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The Impact of HLA-G and HLA-E Polymorphisms on CMV Reinfection in Liver Transplant Recipients

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ABSTRACT

Background: Cytomegalovirus (CMV) reinfection in transplant patients has been associated with graft loss and decreased patient survival. In this regard, the HLA-G molecule has the immunomodulatory characteristic and its soluble isoforms have important roles in immunity to viruses. The 14bp insertion/deletion polymorphism impacts HLA-G mRNA stability. Regarding the HLA-E molecule, two nonsynonymous alleles, HLA-E*0101, and HLA-E*0103 are different in their functions including the affinity of the relative peptide.

Objective: To explore the possible link between HLA-G and HLA-E polymorphisms with CMV reinfection among liver transplant recipients (LTRs).

Methods: In this study, a total of 140 liver transplantations were performed; of which 70 CMV-reactivated LTRs and 70 CMV non-reactivated ones were recruited. The cut-off value of CMV DNA was determined to be 100 copies/mL. PCR evaluated different genotypes for HLA-G and ARMS-PCR for HLA-E*0101 and *0103.

Results: Neither the HLA-G genotypes (-14 bp/-14bp and +14bp/+14 bp homozygous genotypes with the Ps=0.43, and 0.13, respectively, +14 bp/-14 bp heterozygous genotype with P=0.49) nor the HLA-E genotypes (HLA-E*0101/0103, HLA-E*0101/0101, and HLA-E*0103/0103 with the Ps=0.152, 0.249, and 0.391, respectively) had any association with CMV reinfection in the LTRs.

Conclusion: No difference was observed in the HLA-E and HLA-G genotype frequencies between our studied groups. Further studies are needed to explore other genetic variations and evaluate soluble HLA-G and HLA-E levels in the transplant population.

Keywords: Acute Rejection, Cytomegalovirus, Liver Transplantation, Non-classical HLA

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INTRODUCTION

Cytomegalovirus (CMV) is the leading infectious reason of complications in immunocompromised individuals [1, 2]. In this regard, patients needing organ transplants who take immunosuppressive drugs are at high risk of recurrent CMV infections [3, 4]. Like other herpesviruses, CMV elicits a cellular immune response and humoral immunity upon acute infection [5, 6]. CMV has several diverse immune escape mechanisms. For instance, the human leukocyte antigen-G (HLA-G) level can be upregulated through CMV infection on activated macrophages [7, 8] and monocytes [9, 10]. In trophoblast cells, in addition to classical HLA class I molecules, HLA-G a non-classic HLA-I antigen is downregulated. Upon CMV infection, natural killer (NK) cells response could be suppressed through the interaction of KIR2DL4 on NK cells with HLA-G [11, 12]. HLA-G has an essential role in feto-maternal tolerance principally presented at the interface between the maternal and the placenta as the perfect model of a semi-allograft [13]. Notably, the fetus is maintained from the injury of maternal NK cells response through HLA-G [14, 15]. When there is no pathology, HLA-G is expressed in the cornea ,thymus, pancreas, and hematopoietic stem cells [14, 16]. Upon "non-physiological" states, HLA-G molecules are expressed ectopically during virus infection[12, 17] cancer [18], transplantation [19], inflammation, and autoimmune diseases [20, 21]. In pathological settings, HLA-G may display two opposite effects, it could play a protective role in inflammation and autoimmune diseases [22] or a risk factor, for instance, in cancers or microbial infection [23]. HLA-G can comprehensively modulate host anti-graft immune responses in transplant immunology in multiple pathways. Expression of HLA-G leads to impairment of immune cell function. In this regard, the expansion of immune regulatory cells, such as tolerogenic dendritic cells (DCs) and regulatory T cells (Tregs), can be induced and expanded by HLA-G–receptor interactions [24]. Multiple polymorphisms control HLA-G expression, including those in the promoter and 3′ untranslated region (3′ UTR), altering transcriptional and posttranscriptional factor affinity of gene-targeted sequences [25]. In the HLA-G promoter region, twenty-nine single nucleotide polymorphisms (SNPs) play a role in regulating HLA-G expression [12]. In exon eight, an insertion/deletion (INS/DEL) polymorphism of fourteen base pairs affects mRNA production and stability [24]. Notably, the DEL allele results in an increased level of HLA-G through mRNA stabilization [26]. This INS/DEL variant impacts the HLA-G production level. Besides, another non-classic HLA-I molecule is HLA-E, which has a key role in the interaction with CMV peptides, particularly UL40, through NK cells [27]. Two non-synonymous alleles of HLA-E including HLA-E*0103 and HLA-E*0101 differ by a single amino acid (R107G) [28]. Each has a different expression level of HLA-E molecules, which might be caused by their different roles in the peptide affinity. Indeed, there is a difference between the two investigated HLA-E alleles in activity and expression. The evaluation of these two alleles of HLA-E with peptide has demonstrated a greater production of HLA-E*0103 compared with HLA-E*0101 [19], and the HLA-E*0101 allele is less stable than the HLA-E*0103 allele [30]. HLA-E plays a considerable role in regulating immune response through the interaction with CD94/NKG2 receptors expressed on distinct NK cell subsets [31]. Only two receptors of the NKG2 family can interact with HLA-E. It interacts mainly with NK cells through the inhibitory CD94/NKG2A and activating CD94/NKG2C receptors. Both receptors can bind competitively to the same HLA-E epitopes; however, NKG2A/CD94 has a higher affinity to HLA-E than NKG2C/CD94. The interaction between NK cells and their target cells bearing HLA-E depends on the HLA-E and NKG2/CD94 binding. NK cells are the cornerstone of the human

