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Original Article

The Role of CXCLs and CXCR3 mRNA Expression Levels in Viral Hepatitis-Infected Liver Transplant Recipients

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ABSTRACT

Background: Chemokines seriously affecting immune responses to viral infections and allograft rejections.

Objective: Therefore, the current study aims to evaluate the expression levels of CXCLs and CXCR3 in liver transplanted (LT) patients with hepatitis B (HBV), hepatitis C (HCV), HBV/HCV co-infection, and non-infected ones.

Methods: The mRNA expression levels of studied genes were evaluated in LT patients with HBV (n=69), HCV (n=15), HBV/HCV co-infection (n=16), and non-infected (n=48) patients, compared to the control group (n=25). HBsAg and HBeAg were analyzed using ELISA methods. HBV-DNA and HCV-RNA were evaluated using simple PCR and nested RT-PCR protocols.

Results: The mRNA expression level of CXCL10 was significantly up-regulated in HBV-infected and non-infected groups compared to controls ($p \leq 0.05$). The CXCL11 and CXCR3 mRNA expression levels were significantly increased in the patient groups compared with controls ($p \leq 0.05$). The expression levels of CXCL9 and CXCL10 were significantly correlated in the HBV group ($r = 0.6$, $p \leq 0.05$). The correlation between CXCL10 and CXCL11 was also significantly positive ($r = 0.4$, $p \leq 0.05$). Additionally, CXCL11 mRNA expression significantly associated with CXCR3 ($r = 0.4$, $p \leq 0.05$).

Conclusion: The up-regulation of CXCL11, CXCL10, and CXCR3 in HBV and HCV-infected and non-infected liver transplant patients compared to the controls and the direct correlations between the expression levels of CXCLs and CXCR3 in HBV liver transplant patients put more emphasis on the critical function of these molecules in the HBV and HCV infections pathogenesis in post-liver transplantation. However, more investigations are needed.

KEYWORDS: Hepatitis B virus; Hepatitis C virus; CXCL9; CXCL10; CXCL11; CXCR3

INTRODUCTION

Innate and adaptive immune responses against viral infections can determine the outcome of virally infected patients by introducing specific antibody-producing B cells and activating T cells [1]. Numerous cytokines and chemokines participate in the immune system's antiviral response to hepatitis B virus (HBV) and hepatitis C virus (HCV) infections [2,3]. Within the non-ELR CXC chemokine subgroup, which has a unique amino acid sequence of glutamic acid-leucine-arginine, CXCL9, CXCL10, and CXCL11 are included. These chemokines attach to a shared chemokine receptor, CXCR3, which is present on activated T cells, memory T cells, and NK cells [4]. The initiation of CXCR3 signaling has an essential role in the antiviral immune

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response by promoting Th1 activation [5].

The *CXCR3* gene is located at Xq13, and CXCL9, CXCR10, and CXCR11 are located at 4q21.1. The same promoter regulates their expression, but they are expressed independently by different cell types and induced by different Toll-like receptor signaling or cytokines, particularly interferons, during antiviral immune response activation. Furthermore, these chemokines have different efficacy and characteristics when they bind to receptors. CXCR3 and its ligands are expressed at low levels [6,7]. It is worth mentioning that IFN-γ and IFN-α/β induce CXCL10 and CXCL11, but the secretion of CXCL9 is only induced by IFN-γ [2,8]. The induction of these chemokines is up-regulated in different pathologic conditions, such as acute and chronic allograft rejection, viral infections, and autoimmune diseases [4,9]. Furthermore, activation and secretion of CXCL9, 10, and 11 are impaired in persistent chronic HBV (CHB) infection, which can be considered a molecular pattern for functional HBV cure. CXCL10 is highly involved in the antiviral immune response against acute and chronic HCV infections [8].

The CXCR3 ligands are candidate biomarkers for acute rejection after liver, kidney, lung, and heart transplantations [10].

CXCL10 can serve as a reliable molecular indicator for the early identification of fibrosis [10] and LT patients harboring allograft dysfunction [11] in patients with HCV infection who have undergone LT.

Therefore, the current study evaluated the mRNA expression level of the CXCR3 receptor and its ligands (CXCL9, 10, and 11) in LT patients with HBV, HCV, and HBV/HCV co-infections.

