Human Leukocyte Antigen and Killer Immunoglobulin-like receptor genes as outcome predictors of hepatitis C virus-related hepatocellular carcinoma

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Translational relevance

Natural killer (NK) cells play a major role in anti-tumor immune response. The genotypes of NK Killer Immunoglobulin-like receptors (KIRs) and of their HLA ligands are related to the development of hepatocellular carcinoma (HCC) in patients with HCV infection. A better understanding of the role played by the immunogenotypic background over HCC outcome might represent a major breakthrough to improve the clinical management of HCC patients.

Our work shows that the immunogenetic profile is associated with NK-cell function and represents a powerful outcome predictor in HCV-linked HCC. These results support a central role of NK cells in the immune response against HCC with important implications in terms of development of immunotherapeutic approaches and individualized monitoring schemes after treatment of early HCC.
Abstract

Purpose: We evaluated the impact of the Killer Immunoglobulin-like Receptors (KIRs) of natural killer (NK) cells and of their Human Leukocyte Antigen (HLA) ligands over the clinical outcome of HCV-related hepatocellular carcinoma (HCC) after curative treatment by either surgical resection (SR) or radiofrequency thermal ablation (RTA).

Experimental Design: Sixty-one consecutive patients with HCV-related HCC underwent KIR genotyping and HLA typing. A phenotypic/functional characterization of NK-cells was carried out in patients with different KIR/KIR-ligand genotype.

Results: Activating KIR2DS5 was associated with significantly longer time to recurrence (TTR) and overall survival (OS) (p<0.03 each). Homozygous HLA-C1 (p<0.02) and HLA-Bw4I80 (p<0.05) were expressed by patients with significantly better OS, while HLA-C2 (p<0.02) and HLA-Bw4T80 (p<0.01) were associated with a worse OS. Multivariate analysis identified as parameters independently related to TTR the type of treatment (SR vs RTA) (p<0.03) and HLA-C1 (p<0.03), whereas only KIR2DS5 was an independent predictor of longer OS (p<0.05). Compound KIR2DL2-C1 and KIR3DS1-Bw4T80 genotypes were associated with better TTR (p<0.03) and worse OS (p=0.02), respectively. A prevalent cytotoxic (CD56dim) NK phenotype was detected in patients with both longer TTR and OS. Cytotoxic capacity measured by upregulation of CD107a was significantly higher in subjects with HLA-C1 alone or combined with KIR2DL2/KIR2DL3.

Conclusions: These results support a central role of NK-cells in the immune response against HCC, providing a strong rationale for therapeutic strategies enhancing NK response and for individualized post-treatment monitoring schemes.
Introduction

Natural killer (NK) cells recognize target cells through interaction of surface inhibitory and activating receptors with their ligands. NK-cells have been divided into two major populations, one mainly cytotoxic in function (CD56dimCD16+) and the other mainly involved in cytokine secretion (CD56bright, CD16dim or negative) (1).

NK cell receptors belong to two main families: the C-type lectins-like (NKG2) receptors and the immunoglobulin (Ig)-like superfamily, including the killer cell Ig-like receptors (KIRs). KIR genotypes can be grouped into haplotypes A and B, mainly including inhibitory (A) or activating (B) KIRs, respectively (2). The B haplotypes contain variable numbers and combinations of KIR genes.

The fine tuning of KIR-ligand interactions is the result of multi-level regulatory mechanisms relying both on the quantity of KIR/HLA molecules expressed on NK and target cells, and on the affinity of KIR-HLA interactions. Inhibitory KIR2DL1 receptor recognizes alleles of HLA-C with lysine at position 80 (HLA-C2), whereas KIR2DL2 and KIR2DL3, segregating as alleles of a single locus, specifically bind HLA-C alleles with asparagine at position 80 (HLA-C1) (3,4) with different binding affinity (5): KIR2DL3/HLA-C1 interaction is thought to be weaker than both KIR2DL2/HLA-C1 and KIR2DL1/HLA-C2. In addition KIR2DL2, and to a lesser extent KIR2DL3, bind with low affinity HLA-C2 (5). KIR3DL1 recognizes the Bw4 motif of HLA-B alleles (6), generating a stronger inhibitory signal when an isoleucine residue is present at position 80 (Bw4I80). Due to the relevant homology of activating KIR2DS2 and KIR3DS1 to inhibitory KIR2DL2/DL3 and KIR3DL1, respectively, it has been speculated that they might share the same ligands as the inhibitory KIR counterparts, but this assumption has not been demonstrated yet. By contrast, KIR2DS1 has been shown to bind HLA-C2 as KIR2DL1, although with lower affinity (7).

