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TITLE: KIR2DL3 and the KIR ligand groups HLA-A-Bw4 and HLA-C2 predict the outcome of hepatitis B virus infection.

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ABSTRACT

Killer immunoglobulin-like receptors (KIRs) regulate the activation of Natural Killer cells through their interaction with human leukocyte antigens (HLA). KIR and HLA loci are highly polymorphic and certain HLA-KIR combinations have been found to protect against viral infections. In this study we analyzed whether the KIR/HLA repertoire may influence the course of hepatitis B virus (HBV) infection. Fifty-seven subjects with chronic hepatitis B (CHB), 44 subjects with resolved HBV infection, and 60 healthy uninfected controls (HC) were genotyped for KIR and their HLA ligands. The frequency of the HLA-A-Bw4 ligand group was higher in CHB (58%) than subjects with resolved infection (23%) (crude OR, 4.67; $P< 0.001$), and HC (10%) (crude OR,12.38; $P< 0.001$). Similar results were obtained for the HLA-C2 ligand group, more frequent in CHB (84%), than subjects with resolved infection (70%) (crude OR, 2.24; $P< 0.10$), and HC (60%) (crude OR, 3.56; $P< 0.01$). Conversely, the frequency of KIR2DL3 was lower in CHB (81%) than in subjects with

resolved infection (98%) (crude OR,0.10; $P< 0.05$). These results suggest a detrimental role of HLA-A-Bw4 and HLA-C2 groups, which are associated with the development of CHB, and a protective role of KIR2DL3. A stepwise variable selection procedure, based on multiple logistic regression analysis, identified these three predictive variables as the most relevant, featuring high specificity (90.9%), and positive predictive value (87.5%) for the development of CHB. Our results suggest that a combination of KIR/HLA gene/alleles is able to predict the outcome of HBV infection.

INTRODUCTION

Viral hepatitis B affects millions of people worldwide. In the WHO European Region, an estimated 13.3 million adults (1.8% of 898.6 million) have hepatitis B, and two-thirds of them are migrants or live in Eastern Europe.[1]

A chronic course of hepatitis B virus (HBV) infection develops in less than 10% of immune-competent adults with acute HBV infection, while the majority of patients resolve the infection.[1] The reason for this different clinical picture is unknown.

Host immunity, both innate and adaptive, is a key determinant of the natural course of viral hepatitis B. Innate immunity is traditionally viewed as playing a critical role in the initial containment of viremia in acute infections. The contribution of innate effectors, such as Natural Killer (NK) cells, is likely to be of particular relevance in the liver, where their frequencies are greatly enriched.[2] However, whether NK cells are innocent bystanders or potent players, and the hierarchy of importance of these cells in the HBV infected liver is still unknown.[2]

Killer cell immunoglobulin-like receptors (KIRs) on NK cells are involved in the control of virus infection by probing cells for proper expression on Human Leukocyte Antigens (HLA) class I.[3] Different KIRs can transmit inhibitory or activating signals to the cell, through the

interaction with specific HLA ligands.[4] Two broad haplotypes of KIR genes have been defined on the basis of gene content:[5] the B haplotype is characterized by variable numbers of activating and inhibitory genes, whereas the A haplotype contains no functional activating KIR.[5]

Several epidemiological studies have associated KIR/HLA compound genotypes with susceptibility to infectious diseases, such as human immunodeficiency virus (HIV), [6,7] human cytomegalovirus (CMV),[8-11] and hepatitis C virus (HCV).[12] So far there are limited data on the relationship between KIR genes and their HLA ligands and the outcome of HBV infection,[13-16] and no study has been performed on European populations. In this study we investigated whether specific KIR or HLA gene variants influence the natural course of HBV infection.