MATERIALS AND METHODS

Samples

Between 2014 and 2016, 148 liver transplant recipients admitted to the Transplant Unit of Namazi Hospital, affiliated with Shiraz University of Medical Science, Iran, were included in the study. Studied patients include HBV (n=69), HCV (n=15), HBV/HCV-infected (n=16), and non-infected (with stable transplants; n=48) patients. The control group also consisted of 25 healthy individuals who didn't experience of any disease at the time of sampling. Patients with co-infections with other viruses, such as polyomavirus BK, cytomegalovirus (CMV), and immunodeficiency virus (HIV), were excluded from the study. Polyomavirus BK and CMV detection were carried out by molecular assays [11,12].

Rejection episodes were defined as detecting rejection signs in biopsy samples based on Banff criteria and laboratory data such as serum amylase and lipase levels [13]. All patients received immunosuppressive drugs, including tacrolimus, mycophenolate mofetil, and steroids. The demographic data were collected from each studied patient.

Detection of HCV Infection

HCV infection was evaluated by the extraction of RNA from plasma. The cDNA synthesis and nested PCR were executed. RNA extraction was performed from the plasma sample using RNX-Plus (CinnaGen, Iran) based on manufacturer's instructions. The cDNA synthesis step and reverse transcription were performed using a mixture that included 4 µL of 5x reverse transcriptase buffer (Fermentase, Lithuania), 1µL of random hexamer (Fermentase, Lithuania), 0.5 µL of 40u/µL RNase inhibitor (Fermentase, Lithuania), 2.5 µL of 10Mm dNTP mix (CinnaGen, Iran), 0.75 of 200u/µL M-Mulv RT-Enzyme (Fermentase, Lithuania), 8.25 µL of DEPS-water, and 3µL of Extracted RNA. The thermocycling condition of cDNA synthesis was carried out by the initiation step at 25˚C/5 minutes, followed by the second step at 42˚C/60 minutes, and finally at 72˚C/1 minute.

The nested PCR was performed using specific primers for HCV genomic RNA. The sequence of primers is shown in Table 1. The mixture for the first step of nested PCR consisted of 2.5 µL of PCR buffer (10x) (CinnaGen, Iran), 0.75 µL of $MgCl_2$ (50mM) (CinnaGen, Iran),

Table 1: Sequences of primers, annealing temperature, and product length of primers.

Genes	Sequences of primers	Product Length (bp)	Annealing Temperature (˚C)
HCV (Simple Step)	F: CCCCTGTGAGGAACTACTGTC R: TGCACGGTCTACGAGACCTC	225	55
HCV (Nested Step)	F: CACGCAGAAAGCGTCTAGCCATG R: TCGCAAGCACCCTATCAGGCAG	225	64
GAPDH	F: TTCGCCCACAGTCTGACTTC R: AATGGTGCAGTCC TC AGAGC	156	57.5
CXCL9	F: TCCACGTGTTGAGATCATTGCT R: CGATTTTGCTCCCCTCTGGT	148	57.5
CXCL10	F: GAGGACGCTGTCTTTGCATAGG R: AGCCTTGCTTGCTTCGATTTGG	172	61.5
CXCL11	F: GGACTCATGACCACAGTCCA R: CCAGTAGAGGCAGGGATGAT	119	62.5
CXCR3	F: GTGGTGTTCTTTTCCTCTTGG R: ATAGTCCCTTGGTTGGTGCT	112	60

0.5 µL of dNTPs (10mM) (CinnaGen, Iran), 0.25 µL of each forward and reverse primers (12.5 picomol/µL), 0.25 µl of Taq polymerase (5u/µL) (CinnaGen, Iran), 16 µl of DEPS-water, and 2 µL of cDNA. The first step of nested PCR was performed at 95˚C /300 seconds, followed by cycles at 94˚C/50 seconds, 55˚C/40 seconds, and 72˚C/50 seconds. The final extension was 72˚C/180 seconds.

The mixture for the second step of nested PCR comprised 2.5 µL of PCR buffer (10x) (CinnaGen, Iran), 1.5 µL Mgcl2 (50mM) (CinnaGen, Iran), 0.5 µL dNTPs (10mM) (CinnaGen, Iran), 0.5 µL each forward and reverse primers (12.5 picomol/µL), 0.25 µL Taq polymerase (5u/µL) (CinnaGen, Iran), 18.5 µL DEPS-water, and 1 µL PCR products maid in the first step PCR. The second step of nested PCR was initiated at 95˚C/280 seconds, followed by 35 cycles at 94˚C/40 seconds, 64˚C/35 seconds, 72 ˚C/40 seconds, and finally, 72˚C/180 seconds.