NK-cells recognize and eliminate cells that fail to express self HLA molecules, such as virus-infected and transformed cells. In hepatitis C virus (HCV) infection, impaired NK cell frequency and function has been reported (8) and specific KIR/ligand genotypes have been implicated in the
clinical evolution and therapeutic response: the KIR2DL3/HLA-C1 genotype has been associated with the resolution of infection (9-11) as KIR3DS1 and HLA-Bw4, although with a weak protective effect (9). By contrast, homozygous HLA-C2 (9) and KIR2DS3, in the presence of HLA-C2 (12) were more frequent in patients with chronic hepatitis C compared to individuals with spontaneously resolved infection. In patients with acute HCV infection, increased NK-cells cytotoxicity was present in subjects expressing HLA-C1-specific KIR2DL2/3 and in particular in self-limited infection (13). Other studies reported over-representation of homozygous KIR2DL3/HLA-C1 in sustained responders (11,14) and of HLA-C2C2 in patients resistant to treatment (15).

Functional impairment of NK-cells has been observed in patients with hepatocellular carcinoma (HCC) (16). The KIR3DS1/HLA-Bw4I80 genotype and HLA-C1 were interpreted as protective against the development of HCV-related HCC (17), but no data are available about the potential relationship between KIR/HLA genotypes and the prognosis of HCC after treatment. We have evaluated the impact of the immunogenetic host background over the clinical outcome of HCV-related HCC after curative treatment by either surgical resection (SR) or radiofrequency thermal ablation (RTA).
**Materials and Methods**

**Patients**

We evaluated 61 consecutive Caucasian HCC patients with HCV-related liver disease, that underwent curative treatment by either SR or RTA at the University Hospital of Parma, Italy. HCC diagnosis was made by ultrasonography (US) and computed tomography (CT) or magnetic resonance imaging (MRI) in selected cases. The type of treatment was decided on the basis of liver function (Child-Pugh score), comorbidities, age and location of HCC nodules. Patients with early HCC were evaluated by a multidisciplinary group (interventional radiologist, hepatologist and surgeon) in order to decide treatment allocation based on liver function, portal hypertension, number, site and size of HCC lesions. Patients with one or two lesions less than 15 mm in diameter were treated with percutaneous alcohol injection. All patients had not been previously treated for HCC.

All patients undergoing liver resection were within Child-A score. HBsAg and anti-human immunodeficiency virus were negative for all cases. All patients had the same postoperative follow-up based on bidimensional and contrast-enhanced (CE) US every 4 months and dynamic CT or CE MRI in any case of appearance of new liver nodules more than 1 cm in diameter or arterial enhancement at the site of previous ablation. The clinico-pathological features of the patients are shown in Table 1.

The study was approved by the local ethical committee (Comitato Etico Indipendente of the Azienda Ospedaliero-Universitaria of Parma, Italy). Patients gave written informed consent to participate in the study.

**KIR genotyping and HLA typing**

DNA was extracted from frozen peripheral blood mononuclear cells (PBMC) derived from all patients using the QIAamp DNA blood Mini Kit (Qiagen, Hilden, Germany). KIR genotypes were determined by duplex real-time polymerase chain reaction (PCR) (18) on 5 ng DNA in 10 μl
containing 1X SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Hercules, CA), 0.3 μM of each KIR-specific primer, 0.1 μM of each internal control primer, in a Rotor Gene Q thermal cycler (Qiagen) as follows: 2’ 98°C, 5” 98°C and 10” 62 °C for 45 cycles. The PCR products were then discriminated by melting (2’ 98°C, 10” 75°C, and ramping by 0.5°C/s up to 98°C). The KIR genes analyzed are shown in Supplementary Figure S1.

HLA typing was performed in all patients by PCR Sequence Specific Priming on genomic DNA. In all cases showing ambiguities for definition of the HLA-B or C supertypes Cl, C2, Bw4, Bw6 and Bw4 I80/T80 variants, high resolution typing was performed by PCR Sequence Specific Oligonucleotide Probes (Proimmune Co, Oxford, UK).

