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Protective KIR-HLA interactions for HCV infection in intravenous drug users

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Abstract

Intravenous drug use has become the principal route of hepatitis C virus (HCV) transmission due to the sharing of infected needles. In this study, we analyzed the distribution of *HLA-KIR* genotypes among 160 Puerto Rican intravenous drug users (IDUs) with HCV infection and 92 HCV-negative Puerto Rican IDUs. We found a significant association between the presence of different combinations of KIR inhibitory receptor genes (*KIR2DL2* and/or *KIR2DL3*, $pC = 0.01$, OR = 0.07; *KIR2DL2* and/or *KIR2DL3*+*KIR2DS4*, $pC = 0.01$, OR = 0.39) and HLA-C1 homozygous genotypes (HLA-C1+*KIR2DS4*, $pC = 0.02$, OR = 0.43; HLA-C1+*KIR2DL2*+*KIR2DS4*, $pC = 0.02$, OR = 0.40) together with the activating receptor *KIR2DS4* (HLA-C1+*KIR2DS4*+*KIR2DL3* and/or *KIR2DL2*, $pC = 0.004$, OR = 0.38) with protection from HCV infection. Our findings in HCV-infected and non-infected IDUs suggest an important role for KIRs (*KIR2DL2* and *KIR2DL3*) with group HLA-C1 molecules, in the presence of activating *KIR2DS4*, in protection from HCV infection. These results

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support the hypothesis that activator signaling, mediated by KIR2DS4, is a determinant in the regulation of NK cell antiviral-activity.

Keywords

KIR; HLA; NK cells; HCV

1. Introduction

Hepatitis C virus (HCV) infection is a growing worldwide public health problem. For example, in the US, antibodies to HCV are found in 1.8% of blood donor volunteers and are present in much higher percentages in developing countries (Alter et al., 1999; Post et al., 2004). After initial HCV infection, 70–80% of patients develop chronic persistent HCV viremia (CV), and at least 20% of these patients progress to liver cirrhosis and many of them develop hepatocellular dysplasia and, eventually, hepatocellular carcinoma (Davila et al., 2004).

Interestingly, about 20–40% of HCV-infected patients spontaneously clear the virus by mechanisms not completely understood. Furthermore, despite HCV's high infectivity and ability to remain protected inside hepatocytes, some HCV-exposed individuals fail to develop acute or chronic hepatitis C (Post et al., 2004; Davila et al., 2004). Previous studies reported an association between the natural killer (NK) cells' immunoglobulin-like receptors (KIR), MHC class I (Khakoo et al., 2004) and class II (Cramp et al., 1998; Fanning et al., 2000) loci, and the clinical outcome of HCV infection. Previously reported associations between HLA genes, ethnicity, and clinical outcome of HCV infection (Alric et al., 1997; Cramp et al., 1998; Thursz et al., 1999) have come under scrutiny as they may have resulted from either population stratification or from different non-random associations among distinct MHC alleles in different ethnic groups (Pritchard and Rosenberg, 1999; Cardon and Palmer, 2003).

The KIRs are a family of cell surface receptors that recognize MHC class I molecules as ligands and are predominantly expressed on NK cells (Hsu et al., 2002). KIR proteins react with one of two epitopes at amino acid position 80 on HLA-Cw (and a few rare HLA-B) molecules. Those HLA-Cw molecules that have asparagine are group C1, those that have lysine are group 2. In humans, KIRs are encoded by a cluster of genes located in the leukocyte receptor complex on chromosome 19q13.4. At least 14 functional *KIR* genes have been thus far characterized, of which 8 are NK cell inhibitory (*KIR2DL1*, *2DL2*, *2DL3*, *2DL4*, *2DL5*, *3DL1*, *3DL2*, *3DL3*), 6 are activating (*KIR2DS1*, *2DS2*, *2DS3*, *2DS4*, *2DS5*, *3DS1*), and 2 are pseudogenes (*KIR2DP1*, *3DP1*). There are a variable number of KIR inhibitory and activating genes among individuals and extensive allelic polymorphism of the known *KIR* genes has been described (Uhrberg et al., 1997; Hsu et al., 2002). NK cell activity is regulated by the expression array of inhibitory or activating receptors that recognize MHC class I molecules on target cells (Uhrberg et al., 1997; Hsu et al., 2002). Inhibitory *KIR2DL3* genotypes and HLA-C1 homozygotes are thought to be involved in HCV SC (Khakoo et al., 2004), possibly via loss of inhibition of NK cells (Parham, 2004; Romero V et al., 2008). However, it is calculated that *KIR* gene activation conferring either SC or CV accounts for only some (~20%) of the SC outcomes, suggesting that other genes are also involved.

