Int J Clin Exp Pathol 2015;8(3):2603-2614
www.ijcep.com /ISSN:1936-2625/IJCEP0002307

Original Article

Immunity of fungal infections alleviated graft reject in liver transplantation compared with non-fungus recipients

Tonghai Xing[1], Lin Zhong[1], Lihui Lin[2], Guoqiang Qiu[1], Zhihai Peng[1]

[1]Department of General Surgery, Shanghai First People's Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200080, China; [2]Department of Laboratory Medicine, Shanghai First People's Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200080, China

Received September 4, 2014; Accepted October 18, 2014; Epub March 1, 2015; Published March 15, 2015

Abstract: Objective: To evaluate of the immune tolerance in adult LT recipients with Invasive fungal infections (IFIs). Methods: 109 consecutive LT recipients who received LT were included. Percentage of T subsets (CD4 + CD25hiCD127-T cells, CD4 + CD25loCD45RA + T cells, CD4 + CD25loCD45RA- and CD4 + CD45RA-CD45RO + T cells populations), levels of cytokines (IL-1b, IL-2, IL-4, IL-6, IL-8, IL-10, IFN-γ, IL-12p70, IL-17, TNF-α, TNF-β and GM-CSF) were detected by FACS and Bioplex in peripheral blood. Biopsy specimens were fixed, monoclonal antibodies against CD4, Foxp3 and IL-17 were applied to the above sections and FISH was performed. Results: The risk of acute rejection was decreased in fungal infected liver transplant recipients comparing with non-fungal infected group. CD4 + CD25hiCD127T cell population was increased in peripheral blood and memory CD4 + CD45RA-CD45RO + T cell population decreased. There was significant lower levels observed in naïve CD4 + CD25loCD45RA + and CD4 + CD25loCD45RA- T cell populations in fungal infected liver transplant. Moreover, IL-2, IL-6, IL-10 and GM-CSF were decreased. However, no significant difference with IL-4 and IL-8 in serum in two infected LT recipients. Conclusion: The incidence of graft rejection in liver transplantation recipients with fungal infections was lower than the non-fungal group. It is important to assess the risk during pretransplant and postoperation for liver transplantation.

Keywords: Graft rejection, fungal infections, T cell subsets, cytokines, liver transplantation

Introduction

Invasive fungal infections (IFIs) which most commonly occur after orthotopic liver transplantation (LT) are major causes of morbidity and mortality among patients undergoing LT. Although surgical techniques and immunosuppressive regimens have evolved to reduce mechanical complications and rejection episodes in liver transplant recipients (LTRs), IFIs occur in up to 42% of patients after LT with the absence of antifungal agents [1, 2]. Due to the better outcomes with recent years in the literature, it is possibly related to the availability of newer antifungal agents and earlier detection [3].

Antifungal prophylaxis has not indicated a beneficial effect on patient or graft survival [4]. However, it has been associated with increased mortality in patients with developing IFIs [5]. Thus, many treatments have confined antifungal prophylaxis to patients with specific risk factors.

Study had shown previously that suppression of the immune function may lead to a susceptibility to fungal infection, partially due to the increased regulatory T-cells expression [6]. However, the recipient risk assessments of fungal infection have not been evaluated undergoing LT. Prophylactic strategies for antifungal prophylaxis have been contemplated for patients with increased risk of LT [7]. In current study, we collected 109 subjects with IFIs after LT to further define the pathogens and determine their incidence, associated mortality, and risk factors of IFI while considering the risk of allograft rejection. The aim of this study was to elicit the application of a cell immune function

assay in monitoring immune status, and to assess trends in incidence, thus evaluating the development of fungal infections under immunosuppression.

Materials and methods

Study population

We conducted chart review retrospectively for all subjects who were deceased donor of LT from January 2010 to June 2012 at Shanghai First People's Hospital Affiliated to Shanghai Jiao Tong University, a 1586-bed tertiary-care institute in China that specializes in organ transplantation. This study was approved by the institutional review board of the Shanghai Jiao Tong University and was performed in accordance with the Declaration of Helsinki. All LT recipients were evaluated using UNOS MELD scoring system. LTs were accomplished using cadaveric livers and orthotopic liver transplants (OLT) which had end-to-end biliary anastomosis constructed without T-tube drainage [8].

All subjects with less than 18 years of age, dead within 1 week of LT, combined liver/kidney transplant or retransplants and subjects without hospital records or follow-up were excluded. All patients received oral nystatin for mucosal fungal decolonization. Data collected from hospital chart for each liver transplantation recipient included perioperative demographic and clinical characteristics (i.e., age, gender, and Child-Pugh), operative variables (i.e., operation time, blood loss) and clinical events post-transplant (i.e., duration of initial intubation, intensive care unit [ICU] stay, reoperation, dialysis, and rejection).

For the risk factor analysis, control subjects were recruited from the same institution and LT recipients. All LT recipients were divided into 2 groups: non-fungal infected control group including bacterial infection or without any infection (NFI); and invasive fungal infection group (IFI).

IFIs were defined according to the criteria proposed by the European Organization for Research and Treatment of Cancer and the Mycoses Study Group [9]. Briefly, this included histopathological evidence of a fungal infection or a positive culture from a normally sterile site, including blood, cerebrospinal fluid, and peritoneal fluid (not including indwelling drainage catheters). A central venous catheter associated bloodstream infection was identified if *Candida* was grown from blood obtained from a central venous catheter and at least 1 coincident peripherally obtained blood culture and no other source could be identified. *Candida* grown from sputum, the oral cavity, urine, or skin was defined as fungal colonization and was not included in the definition of IFI. Mold infections were defined as proven (consistent histopathological results or a positive culture from tissue obtained by an invasive procedure or autopsy) or probable (a positive sputum culture with compatible radiographic findings such as pulmonary infiltrates or new pulmonary nodules). If there was only one major clinical criterion in a patient without any other clear diagnosis, but being treated effectively for antifungal therapy, patients are considered to have 'possible IFI'. Patients with definite, probable or possible IFI were diagnosed as having a fungal infection in this study. Antifungal susceptibility tests were performed routinely onward for isolating from blood or sterile sites. Death was considered to relate to IFI if the patient had positive cultures from blood or any other normally sterile site within 48 h of expiration. Postmortem evidence of IFI was used to confirm the relationship to death.