**Immunostaining of NK-cells**

In 38 of 61 patients an aliquot of frozen PBMC obtained the same day or the day before treatment was available for phenotypic analysis. PBMC were resuspended in RPMI 1640, and 8% human serum. PBMC (3x10^5) were stained with CD3-PerCP (BD Biosciences-Pharmingen, San Jose, CA) and CD56-APC (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were analyzed on FACSCantoII flow cytometer (BD Biosciences, Franklin Lakes, NJ) by the FACSDiva Software. Frequency of CD56^dim and CD56^bright NK-cells was defined for each patient evaluating different fluorescence intensity of CD3-CD56+ cells.

**Evaluation of cytotoxic function and interferon (IFN)-γ production in NK-cells**

In 31 of 61 patients an aliquot of frozen PBMC obtained the same day or the day before treatment was available for functional analysis. For the evaluation of the cytotoxic function, PBMC (1x10^6) were incubated 16 hours at 37°C in the presence or absence of 1 ng/ml rhIL-15 (R&D Systems, Abingdon, UK). PE/Cy5-5-conjugated CD107a (BD Biosciences) was then added with or without K562 target cells at an E:T ratio of 5:1. After 1 h, 10 μg/ml of brefeldin A (Sigma-Aldrich, St. Louis, MO) was added and cell were incubated further 3 h at 37°C, then harvested and stained for...
NK cell surface markers. Percentage of degranulating NK-cells was calculated by subtracting CD107a staining after incubation without K562 to CD107a staining after incubation with K562 targets.

PBMC (1x10^6) were incubated for 14 h at 37°C with or without 10 ng/ml rhIL-12 and rhIL-18 (Sigma-Aldrich). Brefeldin A (Sigma-Aldrich) (10 µg/ml) was added for the last 3 h of incubation. After staining with CD56-APC (Miltenyi Biotec) and CD3-APC/Cy7 (Biolegend, San Diego, CA), cells were fixed and permeabilised using Fix and Perm medium A and B (Caltag Laboratories, Burlingame, CA) according to the supplier’s instructions. Cells were stained with anti-IFN-γ PerCP/Cy5-5 mAb (Biolegend). The proportion of IFN-γ producing cells was determined by subtracting the percentage of IFN-γ+ cells in unstimulated samples from the one of IL-12/IL-18-stimulated samples.

**Statistical analysis**

The differences between groups of continuous variables were analyzed by t-test for unpaired data. Categorical variables were compared by the Chi-square test or Fisher exact test, as appropriate. Survival curves were estimated by the Kaplan-Meier method and compared by log-rank test. For continuous variables, ROC curve analysis was used to define threshold levels discriminating subgroups of patients with survival/time to recurrence higher or lower than median values, for subsequent analysis by the log-rank test. Cox proportional hazards regression model was performed for multivariate survival analysis. Only variables available for all patients and presenting p<0.1 in the univariate analysis (Table 2) were included in the multivariate model. A p value <0.05 (two-tailed) was considered significant.
Results

Clinical outcome

The study was performed on 61 consecutive Caucasian patients with diagnosis of HCV-related HCC allocated to treatment by either SR or RTA. (Table 1). The median overall survival (OS) was 43 months and the median time to the first HCC recurrence (TTR) was 17 months. By dividing patients according to the treatment received, age was significantly higher and liver function worse in RTA-treated patients, while HCC nodules were on average larger in patients treated by SR. Subjects that performed SR showed a significantly better OS and TTR compared to patients treated by RTA (Tables 1 and 2). Female sex was associated with significantly shorter OS, and Child score with worse TTR; age, number and size of HCC nodules did not affect survival (Table 2). From these results, survival differences related to treatment seem to be most likely due to the treatment allocation criteria, since RTA-treated patients were significantly older and had a worse Child score. Moreover, 3 patients who underwent surgical resection during the same period, died within 2 months from surgery. These patients were not included in the study because the effect of HLA and KIR associations could not be evaluated.

Individual KIRs and HLA expression and their association with disease progression and overall survival

KIRs frequencies identified in our patients cohort were comparable to the ones previously reported in Caucasian patients from Northern Italy (19,20), without any significant difference in the prevalence of each reported KIR (Supplementary Figure S1).