METHODS

Patients

Fifty-seven Caucasoid Italian patients with chronic hepatitis B (CHB),[17] at any stage of liver disease with or without any antiviral treatment were recruited at the University of Palermo Medical Center in Palermo, Italy. As first control group, 44 candidate blood donors screened at the Transfusion Medicine Unit of the same hospital tested negative for anti-HIV I/II antibodies, anti-hepatitis C antibodies, HBsAg and for HIV, HCV and HBV nucleic acids (Tri-NAT assay; NAT, Nucleic Acid Testing), but positive for anti-hepatitis B core total IgG antibodies (anti-HBc), with or without antibodies anti-HBsAg and/or anti-HBeAg were included (resolved infection). The analytical sensitivity of HBV DNA test (Tri-NAT assay) performed on individual plasma samples was 10 copies/mL (Grifols International S.A., TMA, Ultrio Elite). As second control group, 60 blood donors (negative for anti-HIV I/II antibodies, anti-hepatitis C antibodies, HBsAg, anti-HBc antibodies and HIV/HCV/HBV nucleic acids) already enrolled as healthy controls in a previous study were also included (Table 1). [8] These

subjects were all positive for IgG anti-CMV, as marker of a previous asymptomatic CMV infection.[8] Informed consent was obtained for collection of samples from all patients and controls. Consent forms were administered by the physicians involved in the study.

HLA and KIR Genotyping

Peripheral whole blood samples were collected, and genomic DNA was extracted from leukocytes by a commercial kit (PureLink® Genomic DNA, ThermoFisher Scientific, Waltham, MA, USA). Using the polymerase chain reaction sequence-specific primer (PCR-SSP) technique, the DNA of cases and controls was genotyped for the presence of the 3 major KIR ligand groups: HLA-C1, HLA-C2, and HLA-Bw4, both HLA-B and HLA-A loci (Epitop-TYPE kit; BAG Health Care GmbH, Lich, Germany). KIR genotyping was performed for both inhibitory and activating KIR using the KIR-TYPE kit (BAG Health Care GmbH). KIR gene profiles were determined by the presence or absence of each KIR gene.

Statistical Analysis

Crude comparisons of gene frequencies were made using 2 x 2 contingency tables, analyzed by the χ^2 test. A logistic regression model was also considered in analyzing the data, in order to derive a reduced and easily interpretable model for predicting clinical outcomes. The procedure started from a full model, including a set of variables as covariates encompassing all KIR genes and their HLA ligands,[4] except for those covariates having one unique value (zero variance predictors) or covariates having very few unique values relative to the number of samples (details in Supplementary Material). A stepwise procedure that compares nested models by Bayesian Information Criterion (BIC) was carried out to select the final subset of relevant predictors. By randomly splitting the data in two parts, a training set to select and estimate the final model and a test set to measure its performance, we were able to estimate

its ability to predict the clinical course of the infection. We also conducted an internal validation of model predictive accuracy using a 10-fold cross-validation scheme (details in Supplementary Material).[18] The whole analysis was performed by R 3.3.1 (R Core Team, 2013).[19]

RESULTS

The characteristics of the study populations are reported in Table 1. The mean age of the 57 patients with CHB was 52.9±14 years (range 22-79 years), with a prevalence of males (66.6%). Thirteen of them were co-infected with HIV (22.8%), two with HCV (3.5%), one with hepatitis D virus (HDV, 1.7%). The control population (44 subjects), represented by subjects with a previous asymptomatic HBV infection screened for blood donation (resolved infection), was similar for age (mean age, 52.7±8.5 years, range 37-67 years) and sex (males, 66.6%). A second control group (60 subjects; 61.6% males), representing active blood donors without any previous HBV infection, featured lower mean age (39.5 years).[8] This second control population, was added to assess whether there was a different KIR or HLA gene frequency in the general population compared to infected subjects, both with resolved or persistent infection (CHB), considering that KIR and HLA loci are likely under pathogen-mediated selection.

