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Research Article

Association of killer cell immunoglobulin-like receptor polymorphisms with chronic hepatitis C and responses to therapy in Brazil

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Abstract

Soroprevalence for Hepatitis C virus is reported as 2.12% in Northern Brazil, with about 50% of the patients exhibiting a sustained virological response (SVR). Aiming to associate polymorphisms in Killer Cell Immunoglobulin-like Receptors (KIR) with chronic hepatitis C and therapy responses we investigated 125 chronic patients and 345 controls. Additionally, 48 ancestry markers were genotyped to control for population stratification. The frequency of the *KIR2DL2* and *KIR2DL2+HLA-C*^{Asp80} gene and ligand was higher in chronic infected patients than in controls ($p < 0.0009$, OR = 3.4; $p = 0.001$, OR = 3.45). In fact, KIR2DL3 is a weaker inhibitor of NK activity than KIR2DL2, which could explain the association of KIR2DL2 with chronic infection. Moreover, *KIR2DS2* and *KIR2DS2+HLA-C*^{Asp80} ($p < 0.0001$, OR = 2.51; $p = 0.0084$, OR = 2.62) and *KIR2DS3* ($p < 0.0001$; OR = 2.57) were associated with chronic infection, independently from *KIR2DL2*. No differences in ancestry composition were observed between control and patients, even with respect to therapy response groups. The allelic profile *KIR2DL2/KIR2DS2/KIR2DS3* was associated with the chronic hepatitis C ($p < 0.0001$; OR = 3). Furthermore, the patients also showed a higher mean number of activating genes and a lower frequency of the homozygous AA profile, which is likely secondary to the association with non-AA and/or activating genes. In addition, the *KIR2DS5* allele was associated with SVR ($p = 0.0261$; OR = 0.184).The ancestry analysis of samples ruled out any effects of population substructuring and did not evidence interethnic differences in therapy response, as suggested in previous studies.

Keywords: HCV, KIR, HLA-C, hepatitis C, KIR2DL2.

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Introduction

The infection by hepatitis C virus (HCV) is an important cause of chronic liver disease, whose prevalence varies from 0.01% to 26% worldwide (Irshad *et al.*, 2008). In this context, Brazil shows an intermediary prevalence (around 1.23%), with the highest rate (2.12%) in the northern region (Martins *et al.*, 2011). Regarding the clinical evolution of hepatitis C, viral clearance occurs in 20-30% of the cases (Mello *et al.*, 2007), and over 30% of the chronic infections may evolve to severe forms, like cirrhosis and hepatocellular carcinoma (Bode *et al.*, 2008; Paraná *et al.*, 2008).

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The 2002 NIH Consensus Development Conference on the Management of Hepatitis C strongly propagated a combination therapy of ribavirin with pegylated alpha interferon (Peg-IFN). Nonetheless, therapeutic protocols depend on HCV genotype, with genotypes 1 and 4 demanding a more time-consuming therapy (48 weeks) than others which demand only 24 weeks, thus highlighting the role of viral genetic factors in therapy response modulation.

Although the influence of the viral genotype is well established and mainly determining the response to treatment (Vidal-Castiñeira *et al.*, 2010), the influence of the host's genetic background on the evolution of HCV infection and the outcome of hepatitis C treatment is still a matter of current studies (Grünhage and Nattermann, 2010). Concerning the evolution of the infection, polymorphisms

in Killer Cell immunoglobulin-like receptors (KIR) and their HLA-C ligands have consistently been associated with viral clearance and chronic infection (Khakoo *et al.*, 2004; Knapp *et al.*, 2010; Romero *et al.*, 2008), as well as with response to treatment (Carneiro *et al.*, 2010; Knapp *et al.*, 2010; Vidal-Castiñeira *et al.*, 2010). Even though there is a consensus that KIR genes are involved in modulating the course of the HCV infection, the question of exactly which KIR gene (or genes) is responsible for the primary association is still a matter of debate.