The 15 patients carrying the KIR2DS5 gene, that is present in a fraction of the KIR B haplotypes, showed significantly longer TTR and OS (Figure 1). KIR2DS5 is an activating receptor in linkage disequilibrium with KIR2DS1, that also showed a trend towards a protective effect against recurrence (Table 2). No other analyzed KIRs were significantly correlated to clinical outcome. Homozygous HLA-C1 and HLA-Bw4, I80 variant, were associated with significantly better OS,
while HLA-C2 and HLA-Bw4T80 were associated with a worse OS (Figure 1 and Table 2). Multivariate analysis identified as independent parameters associated with outcome the type of treatment (SR vs RTA) and HLA-C1 for TTR, and only KIR2DS5 for OS (Table 3). By analyzing the effect of HLA-C1 copy number on survival, we detected that HLA-C1 homozygous patients showed significantly longer OS compared to HLA-C1 heterozygous patients, and both longer OS and TTR compared to those homozygous for HLA-C2 (Figure 2A).

**Effect of the compound KIR-HLA genotype on disease progression and overall survival**

The inhibitory receptors KIR2DL2 and KIR2DL3 share the same ligand HLA-C1. Activating KIR2DS2 is in strong linkage disequilibrium and highly homologous to KIR2DL2, however experimental evidence of that KIR2DS2 binds HLA-C1 is not available at present. KIR2DL1 and KIR2DS1 bind HLA-C2, while KIR3DL1 binds HLA-Bw4, with higher and lower affinity for I80 and T80 variants, respectively (21). Since KIR3DS1 and KIR3DL1 segregate as alleles of the same locus and show 97% similarity in their extracellular domains, they may share the same HLA ligand. For the remaining KIRs genotyped in this study, the binding HLA molecule is not known. Since almost all our patients were positive for both KIR2DL1 and KIR3DL1 (Supplementary Figure S1), the effect of compound KIR-HLA genotype on disease progression was not evaluated for these molecules. Altogether, combined survival analysis was performed for KIR2DL2/2DL3-C1, KIR2DS1-C2, KIR2DS2-C1 and KIR3DS1-Bw4 (I80 and T80). Significant associations were found for KIR2DL2-C1 and KIR2DS2-C1 with a better TTR and for KIR3DS1-Bw4T80 with a worse OS (Figure 2B and Table 2).

**NK-cell phenotype and function**

First we evaluated whether effector NK-cell phenotype (CD56dim) could be associated with a better clinical outcome. Cut-off values of CD56dim% able to discriminate patients with TTR or OS higher than median values (17 and 43 months, respectively) were identified by ROC curve analysis. Indeed
patients with a frequency of NK cells with a CD56$^{\text{dim}}$ phenotype higher than the above-mentioned cut-offs showed significantly longer TTR and OS (Figure 3 upper left panels). We then compared NK-cell function of patients with specific HLA, KIR and HLA-KIR genotypes showing a significant effect on survival analysis. The distribution of HLA and KIR genotypes in the subgroup of patients with samples available for functional analysis was comparable to the one of the whole cohort (data not shown). Cytotoxic capacity, measured by upregulation of CD107a, was significantly higher in subjects with HLA-C1 alone or associated with KIR2DL2/KIR2DL3 (Figure 3 upper right panel and lower panels). No significant differences could be found for IFN-γ production (data not shown).
Discussion

KIR receptors and their HLA ligands have been implicated as risk factors for several human tumors, chronic inflammatory diseases, and viral infections (22). A recent genome-wide association study (23) identified an association between risk of HCC development in patients with chronic hepatitis and a single nucleotide polymorphism in the gene encoding for MHC-associated chain A (MICA), the ligand of NKG2D, an activating NK receptor (24). This observation strongly supports a relevant role for NK-cells in HCC pathogenesis.

In the present study we evaluated the impact of the immunogenetic profile on the clinical outcome of HCC patients undergoing curative treatment by either SR or RTA. Although both these treatment strategies are able to eradicate neoplastic nodule(s), the relapse rate is high since, in addition to the intrahepatic dissemination of primary tumor, de novo HCC may develop in the pre-tumorous environment of liver cirrhosis. The direct clinical consequences of the underlying liver disease have also a major impact on OS after treatment. Since the biological characteristics of the tumor and the host immune response are both involved in HCC progression (25-27), it is likely that the immunogenetic background determining the NK-cell response may play a role on the risk of relapse and ultimately on the survival of HCC patients.