The aim of this study was to establish the role of different KIR-HLA genetic interactions in protection from or susceptibility to HCV infection in ethnically-matched intravenous drug users (IDUs) with and without HCV infection.

2. Materials and Methods

2.1 Subjects

In this case-control study, a group of 252 IDUs were studied, of which 160 had HCV infection and 92 had no evidence of anti-HCV antibodies or infection. HCV infection was assessed by the presence of anti-HCV IgG detected by both third-generation enzyme immunoassay (EIA) and recombinant immunoblot assay (RIBA). HCV RNA was determined by COBAS Amplicor HCV Kit (Roche Diagnostic Systems, Branchburg, NJ, USA). HCV genotypes were determined by restriction fragment length polymorphism (RFLP) analysis of the product of the reverse transcriptase-polymerase chain reaction (RT-PCR). The 160 HCV-infected patients included in this study had anti-HCV antibodies and detectable HCV RNA in serum for more than 6 months with or without persistently increased alanine aminotransferase levels. Thirty-nine HCV-infected patients (24.2%) were identified as having spontaneous clearance (SC) on the basis of 1) the presence of anti-HCV IgG detected by both third-generation EIA and RIBA and 2) undetectable serum HCV RNA by qualitative assay in three successive samples collected after the first negative HCV RNA result over a minimum 6-month period. Patients with chronic HCV viremia (CV) (N = 121) (75.6%) were diagnosed on the basis of both anti-HCV positivity and persistently detectable serum HCV RNA over a period exceeding six months. All subjects were of Puerto Rican ethnic background and were recruited from the HCV Community Screening and Counseling Programs at the Primary Care Clinic of the Northgate Medical Center in Springfield, Massachusetts, a clinic caring for active IDUs. A group of non-HCV-infected subjects (N = 92) was recruited from the same Hispanic community, had similar socioeconomic background, and also reported by questionnaire that they were of Puerto Rican ancestry (4 grandparents originated from Puerto Rico and none were from Europe or other continents). This study was approved by the relevant Institutional Review Boards.

2.2 HLA class I typing

Genomic DNA from peripheral blood was obtained using a QIAGEN-DNA Blood extraction kit (Qiagen, Leusden, The Netherlands). HLA typing was performed by PCR with published primers to amplify the HLA class I DNA and sequence-specific oligonucleotide probes (SSOP, HLA quick-type kits, Lifecodes, Stamford, CT, USA) for allele detection. To resolve ambiguous PCR-SSOP typing, sequence-specific primer amplification (PCR-SSP) was used (Unitray SSP Pel-Freez, Milwaukee, WI, USA) according to the manufacturer's instructions.

2.3 KIR typing

Briefly, the presence or absence of *KIR* genes was detected by PCR-SSP (Hsu et al., 2002; Gomez-Lozano and Vilches, 2002). The final concentration of each *KIR*-specific primer was approximately 1 μ M. As an internal control, we amplified a 560 bp fragment of the *HLA-DRA* gene using the primers: forward: 5'-ACCTGTCACCACAGG-3' and reverse: 5'-CAGACCCACAGTCAGGCC-3'. Control primers were used at 0.5 μ M final concentration in all PCR-SSP reactions: 100 ng of genomic DNA were amplified in 10 μ l of PCR buffer (67 mM Tris-HCl, pH 8.8, 16 mM (NH₄)₂SO₄, 2 mM MgCl₂, 0.01% Tween-20, 100 μ M dNTP's), 1 μ M for each primer and 0.4 U of *Taq* DNA polymerase (Roche Applied Science, Indianapolis, IN, USA) in a PerkinElmer GeneAmp 9600 system with the following PCR conditions: denaturation for 2 min at 92 °C, then 30 cycles of 10 s at 92 °C, 30 s at 65 °C and 90 s at 68 °C; and final extension at 68 °C for 10 min. Annealing temperatures were modified for primers amplifying *KIR2DL2* (63 °C), *KIR2DS4D* (*KIR2DS4* deletion in exon 5) (63 °C), *KIR2DS5* (63 °C) and *KIR2DS4* (61 °C). Amplification products were electrophoresed in 1.4% agarose gels stained with ethidium bromide.

