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Original Research

CXCL9 expression and polyomavirus BK infectivity in renal transplant patients with nephropathy

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Abstract: Polyomavirus BK is an important risk factor for nephropathy and renal lose after kidney transplantation. CXCL9 is a key immunoregulatory molecule which participates in stimulation and migration of immune cells to the infected sites. Thus, the main aim of this study was to evaluate the expression levels of CXCL9 mRNA and serum levels in the infected polyomavirus BK infected renal transplant patients with and without nephropathy compared with healthy controls. This cross sectional study was performed on three studied groups including: polyomavirus BK infected vs. non-infected renal transplant patients with nephropathy and healthy controls. The mRNA and serum levels of CXCL9 were evaluated on the studied patient and control samples using an in-house comparative real time PCR and ELISA methods, respectively. The mRNA expression and serum levels of CXCL9 were both increased in polyomavirus BK infected compared with non-infected renal transplant patients and also in comparing with healthy controls. This upregulation was significant in the serum level in polyomavirus BK infected vs. non-infected patients and also in comparing with controls. According to these results, polyomavirus BK can induce renal complications via stimulation of inflammatory biomarkers like chemokine. Confirmation of the increasing of the expression and production of CXCL9 as a pro-inflammatory chemokine in renal transplanted polyomavirus BK infected patients with nephropathy need to confirm in further completed studies with longer follow-up.

Key words: Polyomavirus BK, Transplantation, Nephropathy, CXCL9.

Introduction

Chemokines are the main immunoregulatory molecules which participate in immune cells activation and migration through interaction with their corresponded receptors (1). CXCL9 is an important chemokine which performs its function via interaction with CXC (C-X-C motif) R3. CXCL9, which is also known as Monokine induced by gamma interferon (MIG), is a pro-inflammatory chemokine, so, a hypothesis has been aroused regarding the critical roles played by this molecule in several immune system related disorders including: autoimmune diseases such as multiple sclerosis (2) and grave's disease (3), viral infections (4), chronic allograft nephropathy (5), and so on. Due to the roles of CXCL9, its expression is elevated in serum and kidney tissue during renal allograft outcomes and rejection (6). Accordingly, it has been proposed that the viral infections which increase expression of CXCL9 may participate in induction of renal lose indirectly. Polyomavirus BK as a small non-enveloped virion, is a main inducer of post-transplant nephropathy in immunocompromised patients (7). Recent data reveal that, the virus is the main cause of renal loses in the kidney transplanted patients which are under treatment of immunosuppressive conditioning regimen (8). It has been estimated that, polyomavirus BK is prevalent in more than 80% of healthy population (9-12), hence, can be considered as a potential risk factor for graft lose after kidney transplantation. The mechanisms of polyomavirus BK for induction of kidney cell injuries and related inflammation have not yet to be clarified. Based earlier reports, inflammation are the main causes of polyomavirus BK associated nephropathy (BKVAN) (9-11, 13). However, the determinative inflammatory components responsible for renal disorders following polyomavirus BK reactivation are under investigation, recently (14).

Based on the fact that CXCL9 is a determinative chemokine involved in the immune cells chemotaxis and activation, the importance of the up-regulation of CXCL9 during renal graft rejection has been reported previously. However, it may be hypothesized that polyomavirus BK may induce BKVAN by manipulation of expression levels of CXCL9. Therefore, the aim of this study was to evaluate the expression levels of CXCL9 chemokine in peripheral blood mononuclear cells (PBMCs), at mRNA and serum levels, in polyomavirus BK infected renal transplant patients with nephropathy compared with non-infected ones and also with healthy individuals to describe the role of CXCL9 in the pathogenesis of BKVAN.

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Materials and Methods

Subjects

This cross-sectional study was performed on 108 renal transplant patients with nephropathy. The participants had referred to Namazi Hospital, Shiraz University of Medical Sciences, Shiraz, Iran, and have been evaluated regarding the infectivity by polyomavirus BK. After determination of polyomavirus BK infectivity, the patients were divided into two groups including: polyomavirus BK infected patients with nephropathy and non-infected renal transplant ones. The nephropathy was approved according to the following criteria: existence of nephropathy related clinical presentation, creatinine higher than 1.5 mg/dL, and glomeruli filtration rate (GFR) lesser than 30 mL/min/1.73 m2 body surface area. The participants were under treatment of an immunosuppressive conditioning regimen consist of: cyclosporine, 5 mg/kg, as initial therapy and following with 2–2.5 mg/kg; prednisolone, 120 mg/day, and following to 10 mg/day routinely; and mycophenolate mofetil, 1000 mg/day, twice daily. In order to inhibition of herpesvirus primary infection and reactivation, 750 mg/day acyclovir was administrated, from three days before transplantation. Additionally, voluntary sex and age matched non-transplanted subjects, healthy controls, were included in the study. The tubes with and without anti-coagulant were used to collected the blood samples to evaluate mRNA levels of CXCL9 and serum levels of CXCL9 as well as polyomavirus BK infection, respectively. The Ethical Committee of Shiraz University of Medical Sciences have been approved the protocol and all participates have also filled out an informed consent.