immune response to CMV infections [32]. Since the modulation of NK cell's function is done by the interactions of inhibitory or activating receptors with HLA-E, it indicates the necessity of HLA-E variant allele studies, which could be considered in the prediction of CMV reactivation. Notably, during CMV infection, the transition from viral latency to reactivation is an ability of the CMV genome under certain conditions [33]. For instance, the HLA-E/NKG2A axis has been highly jacked by CMV for immune evasion [32]. It drives the interaction between HLA-E and CD94-NKG2 receptors, specially NKG2A, via the expression of the CMV UL40-encoded glycoprotein [34]. Considering the substantial role of HLA-G and HLA-E in the immunity to CMV activation, we aimed to investigate the HLA-G 14-bp INS/DEL and HLA-E genetic variants in the context of liver transplantation.

MATERIALS AND METHODS

Study Population

The sample population included individuals who underwent liver transplantation at Shiraz University of Medical sciences. CMV DNAemia was checked in plasma samples of all the patients, and CMV-reactivated subgroups were selected through quantitative Real-Time PCR data (CMV-DNA>100 copies/mL). Then, HLA-G polymorphism and HLA-E*0101and*0103 genotypes were evaluated in the study population of 140 liver transplant recipients, including 70 CMV-reactivated LTRs and 70 CMV non- reactivated ones through PCR for HLA-G and PCR-ARMS. The Ethics Committee of Shiraz University of Medical Sciences confirmed the protocol, and we obtained written informed consent from all the participants. The clinical characteristics of the studied groups were collected from the electronic folder of the transplant center [14]. Based on Banff criteria, the clinical factors including the increase of bilirubin and transaminase levels and fever with lack of vascular complication, blockade in the biliary system, or tissue sample results, are the current tests for the acute rejection diagnosis.

Immunosuppressive Treatment and Anti-viral Medication

The immunosuppressive medication plan included 1-2 mg of tacrolimus per day and 500 mg of mycophenolate mofetil per day, and prednisone was received at a dose of 120 mg every 12 hrs. The CMV-infected patients took valganciclovir high doses with 900 mg PO q12hr for 21-42 days or until the signs and symptoms resolved [35].