CXCLs and CXCR3 Gene Expression

To extract total RNA from PBMCs, RNX-Plus (CinnaGen, Iran) was utilized. The extracted RNA's quality was assessed through agarose gel electrophoresis (1%). The purity and integrity of the extracted RNA were also evaluated at an optimal density of 260/280. The cDNA synthesis was carried out using the Takara kit (Dalian, Japan) based on manufacturer's instructions.

To measure the gene expression of CXCLs and CXCR3, the Step One Plus Real-time instrument (ABI, Step One Plus, USA) was utilized. Real-time PCR was conducted using a SYBR Premix Ex Taq II kit (Takara, Japan). The GAPDH gene expression was used as a control. The reaction mixture consisted of 5 µL premix, 0.4 µL forward and reverse primers (10 pM), 0.2 µL SYBR Green Dye (50x), 3 µL DEPS-water, and 1 µL cDNA. The forward and reverse primer sequences can be found in Table 1. Real-time PCR was conducted using an initiation step at 95˚C/10 minutes, followed by 40 cycles at 95˚C/30 seconds; the related annealing temperatures for each primer pairs are specified (Table 1) as 20 seconds and 72˚C/30 seconds

Ethical Considerations

This study was approved by Research Ethics Committee (RECs) number: IR.SUMS.REC.1395.S892 in Shiraz University of Medical Sciences.

Table 2: Demographic and laboratory data of liver transplant patients with HBV, HCV, HBV and HCV co-infected, and control group.

		Liver Transplant patients n=103				Control Group n=20
		HBV Group n= 69	HBV and HCV Co-infected Group n= 16	Non-infected Group n= 48	HCV Group n= 16	
Age (mean±SD) (range)		38.54 ± 13 (14-62)	45.58 ± 13 (20-62)	33.66 ± 12.9 (6-57)	44.73 ± 12.2 (23-59)	38.8 ± 11.7 22-72
Gender (n/%)	Female	14 (20.3)	13 (81.3)	19 (39.6)	5 (33.3)	6 (24)
	Male	55 (79.7)	3 (18.7)	29 (60.4)	10 (66.7)	19 (76)
Blood Group	A	21 (30.4)	3 (18.8)	11 (22.9)	3 (20)	—
	B	14 (20.2)	—	10 (20.9)	1 (6.7)	
	AB	12 (17.3)	2 (12.5)	2 (4.2)	—	
	O	22 (31.9)	7 (43.8)	15 (31.3)	7 (46.7)	

Statistical Analysis

The gene expression fold change rate was measured by the Livak ($2^{-\Delta\Delta Ct}$) method. Non-parametric tests, such as Mann-Whitney U analysis, were used to compare the fold change of gene expression level between studied groups. The area under a receiver operating characteristic (ROC) curve of the expression of CXCLs and CXCR3 was analyzed between HBV-infected and non-infected groups. The possible association between the expression levels of studied genes was evaluated using Spearman correlation analysis in the infected and non-infected groups. GraphPad Prism 6.01 (GraphPad Software, Inc., San Diego, CA, USA) was also used to prepare statistical figures. Finally, the level for statistical significance was calculated as $p \leq 0.05$.

RESULTS

All LT patients were divided into four groups: HBV, HCV, HBV/HCV co-infected, and non-infected. Demographic and laboratory data for LT and controls are shown in Table 2. In all four patient groups that were studied, blood group O was the most common (Table 2).

The CXCLs and CXCR3 mRNA Expression Levels in Patient Groups and Controls

A comparison was made between the mRNA expression levels of the analyzed genes in liver transplant patients and controls (Fig. 1). The expression level of CXCL9 had increased in HBV, HCV, and HBV/HCV co-infection groups, compared to both non-infected and control groups, but it was not statistically significant ($p > 0.05$; Fig. 1A).

Compared to the controls, the mRNA expression level of CXCL10 was significantly increased in both the HBV-infected and non-infected groups ($p \leq 0.05$); however, no significant changes in CXCL10 mRNA expression were observed in both HCV and HBV/HCV co-infected groups compared to the controls ($p > 0.05$; Fig. 1B).