Specimens and isolates

Infection of patients was confirmed by a single culture after observing clinical signs of infection (e.g., chills, fever, hypotension or by imaging such as CT or chest X-ray) or isolation of a microorganism in two consecutive cultures associated with signs of infection. Specimens were taken from corresponding infected sites for bacterial species identification. Multiple samples from the same patient were taken at different time points [10]. Species identification for the bacterium was performed using the VITEK 2 System (bioMe´rieux, France) for rapid microbial detection. Antimicrobial susceptibility was determined by the minimal inhibitory concentration (MIC) agar dilution method according to recommendations of the Clinical and Laboratory Standards Institute (CLSI). Regular quality control was performed using the following American Type Culture Collection (ATCC) strains: *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 [11].

All fungal infection episodes occurring within one year of liver transplantation were described regarding the aetiological agent and infection site. Fungal infection was considered "early" with ≤ 90 days after transplantation and "late" with > 90 days after transplantation.

Immunosuppressive and rejection therapy

Immunosuppression characteristically consisted of a 3-drug combination of corticosteroids (methylprednisolone), tacrolimus, and mycophenolate mofetil initiated on the day of transplantation. All patients received methylprednisolone 500 mg as a single intravenous dose before reperfusion during the transplantation procedure, then received 2 doses of intravenous basiliximab 20 mg with the first dose at 6 hours after reperfusion and a second dose on postoperative day 4. Acute rejection episodes were diagnosed by patients' clinical presentations, serum biochemical results, and liver biopsy. All acute rejections were verified by liver biopsy, and confirmed using the criteria of the fifth Banff Consensus conference. Rejection episodes were mainly treated with methylprednisolone and increasing FK506 blood concentrations. Postoperative anti-hepatitis B virus protocol included administration of lamivudine plus low-dose intramuscular HBV immunoglobulin therapy.

Prophylactic anti-infective treatment

Most patients received caspofungin intravenously at 50 mg daily (after a 70-mg loading dose of caspofungin on day 1). The daily dose of caspofungin was reduced from 50 to 35 mg in patients with moderate hepatic insufficiency (defined as a Child-Pugh score of 7-9) at onset of study therapy or during caspofungin administration [12].

Flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated by Histopaque (Sigma-Aldrich, UK) density centrifugation then separated through a 50% percoll (Sigma-Aldrich, UK) gradient (30 min at 300 g). Surface antigen expression was performed with the FC500 Flow cytometry and analyzed by CXP software (Beckman Coulter). Fluorochrome-conjugated anti-human mAbs were anti-CD3-FITC clone, anti-CD4-PE-Cy5 clone, anti-CD45RA-FITC clone, anti-CD45RO-PE clone, anti-CD25-FITC clone, anti-CD127-PE clone, anti-CD28-FITC clone (Beckman Coulter). Purified PBMCs were pelleted then resuspended in 200 μL of FACS buffer (PBS containing 2% FCS, 2 mM EDTA, and 0.05% NaN3), stained on ice with fluorescent antibodies for 30 minutes, washed with FACS buffer and then fixed with 4% parafomaldehyde in PBS. Appropriate isotype control antibodies were used to assess the level of specific labelling. CD4 + CD127lo/- CD25high subpopulation were defined as Treg cells. The naïve and memory T-cell subsets were analyzed by CD45RA + andCD45RO + respectively [13].

Bioplex

Bio-Plex human cytokine multi-plex kits and Bio-Plex cytokine reagent kits were purchased from Bio-Rad Laboratories, CA, USA. The supernatants were analyzed simultaneously for 12 cytokines, including IL-1b, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-17, TNF-α, IFN-g, TNF-β and GM-CSF with a Bio-Plex machine, which employed a bead-based sandwich immunoassay [14]. For the detection of multiple cytokines, a monoclonal antibody specific for each cytokine was coupled to a particular set of beads with known internal fluorescence. Multiple cytokine antibody-coated beads were pooled together to allow the cytokines to be measured simultaneously. The assay was performed according to the manufacturer's instructions (Bio-Rad Laboratories) and analyzed with the Bio-Plex manager software (version 4.0). The sensitivity of this method was less than 10 pg/ml and the assay could accurately detect cytokines in the range of 1-32,000 pg/ml.

Histological observation

Biopsy specimens were fixed in 10% buffered formalin, embedded in paraffin, sectioned serially at a 4 μm thickness, and de-waxed. H&E was performed routinely. Four-micrometer liver sections were deparaffinized in xylene and hydrated in graded ethanol. After deparaffinization, rehydration, and heating in 95°C buffer, sections were incubated with each antibody and subsequently with Histofine Simple Stain MAX-PO (MULTI) (Nichirei, Japan). Incubation was performed overnight at 4°C and followed by a wash in three changes of phosphate buffered saline (PBS) for 5 min. For all staining, the reaction product was developed with the use of 3-diaminobenzidine tetrahydrochloride and H2O2. The sections were counterstained with

Table 1. Perioperative demographic and clinical characteristics of liver transplantation with non-fungal infected and fungal infected group in this study