The KIR gene complex, which has 14 genes and is localized in a region of approximately 150 kb within the Leukocyte Receptor Complex, exhibits considerable polymorphism, greatly due to large gene rearrangements that generate differences in gene composition among chromosomes. The presence or absence of specific KIR genes has been a major focus in association studies. For instance, the presence of *KIR2DL3* was frequently associated with viral clearance (Khakoo *et al.*, 2004; Knapp *et al.*, 2010; Romero *et al.*, 2008), except for one study (Montes-Cano *et al.*, 2005). Additional phenotypes, such as the presence and *KIR2DS5*, and the absence of *KIR2DS2* have also been described as associated with HCV clearance, but these associations are not as clear (Paladino *et al.*, 2007). The influence of KIR on the modulation of the response to therapy was only recently approached, and two studies reported an association of *KIR2DL3* homozygosis, in combination with their respective HLA-C ligand, with response to treatment (Knapp *et al.*, 2010; Vidal-Castiñeira *et al.*, 2010), while a third study failed to do so and instead reported an additional association of the presence of *KIR2DL5* with non-responsiveness (Carneiro *et al.*, 2010).

Such disagreements may reflect differences in linkage disequilibrium patterns among the populations investigated in the different studies, as well as an effect of population substructuring or stratification. However, since linkage disequilibrium trends have been demonstrated to be mostly homogeneous across different regions of the world (Single *et al.*, 2008), substructuring remains as the likely cause of disagreement between such studies.

In our study we investigated 125 chronic hepatitis C patients, infected with HCV genotypes 1 and 3, and compared them to 345 healthy and non HCV-infected controls in order to investigate the association of HCV chronic infection and response to therapy with KIR polymorphism. To our knowledge, the present study is the first one that controls for population stratification when evaluating the association between response to therapy and KIR gene cluster polymorphism. Moreover, we also controlled for the independence of the associations observed, thus ruling out putative linkage disequilibrium biases. Finally, the use of ancestry estimates allowed us to testing for putative ethnic differences in the response to therapy, as reported in the literature (Liu *et al.*, 2008; Ge *et al.*, 2009).

Subjects and Methods

Patients and controls

Blood samples of HCV patients and control were collected from admixed population from the city of Belém, Pará, in the Northern Region of Brazil. HCV patients comprised 125 individuals diagnosed with chronic hepatitis C from a special Chronic Liver Disease Program of the Santa Casa de Misericórdia do Pará, a public hospital. Thus, diagnosis and treatment were always conducted by the same group of physicians (Liver Workgroup) following homogeneous and well established diagnosis criteria and therapeutic protocols. All patients had a chronic HCV infection. The characterization of chronic infection comprised the detection of seropositivity by means of an ELISA using anti-HCV antibodies, detection of RNA-HCV by PCR, and determination of viral load by quantitative real time PCR (Miranda *et al.*, 2004) Besides serological and molecular detection of HCV, all patients had chronic hepatitis diagnosed according to clinical and histopathologic criteria, as well as laboratorial evaluation of liver function. Co-infection with hepatitis B virus and/or HIV, and association with metabolic or autoimmune disorders were used as exclusion criteria in this study.

All patients had their HCV genotype determined by reverse hybridization (VERSANT HCV Genotype Assay LiPA). Genotype 1 was detected in 82% of the patients, while genotypes 3 (17%) and 2 (1%) constituted a small fraction of the sample. Following the Brazilian therapeutic protocol, all patients carrying genotype 1 were treated with peguilated interferon (PEG-IFN) associated with Ribavirin (RBV) for 48 to 72 weeks, while those bearing genotype 2 or 3 were treated with conventional IFN also associated to RBV for 24 weeks. The response to treatment was evaluated afterwards, allowing to classify the patients according to the following groups: i) Sustained Viral Response (SVR), when HCV RNA was no longer detectable by qualitative PCR at 24 weeks after the end of the treatment; ii) Non-respondents (NR), when HCV RNA was still detected at 24 weeks after treatment (Brasil, 2011). The control sample consisted of 345 healthy unrelated individuals, representative of the same population as the patients. The control sample was the same as the one used in a previous study from our group (Pedroza *et al.*, 2011). The mean age and proportion of females were, respectively, 41.7 years and 59% in controls, and 57.4 years and 40% in patients. Informed consent was obtained from all individuals and the study protocol met the ethical demands defined by the Brazilian legislation and was approved by the Ethic Committee from the Health Sciences Institute from the Federal University of Pará: registration 148/09.