Compared to the control group ($p \leq 0.05$), the expression level of CXCL11 was significantly elevated in all patient groups. Additionally, compared to the non-infected group, the expression level of CXCL11 mRNA was notably down-regulated in the HBV/HCV co-infection and HCV groups ($p \leq 0.05$; Fig. 1C). Compared to the control group, the mRNA expression level of CXCR3 was significantly elevated in the HBV, HCV, and HBV/HCV co-infected groups ($p \leq 0.05$; Fig. 1D).

Roc curve analysis showed no significant AUC and specific cut-off values in all CXCLs and CXCR3 expression between the HBV-infected and non-infected groups (Table 3; Fig. 2).

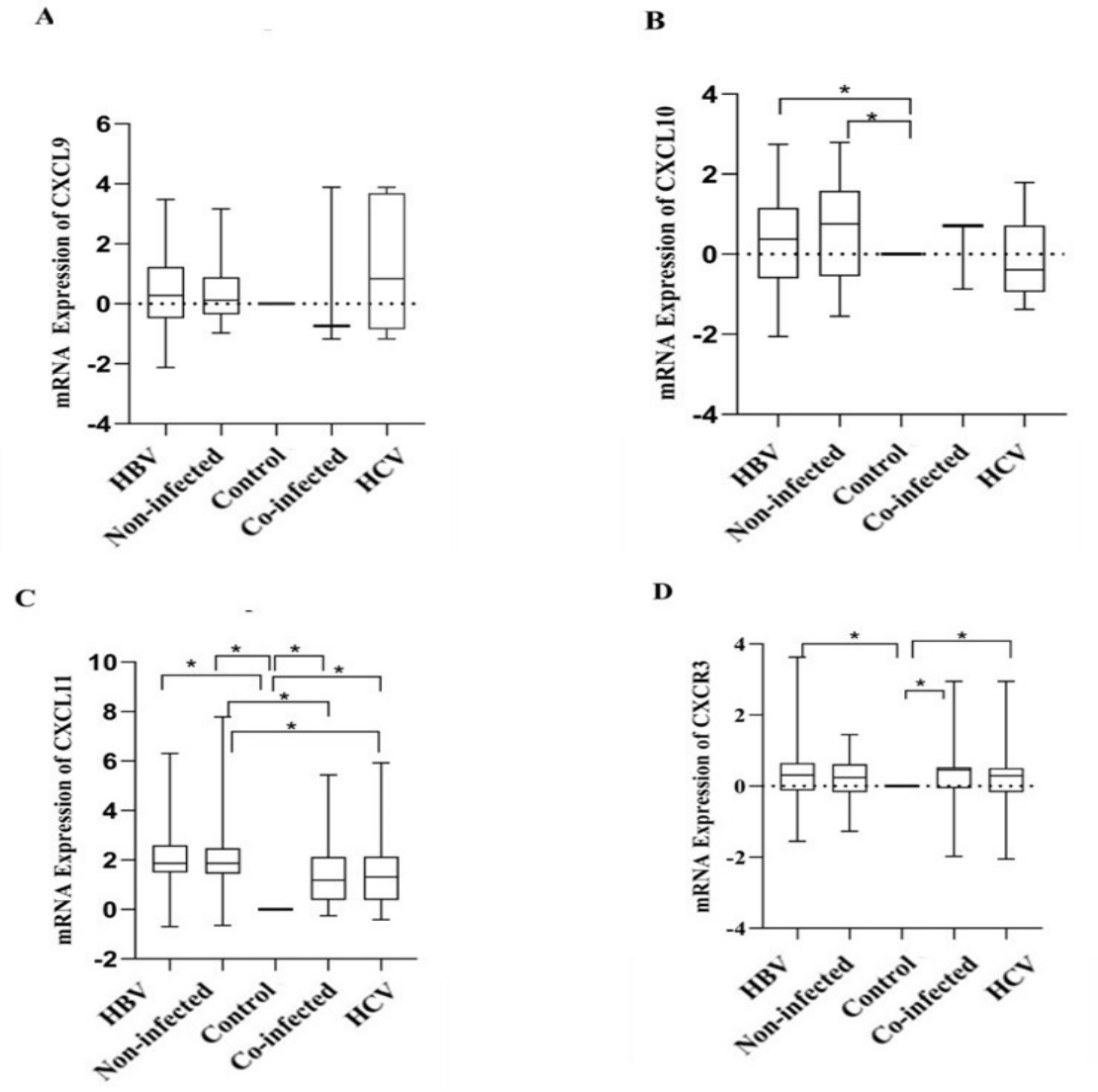

Figure 1: The comparison of the mRNA expression levels of CXCLs and CXCR3 in different groups. Expression of CXCL9 (**A**), CXCL10 (**B**), CXCL11 (**C**), and CXCR3 (**D**) was compared between HBV, HCV, HBV and HCV co-infected, non-infected, and control groups by Mann-Whitney U analysis. All data showed at log scale. *: p≤0.05, Co-infected group: HBV and HCV co-infected group.