Polyomavirus BK detection

One EDTA-treated blood sample was collected from each studied renal transplant patient. DNA of polyomavirus BK was extracted from plasma samples using the Invisorb®Spin Virus DNA Blood Mini kit (Invitek, Germany) according to the manufacturer's instruction. Polyomavirus BK genomic DNA quantification was done using Genesig BKV Real-Time PCR kit (Primer Design Ltd TM, Advanced kit,UK). The protocol was optimized based on in-house instruments. polyomavirus BK PCR mixture in a final reaction volume of 20 µL containing: 5 µL of the DNA, 10 µL Precision™ Master Mix (Applied Biosystems), 1 µL primers and a probe targeting the polyomavirus BK NCCR sequence, 1 µL primers and a probe targeting the internal control (IC) gene, and 3 µL DEPS water. The thermocycling condition for polyomavirus BK included one cycle at 95°C for 10 min followed by 50 cycles at 95°C for 5 sec and 60°C for 60 sec using Step One Plus Real-Time thermocycler (Applied Biosystems, USA). This quantitative PCR assay was sensitive enough to detect 10 copies of polyomavirus BK genomic DNA / mL of plasma samples.

Evaluation of the CXCL9 Gene Expression

CXCL9 mRNA level was quantified using a competitive Sybr Green Real-Time PCR protocol. Peripheral blood mononuclear cells (PBMCs) were used for extraction of total mRNA by RNX plus solution (CinnaGen, Iran). The mRNA integrity and purity were evaluated by running on agarose gel (1%) electrophoresis and measuring the optical density 260/280, respectively. DNase (1u/µg of RNA) was used to treat the purified mRNA to remove genomic DNA and then 5 µg of total mRNA was reversely transcribed to complementary DNA (cDNA) using reverse transcriptase (RT) enzyme in association with random hexamer (Vivantis, Malaysia). Briefly, mRNA (5µg), dNTPs (1µ/10mM) and random hexamer (1µl/0.2µg) were mixed and incubated at 65°C for 7 minutes. After incubation on ice for 2 minutes, the following material were added to the tubes: M-MLV RT enzyme (1µl/200U), RT-buffer (2µl/10x), RNase inhibitor (1.3µl/ 60U) and appropriate levels of nuclease free water and was incubated at 42 ℃ for 60 minutes and at 85 ℃ for 5 minutes. Appropriate primers (CXCL9 F: 5`- GTGGTGTTCTTTTCCTCTTGG-3`, CXCL9 R: 5`- ATAGTCCCTTGGTTGGTGCT-3`, GAPDH F: 5`- GACTCATGACCACAGTCCA-3` and GAPDH R: 5`-CCAGTAGAGGCAGGGATGAT-3`) and a commercial Real-Time Sybr Green master mix (Takara, Tokyo, Japan) was used for quantification of the expression levels of CXCL9 and GAPDH (as the housekeeping gene) in Exicycler™ 96 Quantitative Real-Time PCR System. The PCR program which was used for evaluation of the expression levels of CXCL9 and GAPDH in synthesized cDNA was as follow: 5 min at 95°C following by 40 cycles of denaturation at 95°C for 1 min, 56 for 55 sec and extension at 72°C for 1 min. The $2^{-\Delta\Delta C(T)}$ formula, based on the Livak method was used for calculation of the results(15).

Quantification of the serum level of CXCL9

The CXCL9 serum level was measured in polyomavirus BK infected renal transplant patients with nephropathy vs. non-infected ones and healthy controls using a commercial ELISA kit (Booster Immunoleader, USA) according to the manufacturer's instruction.

Statistical analyses

The statistical differences in the expression and serum levels of CXCL9 and the fold changes between patient groups and controls were compared via analysis of variance (ANOVA) and the Livak ($2^{-\Delta\Delta CT}$) methods. Statistical analyses were performed with SPSS software (SPSS: An IBM Company, version 18.0, IBM Corporation, Armonk, NY, USA). *P* values less than .05 were considered.