To assess if genetic variants of KIR and their HLA ligands may influence the outcome of the HBV infection we first compared the frequencies of these gene families in subjects with CHB and subjects with resolved infection (Table 2). The inhibitory KIR2DL3 gene was less frequent in CHB (81%) than in subjects with resolved infection (98%) (crude-OR, 0.10; $P<0.05$). The only other KIR gene expressed differently between CHB and subject with resolved infection was the KIR2DS4-Del, which codes for an inactive receptor (crude-OR, 4.36; $P<0.01$). No difference was reported in the frequency of KIR haplotypes between the

groups, suggesting that activating receptors likely do not play a role in the control of the infection (crude-OR, 1.23; P=0.67).

Regarding the KIR-ligand groups, the frequency of individuals with HLA-A-Bw4 alleles was higher in the group with CHB (58%) relative to the group that had resolved infection (23%) (crude-OR, 4.67, P<0.001). The frequency of HLA-B-Bw4 alleles did not differ significantly between the groups, while the frequency of HLA-C1 alleles was lower in the group with persistent infection (CHB, 65%) than with resolved infection (86%) (crude-OR, 0.29; P<0.05) (Table 2). Regarding the KIR-HLA ligand interactions, the frequency of the interaction between HLA-C1 and KIR2DL3 was lower in persistent infection (CHB, 53%) than in resolved infection (84%) (crude-OR, 0.21, P<0.001), while the interaction between HLA-C1 and KIR2DL2 did not differ between the groups. The frequency of the interaction between HLA-A-Bw4 and KIR3DL1 was the same as HLA-A-Bw4 since KIR3DL1 was expressed in almost all subjects (crude-OR, 4.67, P<0.001).

Stepwise logistic regression analysis supported the effect of HLA-A-Bw4 (Adj.-OR, 6.52, P<0.05) and KIR2DL3 (Adj.-OR, 0.04, P<0.01) (Table 3). Furthermore, the HLA-C2 alleles, which were more frequent in subjects with persistent infection (CHB, 84%) than in those with resolved infection (70%) with marginal statistical significance at the univariate analysis (crude-OR, 2.24, P<0.10) (Table 2), were significantly associated with the presence of persistent infection (Adj.-OR, 8.17, P<0.05) (Table 3). Conversely, the effect of HLA-C1 was weakly significant (P<0.10) in the multiple logistic regression analysis, and it was readily removed during the stepwise selection procedure, as well as KIR2DS4 genes (not significant). Similarly, sex and age, analyzed as conditioning variables did not show any statistically significant difference between the groups in the multiple variable logistic regression analysis, and they were removed during the stepwise selection phase as well.

Stepwise selection of relevant genes was conducted on a training set, obtained by randomly

splitting the whole dataset in two parts: one part was used as training set, the other as test set (75/25 split, 75% of data for training set, 25% for test set). The normalized effect sizes of each variable are reported in Table 4. The most related variables with the risk of developing CHB were ranked by the importance values from the highest (100%) to the smallest (0.00%) (Table 4). At variance with odds ratios (Table 3), such normalized effect sizes are expressed on a common scale of variation (0%-100%), and they provide, for each gene, information about the relative impact in explaining the probability that a randomly selected patient will develop CHB (Supplementary Materials for details). HLA-A-Bw4 featured the highest relative impact on the outcome prediction (effect size: 100%), followed by KIR2DL3 (16,64%), and HLA-C2 (0.00%). Using the test set, we were able to estimate the ability of the model to predict the clinical course. The balanced accuracy of the predictive model was fairly good (70.5%), with low sensitivity (50.0%), but high specificity (90.9%) and positive predictive value (87.5%) (Table 4). According to the predictive model the presence of KIR2DL3 gene is highly protective (predicts resolved infection anytime it is present) if only one out of the two detrimental HLA-ligand groups (HLA-A-Bw4, HLA-C2) is present, but is unable to confer protection when both HLA-A-Bw4 and HLA-C2 are present (Table 1 in Supplementary Material). In contrast, when KIR2DL3 is absent only one of the two detrimental genes is able to predict the development of persistent infection. A tenfold cross-validation procedure was used to internally validate the predictive accuracy of the final model, producing an accurate and unbiased estimate of how well the proposed classifier correctly identifies or excludes CHB. The cross-validated accuracy was 70.0%, strongly confirming the goodness of our analysis (Table 4).