2.4 Short tandem repeats typing

Fifteen autosomal short tandem repeats (STR) markers (CSF1PO, FGA, THO1, TPOX, VWA, D3S11358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, D19S433, D2S1338 and amelogenin) were typed using the Applied Biosystems AmpFI STR Identifier Kit (Applied Biosystems, Carlsbad, CA, USA). PCR amplification was carried out on a Gene Amp 9600 thermocycler using 1 ng of DNA according to the manufacturer's protocol. The PCR conditions were: 95 °C for 11 min followed by 28 cycles of 94 °C for 1 min, 59 °C for 1 min, 72 °C for 1 min followed by a hold at 60 °C for 60 min. PCR products were diluted 1:15 in Hi-Di formamide and GS500-LIZ internal size standard and analyzed on the ABIPrism 3100 Genetic Analyzer (Applied Biosystems). Allele assignments were made using Genotype 3.7 software by comparison with kit allelic ladders (Applied Biosystems). Gene frequencies, expected heterozygosity and fit to the Hardy–Weinberg (HW) equilibrium were calculated by Arlequin population genetics software v2.1. *p* Values < 0.003 after Bonferroni correction were considered significant. Previously published STR data on parental populations (Europeans from Spain (Sanz et al., 2003), West Africans from Angola (Goncalves et al., 2002) and Amerindian populations (Barrot et al., 2005) were used for the analyses of admixture.

2.5 Statistical Methods

The differences in the distribution of KIR-HLA genetic interactions between groups were analyzed by chi-square test with Yates' correction or Fisher's exact test when necessary. Odds ratios (OR) and 95% CI were also calculated. The Bonferroni correction for multiple comparisons was applied. Corrected *p* values (*p*C) < 0.05 were considered statistically significant.

2.6 Population Genetics and Population Stratification Analysis

The allelic frequencies of the STR markers were used to estimate the admixture of Puerto Rican Americans by the maximum likelihood method and Arlequin v2.1 population genetics software. Spaniards, Angolans and Amerindians were considered parental populations. In addition, individual admixture estimates were obtained using the Structure 2.0 program. To further test for population stratification, we compared the frequencies of STR markers in the test populations by chi-square analysis (Pritchard and Rosenberg, 1999) under the null hypothesis that there were no differences in the frequency of the STR markers between HCV-infected patients and non-infected IDUs. *p* values < 0.05 were considered statistically significant.

3. Results

3.1 Admixture Estimation and Population Structure Analysis

No significant deviations from the Hardy-Weinberg equilibrium of the STR genotypes from those predicted by allele frequencies were detected (data not shown). Admixture estimations, using STR markers in a tri-hybrid model, demonstrated an important contribution of Caucasian European (73.5%), African (16.0 %) and American Indian genes (10.4%) in Puerto Ricans. In addition, we did not detect significant differences in STR distribution between HCV-infected and non-infected IDUs, suggesting that our studied groups were comparable. Bayesian analysis with the software Structure 2.0 also confirmed the lack of population stratification in the studied groups. The individual admixture estimates of HCV-infected and non-HCV-infected individuals are shown in Figure 1.

3.2 HLA-C group and *KIR* inhibitory and activating genotypes associated with protection from infection

HLA-C group, *KIR* inhibitory and *KIR* activating genotypes were determined in 160 IDUs infected with HCV and a group of 92 ethnically matched IDUs with no markers of HCV infection (Table 1). *HLA-Cw* alleles were classified as group C1 (encoding asparagine at amino acid position 80 of the heavy chain of *HLA-Cw* or a few rare *HLA-B* alleles) or group C2 (lysine).

The frequency of the combinations *HLA-C1+KIR2DS4* ($pC = 0.02$, $OR = 0.43$); *KIR2DL2* and/or *KIR2DL3* ($pC = 0.01$, $OR = 0.07$); *KIR2DL2* and/or *KIR2DL3* together with *KIR2DS4* ($pC = 0.01$, $OR = 0.39$) and *HLA-C1+KIR2DL2+KIR2DS4* ($pC = 0.02$, $OR = 0.40$) were significantly increased in HCV non-infected compared with infected IDUs.

However, the presence of genes for the inhibitory receptors *KIR2DL2* and/or *KIR2DL3* and *HLA-C1* together with the activating receptor *KIR2DS4* ($pC = 0.004$, $OR = 0.38$) was the combination most strongly associated with protection from HCV infection (frequency in HCV-infected IDUs (31.8% versus 55.48% in non-infected IDUs). Also, there was a trend in the interaction of *HLA-C2* and *KIR2DS4/KIR2DL3* and/or *KIR2DL2* ($pC = 0.04$) associated with protection from HCV infection. No significant differences were detected in other combinations that included group *HLA-C2* alleles.

