, oxidase negative) Acinetobacter, Stenotrophomonas and similar organisms. Philadelphia: Elsevier press (2014) (13): 329- 335.
10. Winn W, Allen S, Janda W, Koneman E and Procop G. Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th Edition, Lippincott Williams and Wilkins (2006), New York.
11. Kjeldsberg C, Johnson KE and Hussong J. Flowcytometry molecular diagnosis, fluorescent in-situ hybridization and cytogenetics. (2006) Chap. 48: 861-885.
12. Zhu JH, Hussaini T, Erb SR, Marquez V and Yoshida EM. Medical complications of liver transplantation. AME Med J (2018) 3(1):2520-0518.
13. Żabińska M, Krajewska M and Kościelska-Kasprzak K. CD4$^+$CD25$^+$CD127$^-$ and CD4$^+$CD25$^+$Foxp3$^+$ regulatory T Cell subsets in mediating autoimmune reactivity in systemic lupus erythematosus patients. Arch Immunol Ther Exp (2016) 64(5): 399–407.
14. Khuja Z, Parry M, Rasool R and Reshi AR. Association of Forkheadbox P3 gene polymorphisms with allograft rejection episodes in kidney transplant patients: a study from Kashmir, North India. Iran J Kidney Dis (2018)12(5):305-311.
15. Wen PH, Lu CL, Strong C, Lin YJ, Chen YL, Li CY and Tsai CC. Demographic and urbanization disparities of liver transplantation in Taiwan. Int J Environ Res Public Health (2018) 15(2): 177.
16. Choudhary NS, Saigal S, Bansal RK and Saraf N. Acute and chronic rejection after liver transplantation: What a clinician needs to know. J Clin Exp Hepatol (2017) 7(4):358-366.

17. George JF, Pamboukian SV, Tallaj JA, Naftel DC and Myers SL. Balancing rejection and infection with respect to age, race, and gender: Clues acquired from 17 years of cardiac transplantation data. J Heart Lung Transplant (2010) 29:966 –72.
18. Amer KE and Marwan I. Living donor liver transplantation in Egypt. HepatoBiliary Surg Nutr (2016) 5(2):98–106.
19. Hernandez MDP, Paul Martin P, and Jacques Simkins. Infectious complications after liver transplantation. Gastroenterol Hepatol (2015) 11(11): 741-753.
20. Santoro-Lopes G and de Gouvêa EF. Multidrug-resistant bacterial infections after liver transplantation: an ever-growing challenge. World J Gastroenterol. (2014) 20(20):6201-6210.
21. Islam MS, Yusuf MA, Islam MB, and Jahan WA. Frequency of ESBL in surgical site infection at a tertiary care hospital. JC Adv Med Res (2014)1(2): 25-29.
22. Silveira FP and Kusne S. Candida infections in solid organ transplantation. Am J Transplant. (2013)13(4):220-227.
23. Germani G, Rodriguez-Castro K, Russo FP Marco and Senzolo M. Markers of acute rejection and graft acceptance in liver transplantation. World J Gastroenterol (2015) 21(4): 1061-1068.
24. Wang Y, Zhang M, Liu Z-W, Ren W-G and Shi Y-C The ratio of circulating regulatory T cells (Tregs)/Th17 cells is associated with acute allograft rejection in liver transplantation. PLoS ONE (2014) 9(11): 112-135.
25. Thude H, Tiede P, Sterneck M and Nashan B. Impact of TBX21, GATA3, and *FOXP3 gene* polymorphisms on acute cellular rejection after liver transplantation HLA (2019) 93:97–101.
26. Misra MK, Mishra A, Pandey SK and Kapoor R. Association of functional genetic variants of transcription factor Forkhead box P3 and nuclear factor-kappaB with end-stage renal disease and renal allograft outcome. Gene (2016) 581(1):57–65.
27. Pereira LMS, Amoras ESG, da Silva Conde SRS and Demachki S. The −3279C>A and −924A>G polymorphisms in the *FOXP3 gene* are associated with viral load and liver enzyme levels in patients with chronic viral liver diseases. Front. Immunol (2018) 9:2014.
28. Wu Z, Xu Q, Qiu X, Jiao Z, Zhang M and Zhong M. *FOXP3 rs3761548* polymorphism is associated with tacrolimus-induced acute nephrotoxicity in renal transplant patients. Eur J Clin Pharmacol (2017) 73:39–47.