HLA-G & HLA-E Genotypic Variations

The DNA was isolated from peripheral leukocytes by the commercial extraction kit (DNP Extraction Kit, Cinagene Company, Tehran, Iran). Besides, the PCR was used for the amplification of the HLA-G 14 bp INS/DEL variant (rs16375) [36] with the following primers: forward HLAG1 5′-GTGATGG GCTGTTTAAAGTGTCACC-3′ and reverse HLAG2 5′-GGAAGGAATGC AGTTCAGCATGA-3. PCR reaction included 25 µl, 100 ng DNA, 10 pM of each primer, 1X Go Taq buffer including 1.5 mM MgCl2, 1.25 U GoTaq DNA polymerase (Cinagene Company, Tehran, Iran), and 0.2 mM each dNTP (Cinagene Company, Tehran, Iran). Based on the insertion or deletion of 14 bp in exon 8 of the HLA-G gene, two fragment sizes were observed 224 or 210 bp (Figure 1). The PCR procedure was performed using the following conditions: primary denaturation (92 °C, 5 min) followed by 30 cycles of denaturation (92 °C, 30 seconds), annealing (64 °C, 1 min), and synthesis (72 °C, 2 min). The amplification products were evaluated by electrophoresis via 4% agarose gel and then observed under ultraviolet light. HLA-E genotyping was performed using an amplification-refractory mutation system (ARMS) PCR method. Twenty-five µl reaction mixture contained Genomic DNA (100 ng), with a 10 pM concentration of each primer set, including forward

5′-ATACCCGCGGAGGAAGCGCCT-3′ and reversed 5′-TCCCAGATTCACCCCAAG-3′ primers, 1×PCR buffer, 200 µl of dNTP mixture, MgCL2 with 1.5 mM concentration and 2.5 U of Taq DNA polymerase (Cinagene Company, Tehran, Iran). The PCR cycling was carried out by Eppendorf master cycler (Applied Biosystems, California, USA) as initial denaturation (95 °C, 2 min), and 35 cycles with denaturation (94 °C, 45 s), annealing 1 min in 61 °C, extension (72 °C, 1 min), and final extension (72 °C, 5 min). Then the result was electrophoresed on 1.5% agarose gel (Figure 2).

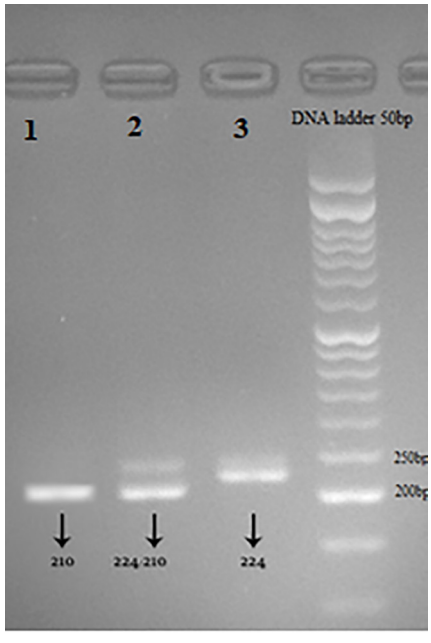

Figure 1. PCR reactions for detection of HLA-G 14 bp (INS/DEL) polymorphism (rs16375). Based on the insertion or deletion of 14 bp in the HLA-G exon 8, two fragment sizes were observed 224 or 210 bp.

Statistical Analysis

All analyses were conducted using the SPSS, v16.0 (SPSS Inc., Chicago, IL, USA), and Epi info 2000. The data were presented as the mean±SD. The normality test (Kolmogorov-Smirnov test) was done to analyze the normal and non-normal distribution data. For the comparison of continuous variables, Student T-test and Mann-Whitney were done. In addition, Pearson's χ^2 test and Fisher's exact test were used to study the differences among the studied patient groups in the clinical results and the frequency of alleles and genotypes in CMV-reactivated recipients and non-reactivated ones. The $P<0.05$ was defined as statistically significant.

RESULTS

Tables 1 and 2 indicate the demographic and clinical data of liver transplant donors/recipients. No significant difference was found regarding gender, age, or rejection events