Correlation between Expression of CXCLs and CXCR3 in Different Groups

The correlation between expression levels of studied genes was measured using Spearman's rho in all studied patient groups (Fig. 3). In the HBV group, the expression levels of CXCL9 and CXCL10 were significantly correlated (r= 0.6, p≤ 0.05) (Fig. 3A). Also, the correlation between CXCL10 and CXCL11 was significantly positive (r= 0.4, p≤ 0.05) (Fig. 3B). Furthermore, there was a significant correlation between the expression levels of CXCL11 and CXCR3 (r= 0.4, p≤ 0.05) (Fig. 3C).

In non-infected patients, correlations between CXCL10 and CXCL11, as well as CXCL11 and CXCR3, were significant (r= 0.56, p≤ 0.05, and r= 0.3, p≤ 0.05, respectively) (Fig. 3D, 3E).

The expression level of CXCR3 and its ligands did not significantly correlate in the HCV patient group, while a significant correlation was found between CXCR3 and CXCL11 expression levels in the HBV/HCV co-infection group (r= 0.5, p≤ 0.05) (Fig. 3F).

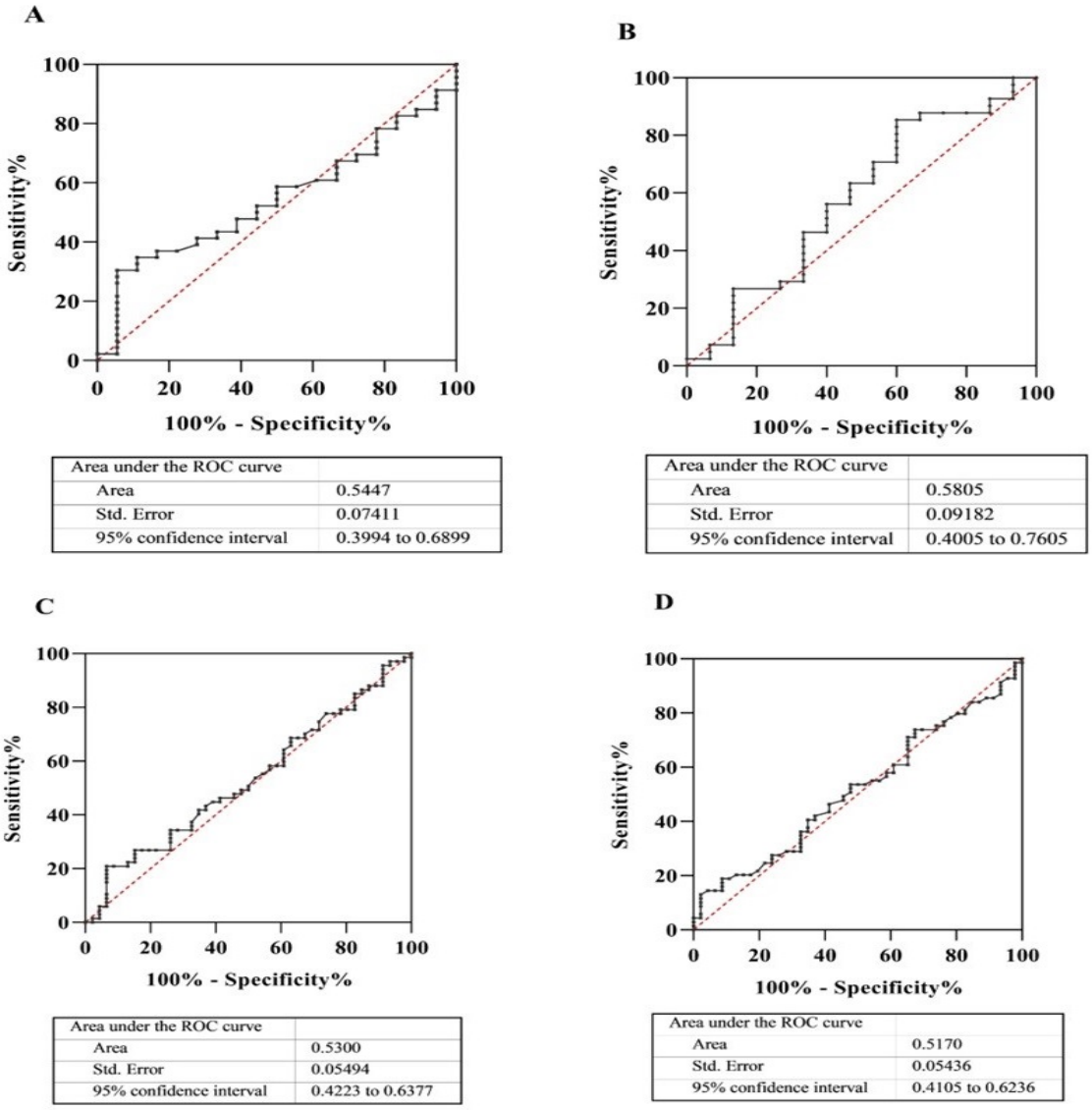

Figure 2: ROC curve for CXCLs and CXCR3 in HBV and non-infected group. ROC curve analysis was used for the expression of CXCL9 (**A**), CXCL10 (**B**), CXCL11 (**C**) and CXCR3 (**D**) in HBV group, compared with non-infected ones. All data showed at log scale. Co-infected group: HBV and HCV co-infected group.

Additionally, acute rejection was observed in only eight individuals in the HBV-infected group and four patients in the non-infected group. None of the liver transplant patients in the HCV and HBV/HCV co-infection groups showed acute rejection. The expression levels of CXCLs and CXCR3 were compared between the HBV and non-infected groups with acute rejection. In the non-infected group, the expression level of CXCL9 was not significantly higher compared to the HBV-infected group that experienced acute rejection.

DISCUSSION