Variables	Non-fungal infected group (NFI) (n = 123)	Fungal infected group (IFI) (n = 45)	*P* Value
Gender (male, %)	104 (84.6)	37 (82.2)	0.716
Age (yrs)	48.07 ± 9.96	51.72 ± 8.18	0.029
Weight (kg)	66.66 ± 10.82	66.29 ± 11.74	0.681
Creatinine (μmol/L)	67.98 ± 26.69	66.49 ± 20.16	0.794
Albumin (g/L)	36.74 ± 5.98	35.07 ± 5.67	0.108
Total bilirubin (μmol/L)	77.13 ± 155.5	102.8 ± 167.0	0.187
Int'l normalized ratio (INR)	1.347 ± 0.96	1.362 ± 0.49	0.188
Alpha-fetoprotein (AFP) (ng/mL)	1058 ± 5632	227.3 ± 405.7	0.511
MELD score	12.38 ± 8.69	13.42 ± 7.25	0.224
Child-Pugh score	6.89 ± 2.04	7.73 ± 2.64	0.099
Cold ischemia time (h)	8.47 ± 2.71	9.111 ± 2.29	0.126
Hypertension (%)	15 (12.2)	3 (6.7)	0.405
Diabetes mellitus (%)	17 (13.8)	6 (13.3)	0.935
Progressive hyperbilirubinemia	35 (28.5)	23 (51.1)	0.006
High PT, low albumin (%)	46 (37.4)	26 (57.8)	0.018
HBsAg positive (%)	22 (22.2)	10 (17.9)	0.526
Refractory ascites/Fluid retention (%)	48 (39.0)	24 (53.33)	0.097
Hepatic encephalopathy history (%)	11 (8.9)	5 (11.1)	0.672
Tumor (%)	72 (58.5)	24 (53.3)	0.546
Donor ABO match (%)	105 (85.4)	41 (91.1)	0.328
Ascites (%)			0.425
None	74 (60.2)	22 (48.9)	
Mild	32 (26.0)	15 (33.3)	
Severe	17 (13.8)	8 (17.8)	
Main reasons leading to LT (%)			0.891
Cirrhosis related to HBV	102 (82.9)	37 (82.2)	
Autoimmune cirrhosis	6 (4.9)	3 (6.7)	
Other etiologies	15 (14.2)	5 (11.1)	
Pathological diagnosis			0.556
Cirrhosis	99 (80.5)	36 (80.0)	
Tumor (no cirrhosis)	10 (8.1)	2 (4.4)	
Fulminant hepatic failure	7 (5.7)	5 (11.1)	
Other	7 (5.7)	2 (4.4)	

Meyer hematoxylin-eosin. The RAI for each specimen was scored according to the Banff consensus by two independent qualified transplant pathologists unaware of the clinical data of the patients [15].

Immunohistochemical analysis

Monoclonal antibodies against CD4, Foxp3 and IL-17 (DAKO, Glostrup, Denmark) were applied to the above sections. Antigens were retrieved in citrate buffer in a microwave oven and endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Then, sections were incubated at 4°C with primary antibodies (with a dilution of 1:200 for CD4, 1:200 for Foxp3 and 1:100 for IL-17) overnight and the Envision™ staining (DAKO, Glostrup, Denmark) procedure was performed. Sections with the primary antibody application omitted served as a negative control. Brown granular staining was considered to be a positive signal for the IHC assay. The positive cells were counted under light microscope according to the previous description with some modifications [16]. The positive cells in the portal tract area and lobules were counted separately to investigate the distribution of the molecules. For each slide, the posi-

tive cells in least 10 portal tracts and 10 high power fields (HPF) in the lobules were counted under the light microscope (Olympus BX51, Japan). The average positive cells per portal tract and per HPF in the lobules were calculated.

Fluorescent in situ hybridization (FISH)

FISH was performed in our laboratory [17]. Sections from paraffin-embedded biopsied liver tissues were placed on silane-coated glass slides. The slides were deparaffinized immediately in two rinses of 1,000 g/l xylene for 10 min each. Each slide was rehydrated in an ethanol series for 5 min. The slides were then treated with 0.2 mol/l HCl for 20 min, followed by 2 9 SSC (0.3 mol/l sodium chloride and 0.03 mol/l sodium citrate) for 20 min at 80°C, treated with 0.05 mg/ml proteinase K in TEN [0.05 mol/l Tris-HCl, pH 7.8, 0.01 mol/l ethylenediamine tetraacetic acid (EDTA), and 0.01 mol/l sodium chloride] for 10 min at 37°C, and placed in 40 g/l formaldehyde in PBS for 10 min. Both FISH probes and target DNA were denatured simultaneously for 10 min at 90°C, and the slides were incubated overnight at 42°C, placed in 2 9 SSC for 10 min at 42°C, washed twice in 2 9 SSC/500 g/l formaldehyde formamide for 5 min each at 42°C, washed 2 9 SSC for 5 min at 42°C, and counterstained in 2 9 SSC/0.03 lg/ml 4',6-diamidino-2-phenylindole (DAPI).

Statistical analysis

We performed a descriptive analysis of all variables and analyzed the qualitative variables by absolute and relative frequencies, whereas quantitative variables were studied with means, standard deviations. Univariate analyses of categorical data were performed with the chi-square test or Fisher's exact test for variables between two groups, and univariate analyses of continuous data were performed with the either the Student t test or the Wilcoxon nonparametric test. All univariate analyses were performed with SPS 17.013. The logistic regression method was applied in the multifactorial analysis. All logistic and survival analysis was performed with SAS 9.2.

Results

Characteristics of study population

A total of 177 deceased donor LT procedures were performed between January 2010 and June 2012. Five of these patients were excluded because of retransplant, and 4 died within 1 week of transplantation. There were no split LT procedures performed during this time period. Thus, 168 subjects were included in the present analysis with average age of 48.07 (SD, ± 9.96) in non-fungal infected group (NFI) and average age of 51.72 (SD, ± 8.18) in fungal infected group (IFI). Percentage of progressive hyperbilirubinemia and high prothrombin time test (PT) with low albumin (%) in fungal infected group were significantly higher than in non-fungal infected group (**Table 1**). Hepatitis B with or without hepatocellular carcinoma was the most common underlying liver disease. Pretransplant fungal colonization was identified in several patients; based on our partial collected data, the major pathogen including fungal pathogens which were involved in IFIs and bacterial infections in this study were identified mostly as fungi with Yeasts. Albicans (**Table 2**). Immunosupressant therapy was used postoperation in non-fungal infected group and fungal infected group (**Table 3**) while no difference was found between these two groups. Antifungal prophylaxis with caspofungin was used in 55 patients (50%). Other operative variables (i.e., operation time, blood loss) and clinical events posttransplant (i.e., duration of initial intubation, intensive care unit [ICU] stay, reoperation, dialysis, and rejection) were measured (data not shown).

Association of fungal infected liver transplant and risk of acute rejection

Twenty-six graft reject existed in non-fungal infected group with 21.1% percentage while three graft rejects were in fungal infected group with 7.6%. The risk of acute rejection was significantly decreased in fungal infected liver transplant recipients comparing with non-fungal infected group in the liver transplant recipients after surgery (**Table 4**). In addition, by using multifactorial analysis with logistic regression, we found that graft reject in fungal infected group was also lower compared with non-fungal infected group after surgery; meanwhile, graft reject in non-diagnosis with tumor was lower compared with diagnosis with tumor group after surgery, however, no difference was found between two groups with or without diabetes melitus (**Table 5**).