Individual ancestry markers

The Brazilian population is highly admixed, displaying a main European ancestry with variable Amerindian

and African contributions. Four decades of studies done in Belém, Pará, revealed proportions of African, Amerindian and European ancestry ranging from 0.12 to 0.33, 0.16 to 0.3 and 0.48 to 0.61, respectively (Ayres *et al.*, 1976; Schneider and Salzano, 1979; Guerreiro and Chautard-Freire-Maia, 1988; Santos *et al.*, 2010). Hence, to avoid a bias in population substructuring we evaluated the individual proportion of ethnic admixture using 48 unlinked insertion/deletion polymorphisms, previously validated as optimal ancestry markers for the population of Belém (Santos *et al.*, 2010).

Ancestry markers met two main criteria: i) great differences in allele frequencies (≥ 40%) between African, European and/or Amerindian ethnicities, and ii) location in different chromosomes or in distinct physical regions on the same chromosome (Santos *et al.*, 2010). Primer concentrations and sequences, as well as multiplex PCR conditions and capillary electrophoresis on ABI PRISM 3130 Genetic Analyzer (Applied Biosystems), are described elsewhere (Santos *et al.*, 2010).

Laboratory procedures

Venous blood samples (5 mL) were collected from all individuals, and DNA was isolated from white cells following a protocol by Sambrook *et al.* (1989).

KIR phenotypic profiles were obtained by performing 29 PCR-SSP reactions for each individual: two different pairs of primers for 13 KIR loci (*KIR2DL1*, *KIR2DL2*, *KIR2DL3*, *KIR2DL4*, *KIR2DL5*, *KIR2DS2*, *KIR2DS3*, *KIR2DS4*, *KIR3DL1*, *KIR3DS1*, *KIR3DS2*, *KIR3DS3*, *KIR2DP1*), a pair of primers for *KIR2DS1* and *KIR2DS5*, and an additional pair of internal control primers (Martin *et al.*, 2002). Each reaction contained 15 ng of DNA, 0.1 mM dNTPs, 1.5 mM $MgCl_2$, 67 mM Tris-HCl (pH 8.8), 16 mM $(NH_4)_2SO_4$, 0.25 mM of each primer, and 1 U *Taq* polymerase (Invitrogen®) to a total volume of 10 μL. Negative reactions without sample DNA were done to confirm non-amplification, while 30% of samples were randomly repeated in order to control for the accuracy and reproducibility of genotyping. No discordances were observed and all genotyping reactions were validated. Visualization of the PCR products was done by electrophoresis (120 V, 2 h) in polyacrylamide gels followed by staining with silver nitrate.

The HLA-C ligand groups (HLA-C^{Asp80} and HLA-C^{Lys80}) were genotyped by real time PCR according to Hong *et al.* (2011).

Statistical analysis

Frequencies of KIR genes in controls and patients were compared utilizing a chi-square test with the p value obtained by Monte Carlo simulations (Sham and Curtis, 1995) equivalent to Fisher's Exact Test. A correction for multiple testing was done whenever necessary by multiplying the p value by the number of tests, according to the hypothesis. The *Odds Ratio* (OR) was estimated for significant associations. Differences in the number of activating receptors between samples and subsamples were tested by Mann-Whitney test.