Results

CXCL9 Gene Expression in Renal Transplant Patients With and Without Polyomavirus BK Infection and Controls

The CXCL9 gene expression level (based on ΔCT and $2^{-\Delta\Delta CT}$ analysis) in polyomavirus BK infected renal transplanted patients vs. non-infected ones and controls were presented in figure 1.A and 1.B, respectively. The findings have been shown that CXCL9 gene expression level was upregulated in polyomavirus BK infected patients compared with non-infected ones and also in comparing with controls. Also the CXCL9 gene expression level was upregulated in non-infected renal transplant patients vs controls (figure 1.A). Based on $2^{-\Delta\Delta CT}$ statistical analysis, CXCL9 was increased 116 vs 61.6

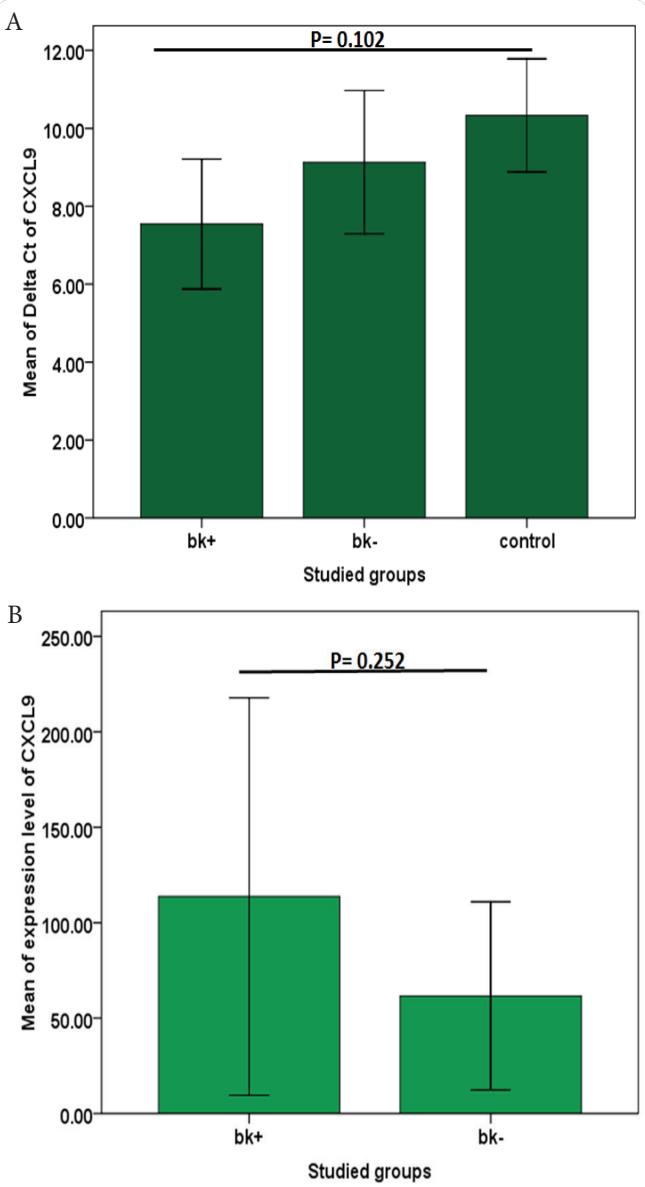

Figure 1. Delta Ct and mRNA levels of CXCL9 in the studied groups. The figure shows that delta Ct (*P*= 0.252, 95%CI= 0.173- 0.188) and mRNA levels (*P*= 0.252, 95%CI= 0.509- 0.529) of CXCL9 was not significantly differ among studied groups.

fold change in polyomavirus BK infected renal transplant patients with nephropathy compared with non-infected ones (figure 1.B). However, the results revealed that mean of CXCL9 mRNA level were not significantly differ among polyomavirus BK infected patients with nephropathy, compared with non-infected ones and in comparing with healthy controls (*P*= 0.252, 95%CI= 0.250- 0.268).

CXCL9 serum level in Renal Transplant Patients With and Without Polyomavirus BK Infection and Controls

Analytical results of CXCL9 serum level between polyomavirus BK infected renal transplant patients with nephropathy and non-infected ones as well as controls were presented in Figure 2. The CXCL9 serum levels was significantly higher in the polyomavirus BK infected (1309 ± 264.46 pg/mL) vs. non-infected (549.04 ± 42.09 pg/mL) renal transplanted patients with nephropathy as well as controls (264.33 ± 53.74 pg/mL), (*P*< 0.001, 95%CI= 0.00- 0.00). The CXCL9 serum level was also significantly increased in non-infected polyomavirus BK patients compared with healthy controls (*P*< 0.001, 95%CI= 0.00- 0.00) (Figure 2). In spite of upregulation of CXCL9 gene expression and serum levels in virus infected compared with non-infected renal transplant patients and vs. controls, significant association was not found between polyomavirus BK load with CXCL9 serum (r= 0.150, *P*= 0.326) and mRNA (r= -0.113 *P*= 0.608) levels.