T cell subset flow cytometry analysis

Peripheral blood mononuclear cells (PBMCs) were isolated by Histopaque density centrifuga-

Table 2. Characteristics and sites of pathogens including fungal pathogens involved in IFIs and bacterial infections in this study

Pathogen	Bloodstream	Peritoneal/Abdominal	Respiratory	Other infection	*All*
Candida species					
C. albicans	1	1	12	1	15
Non-albicans	0	2	3	0	5
Aspergillus species	0	2	5	0	7
Bacterial infections	0	2	23	1	26

Table 3. Immunosupressant therapy postoperation between non-fungal infected group and fungal infected group

Therapy plans	Non fungal infection (n = 123)	Fungal infection (n = 45)	*P* value
Tacrolimus + MMF* + Hormone	42	12	0.358
Tacrolimus + MMF	46	17	0.964
Tacrolimus + Hormone	5	3	0.770
Tacrolimus	100 (single use: 7)	36 (single use: 4)	0.849
Cyclosporine + MMF + Hormone	2	0	1.000
Cyclosporine + MMF	2	2	0.291
Cyclosporine + Hormone	0	1	0.268
Cyclosporine	5 (single use: 1)	5 (single use: 2)	0.180
MMF + Hormone	4	3	0.586
MMF	9	1	0.385
Rapamune or others	5	0	0.326

*MMF = Mycophenolate mofetil.

Table 4. The risk association between fungal infection and graft reject in the liver transplant recipients after surgery

Stratum	Graft Reject (%)	Non-Graft Reject (%)	OR (95% *CI*)
Non-fungal infected group (NFI)	26 (21.1)	97 (78.9)	0.267 (0.076-0.929)§
Fungal infected group (IFI)	3 (6.7)	42 (93.3)	-
Yeast infection	3	32	-
Aspergillus infection	0	10	-

§*P* = 0.028, graft reject was decreased in fungal infected group compared with non-fungal infected group after surgery.

Table 5. The risk analysis of graft reject of fungal infection, diabetes melitus and diagnosis with tumor using multifactorial analysis (Logistic regression)

Variables		OR	Lower 95% *CI*	Upper 95% *CI*	*P* value
Fungal infection	Yes vs No	0.250	0.070	0.895	0.03§
Diabetes melitus	Yes vs No	5.895	0.746	46.584	0.09
Diagnosis with tumor	No vs Yes	2.552	1.094	5.953	0.03*

§*P* = 0.03, graft reject was lower in fungal infected group compared with non-fungal infected group after surgery. **P* = 0.03, graft reject was lower in non-diagnosis with tumor compared with diagnosis with tumor group after surgery.

tion and then separated through a 50% percoll gradient. Using the same anti-rejection scheme (FK506 concentration) and comparing with the non-fungal infected group, T lymphocytes CD4 + CD25highCD25127-T cells (Treg), CD4 + CD25-lowCD45RA-T (memory) cells, CD4 + CD45RA-CD45RO + T cells (human IL-17-producing T cells) in fungal infected group were increased significantly while CD4 + CD25lowCD45RA + T cells (naive) and CTL (CD8 + CD28 +) cells had

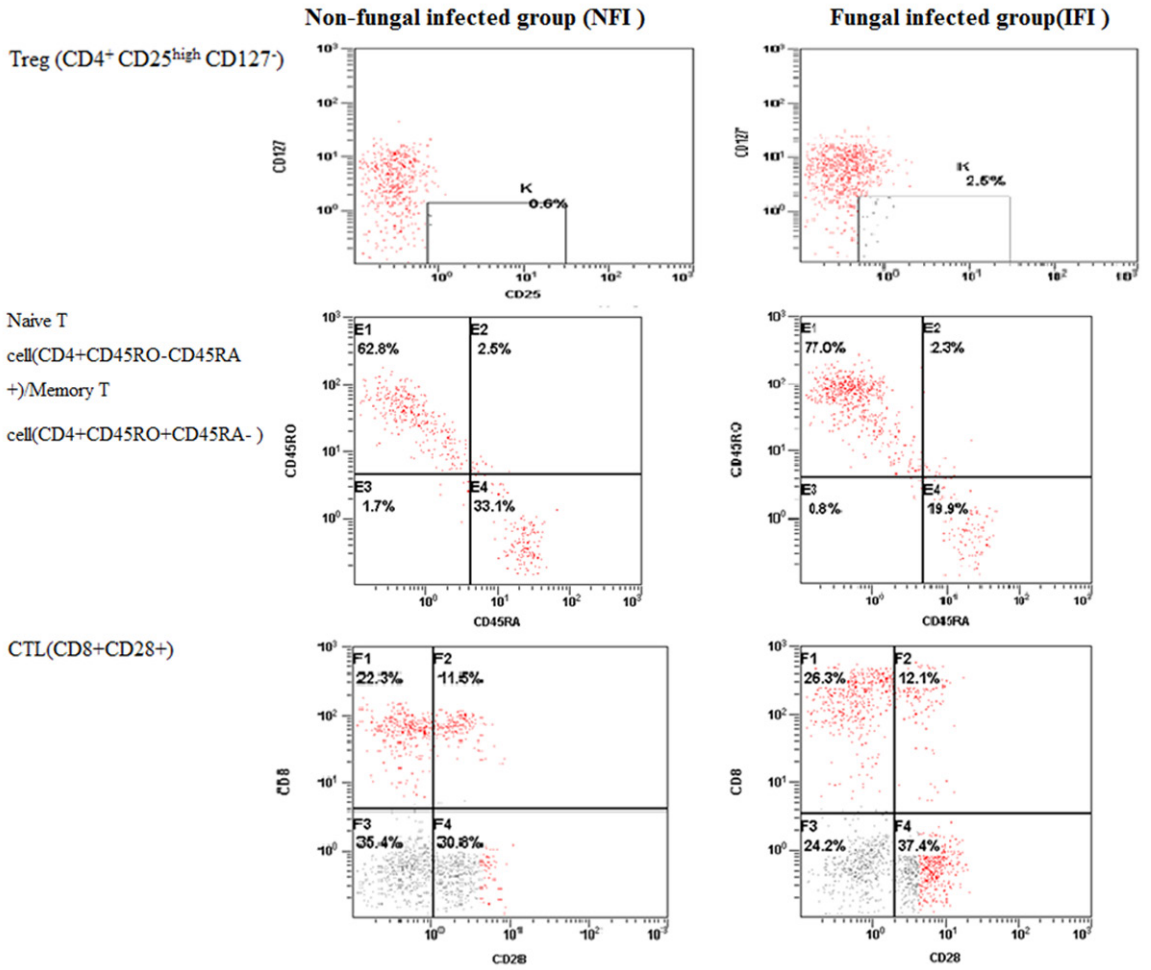

Figure 1. T cell subset flow cytometry analysis of peripheral blood mononuclear cells.

significant lower level compared to non-fungal infected group (**Figure 1**).