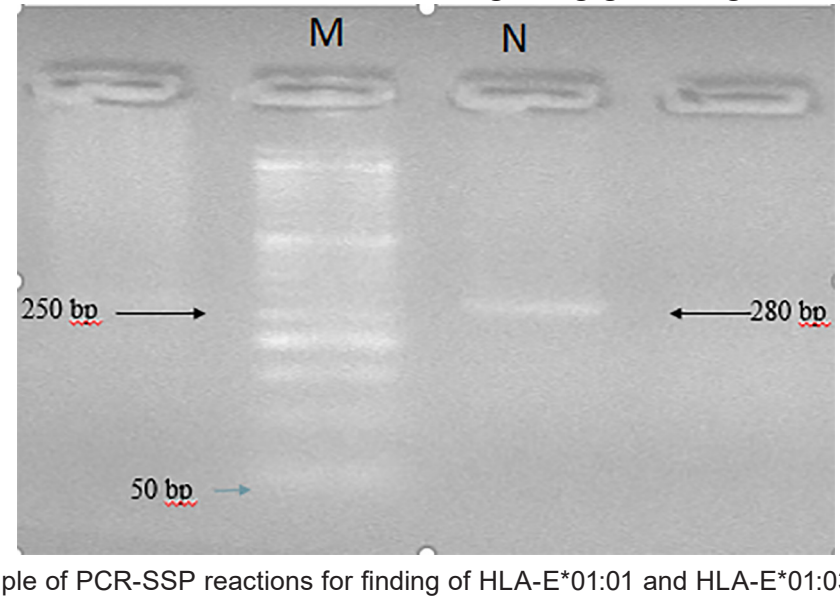

Figure 2. Sample of PCR-SSP reactions for finding of HLA-E*01:01 and HLA-E*01:03 (Lane N). The amplified PCR product is a fragment length of 280 bp. The resulting 280 bp fragment was assessed by electrophoresis on 1.5% agarose gel. Lane M: 2,000 ladder. ARMS-PCR, amplification refractory mutation system polymerase chain reaction.

Table 1. Demographics of liver graft Donor /recipients

Parameters	CMV reactivated group %		CMV non-reactivated group%	
	Donor	Recipient	Donor	Recipient
Number of patients	(n=70)	(n=70)	(n=70)	(n=70)
Female	26 (37.1)	23 (32.9)	24 (34.3)	23 (32.9)
Male	44 (62.9)	47 (67.1)	46 (65.7)	47 (67.1)
Age	34.24±17.45	30±20.7	33.72±16.24	36.45±18.39
Rejection	29 (41.4)		20 (28.6)	
Non-rejection	41 (58.6)		50 (71.4)	
CD	70 (100)		70 (100)	
LD	0		0	

CD: Cadaver donor, LD: Living donor; CMV: Cytomegalovirus

Table 2. Liver transplantation by etiology of liver diseases in liver transplant recipients

Primary liver diagnosis	CMV reactivated LTRs%	CMV non-reactivated LTRs%
HBV/HCC/HCV	18 (25.7)	19 (27.1)
A. hepatitis	4 (5.7)	14 (20)
PFIC /BA	12 (17.1)	4 (5.7)
Cryptogenic	10 (14.3)	8 (11.4)
ALF	6 (8.6)	2 (2.9)
Cryptogenic	10 (14.3)	8 (11.4)
Wilson disease/ Tyrosinemia/ CNs	6 (8.6)	11 (15.8)
Alcoholism/ NAFLD	4 (5.7)	4 (5.7)

LTRs: liver transplant recipients, ALF: Acute Liver Failure, CNs: Crigler Najjar syndrome, NAFLD: Nonalcoholic fatty liver disease. A. hepatitis: Autoimmune hepatitis, BA: Biliary atresia; CMV: Cytomegalovirus

upon CMV infection, between the CMV-reactivated LTRs and the non- reactivated ones. Our findings of different HLA-G genotypes analysis indicated that there was the homozygous−14/−14 bp genotype in 43 recipients, and the heterozygous−14/+14 bp genotype was found in 61 recipients, in addition, the non-deleted homozygous (+14/+14 bp genotype) was detected in 36 recipients (Table 3). The frequencies of the homozygous genotypes (−14 bp/−14 bp (P=0.43) and +14bp/+14 bp (P=0.13), and the frequency of the +14 bp/−14 bp heterozygous genotype (P=0.49) were not different among the CMV-reactivated LTRs than those of the non-reactivated ones (Table 3). Furthermore, there were no differences in allele frequencies of 14-bp polymorphism between the two groups (allele +14 P=0.25, and allele -14 P=0.3). Notably, the HLA-E*0101/0103 alleles genotype was found to be the most frequent genotype and revealed to be of the highest frequency (100%), followed by the HLA-E*0101⁄0101 genotype (0%) and finally, HLA-E*0103⁄0103 genotype (0%) in each group (Table 4). The difference in the frequencies of HLA-E*0101/0103, HLA-E*0101⁄0101, and HLA-E*0103/0103 in LTRs with CMV-reactivation were not statistically significant compared with those in the non-reactivated ones (Ps=0.152, 0.249, and 0.391, respectively).