Considering that a high proportion of subjects who resolve the acute infection characterizes the natural history of HBV infection, we further assessed the frequency of KIR and their HLA ligands in the second control population represented by active blood donors without

any previous HBV infection. This second control group (60 subjects) that represents the general population was compared to infected subjects, both with resolved or persistent infection (CHB). This group, referred as healthy controls (HC), was represented by subjects already enrolled in another study with a previous CMV asymptomatic infection, and featured lower mean age (39.5 years).[8] The different age was considered not relevant for this analysis since the variable "age" was not shown to be associated with the outcome. The presence of a previous asymptomatic CMV infection in the whole HC group was considered unlikely to influence the comparability with the other groups (CHB, resolved infection) owing to the high prevalence of CMV positivity in the general population (about 80% in Europe and the USA).[20]

Table 5 shows the comparisons, among individuals with CHB, subjects with resolved infection, and HC, of the frequencies of the three KIR and HLA variables (HLA-A-Bw4, HLA-C2, KIR2DL3) that were identified as the most relevant to predict CHB in the models shown in Tables 3 and 4. The gene group HLA-A-Bw4 identified by the predictive model as detrimental is significantly underrepresented in the general population (HC, 6 out of 60, 10%) compared to CHB (58%) (crude-OR: CHB vs. HC, 12.38, $P<0.001$) (Table 5). Interestingly, if we also included in the group of 60 healthy controls the 60 subjects with CMV symptomatic infection enrolled in the previous study (HBV uninfected subjects),[8] as representative of the general population as well, the frequency of HLA-A-Bw4 does not change (10 out of 120, 8.3%), again confirming the reported HLA-A-Bw4 frequency estimate in a bigger sample, markedly different from that of the CHB patients (Table 5).

Similar findings were reported for the other detrimental gene group, HLA-C2, underrepresented in the general population (HC, 60%) compared to CHB (84%) (crude-OR: CHB vs. HC, 3.56, $P<0.01$). In contrast, no significant difference is reported in the KIR2DL3 frequencies between CHB and HC (crude-OR, 1.26, $P=0.60$) (Table 5). Conversely, the

comparison between subjects with resolved infection and HC shows no significant difference between the groups except for the protective KIR2DL3 gene that was more frequent in subjects with resolved infection (98%) than in HC (77%) with a significant OR (crude-OR, 13.09, $P<0.01$) (Table 5).

Finally, the subgroup of 13 CHB patients with concomitant HIV infection had a higher frequency of HLA-A-Bw4 (69%) compared to CHB with only HBV infection (58%) (Table 5). The differences between this subgroup of CHB (HIV co-infected) and subjects with resolved infection (crude-OR, 7.65; $P<0.01$), and HC (crude-OR, 20.25; $P<0.001$) were more marked than those observed with CHB (All). These findings are not shown for HLA-C2 and KIR2DL3 (Table 5).

DISCUSSION

This genetic association study shows that the KIR2DL3 inhibitory receptor gene and the KIR-ligand group HLA-A-Bw4, and HLA-C2 alleles predict the outcome of HBV infection. Subjects possessing HLA-A-Bw4 or HLA-C2 alleles are more susceptible to develop CHB, while the presence of KIR2DL3 seems to confer protection.