Cytokines levels and infection after liver transplantation

Compared with non-fungal infected group (armA), IL-2, IL-6, IL-10 and GM-CSF levels were significantly decreased in fungal infected group (armB) after one week of liver transplantation. However, IL-4 and IL-8 were no significant difference between these two groups. Moreover, IL-2, IL-6, IL-10 and GM-CSF were decreased. However, no significant difference with IL-4 and IL-8 in serum in two infected LT recipients (**Figure 2**).

Immunohistochemial analysis for liver biopsy specimens after liver transplant

Using the same anti-rejection scheme (FK506 concentration), compared with non-fungal infected group, staining of CD4, IL-17 and Foxp3 in fungal infected group had significantly lower density after liver transplant (**Figure 3**).

Fluorescent in situ hybridization (FISH) analysis for liver biopsy specimens

Using the same anti-rejection scheme (FK506 concentration), compared with non-fungal infected group, the fluorescent contrast of counterstaining of CD4, IL-17 and Foxp3 in fungal infected group had significantly lower density after liver transplant (**Figure 4**).

Discussion

IFI is a potentially life-threatening complication post-LT. To understand its epidemiology and the evolving trends in the particular locality is beneficial for the proper prophylactic treatment of post-LT recipients. In this retrospective analysis of our 109 liver recipients, overall, the

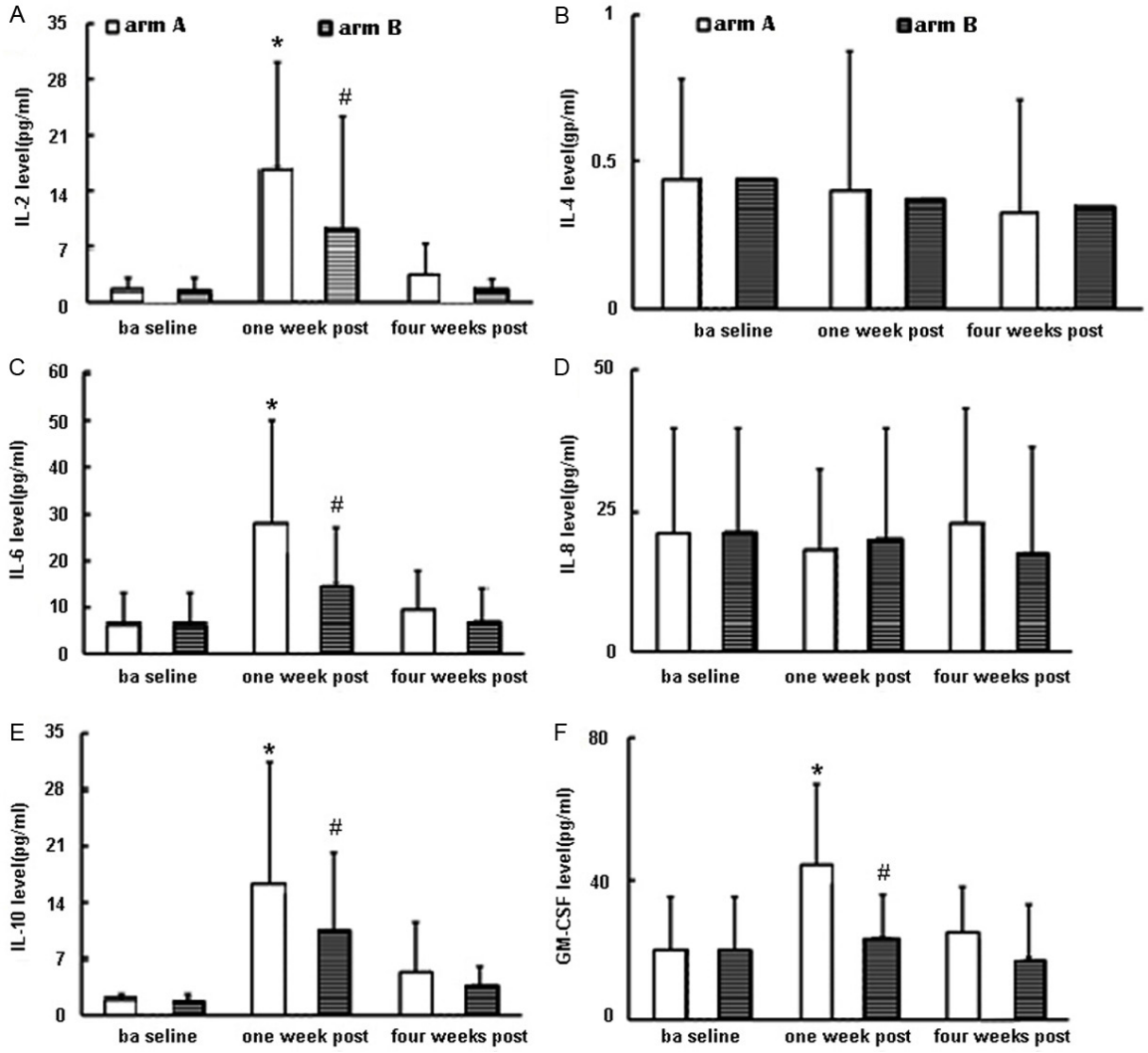

Figure 2. Cytokines levels and infection after liver transplantation.

infections were bacterial in 23.9% (26/109) of the cases, fungi in 24.8% (27/109).