A significant association was observed between KIR2DS5, an activating receptor part of haplotype B, and longer OS and TTR, but the implications of our results could not be further investigated since the ligand of KIR2DS5 has not been identified yet. Whether KIR2DS5 is a marker for a haplotype involved in HCC outcome, or whether NK-cells bearing KIR2DS5 play a direct role in the clinical outcome due to specific functional characteristics remains to be determined. A survival advantage of patients homozygous for HLA-C1 (HLA-C1C1) and therefore lacking HLA-C2 was also shown, together with an association of HLA-Bw4 variants I80 and T80 with longer or shorter OS, respectively. Homozygous HLA-C1, as well as HLA-Bw4I80, has already been reported as protective against development of HCV-related HCC (17). Our results further support the role of these genotypes in human HCC, showing their association with survival after curative treatment.
Multivariate survival analysis identified as independent parameters associated with outcome the type of treatment and HLA-C1 for TTR, and only KIR2DS5 for OS. These observations suggest that the therapeutic approach is a major determinant for disease recurrence, together with the HLA-C genotype, but does not represent an independent risk factor for OS, where the immunogenetic profile appears to be the only independent predictive variable.

Previous studies mainly analyzed the implications of the co-expression of inhibitory KIRs and their HLA ligands, that leads to the maturation of fully competent (“licensed”) NK-cells both in mice and in humans (28,29). The opposite situation may occur when activating KIRs are co-expressed with their ligands, as it was shown for KIR2DS1 that recognizes HLA-C2 (30). Indeed the compound KIR2DS1/HLA-C2 immunogenetic profile would lead to reduced functional competence of NK-cells. In HCC patients we observed that the concurrent presence of KIR2DL2 and HLA-C1 was associated with longer TTR, possibly due to the protective effect of NK-cells that had reached full functional competence upon maturation. Consistent with this view, HLA-Bw4I80 (a high-affinity ligand of inhibitory KIR3DL1, expressed by 60/61 patients) was associated with longer survival, whereas the opposite was observed in patients with HLA-Bw4T80, a low affinity ligand of KIR3DL1 (21,31). In addition, the combined presence of KIR3DS1 and HLA-Bw4T80 was associated with reduced OS. Previous reports suggested a protective effect of HLA-Bw4I80 in association with activating KIR3DS1 towards the development of HCV-linked HCC (17), the progression of acquired immunodeficiency syndrome (32) and, with weak effect, the development of chronic HCV infection (9). Although the binding of KIR3DS1 to HLA-Bw4 has not been convincingly demonstrated so far, these results suggest that a less effective interaction of KIR3DS1 and HLA-Bw4T80 in HCC patients might result in reduced NK function and impaired survival. The significance of the association between KIR2DS2/HLA-C1 compound genotype and longer TTR is uncertain: to date, no experimental evidence of HLA-C1 binding by KIR2DS2 is available. Therefore, it cannot be ruled out that this association only derives from the strict linkage disequilibrium between KIR2DL2 and KIR2DS2.
Activating KIR–HLA genotypes appear to increase the risk of developing some virus-related tumors, such as cervical cancer due to papilloma virus infection and nasopharyngeal carcinoma associated with Epstein Barr virus infection (33,34). An increased risk of developing HCC was also reported in patients with chronic HBV infection carrying HLA-C1C1, HLA-Bw4I80 and 22 bp-deleted form of KIR2DS4 (KIR2DS4/1D) (35), thus suggesting that increased NK cell function might represent a risk factor for HBV-related HCC. The apparent discrepancy between these observations and those reported in HCV-linked HCC (17) might derive from differences in the specific immunopathogenic mechanisms. Since NK-cells exert both antiviral and anti-tumor functions, an enhanced effector phenotype might be protective against tumor development altogether increasing the pro-inflammatory anti-viral environment and the consequent tissue damage, both favouring a pre-neoplastic condition. However, in our study the tumor had already developed and immunosurveillance by NK-cells would represent an immunological mechanism affecting clinical outcome.

The impact of the immunogenetic background was further analyzed by phenotypic and functional characterization of NK-cells. Higher percentage of CD56dim NK cells, indicative of prevalent effector cytotoxic phenotype, was associated with both longer TTR and OS, and NK-cells from patients carrying HLA-C1 had significantly higher cytotoxic activity. In addition, the combined presence of KIR2DL2/KIR2DL3 and HLA-C1 was associated with increased cytotoxic function, further supporting the relevance of NK-cell licensing for the achievement of full functional competence. The same association was reported in patients with spontaneously resolving acute HCV infection (13).