4. Discussion

Injection drug users (IDUs) are at increased risk of acquiring HCV infection. In previous studies we have reported (Romero et al., 2008) the association between *KIR* genotypes and *HLA*-class II alleles in the outcome of HCV infection and confirm the interaction between *2DL3/2DL3*, *HLA-C1* as markers of spontaneous viral clearance. In the present study, we compared the entire cohort of HCV infected individuals with a novel cohort of IDUs without evidence of HCV or HIV infection. A significant association was found between protection from HCV infection and the presence of the inhibitory receptors *KIR2DL2* and/or *KIR2DL3* and the *HLA-Cw1* homozygous genotype together with the activating receptor *KIR2DS4* in the protection from HCV infection.

The interaction between *KIRs* and *HLA-C* ligands in the control of HCV infection suggests that these receptors play an important part in the regulation of the cytolytic activity characteristic of the antiviral innate immune response of NK cells (Lodoen and Lainer, 2006). The repertoire of NK cells is extensive in the liver (Crispe and Mehal, 1996) and previous functional and genetic studies (Husain et al., 2002a; Husain et al., 2002b) have demonstrated that the repertoire of NK cells is influenced by the MHC class I phenotype in humans (Parham, 2005).

Therefore, it is not clear whether *KIR* haplotypes that carry several activating *KIRs* would necessarily favor a proactivating profile of NK cells. In this context, some authors have suggested that the hepatic lymphocyte repertoire determines the immune responses against HCV and the clinical outcome of HCV infection (Doherty and O'Farrelly, 2000; Khakoo et al., 2004, Romero et al., 2008).

Several population-based studies of viral infection have revealed an influence of specific *HLA-KIR* genetic interactions on the clinical outcomes. In HIV infection, a significant association between the interaction of *KIR3DS1* and *HLA-Bw4* alleles (*HLA* alleles encoding Ile at position 80 of the beta chain) with protection from AIDS progression was described recently (Martin et al., 2002). Interestingly, other studies have associated the coexpression of *KIR3DL1* and *HLA-B57* with slow progression to AIDS and with lower risk of HIV infection in multi-

exposed non-infected individuals (Boulet et al., 2008). Also, HLA–KIR interactions have been described in the susceptibility to autoimmune disorders (van der Silk et al., 2003; Luszczek et al., 2004; Nelson et al., 2004).

As previously mentioned, HLA-C group and *KIR2DL3* homozygosity have been associated with spontaneous clearance of HCV infection (Khakoo et al., 2004). The mechanism of viral control proposed is that *KIR2DL3* interacts with HLA-C molecules with lower physicochemical affinity than *KIR2DL1* or *KIR2DL2* receptors. The lower affinity of the *KIR2DL3*–HLA-C interaction causes reduced NK cell inhibition, promoting a greater capacity of NK cells to control the infection. In this regard, no differential affinity of different inhibitory *KIR2DL* molecules for specific HLA-C ligands has been documented.

Our results indicate that inhibitory receptors, in the presence of HLA-C1 and *KIR2SD4*, interact in conferring protection from HCV infection. It is important to mention that the activator *KIR2DS4* is the ligand for *HLA-Cw*04*-encoded (group 2) molecules; however, the existence of yet unidentified ligands for *KIR2DS4* has been suggested (Katz et al., 2004). In addition, our results suggest that *KIR2DS4* might be important in the stimulation of NK cell activation together with the previously described phenomenon of *KIR2DL3*–HLA-C1-induced loss of inhibition (Parham, 2004).

Population genetic studies have revealed that the distribution and frequency of *KIR* genes varies with ethnicity. Our genetic admixture analysis showed that Puerto Ricans have 75% European Caucasian genes, which differs significantly from the estimates in the Mexican Mestizo population, in which genetic population studies showed only 40% of Caucasian genes (Lisker et al., 1990). It is noteworthy that IDU's with and without HCV infection were ethnically matched since we did not detect significant population stratification using unlinked STR genetic markers and chi-square association and model-based clustering analysis (Pritchard and Rosenberg, 1999).

It is well-established that alterations in the NK cell subsets and cytolytic function contribute to the development of chronic viremia in HCV-infected patients (Nattermann et al., 2006; Morishima et al., 2006). Significant alterations in NK subsets in patients with chronic HCV infection compared with individuals who resolved the infection and healthy controls have been described (Golden-Mason et al., 2006). Phenotypic analysis of NK cells in HCV non-viremic and viremic groups suggests that elevated levels of CD56^{Bright} NK cells, together with pre-NK cells and activated CD4⁺ T-cells in combination with CD4⁺CD25^{High} T-cells, is associated with viremia control (Zarife et al., 2009).