To our knowledge, this is the first study to show that the KIR-ligand group HLA-A-Bw4 can influence the immune control of HBV. This effect may be mediated by the activation of the inhibitory KIR3DL1,[21] although an association with HLA-B-Bw4 alleles,[5] that bind the same KIR gene, was not reported in this study. This may imply different binding affinity and, consequently inhibitory ability between the HLA-A-Bw4 and HLA-B-Bw4 alleles. Furthermore, KIR3DL1 is among the most polymorphic of the KIR loci, and KIR3DL1 alleles show high variability in the level of their expression on cell surfaces, with functional repercussions.[22]

The results of the main analysis comparing CHB to subjects with resolved infection showed that the frequency of HLA-A-Bw4 in CHB is about three times higher than in subjects with resolved infection (58% vs. 23%), and this suggests a detrimental role of this ligand group. This possibility seems to be confirmed by the observation that in the general population the frequency of HLA-A-Bw4 is much lower (10%) than in CHB (58%; OR, 12.38; $P<0.001$) (Table 5). This may be the result of a pathogen selective pressure leading to a decreased frequency of this detrimental ligand group in the general population. Indeed, the general population seems to be relatively resistant to HBV, since less than 10% of subjects develop CHB after HBV infection. This percentage is very close to the frequency of the HLA-A-Bw4 in the general population (HC, 10%), and this gene was precisely the one identified by our cross validation model as the most important to predict the persistence of the infection (Table 4). A descriptive subgroup analysis was carried out comparing only the CHB patients co-infected with HIV (22.8%) with subjects with resolved infection and HC. This analysis showed that in the CHB/HIV co-infected group the frequency of the HLA-A-Bw4 ligand was even higher (69%) than in the entire CHB group (58%), with a remarkably different OR compared to resolved infection (crude-OR = 7.65; $P < 0.01$), and HC (OR = 20.25; $P < 0.001$) (Table 5). Despite the small sample size of this subgroup that limits the reliability of the analysis, these findings may suggest that in a condition of immune suppression NK cells may play a major role in the control of HBV infection. Further studies are needed to confirm these data, because the small sample size of this subgroup hampered the possibility to correlate the outcome to the CD4 T-cell count. However, for CMV infection it was shown that, in patients who had undergone bone marrow, HSCT or solid organ transplantation, specific KIR genes are associated with a low rate of CMV reactivation, suggesting that NK cells likely play a major role during immune suppression.

Also HLA-C2 alleles were more frequent in CHB (84%) than in subjects with resolved infection (70%), and in healthy controls (60%). Even in this case a linear increase from healthy controls to CHB was reported, suggesting a possible pathogen selective pressure. The difference in the frequencies of HLA-C2 between the groups is less marked than HLA-A-Bw4, and, accordingly the relative predictive importance of this variable shown by the cross validation model is lower than HLA-A-Bw4 (Table 4).

The KIR2DL3 gene is present in almost all the subjects who resolved the infection (98%), while less frequent in CHB (81%) and in healthy controls (77%). This difference may explain the different behavior of the subjects after the infection, with higher chance of resolving the infection in subjects possessing this protective gene (resolved infection group). The mechanism responsible for the protective role of KIR2DL3 is unclear, as this gene codes for an inhibitory receptor. Khakoo et al. showed that in HCV infection the presence of the KIR2DL3-HLA-C1 interaction is associated with resolved infection.[12] KIR2DL3 binds HLA-C1 with a weaker affinity compared to KIR2DL2. Therefore, the authors hypothesized that this weaker inhibitory receptor-ligand interaction (KIR2DL3-HLA-C1) would be protective, because it should be more easily overridden by activating signals than a stronger inhibitory interaction such as KIR2DL2-HLA-C1.[12] In the multiple logistic regression analysis we did not analyze the combined KIR-HLA interactions with this set of data since adding interaction terms to the full model would have led to strong over-parameterization (with respect to the limited sample size), and poor statistical inference. However, the descriptive univariate analysis (Table 2) showed that the KIR2DL3-HLA-C1 combination was indeed more frequent in CHB than in resolved infection, despite the loss of significance of the HLA-C1 in the multiple logistic regression analysis, supporting the role of this receptor-ligand association. Our findings are consistent with three other studies, one from Brazil,[14] one from China,[15] and one from Turkey,[16] which reported a protective effect of KIR2DL3 or the

interaction between KIR2DL3 and HLA-C1, suggesting a possible generalizability of the protective role of KIR2DL3 to different populations.