The criterion to designate an individual as homozygous for haplogroup A (AA) was the absence of *KIR2DL2*, *KIR2DL5*, *KIR3DS1*, *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, and *KIR2DS5* genes, according to what is preconized by Gonzalez-Galarza *et al.* (2011). Control and patient samples were tested for differences between individual ancestry proportions using the Mann-Whitney test. The same procedure was used to test for differences in African, European, and Amerindian ancestry proportions between SVR and NR subgroups of patients; these also being matched for genotype 1 HCV and treatment protocol. Individual ancestry proportions were estimated using the program Structure ver. 3.2.

Results

Association between KIR polymorphism and chronic hepatitis C

Three KIR genes showed an association with chronic hepatitis C: *KIR2DL2* ($p = 0.0009$ $p_c = 0.0126$; OR = 3.4 95%CI 1.64-7.05), *KIR2DS2* ($p < 0.0001$ $p_c = 0.0014$; OR = 2.51 95%CI 1.58-4.00), and *KIR2DS3* ($p < 0.0001$ $p_c = 0.0014$; OR = 2.57 95%CI 1.63-4.05). The joint analysis of KIR2DL2 and KIR2DS2 with their HLA-C^{Asp80} ligand revealed an association of both combinations with a predisposition to HCV infection (Table 1). However, after correction for multiple testing the association with *KIR2DS2+HLA-*C^{Asp80} was no longer statistically significant.

Since *KIR2DL2* was already reported as associated with chronic hepatitis C, and both *KIR2DS2* and *KIR2DS3* have been described as being in linkage disequilibrium with *KIR2DL2* (Single *et al.*, 2008), the association of both activating genes with chronic hepatitis C were re-tested using a subsample of *KIR2DL2* positive controls (257 individuals) and patients (91). The associations remained significant for both *KIR2DS2* ($p = 0.0083$ $p_c = 0.1162$; OR = 2.06; 95%CI = 1.2-3.4) and *KIR2DS3* ($p = 0.0008$ $p_c = 0.0112$; OR = 2.37; 95%CI = 1.4-3.9), and the KIR profile composed of all three genes was also highly significant, showing an even higher OR ($p < 0.0001$ $p_c = 0.0014$; OR = 3 95%CI 1.8-4.7). In addition, the frequency of AA profiles was higher in controls (8%) than in patients (2%). Nonetheless, the statistical significance of this association was modest ($p = 0.0267$; OR = 0.222 95%CI 0.05-0.95), probably due to the absence of the *KIR2DL2*, *KIR2DS2*, and *KIR2DS3* genes in the haplogroup A profile.

These associations, in particular those involving *KIR2DS2* and *KIR2DS3*, were further corroborated by differences in the number of activating genes. Hence, the average number of activating receptors in patients (3.94) was

Table 1 - Frequencies of KIR genes and profiles in controls, patients, and subsamples according their response to therapy.

	Patients N (%)			Controls N (%)
	Total	SVR	NR	
KIR2DL1	99 (99)	17 (100)	25 (96)	332 (99)
KIR2DL2	91 (91)[a]	16 (94)	22 (96)	258 (75)[a]
KIR2DL3	91 (91)	16 (94)	25 (96)	311 (90)
KIR2DL4	99 (99)	17 (100)	25 (96)	336 (97)
KIR2DL5	78 (78)	16 (94)	20 (77)	241 (70)
KIR2DS1	54 (54)	11 (65)	11 (42)	148 (43)
KIR2DS2	65 (65)[b]	10 (59)	19 (73)	146 (43)[b]
KIR2DS3	57 (57)[c]	10 (59)	19 (73)	115 (34)[c]
KIR2DS4	95 (95)	17 (100)	26 (100)	301 (87)
KIR2DS5	57 (57)	14 (82)[f]	12 (46)[f]	188 (55)
KIR3DS1	66 (66)	15 (88)	19 (73)	193 (56)
KIR3DL1	96 (96)	17 (100)	25 (96)	319 (93)
KIR3DL2	100 (100)	17 (100)	26 (100)	342 (99)
KIR3DL3	97 (97)	17 (100)	24 (92)	196 (99)
KIR2DL2+KIR2DS2+KIR2DS3	51(51)[d]	7 (41)	18 (69)	89(26)[d]
KIR2DL2+ HLA-C^{Asp80}	45(73)[g]	16(64)	29(78)	33(43)[g]
KIR2DS2+ HLA-C^{Asp80}	21(28) [h]	8(32)	23(62)	31(50)[h]
Haplogroup A (homozygous)	2 (2)[e]	0 (0)	0 (0)	29 (8)[e]