This is to our knowledge the first study addressing the relevance of KIR genotype on the prognosis of HCV-related HCC after curative treatment. Results suggest a central role of NK-cells in the immune response against HCC, thus supporting the rationale for developing immunomodulatory strategies aimed at the enhancement of NK response (36) especially in patients showing a favorable immunogenotypic background. In addition, NK cell phenotype and function may provide information
useful to predict outcome and tailor follow-up strategies after HCC treatment. Thus our results, if confirmed on larger series of patients, will be relevant for the implementation of individualized post-treatment monitoring schemes and novel therapeutic strategies in patients with HCC.
**Figure Legends**

**Figure 1.**

Individual KIRs and HLA molecules associated with better TTR and/or OS.

Only KIR and HLA ligands significantly associated with TTR and/or OS are shown. p values were determined by the log-rank test.

Of 61 patients, 15 were carriers of the \textit{KIR2DS5} gene and 19 were homozygous for HLA-C1. \textit{KIR2DS5} gene carriers presented better TTR and OS. Homozygous expression of HLA-C1 was associated with better OS. Among 48 patients positive for HLA-Bw4, at least one copy of the I80 or T80 variants was present in 31 and 23 patients, respectively. The I80 variant (either one or two copies) was associated with better OS, while opposite behavior was shown by the T80 variant.

**Figure 2.** Effect of HLA-C1 and compound KIR/HLA genotypes on TTR and OS.

**A:** Homozygous HLA-C1 (HLA-C1C1; 2 copies, detected in 19 patients) was associated with significantly longer OS compared to heterozygous HLA-C1 (HLA-C1C2; 1 copy, detected in 29 patients) and with significantly longer TTR and OS compared to homozygous HLA-C2 (HLA-C2C2; 0 copies, detected in 13 patients). Survival curves are referred to each of the 3 possible genotypes (HLA-C1 homozygous, heterozygous or null), while bar panels show the progressive effect of HLA-C1 copy number on median TTR and OS. **B:** Effect of the compound KIR2DL2-KIR2DS2/HLA-C1 and KIR3DS1/HLA-Bw4T80 genotypes on TTR and OS, respectively. KIR2DL2 was detected in 34 patients, 2 of whom homozygous, and 26 also positive for HLA-C1 (homozygous or heterozygous); KIR2DS2 was detected in 33 patients, 25 also positive for HLA-C1 (homozygous or heterozygous); KIR3DS1 (homozygous or heterozygous) was detected in 18 patients, 9 of whom also positive for HLA-Bw4T80 (all heterozygous, either Bw4T80/BwI80 or Bw4T80/Bw6).
**Figure 3.** Analysis of NK-cell phenotype and cytotoxicity.

Upper panel: left, Kaplan-Meier curves of TTR and OS of HCC patients with different \%CD56^{dim} NK-cells. The cut-off value between high or low CD56^{dim} was determined by ROC curve analysis. The area under the ROC curve (AUC) for TTR was 0.73 (0.56-0.90), and a cut-off value of 88.95% CD56^{dim} showed 75% sensitivity and 76.2% specificity. The AUC for OS was 0.77 (0.66-0.96), with 72.2% sensitivity and 78.6% specificity for a cut-off of 89.25% CD56^{dim}; right, cytotoxic potential measured by upregulation of CD107a in CD3-CD56^{+} cells upon stimulation (mean±standard error) in subjects with or without HLA-C1/C2 or combined KIR2DL2/DL3-C1.

Lower panel: Flow cytometry analysis of NK cells cytotoxic potential is shown for a representative patient (RTA14), positive for HLA-C1C2 and HLA-Bw4(T80)/Bw6. The KIR genotype of patient RTA14 is shown in Supplementary Figure S1. The two plots on the left show gating of NK cells after overnight PBMCs activation with IL-15, while the remaining plots show staining for CD107a on gated NK-cells (CD3-CD56^{+}) after incubation with or without K562 target cells.
References


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**Table 1.** Clinical characteristics of patients.