As already mentioned, HLA-A-Bw4, HLA-C2, and KIR2DL3 were identified as the best variables to predict the outcome of HBV infection by the stepwise covariate selection. According to the model, patients with at least one of the two detrimental ligand groups (HLA-A-Bw4 or HLA-C2) are going to be classified as at risk of developing CHB, unless they do not possess the protective KIR2DL3 gene (Table 1 in Supplementary Material). Furthermore, if both the detrimental genes are present, the subject has a high chance of developing CHB even with the presence of the protective gene KIR2DL3 (Table 1 in Supplementary Material). The performance of this model was low for sensitivity (50%), but high for specificity (90.1%) and positive predictive value (87.5%). This means that using the three predicting variables a subject classified by the model as at risk of developing CHB has a chance of about 88% of actually developing a chronic disease. This model is consistent with the assumption that NK cell activity is influenced by a balance between signal with variable activating and inhibitory activity. Therefore, this approach seems to be particularly suitable for facing the complexity of activating vs. inhibitory KIR (and their HLA-ligands) that determine NK cell function, since it is not based on the analysis of a single gene, but a combination of genes variably influencing the outcome.

A limitation of this analysis is the relatively small sample size. This may reduce the statistical power and limit the ability to identify some relevant association between KIR or HLA ligand groups with the outcome. On the other hand, the differences between the groups reported in this study are remarkable and highly statistically significant, strengthening the importance of this exploratory analysis. It is worth noting that the inflammatory infiltrate in the liver infected by HBV is particularly rich in NK cells (30–40% of total intrahepatic lymphocytes),

and NK activity is high early in HBV infection and during the incubation time, suggesting that these cells are important in the pathogenesis of CHB.[23-26] Although the ability of NK cells to contribute to control of HBV has been demonstrated in mouse models, their importance in human HBV-infected liver is still unclear (innocent bystanders or potent players?).[2] Considering the complexity of the immune system, the highly significant results reported here analyzing only a single family of NK receptors and their HLA ligands suggest that NK probably play an active role in HBV control.

Another important limitation is that an external validation of these data is needed to assess the generalizability of our findings to other populations, since we studied subjects of the same geographic area and ethnicity.

In conclusion, we used a genetic approach to provide evidence for a protective effect of the KIR2DL3 gene and a detrimental effect of the HLA-A-Bw4 and HLA-C2 alleles on the outcome of primary HBV infection, suggesting that KIR and HLA polymorphisms play a primary role in the control of the infection. Genetic differences are present between subjects with different abilities to respond to primary HBV infection, suggesting that specific KIR and HLA gene segregations were likely the result of a pathogen selective pressure.

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AUTHORSHIP CONTRIBUTIONS: C.Co., R.R., L.G., A.T., A.C., S.R, G. DL. G.M. collected the data; A.A. and G.A. performed the experiments and compiled the data for summation and analysis; D.DB, C.Ca and C.Co contributed to the study design; D.DB. wrote the first draft of the manuscript; M.B. performed statistical analyses; all authors reviewed and revised the manuscript, approved the final version, and agreed to submit the manuscript for publication. D.DB.and A.A. equally contributed.

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Table 1. Clinical characteristics of the study populations.

Characteristic	CHB N_{tot} = 57	Resolved Infection N_{tot} = 44	HC N_{tot} = 60
Mean Age, Y ± SD	52.9 ± 14.0	52.7 ± 8.5	39.5 ± 11.7
Y (range)	(22 - 79)	(37 - 67)	(18 - 65)
Sex, M (%)	38 (66.6)	30 (66.6)	37 (61.6)
Other infections, *N* (%)			
HDV	1 (1.7)	0	0
HCV	2 (3.5)	0	0
HIV	13 (22.8)	0	0

CHB, chronic hepatitis B; Resolved infection, subjects with resolved HBV infection; HC, healthy controls: blood donors; HDV, hepatitis D virus; HCV, hepatitis C virus, HIV, human immunodeficiency virus; N_{tot}, total number of subjects; Y, years; M, males, SD, standard deviation.