Cytokines and chemokines mediate the activation and maintenance of immune responses in various pathological processes such as infections and injuries [14]. These immune molecules could stimulate different immune cell subsets, such as T cells, B cells, neutrophils, and monocytes, to activate antiviral responses to viral infections such as hepatitis [15,16]. CXCR3 receptor and its ligands (CXCL9, 10, and 11) contribute to inflammation and immune response during infections [4].

Table 3: .ROC Curve analysis of CXCLs and CXCR3 expression in HBV and non-infected groups.

Gene Expression	AUC/95%CI	P-value	Cut-off Value	Sensitivity/95%CI	Specificity/95%CI
CXCL9	0.5447	> 0.5	> 0.96	35.29 19.7 - 53.5	88.89 65.3 - 98.6
CXCL10	0.5805	> 0.5	≤ 1.29	86.21 68.3 - 96.1	40.00 16.3 - 67.7
CXCL11	0.53	> 0.5	> 2.84	26.53 14.9 - 41.1	93.48 82.1 - 98.6
CXCR3	0.5170	> 0.5	> 0.99	16.00 7.2 - 29.1	97.83 88.5 - 99.9

CXCL9-11 signaling plays a significant role in liver transplantation [17] and has been identified as an antifibrotic pathway members in liver fibrosis *in vivo* and *in vitro* [18]. The CXCL9-11 ligands are angiostatic, and it was detected that in vivo use of CXCL9 can prevent fibrosis and inhibit neoangiogenesis. The CXCL9-11/CXCR3 axis has different pathways for regulating each of the three ligands, but all pathways are induced through IFN-γ. Principally, CXCR3 expresses on $CD4^+$ Th1 cells, $CD8^+$ cytotoxic lymphocytes, and the immune cells of innate immunity such as dendritic cells, NK, and NKT cells. The protecting or damaging function of the CXCL9-11/CXCR3 axis during LT still needs more investigations [17]. In LT patients, the CXCL9-11/CXCR3 axis plays a crucial role in the recruitment of effector T cells, which can lead to acute allograft rejection. When CXCR3 is not present, the amount of Tregs located in the spleen and lymph nodes increases, suggestinghat they are incapable of migrating to the liver [17].