Risk factors for development of IFI have been assessed in several studies [7, 8] and consist of three main factors: (i) poor allograft function or primary graft failure; (ii) renal dysfunction; and (iii) overimmunosuppression. Correspondingly, our results also showed that classic risk factors, such as a large volume of blood infusion, prolonged ICU duration (catheters and surgical drains), a high immunosuppressant trough level, post-treatment neutrocytopenia and hyperglycemia, as well as low CD4 + T-cell activity were associated with IFI by univariate analysis. Most IFIs were found to occur within the first 2 to 4 weeks after transplantation, and this is in agreement with previously published data. The one-year survival rate of subjects with IFIs was much lower than the rate of those without IFIs [14-16].

In this study, immunological parameters were used including T cell subsets, cytokines, complement the change of fungal infection in liver transplant patients. We selected 109 cases of sequential liver transplant patients among bacterial infection, fungal infection and non-infection. And the clinical diagnosis was divided into a stable state or infection status. Retransplant patients from the overall analysis were excluded due to their unique anatomical considerations, their existing immune impairment and their reduced overall posttransplant survival which likely affected the overall results. Compared with the non-fungal control group,

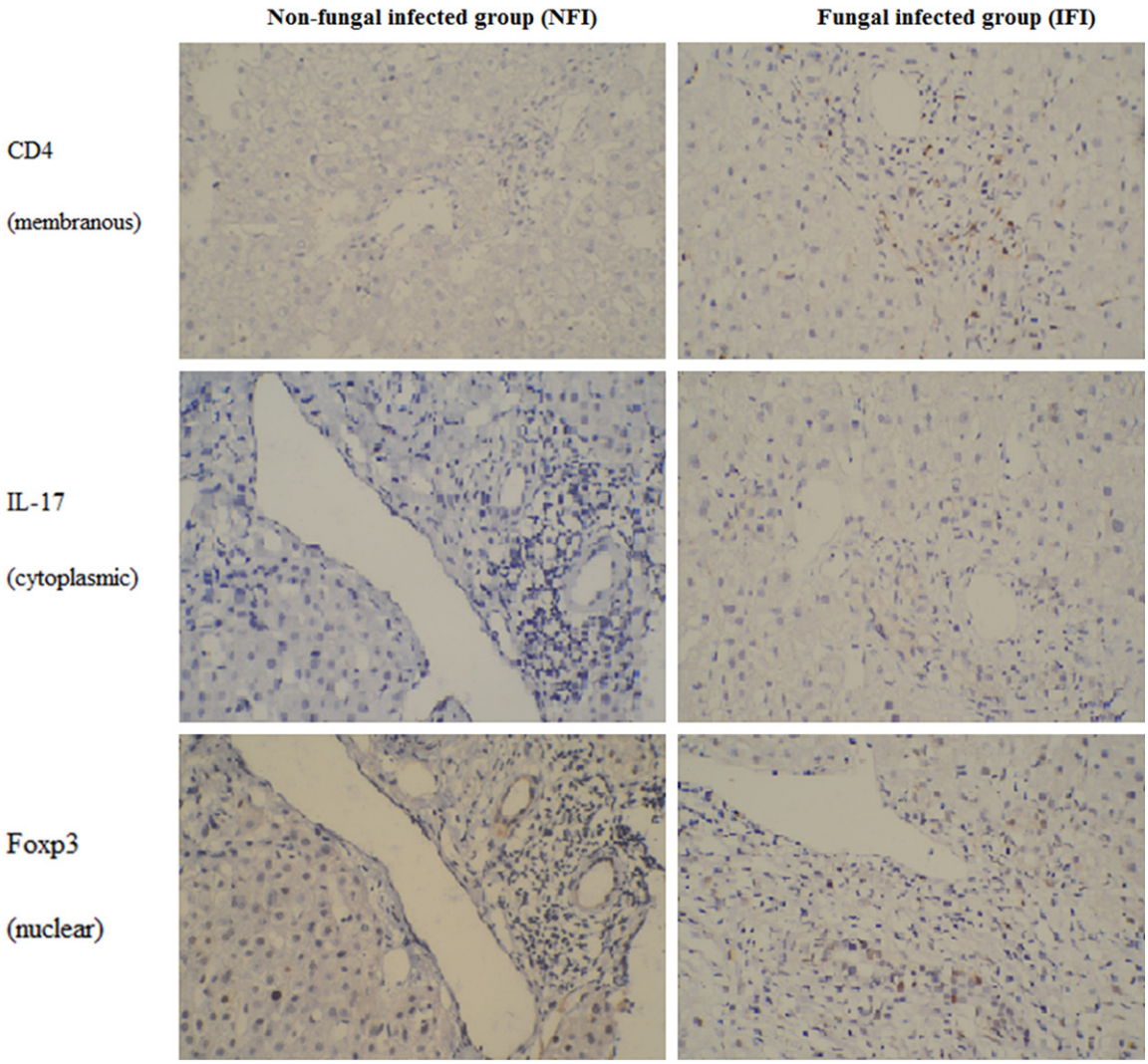

Figure 3. Immunohistochemistry analysis for liver biopsy specimens after liver transplant.

fungal infection group in patients with CD4 + 25 high T cells increased, CD4 + CD45RA-CD45RO + T cells (human IL-17-producing T cells) decreased, CD4 + CD25low CD45RA + (naive)/CD4 + CD25low CD45RA-(memory) increased significantly. Meanwhile, cytokines, complement and T cell subsets were correlated to infection.

Monitoring immune function can predict the risk of infection including T cell subsets, cytokines, complement and invasive fungal infection in liver transplantation. Approximately 24.8% of patients were diagnosed with invasive fungal infections. Compared with non-invasive fungal infections group, the average value of T cell subsets and cytokines in IFIs were shown as independent risk factor by Logistic regression analysis. We concluded that the T cell subsets, cytokine changes can be monitored in patients with immune function.