[a]$p = 0.0009$ $p_c = 0{,}0126$ OR = 3.4 CI 95%1.64-7.05; [b]$p < 0.0001$ $p_c = 0.0014$ OR = 2.51 CI 95% 1.58-4.00; [c]$p < 0.0001$ $p_c = 0{,}0014$ OR = 2.57 CI 95% 1.63-4.05; [d]$p < 0.0001$ $p_c = 0.0014$ OR = 3 CI 95% 1.8-4.7; [e]$p = 0.0267$ OR = 0.222 CI 95% 0.05-0.95; [f]$p = 0.0261$ OR = 0.184 CI 95% 0.042-0.795; [g]$p = 0.001$ OR = 3.45 CI 1.68-7.1; [h]$p = 0.0084$ OR = 2.62 CI 1.29-5.31. NR=non-responders; SVR= sustained virological response.

significantly higher than in controls (3.16; Mann-Whitney $Z(U) = 4.07$, $p < 0.0001$).

KIR genes and therapeutic response

Concerning the response to treatment, 43% of the patients had SVR. However, when stratifying according to virus genotype, SVR was observed among 43% of the genotype 1 infected patients and among 20% of the genotype 3 infected patients. Among the genotype 1 infected ones, the *KIR2DS5* gene was more frequent in the SVR subsample ($p = 0.0261$ $p_c = 0.3654$; OR = 0.184 95%CI 0.042-0.795).

Ethnic heterogeneity between controls and patients

The average proportions of European, African and Amerindian ancestries were identical in the patient (0.49, 0.21 and 0.28) and control group (0.5, 0.21 and 0.28, respectively). This was also the case for the SVR (0.48, 0.22 and 0.29) and NR (0.49, 0.22 and 0.286) subsamples.

Discussion

The association of the presence of the *KIR2DL2* gene with chronic hepatitis C, observed in our study, agrees with what is established in the literature. Moreover, the association of the combination *KIR2DL2+HLA-C^{Asp80}* with HCV infection corroborates previous studies (Vidal-Castiñeira *et al.*, 2010; Vejbaesya *et al.*, 2011) and highlights the role of this receptor in the predisposition to HCV infection. This has been interpreted as the result of the higher affinity of KIR2DL2 for its HLA-C ligand when compared to the affinity of KIR2DL3 for the same ligand, which confers higher inhibitory activity against the cytolytic function of natural killer (NK) cells (Khakoo *et al.*, 2004; Parham, 2005). The presence of *KIR2DL2* is, thus, used as a marker for indicating a worse prognostic for the hepatitis infection followed by chronification and HCV persistence (Khakoo *et al.*, 2004; Knapp *et al.*, 2010; Romero *et al.*, 2008).