DISCUSSION

Immunity regulation is affected by the HLA-G molecule [37]. The expression of this molecule has been studied in detail in pathological conditions such as cancers and infectious diseases. The relevance of studies lies in the inhibitory effect of this molecule and its increased expression in pathological conditions [38]. The 14-bp polymorphism

Table 3. Distribution of HLA-G 14 bp INS/DEL (rs16375) genotypes/alleles among LTRs with CMV reinfection and without CMV reinfection

	CMV reactivated LTRs% (n=70)	CMV non-reactivated LTRs% (n=70)	P value
1) Genotype			
-14/-14	17 (24.3)	26 (37.1)	0.430
-14/+14	36 (51.4)	25 (35.8)	0.496
+14/+14	17 (24.3)	19 (27.1)	0.139
2) Allele			
+14 allele	70 (50)	77 (55)	0.259
-14 allele	70 (50)	63 (45)	0.321

LTRs: Liver transplant recipients; CMV: Cytomegalovirus

Table 4. Frequencies of HLA-E*0101/0103 genotypes/alleles in LTRs with CMV reinfection and without CMV reinfection

	CMV reactivated LTRs% (n=70)	CMV non-reactivated LTRs% (n=70)	P value
1) Genotype			
0101/ 0101	0 (0)	0 (0)	0.249
0103/ 0103	0 (0)	0 (0)	0.391
0101/ 0103	70 (100)	70 (100)	0.152
2) Allele			
0101allele	70 (50)	70 (55)	0.359
0103allele	70 (50)	70 (55)	0.431

LTRs: Liver transplant recipients; CMV: Cytomegalovirus

in exon 8 of the HLA-G gene has recently been studied with genetic variations. Studies reported that the deleted variant of 14-bp occurred more than the non-deleted homozygous (+14/+14 bp) genotype [39]. Literature data found an association between the high expression of HLA-G and a low incidence of rejection episodes in transplanted patients [40]. In addition, HLA-G has been assigned to be a good tolerance factor involved in better graft results [41]. Otherwise, there is a negative correlation between the expression of this molecule and protection against viral infections [38]. Several studies have indicated that the alteration of HLA expression shows an essential role in the CMV infection [39]. Consistent with this, Zheng Xq et al. [42] studied the role of HLA-G 14 bp INS/DEL variants in the reactivation of CMV .They observed that the occurrence of deleted homozygous -14 bp genotype among CMV-reactivated individuals enhanced (P=0.00034), and a difference was found in the frequency of the -14 bp allele (P=0.0023) compared with the healthy control individuals. Moreover, the -14 bp/ -14 bp genotype in urine CMV DNA of patients was significantly greater than in the group with the non-deleted homozygous genotype of 14 bp (P=0.041). Myriam Onno et al. [7] based on their investigation, suggested that the stimulation of HLA-G molecules might be a helpful element that causes CMV to escape the host immune system . Another study by Guberina et al. [43] monitored the recipients with genotyping of HLA-G +3142 CC and HLA-G level of plasma impacting the incidence of CMV activation after a kidney transplant procedure. Their result confirmed that HLA-G establishes CMV infection post-kidney transplantation with a living donor. The genotype of HLA-G +3142 CC and serum HLA-G concentrations are considered risk factors for the prediction of CMV reinfection. In the present study, the frequency of 14-