Recent studies have explored the T cell responses in a cohort of long-term IDUs who remain uninfected by HCV, so-called "exposed uninfected." The production of IFN- γ by T cells in response to different structural HCV antigens (core, E1, NS3, NS4, and NS5) was found in 58% of the exposed uninfected IDUs (Thurairajah et al., 2008). These results, together with the findings described by us in this report, suggest that innate immunity via NK receptors and acquired immunity via T cell responses are both important in protection from HCV infection.

In summary, our findings in a group of HCV-infected and exposed non-infected IDUs of Puerto Rican ancestry suggest an important role for the combination of inhibitory *KIR* genes (*KIR2DL2* and *KIR2DL3*) with the genes that encode group HLA-C1 molecules in the presence of activating *KIR2DS4*, in protection from HCV infection. We hypothesize that the activation signaling mediated by *KIR2DS4* regulates NK cell activity. Our findings in regard to the potential role played by the NK cell-mediated responses in protection from HCV infection in exposed non-infected individuals offer clinically relevant opportunities for disease management. Functional studies are still needed to understand a) the full repertoire of stochastically expressed *KIR* molecules in the subsets of NK cells in individuals with

haplotypes with different activating genes and b) functional differences in the NK cell subsets in chronic HCV and exposed non-infected individuals and c) the role of T cell responses in protection from HCV infection together with (or independent of) that produced by KIRs.

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Figure 1.

Individual admixture estimates in HCV-infected IDU's (red circles) and non-infected IDU's (dark blue circles) of Puerto Rican ethnic background. Admixture estimates, using STR markers in a tri-hybrid model, demonstrated 73.5 % of European, 16% of African, and 10.4% of American Indian contribution in Puerto Ricans living in Massachusetts. The parental populations are represented in each cluster (corners of the triangle): Cluster 1: represents European parental population (green circles); Cluster 2: West African parental population (light blue circles) and; "All others": The indigenous parental population (Yellow circles).

Table 1Frequency of group 1 and 2 HLA-C ligands and *NK* genes in HCV-infected and Non-infected IDU's.

HLA, KIR genes	HCV ⁺ IDUs N = 160	Non-HCV ⁺ IDUs N = 92	<i>p</i> C	OR (95% CI)
	n (%)	n (%)		
C1	122 (76.2)	76 (82.6)	ns	
KIR2DS4	73 (45.6)	58 (63.0)	ns	
KIR2DL2	70 (43.7)	48 (52.2)	ns	
KIR2DL3	116 (72.2)	79 (85.9)	ns	
KIR2DL2 and/or KIR2DL3	137 (85.6)	91 (98.9)	0.01	0.07 (0–0.47)
KIR2DL2 and/or KIR2DL3/KIR2DS4	68 (42.5)	58 (63.0)	0.01	0.39 (0.22–0.68)
C1+KIR2DL3	88 (55.0)	65 (70.6)	ns	
C1+KIR2DL2	52 (32.5)	40 (43.5)	ns	
C1/KIR2DL3 and/or KIR2DL2	103 (64.4)	75 (81.5)	ns	
C1+KIR2DS4	54 (33.7)	50 (53.3)	0.02	0.43 (0.24–0.75)
KIR2DL3+KIR2DS4	58 (36.2)	47 (51.1)	ns	
KIR2DL2+2DS4	37 (23.1)	36 (39.1)	ns	
C1+KIR2DL3+KIR2DS4	43 (26.7)	39 (42.4)	ns	
C1+KIR2DL2+KIR2DS4	29 (18.1)	33 (35.9)	0.02	0.40 (0.21–0.74)
C1/2;KIR2DS4/KIR2DL3 and/or KIR2DL2	51 (31.8)	51 (55.4)	0.004	0.38 (0.21–0.74)
C2	109 (68.1)	58 (63.0)	ns	
C2+KIR2DS4	44 (27.3)	35 (38.0)	ns	
C2+KIR2DL3	74 (46.2)	51 (55.4)	ns	
C2+KIR2DL2	49 (30.6)	29 (31.5)	ns	
C2+KIR2DS4+KIR2DL3	32 (20.0)	28 (30.4)	ns	
C2+KIR2DS4+KIR2DL2	23 (14.4)	21 (22.8)	ns	
C2/2;KIR2DS4/KIR2DL3 and/or KIR2DL2	40 (25)	34 (36.9)	ns*	

HCV⁺ = Hepatitis C-infected; ns= not significant*p*C: The *p* values were Bonferroni-corrected by multiplying by the number of comparisons (n = 21).* non-corrected *p* value = 0.04.