The CXCR3 and its ligands were designed to evaluate the mRNA expression level in LT patients with HBV, HCV, and HBV/HCV coinfection compared to non-virally infected LT patients and healthy controls [19]. The results showed that the mRNA expression levels of CXCR3, CXCL10, and CXCL11 were significantly up-regulated in the HBV group compared to the control ones. Studies have verified that there is a connection between acute and chronic HBV infections with CXCR3 and its ligands. To illustrate, immunohistochemistry has revealed that the level of CXCR3 and CXCL10 expression had progressively risen in CHB patients with varying degrees of liver inflammation and fibrosis when compared to individuals without the condition. They also found a positive correlation between CXCR3 and CXCL10 and the inflammation and fibrosis grade in CHB patients, suggesting the potential role of CXCR3 and CXCL10 in CHB clinical complications [20]. Furthermore, the expression level of CXCL9, 10, 11, and IL-10 was up-regulated in CHB compared to healthy controls and asymptomatic HBV-infected patients [21].

The expression levels of CXCL9 and 10 correlated in HBV-infected patients, suggesting their simultaneous contribution to the antiviral response against HBV infection. A correlation between CXCL10 and 11 and CXCL11 and CXCR3 levels was observed in both LT patient groups, with and without HBV infection. These results indicated that CXCL10, 11, and CXCR3 levels are critical in LT patients with and without HBV infection. These results were confirmed in earlier reports in which the expression levels of CXCR3 and CXCL10 positively correlated in CHB patients [17]. A positive correlation between CXCL9, 10, and 11 was also reported in acutely HBV-infected patients, suggesting their importance during anti-HBV responses in LT patients [8].

In acute and chronic HBV infections, a direct relationship was noted between the serum presence of CXCL9, 10, and 11 and the levels of alanine aminotransferase (ALT), which shows that these molecules are involved in