SR: surgical resection; RTA: radiofrequency thermal ablation; NA: not applicable; NS: not significant; M: males; F: females; SD: standard deviation.
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<td>HLA-C1</td>
<td>0.057</td>
<td>0.44 (0.19-1.03)</td>
</tr>
<tr>
<td>HLA-C2</td>
<td>0.093</td>
<td>1.65 (0.92-2.98)</td>
</tr>
<tr>
<td>KIR2DL2+/C1+ vs C1-</td>
<td>0.027</td>
<td>0.29 (0.09-0.86)</td>
</tr>
<tr>
<td>KIR2DL3+/C1+ vs C1-</td>
<td>0.073</td>
<td>0.42 (0.16-1.08)</td>
</tr>
<tr>
<td>KIR2DS2+/C1+ vs C1-</td>
<td>0.026</td>
<td>0.28 (0.09-0.86)</td>
</tr>
<tr>
<td>KIR3DS1+/I80+ vs I80-</td>
<td>0.91</td>
<td>1.08 (0.27-4.36)</td>
</tr>
<tr>
<td>KIR3DS1+/T80+ vs T80-</td>
<td>0.81</td>
<td>1.17 (0.32-4.30)</td>
</tr>
</tbody>
</table>

**Table 2.** TTR and OS of HCC patients according to clinical characteristics, KIRs, KIR ligands, and compound KIR-HLA genotypes. Only parameters presenting p<0.1 in at least one analysis (TTR and/or OS) are shown.

* Age ≥ vs < median (71 years); HR: hazard ratio; CI: confidence interval.
<table>
<thead>
<tr>
<th></th>
<th>TTR</th>
<th></th>
<th>OS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>p value</strong></td>
<td><strong>HR (95% CI)</strong></td>
<td><strong>p value</strong></td>
<td><strong>HR (95% CI)</strong></td>
</tr>
<tr>
<td>Gender (M vs F)</td>
<td>NA</td>
<td>NA</td>
<td>0.342</td>
<td>0.70 (0.33-1.46)</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td>0.106</td>
<td>1.67 (0.89-3.12)</td>
<td>0.114</td>
<td>1.85 (0.86-3.96)</td>
</tr>
<tr>
<td>(≥71 vs &lt;71 years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Child (A vs B)</td>
<td>0.106</td>
<td>0.43 (0.160-1.19)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Treatment (SR vs RTA)</td>
<td>0.021</td>
<td>0.43 (0.21-0.88)</td>
<td>0.992</td>
<td>0.99 (0.42-2.34)</td>
</tr>
<tr>
<td>KIR2DS5 (pos vs neg)</td>
<td>0.664</td>
<td>0.74 (0.19-2.91)</td>
<td>0.047</td>
<td>0.408 (0.17-0.99)</td>
</tr>
<tr>
<td>KIR2DS1 (pos vs neg)</td>
<td>0.698</td>
<td>0.78 (0.22-2.79)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>HLA-C1 (pos vs neg)</td>
<td>0.023</td>
<td>0.40 (0.18-0.88)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>HLA-C2 (pos vs neg)</td>
<td>0.961</td>
<td>1.02 (0.50-2.09)</td>
<td>0.067</td>
<td>2.17 (0.99-4.98)</td>
</tr>
</tbody>
</table>

**Table 3.** Multivariate analysis of TTR and OS.

NA: not applicable (p>0.10 in the univariate analysis).
Fig. 1

- KIR2DS5- vs KIR2DS5+ (TTR months)
  - p = 0.0244

- HLA-C1C1 vs HLA-C1C2/HLA-C2C2 (OS months)
  - p = 0.0105

- HLA-Bw4 I80- vs HLA-Bw4 I80+ (OS months)
  - p = 0.0499

- HLA-Bw4 T80- vs HLA-Bw4 T80+ (OS months)
  - p = 0.0075
**Fig. 2**

A. Survival curves for different HLA-C genotypes.

- **HLA-C2C2**: Median (range) of TTR (Total Time to Relapse) months is 115 (90-140) with a p-value of 0.034.
- **HLA-C1C2**: Median (range) of TTR months is 110 (85-135) with a p-value of 0.022.
- **HLA-C1C1**: Median (range) of TTR months is 105 (80-125) with a p-value of 0.018.

B. Survival curves for different KIR genotypes.

- **KIR2DL2+/C1- vs. KIR2DL2+/C1+**: Median (range) of TTR months is 120 (100-140) with a p-value of 0.0265.
- **KIR2DS2+/C1- vs. KIR2DS2+/C1+**: Median (range) of TTR months is 115 (90-140) with a p-value of 0.0262.
- **KIR3DS1+/Bw4T80- vs. KIR3DS1+/Bw4T80+**: Median (range) of OS months is 130 (110-145) with a p-value of 0.0197.

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KIR: Killer Immunoglobulin-like Receptor; HLA: Human Leukocyte Antigen; TTR: Total Time to Relapse; OS: Overall Survival.
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