Table 2. Frequencies of KIR, HLA and KIR-HLA combinations among individuals with CHB and subjects with resolved infection.

Genetic Factor	Frequency CHB N_{tot} = 57 *N* (%)	Frequency Resolved Infection N_{tot} = 44 *N* (%)	Crude OR	*P*-value
KIR haplotypes				
AA	11 (19)	10 (23)	-	-
AB+BB	46 (81)	34 (77)	1.23	0.67
KIR alleles				
2DL1	54 (95)	44 (100)	N.A.	N.A.
2DL2	34 (60)	23 (52)	1.35	0.46
2DL3	46 (81)	43 (98)	0.10	< 0.05[b]
2DL4	57 (100)	44 (100)	N.A.	N.A.
2DL5A	27 (47)	23 (52)	0.82	0.63

2DL5B	18 (32)	20 (45)	0.55	0.16
2DS1	25 (44)	26 (59)	0.54	0.13
2DS2	33 (58)	22 (50)	1.38	0.43
2DS3	19 (33)	18 (41)	0.72	0.43
2DS4 Full	4 (7)	8 (18)	0.34	< 0.10[a]
2DS4 Del	52 (91)	32 (73)	4.36	< 0.01[c]
2DS4 Del*008	0 (0)	1 (2)	N.A.	N.A.
2DS5	21 (37)	20 (45)	0.70	0.38
3DL1	57 (100)	40 (91)	N.A.	N.A.
3DL2	57 (100)	44 (100)	N.A.	N.A.
3DL3	57 (100)	44 (100)	N.A.	N.A.
3DS1	26 (46)	23 (52)	0.77	0.51
2DP1	53 (93)	44 (100)	N.A.	N.A.
3DP1	4 (7)	0 (0)	N.A.	N.A.
3DP1*003	53 (93)	44 (100)	N.A.	N.A.
HLA alleles				
HLA-C1	37 (65)	38 (86)	0.29	< 0.05[b]
HLA-C2	48 (84)	31 (70)	2.24	< 0.10[a]
HLA-B-Bw4^{T}	14 (25)	12 (27)	0.87	0.76
HLA-B-Bw4^{I}	32 (56)	29 (66)	0.66	0.32
HLA-A-Bw4	33 (58)	10 (23)	4.67	< 0.001[d]
KIR-HLA combinations				
2DL3/HLA-C1	30 (53)	37 (84)	0.21	< 0.001[d]
2DL2/HLA-C1	21 (37)	21 (48)	0.64	0.27
2DS2/HLA-C1	20 (36)	20 (45)	0.65	0.29
2DL1/HLA-C2	46 (81)	31 (70)	1.75	0.23
2DS1/HLA-C2	23 (40)	20 (45)	0.81	0.61
3DL1/HLA-B-Bw4^{I}	32 (56)	26 (59)	0.89	0.77
3DS1/HLA-B-Bw4^{I}	12 (21)	14 (32)	0.57	0.22
3DL1/HLA-B-Bw4^{T}	14 (25)	12 (27)	0.87	0.76
3DL1/HLA-A-Bw4	33 (58)	10 (23)	4.67	< 0.001[d]

The KIR-HLA interaction suggested by literature were analysed and reported in the table. Abbreviations: CHB, Chronic Hepatitis B; Resolved infection, subjects with resolved HBV infection; HLA, human leukocyte antigen; KIR, killer immunoglobulin-like receptor; crude OR, unadjusted odds ratio derived from a 2 x 2 table. Statistically significant *P*-values have been expressed in relational terms with the statistical significance threshold, in the sense that '$P < \alpha$' means that the null hypothesis of crude OR = 1 can be rejected when the chosen significance threshold is equal to α. We report the following degree of evidence against the null hypothesis: [a]weak statistical significance ($P < 0.10$); [b]moderate statistical significance ($P < 0.05$); [c]strong statistical significance ($P < 0.01$); [d]very strong statistical significance ($P < 0.001$).