bp INS/DEL polymorphism in recipients of liver allografts with CMV reinfection and the recipients without reinfection of CMV were evaluated. Since no significant differences were detected between our studied groups, it should be noted, many factors may influence the reinfection of CMV. Patients with impaired immunological defense due to immune suppressive therapy are particularly prone to opportunistic infections such as CMV [3, 44]. CMV expresses several genes and microRNA to escape the immune response [45]. Therefore, many elements from the virus and the host interfere with the CMV reinfection. Furthermore, HLA-E could be another key in regulating the immune response to CMV infection. Previous studies of HLA-E gene polymorphism have reported that the two main HLA-E alleles, E*0101, and E*0103, may be conserved by natural selection in most populations, indicating a distinct functional difference between these alleles. There is a link between HLA-E gene variants and some diseases, including viral infections, malignant, and autoimmunity [46, 47]. In this regard, some studies reported that E*01:03 has a higher affinity to peptides, higher surface expression, and higher thermal stability compared with E*01:01 [48]. In addition, some other studies evaluated the association between HLA-E polymorphisms and transplantation consequences after HLA-matched allogeneic hematopoietic stem cell transplantation (HSCT). They found an association between HLA-E*0103 homozygosity and a better survival in their patients [49, 50]. On the other hand, the HLA-E*0101 allele, unlike HLAE*0103, might have a better affinity to CD94/NKG2A, an inhibitory receptor on NK cells and CD8+T cells causing higher cytotoxicity in the inflammatory environment, which mediates tissue damage in the context of transplantation [49]. Recently, Vietzen H. et al. [51] highlighted the association between heterozygous HLA-E*0101/0103 variants and high-level viremia in lung transplant patients. A recent investigation revealed that there are CMV-specific NK cells with memory features resulting in the limitation of CMV replication after transplantation. These memory-like NK cells (NKG2C+NK cells) could efficiently control the CMV replication in transplant patients, even under immunosuppressive therapy. NKG2C receptor, an activating receptor on NK cells, interacts with HLA-E molecules on the surface of CMV-infected cells inducing NK cell expansion. CMV also appears to specifically influence the interaction between HLA-E and two distinct CD94-NKG2 receptors (NKG2A, NKG2C) via the expression of the UL40 glycoprotein. Still, this study has shown that CMV-encoded UL40 peptides stabilize the interaction between HLA-E and NKG2C. Thus, the interaction between different UL40-derived HLA-E-binding peptides and NKG2C receptor regulate the NK-cell response against CMV infection [52, 53]. To characterize the effect of HLA-E alleles as a predictive factor on CMV active infection in our liver transplant cases, we compared the numbers of HLA-E alleles (E*0101, E*0103) between CMV-reactivated LTRs and CMV non- reactivated ones. Our finding demonstrated that HLA-E*0101/0101 and HLA-E*0103/0103 frequencies were very low compared with the frequencies of HLA-E*0101/0103 (heterozygote) in the total liver transplant population, and no significant difference was found in HLA-E alleles (E*0101, E*0103) among the two groups. One reason for different HLA-E allele distributions could be related to the fact that different populations present varied immunogenetic profiles depending on race and ethnicity. In our region, the allele frequency of HLA-E*0101/0103 might be the highest. However, there is no adequate evidence to prove this hypothesis. In this line, future studies are necessary to determine HLA-E genotyping in a larger sample size. To clarify the role of HLA-E in liver transplantation results, immunophenotyping of patients' peripheral blood lymphocyte subpopulations could be helpful. In addition, it is valuable to evaluate the influence of the donor HLA-C genotyping on transplantation consequences.

The present study's findings revealed no significant association between HLA-E alleles and the insertion or deletion variants of HLA-G 14 bp with CMV reinfection post liver transplant. Although earlier studies found the key effect of 14 bp polymorphism in the post transcription regulation of the HLA-G gene, further studies are suggested to be assessed on other gene variants sites located at 5 ′ untranslated regions (5′UTR) or 3′UTR, around the transcription factor binding sites that impact HLA-G mRNA accessibility. Besides, the 3′UTR, the HLA-G polymorphic promoter region seems to have a substantial role in regulating its expression[54]. Hence, functional studies on immune regulation are also needed to clarify the role of HLA-G in CMV infection in the context of transplantation. Ultimately, differential expression of HLA-G might be affected by the variations of other genes. Altogether, this investigation indicates that the variants of HLA-G14-bp and HLA-E*0103/0101 heterozygotes are not the predictive factors exclusively responsible for CMV reinfection in liver transplant patients.

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Conflicts of Interest: None declared.

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