The association of *KIR2DS2* and *KIR2DS3* with chronic hepatitis C is not consensual. For example, Marangon *et al.* (2011) did not find any statistically significant association with KIR, despite the fact that *KIR2DS2* and *KIR2DS3* showed slightly higher frequencies in patients. Nonetheless, viral non-clearance turned out to be associated with *KIR2DS2* (Paladino *et al.*, 2007) and *KIR2DS3* (Dring *et al.*, 2011) in different populations. Such disagreement could theoretically be attributed to substructuring and heterogeneous linkage disequilibrium (LD) patterns across different populations. However, Single *et al.* (2008) showed that LD patterns are homogeneous across different regions of the world, making population substructuring the more probable cause of this lack of consensus. Single *et al.* (2008) reported a strong positive LD between *KIR2DL2* and *KIR2DS2* worldwide and a weak but constant positive

LD of *KIR2DS3* with both *KIR2DL2* and *KIR2DS2*. So as to investigate the influence of these LD patterns on the associations we found herein, and to discriminate possible secondary or non-independent associations we tested whether the association of *KIR2DS2* and *KIR2DS3* with chronic infection was independent of the presence of *KIR2DL2*. As it turned out, *KIR2DS2* and *KIR2DS3* associations remained indeed significant in a subsample of the *KIR2DL2* carriers, ruling out the influence of *KIR2DL2* in the *KIR2DS2* and *KIR2DS3* associations. Additionally, the association of the profiles resulting from the presence of these three genes highly significant, reinforcing the independence of the associations observed for those genes separately. The AA profile was also associated with a protection against HCV infection. It is worthy of note that the KIR2DL2, KIR2DS2 and KIR2DS3 genes are absent in AA. The strength of association with these genes and/or with a combination of these is far more significant than those observed for AA and non-AA profiles. Thus it is inferred that the association with AA merely reflects the role of *KIR2DL2*, *KIR2DS2*, and *KIR2DS3*.

The higher frequencies of the activating KIR2DS2 and KIR2DS3 gene products in patients could also be reflected in the mean number of activating receptors, which was greater in patients than in controls.

With respect to the response to therapy, the presence of *KIR2DS5* was associated with SVR. Interestingly, the presence of this gene was associated with viral clearance in a previous study (Paladino *et al.*, 2007). In addition, *KIR2DS5* was not the sole gene related with viral clearance that was also associated with SVR; particularly the presence of *KIR2DL3* was also reported as associated with SVR (Knapp *et al.*, 2010; Vidal-Castiñeira *et al.*, 2010). Hence, KIR with a stimulatory trend and involved in the HCV clearance could also play a key role in the response to therapy. In agreement with this idea, the inhibitory KIR2DL5 was associated with non-response in a recent study (Carneiro *et al.*, 2010).

Population stratification is a common source of bias in association studies. Only one previous study (Romero *et al.*, 2008) reported an association between KIR polymorphisms and HCV clearance while controlling for population substructuring. In our study we used a rigorous control to exclude the influence of the stratification in the associations with chronic infection and response to therapy, clearly showing that all samples and subsamples were practically identical in their ethnic composition. Thus the ancestry analyses also allowed testing whether ethnicity had any influence on the response to therapy. Data reported by Ge *et al.* (2009) suggested that African descendents have the lowest rate of response to therapy and East Asians the highest ones, while European descendents and Hispanics showed intermediary rates. Our results did not show any evidence of differences in the average ethnic proportions of African, European, and Amerindian in both SVR and non responsive subsamples. Non-genetic host factors could explain such disagreement, like socio-economic differences among the samples (Ge *et al.*, 2009). Our study carefully controlled for such bias. Because treatment in Brazil is public and free of costs for the patient and all the patients were evaluated by a defined number of physicians, it was implicitly controlled for both socio-economic level and access to health care.

In conclusion, our study strengthens the reports on the association of the *KIR2DL2* gene with chronic hepatitis C and corroborates previous reports on the association of KIR genes and their profiles. Moreover, *KIR2DS5* was associated for the first time with SVR. The analysis of the ethnic composition of the samples ruled out any population substructuring as a possible source of bias, anf furthermore showed that there were no interethnic differences in response to therapy.

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Internet Resources

Software package for using multi-locus genotype data to investigate population structure, STRUCTURE ver. 3.2, http://pritch.bsd.uchicago.edu/software.html.

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