CD4 cell-mediated immune responses after transplantation rejection and infection play an important role in the process. Therefore, determination of CD4 T lymphocyte immune function can be used as organ allograft function monitoring [18, 19], depending on the patient's immune status and individual immunosuppressive regimen, appropriate individualized program to combat fungal infections. Since the application of high-dose corticosteroids in liver transplantation, postoperative CD4 T lymphocyte decreased significantly, then gradually increased after 1 week. CD4 T lymphocyte and CD4/CD8 ratio may determine fungal infection

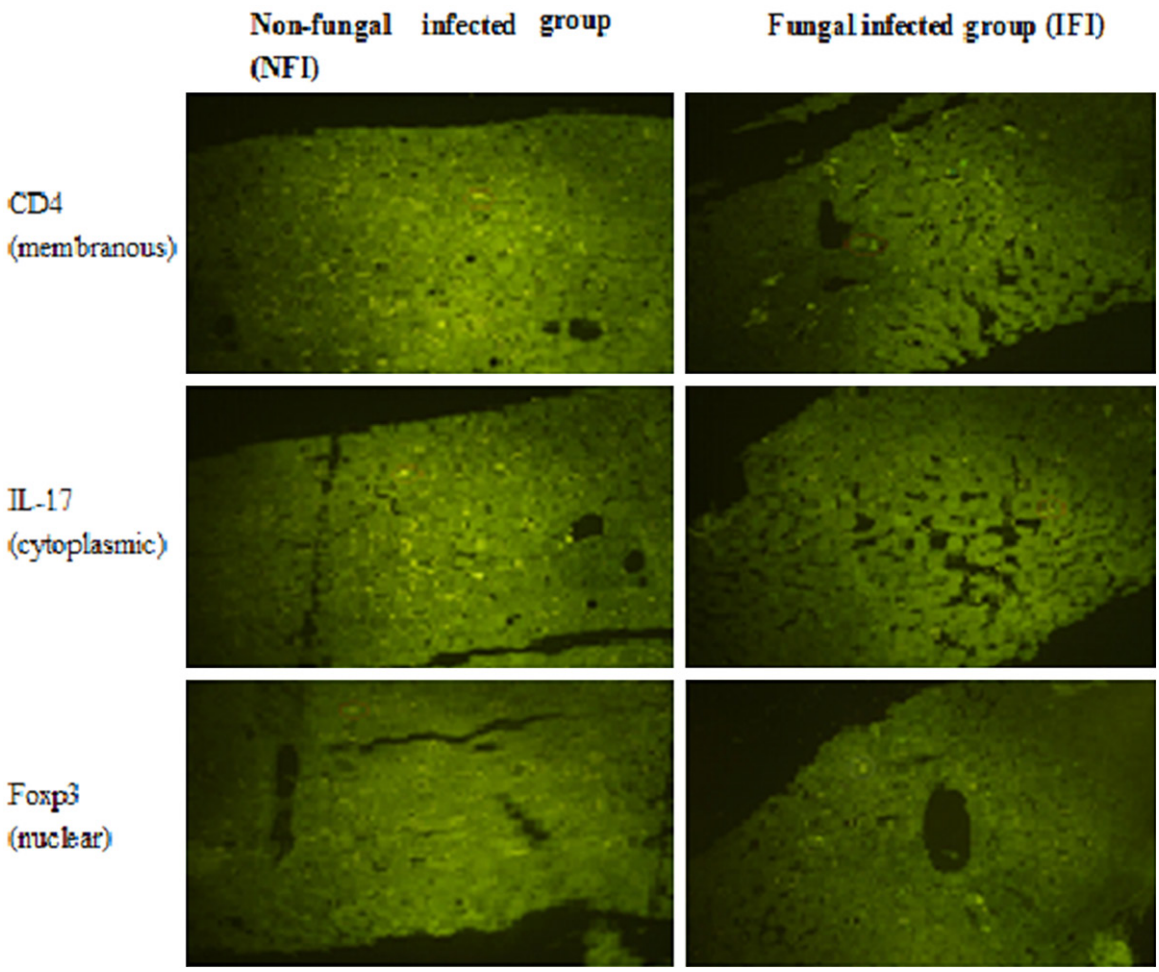

Figure 4. Fluorescent in situ hybridization (FISH) analysis for liver biopsy specimens after liver transplant.

and disease control as an individualized indicator. It is important to have dynamic monitoring of CD4 T lymphocyte and CD4/CD8 ratio. Occurrence of rejection will be existed in their fast rising whereas if the CD4 T lymphocyte counts remained low (< 200 × 10/L), and CD4/CD8 ratio < 1.0, it will have to consider reducing the dosage of immunosuppressive drugs to prevent fungal infections. When fungal infection in liver transplant recipients sustained CD4 T lymphocyte count < 100 × 10/L, mortality will be as high as 50%. However, the CD T lymphocyte count and CD4/CD8 ratio may not fully reflect the cellular immune function of T lymphocytes in this activity.

As the complexity of the human immune system, immune regulation constitutes intricate network, CD4 T lymphocyte immune function may not fully reflect the immune status. However, it is to observe the immune status of a window. According to T lymphocyte subsets CD4 T lymphocyte count and quantitative assessment of the value of ATP in liver transplant recipients cellular immune function, the individual immune regulation may be necessary to develop individualized early postoperative fungal infection prevention and reduce early postoperative fungal infection for liver transplantation, fungal infection mortality.

However, there are still limitations to our study. First, it was a retrospective study from a single center and it is important to know whether the associations between low ImmuKnow ATP and IFI identified in our study can be confirmed by those of other centers. Second, due to the small number of cases, we did not compare fungal infection with CMV and other viral infections in the study, even though CMI is crucial for both types of infection. The third limitation is the failure to include cases of IFI not reported

by clinicians but treated empirically without microbiological confirmation. Consistent application of standardized definitions and methods to identify and to risk-stratify IFI will be required for further investigations.

In conclusion, it is crucial to identify those individual transplant candidates who may subsequently develop fungal infections. Our results suggest that antifungal prophylaxis should be given to high-risk LT patients, such as patients with massive hemorrhage during operation and with a prolonged ICU stay. In addition, the adjustment of immunosuppression based on the measurement of global immunity is of similar importance, and an ImmuKnow assay may be useful in both antifungal prophylaxis and close monitoring of immunosuppression. With the advent of this technique, we anticipate that an improved assessment of patients' immune status may well lead to better patient management and evidence-based individualization of treatment.

Clinicians constantly face a dilemma when treating post-LT recipients with abnormal graft function. Too little immunosuppression may lead to ACR, whereas too much immunosuppression will increase the risk of recurrent infection and other life-threatening complications [20].