Table 3. Logistic regression model to predict the effect of KIR and HLA alleles on the persistence of HBV infection (CHB).

Variable	Code	Est. β	SE	Adj. OR (95%CI)	*P*-value
HLA-A-Bw4	0: absent 1: present	1.88	0.63	6.52 (1.88, 22.61)	< 0.05
HLA-C2	0: absent 1: present	2.10	0.93	8.17 (1.33, 50.26)	< 0.05
KIR2DL3	0: absent 1: present	-3.19	1.34	0.04 (0, 0.57)	< 0.01

Sex and age did not enter into the final model after the stepwise procedure. CHB, Chronic Hepatitis B; CI, confidence interval; Adj. OR, adjusted odds ratio; SE, standard error. The stepwise procedure has been carried out on a training set comprising randomly selected subjects. The training set dimension is 75% of the overall sample dimension (with $N_{train} = 0.75 * (44 + 57) \simeq 76$).

Table 4. Predictive model for persistence of HBV infection (CHB).

Relative Variable Importance*	
1. HLA-A-Bw4	100.0%
2. KIR2DL3	16.6%
3. HLA-C2	0.00%
Sensitivity	50.0%
Specificity	90.9%
Positive Predictive Value	87.5%
Negative Predictive Value	58.8%
Balanced accuracy**	70.5%
Cross-validated accuracy***	70.0%

The dataset was randomly splitted into a training and a test set (75/25 split, 75% of data for training set, 25% for test set). The training set was used for stepwise selection and effect size estimation of most relevant genes. The test set was used the assess sensitivity, specificity and other predictive accuracy measures. *The variable relative importance values of the predictive model used for classifying future patients. The values were normalized to scale of 0%–100%. CHB, Chronic Hepatitis B. **Balanced accuracy is obtained as (sensitivity + specificity)/2, which turns out to be a more accurate estimator when one of the classes (the minority class, subjects with resolved infection) is under-represented as regards the other class (subjects with CHB)[27]. ***Cross-validated accuracy was calculated by means of 10-fold cross-validation. See the Supplementary Materials for further details.

Table 5. **Frequencies of KIR2DL3, HLA-C2 and HLA-A-Bw4 among individuals with CHB, subjects with resolved infection, and healthy controls.**

Genetic Factor	Frequency CHB *N* (%)	Frequency Resolved *N* (%)	Frequency HC *N* (%)	Crude-OR (CHB vs. Resolved)	*P*	Crude-OR (CHB vs. HC)	*P*	Crude-OR (Resolved vs. HC)	*P*
HLA-A-Bw4									
All	33/57 (58)	10/44 (23)	6/60 (10)	4.67	<0.001	12.38	<0.001	2.56	0.07
HIV-co-infected	9/13 (69)			7.65	<0.01	20.25	<0.001		
HLA-C2									
All	48/57 (84)	31/44 (70)	36/60 (60)	2.24	0.09	3.56	<0.01	1.59	0.27
HIV-co-infected	11/13 (85)			2.31	0.30	3.67	0.09		
KIR2DL3									
All	46/57 (81)	43/44 (98)	46/60 (77)	0.10	<0.05	1.26	0.59	13.09	<0.01
HIV-co-infected	10/13 (77)			0.08	<0.01	1.01	0.98		

CHB, Chronic Hepatitis B; Resolved, subjects with resolved HBV infection; HC, healthy controls; OR, Odds ratio; All: All 57 patients with chronic hepatitis B; HIV-co-infected: a subgroup of 13 patients with HBV/HIV co-infection out of the 57 CHB patients. The HIV-co-infected subgroup was compared to the Resolved infection, and the HC groups.

The balance between signals with variable activating or inhibitory activity on NK cell function influences the outcome of HBV infection.