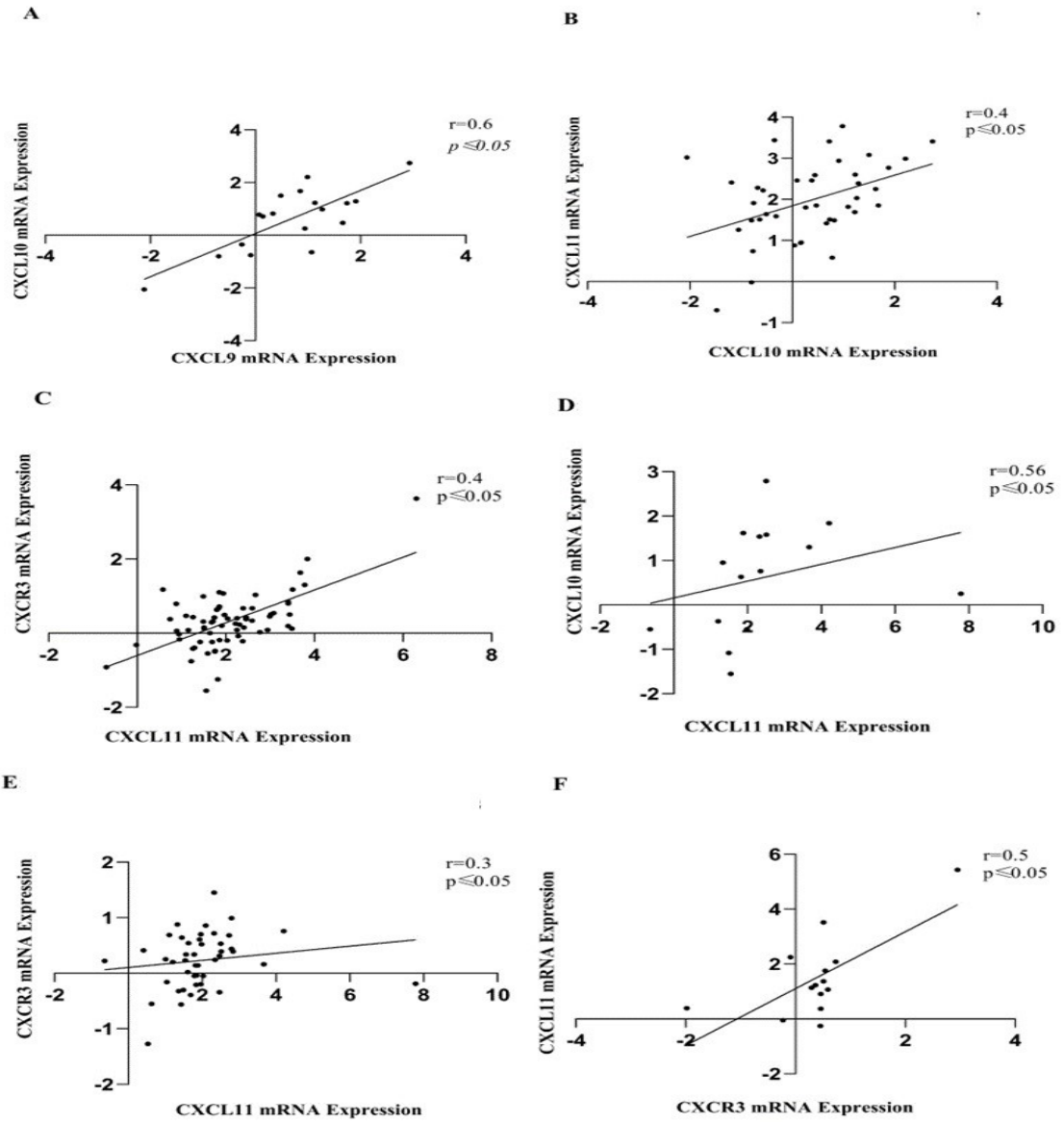

Figure 3: The correlation between CXCL9 and CXCL10 (**A**), CXCL10 and CXCL11 (**B**), CXCL11 and CXCR3 (**C**) in HBV group, and between CXCL10 and CXCL11 (**D**) in non-infected group, and between CXCL11 and CXCR3 (**E**) in HCV group, and between CXCR3 and CXCL11 (**F**) in HBV and HCV co-infected group was determined by Spearman rank analysis. All data showed at log scale.

liver inflammation. A study showed that levels of CXCL9, 10, 11, and 13 and IL-21 were over-expressed when the ALT was at its highest level and decreased during the reduction of HBV-DNA load in acute HBV infection [8]. Moreover, the correlation between the serum level of these chemokines and ALT was reported in HCV-infected patients [21].

Acute rejection remains a serious issue after liver transplantation, and up-regulation of CXCR3 and its ligands (CXCL9, 10, and 11) have been detected to be associated with Th1 cell trafficking into the allograft in the cellular immune response during acute rejection [22–24]. An increase in the serum level of CXCL9, 10, and 11 in early liver dysfunction after transplantation [24] suggests they are noninvasive biomarkers for rejection after transplantation [25]. Another study found that CXCL9 was up-regulated on the first day post-liver transplant and could be used as a noninvasive biomarker for rejection [26].

In contrast, in the current study, a significant association was not observed between the CXCR3 mRNA expression level and its ligands in LT patients with rejection episodes and HBV infection, compared to non-infected patients who experienced rejection. This difference might be related to the small population group.

The effective role of CXCLs in HCV infection was reported earlier [27]. Therefore, in the present research, the CXCL11 mRNA expression level in the HCV and HBV/HCV co-infection groups significantly increased compared to the controls. The increased plasma level of CXCL10 is detected as a hallmark for detecting liver fibrosis progression in patients suffering from chronic HCV infection [28]. The association of both CXCL10 and CXCL11 was reported with mix-HCV and cryoglobulinemia [29]. Pro-apoptotic effects of CXCL10 in hepatocytes were established during HCV infection [30]. Also, the association between CXCL9 and advanced inflammation in HCV infection was also observed [28]. Serum levels of CXCL10, 11, and 12 were considered to be allied with liver cirrhosis in chronic HCV-infected patients, which can be defined as new biomarkers for liver inflammation and fibrosis [29].

CXCL9, 10, and 11 were found to be associated with both the mRNA and protein levels in the course of polyomavirus BK infection post-kidney transplantation (KT). These results presented that CXCL9 mRNA and serum levels up-regulated in KT patients during active polyomavirus BK infection. Also, the mRNA level of CXCL11 and serum level of CXCL10 were evaluated during polyomavirus BK infection, suggesting this infection might induce inflammation via inflammatory chemokines, leading to an increased risk of rejection after transplantation [31,32]. This research suffered from some restrictions, including an inadequate sample size and limitations in patients' follow-up post-transplantation.

In conclusion, CXCR3 and two of its ligands (CXCL10 and 11) are up-regulated in LT patients, especially in HBV and HCV-infected ones. Also, finding direct correlations between the expression levels of CXCLs/CXCR3 and HBV infection in LT patients reveals their possible regulatory role in the pathogenesis of viral hepatitis agents in LT patients. These findings suggest that these chemokines should be studied further for their potential importance as prognostic and diagnostic markers in liver transplant complications.

CONFLICTS OF INTEREST: None to be declare.

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