Discussion

Polyomavirus BK reactivation is one of main viral causes of renal lose and BKVAN after renal transplantation (16). On the other hand, chemokines are determinative immunoregulatory molecules which participate in the recruitment and activation of adaptive and innate immune cells against viral infected and inflamed tissues (1, 17). Based on the fact that, CXCL9 is a pro-inflammatory chemokine and play a key role in induction of immune responses, the aim of this study was to evaluate the expression levels of CXCL9 in both mRNA and serum levels among polyomavirus BK infected renal transplant patients with nephropathy, non-infected ones and healthy controls. The results showed that CXCL9 mRNA levels were upregulated in polyomavirus BK infected renal transplant patients with nephropathy in comparing with non-infected ones and also with healthy controls. The CXCL9 at serum level was also elevated especially significant in virus infected patients compared with non-infected once and controls and also in the non-infected renal transplant patients when compared with healthy controls. Due to these results, it appears that post renal transplant nephropathy is associated with upregulation of CXCL9 expression and serum levels and infectivity of the polyomavirus BK. Although, the mRNA expression was not significantly altered, but also the serum level was elevated more in the polyomavirus BK infected patients with nephropathy and non-infected ones, may related to this fact that polyomavirus BK may increased CXCL9 production by increasing the CXCL9 mRNA stability or increased activities of ribosome. Other possibility may relate to negative inhibitory that

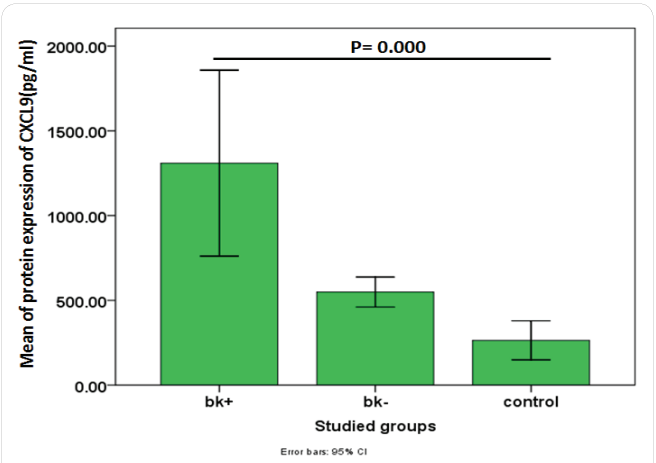

Figure 2. The protein expression of CXCL9 in studied groups. The figure revealed that CXCL9 serum levels were significantly increased in the BK virus positive and negative renal transplanted patients BK in comparison to healthy controls (*P*< 0.001, 95%CI= 0.00- 0.00).

suppress the increase of CXCL9 expression at mRNA levels. Additionally, previous investigations demonstrated that CXCL9 can be produced by other cells like renal tubular epithelial cells (18). It may also be concluded that elevation of the CXCL9 serum levels in polyomavirus BK infected and non-infected patients may be related to induction of CXCL9 production by other cell systems (19). Altogether, it appears that CXCL9, as a pro-inflammatory chemokine, may be considered as an important risk factor in the pathogenesis of renal transplant associated nephropathy, polyomavirus BK reactivation may also be a main inducer of CXCL9 expression and subsequently deteriorate the pathologic condition and induce BKVAN in CXCL9 dependent manner. In another word, CXCL9 induces nephropathy in polyomavirus BK infected and non-infected patients by infiltration of immune cells and stimulation of inflammatory biomarkers. Previous investigations approved the crucial roles of CXCL9 in the pathogenesis of nephropathy and also BKVAN. Similar to these results, Panzer and colleagues reported the increased number of CXCR3, the main receptor for CXCL9, that bearing T cells in the renal transplanted patients with nephropathy (6). Interestingly, other investigators have also approved the significant roles of CXCR3 in nephropathy outcomes following renal transplantation (20-23). Based on our results increased expression of CXCL9 in polyomavirus BK infected compared with non-infected patients, may be hypothesized that polyomavirus BK has a determinative direct and indirect role in introducing of nephropathy after kidney cell lysis and up-regulation of pro-inflammatory molecules such as CXCL9 (24). This hypothesis has also been confirmed by Hu *et al.,* which have reported that urine levels of CXCL9 can be considered as an important risk factor for acute renal-allograft dysfunction (19). Jackson and colleagues have also revealed that urinary levels of CXCL9 are the markers of renal allograft rejection in polyomavirus BK infected patients (25).

Based on these findings, it seems that CXCL9 as an important immunoregulatory molecule participates in the pathogenesis of BKVAN. However, targeting of CXCL9 with more biochemical details in polyomavirus BK infected renal transplant patients are needed to confirm this determinative role in further completed studies with longer follow-up.

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