Acknowledgements

This work was sponsored by a grant from Shanghai Nature Science Fund project (No. 10ZR1423900) and a grant from Medicine and Technology Fund of Shanghai Jiao Tong University (No. YG2013MS01).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zhihai Peng, Department of General Surgery, Shanghai First People's Hospital, School of Medicine, Shanghai Jiao Tong University, 100 Hai-Ning Road, Shanghai 200080, China. Tel: +8613621691786; Fax: 021-61317959; E-mail: xingth422@163.com

References

[1] Raghuram A, Restrepo A, Safadjou S, Cooley J, Orloff M, Hardy D, Butler S, Koval CE. Invasive fungal infections following liver transplantation: incidence, risk factors, survival, and impact of fluconazole-resistant Candida parapsilosis (2003-2007). Liver Transpl 2012; 18: 1100-1109.

[2] Hadley S, Huckabee C, Pappas PG, Daly J, Rabkin J, Kauffman CA, Merion RM, Karchmer AW. Outcomes of antifungal prophylaxis in high-risk liver transplant recipients. Transpl Infect Dis 2009; 11: 40-48.

[3] Pappas PG, Alexander BD, Andes DR, Hadley S, Kauffman CA, Freifeld A, Anaissie EJ, Brumble LM, Herwaldt L, Ito J, Kontoyianis D, Lyon GM, Amarr KA, Morrison VA, Park BJ, Patterson TF, Perl TM, Oster RA, Schuster MG, Walker R, Walsh TJ, Wannemuehler KA, Chiller TM. Invasive fungal infections among organ transplant recipients: results of the Transplant-Associated Infection Surveillance Network (TRANSNET). Clin Infect Dis 2010; 50: 1101-1111.

[4] Cruciani M, Mengoli C, Malena M, Bosco O, Serpelloni G, Grossi P. Antifungal prophylaxis in liver transplant patients: a systematic review and meta-analysis. Liver Transpl 2006; 12: 850-858.

[5] Husain S, Tollemar J, Dominguez EA, Baumgarten K, Humar A, Paterson DL, Wagener MM, Kusne S, Singh N. Changes in the spectrum and risk factors for invasive candidiasis in liver transplant recipients: prospective, multicenter, case-controlled study. Transplantation 2003; 75: 2023-2029.

[6] Moreira AP, Cavassani KA, Massafera Tristão FS, Campanelli AP, Martinez R, Rossi MA, Silva JS. CCR5-dependent regulatory T cell migration mediates fungal survival and severe immunosuppression. J Immunol 2008; 180: 3049-3056.

[7] Reed A, Herndon JB, Ersoz N, Fujikawa T, Schain D, Lipori P, Hemming A, Li Q, Shenkman E, Vogel B. Effect of prophylaxis on fungal infection and costs for high-risk liver transplant recipients. Liver Transpl 2007; 13: 1743-1750.

[8] Zhong L, Men TY, Li H, Peng ZH, Gu Y, Ding X, Xing TH, Fan JW. Multidrug-resistant gram-negative bacterial infections after livertransplantation-spectrum and risk factors. J Infect 2012; 64: 299-310.

[9] Ascioglu S, Rex JH, de Pauw B, Bennett JE, Bille J, Crokaert F, Denning DW, Donnelly JP, Edwards JE, Erjavec ZE, Fiere D, Lortholary O, Maertens J, Meis JF, Patterson TF, Ritter J, Selleslag D, Shah PM, Stevens DA, Walsh TJ. Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: an international consensus. Clin Infect Dis 2002; 34: 7-14.

[10] Zhong L, Men TY, Li H, Gu Y, Ding X, Xing TH, Fan JW, Peng ZH. Prevalence and risk factor for

MDR-GNB infection in liver transplantation. Front Biosci 2013; 18: 366-370.
[11] Men TY, Wang JN, Li H, Gu Y, Xing TH, Peng ZH, Zhong L. Prevalence of multidrug-resistant gram-negative bacilli producing extended-spectrum β-lactamases (ESBLs) and ESBL genes in solid organ transplant recipients. Transpl Infect Dis 2013; 15: 14-21.
[12] Fortún J, Martín-Dávila P, Montejo M, Muñoz P, Cisneros JM, Ramos A, Aragon C, Blanes M, Juan RS, Gavalda J, Llinares P. Prophylaxis with caspofungin for invasive fungal infections in high-risk liver transplant recipients. Transplantation 2009; 15; 87: 424-435.
[13] Almerighi C, Bergamini A, Lionetti R, Sinistro A, Lenci I, Tariciotti L, Tisone G, Angelico M. Vitamin D3 modulates T lymphocyte responses in hepatitis C virus-infected liver transplant recipients. Dig Liver Dis 2012; 44: 67-73.
[14] Liu Z, Yuan X, Luo Y, He Y, Jiang Y, Chen ZK, Sun E. Evaluating the effects of immunosuppressants on human immunity using cytokine profiles of whole blood. Cytokine 2009; 45: 141-147.
[15] Banff schema for grading liver allograft rejection: an international consensus document. Hepatology 1997; 25: 658-663.
[16] Cheng L, Tian F, Tang L, Wang S, Chen G, Duan G, Yan XC. Local distribution analysis of cytotoxic molecules in liver allograft is helpful for the diagnosis of acute cellular rejection after orthotopic liver transplantation. Diagn Pathol 2012; 7: 148.
[17] Eguchi S, Takatsuki M, Yamanouchi K, Kamohara Y, Tajima Y, Kanematsu T. Regeneration of graft livers and limited contribution of extrahepatic cells after partial liver transplantation in humans. Dig Dis Sci 2010; 55: 820-825.
[18] Israeli M, Klein T, Sredni B, Avitzur Y, Mor E, Bar-Nathen N, Steinberg R, Dinari G, Shapiro R. ImmuKnow: a new parameter in immune monitoring of pediatric liver transplantation recipients. Liver Wran Spl 2008; 14: 893-898.
[19] Israeli M, Yussim A, Mor E, Sredni B, Klein T. Preceeding the rejection: in search for a comprehensive post-transplant immune monitoring platform. Transpl Immunol 2007; 18: 7-12.
[20] Cabrera R, Ararat M, Soldevila-Pico C, Dixon L, Pan JJ, Firpi R, Machicao V, Levy C, Nelson D, Morelli G. Using an immune functional assay to differentiate acute cellular rejection from recurrent hepatitis C in liver transplant patients. Liver Transpl 2009; 15: 216-222.