HLA and Killer Immunoglobulin-like Receptor Genes as Outcome Predictors of Hepatitis C Virus–Related Hepatocellular Carcinoma

Elisabetta Cariani1, Massimo Pilli2, Alessandro Zerbini4, Cristina Rota1, Andrea Olivani2, Paola Zanelli2, Adele Zanetti3, Tommaso Trenti1, Carlo Ferrari2, and Gabriele Missale2

Abstract

**Purpose:** We evaluated the impact of the killer immunoglobulin-like receptors (KIR) of natural killer (NK) cells and of their HLA ligands over the clinical outcome of hepatitis C virus (HCV)–related hepatocellular carcinoma after curative treatment by either surgical resection or radiofrequency thermal ablation (RTA).

**Experimental Design:** Sixty-one consecutive patients with HCV-related hepatocellular carcinoma 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, whereas 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 (surgical resection 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 (CD56^dim) 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 hepatocellular carcinoma, providing a strong rationale for therapeutic strategies enhancing NK response and for individualized posttreatment 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 (CD56^dim,CD16^+) and the other mainly involved in cytokine secretion (CD56^bright, CD16^dim, or negative; ref. 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 Ig-like receptors (KIR). 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 multilevel 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 to HLA-C alleles with asparagine at position 80 (HLA-C2, refs. 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
Translational Relevance

Natural killer (NK) cells play a major role in antitumor immune response. The genotypes of NK cell immunoglobulin-like receptors (KIR) and of their HLA ligands are related to the development of hepatocellular carcinoma in patients with hepatitis C virus (HCV) infection. A better understanding of the role played by the immunogenotypic background over hepatocellular carcinoma outcome might represent a major breakthrough to improve the clinical management of patients with hepatocellular carcinoma. Our work shows that the immunogenetic profile is associated with NK-cell function and represents a powerful outcome predictor in HCV-linked hepatocellular carcinoma. These results support a central role of NK cells in the immunoreponse against hepatocellular carcinoma with important implications in terms of development of immunotherapeutic approaches and individualized monitoring schemes after treatment of early hepatocellular carcinoma.

present at position 80 (Bw4I80). Because of 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 shown yet. In contrast, KIR2DS1 has been shown to bind to 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). In contrast, homozygous HLA-C2 (9) and KIR2DS3, in the presence of HLA-C2 (12) were more frequent in patients with chronic hepatitis C compared with 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 overrepresentation of homozygous KIR2DL3/HLA-C1 in sustained responders (11, 14) and of HLA-C2-C2 in patients resistant to treatment (15).

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

Materials and Methods

Patients

We evaluated 61 consecutive Caucasian patients with hepatocellular carcinoma with HCV-related liver disease that underwent curative treatment by either surgical resection or RTA at the University Hospital of Parma (Parma, Italy). Hepatocellular carcinoma diagnosis was made by ultrasonography and computed tomography or MRI in selected cases. The type of treatment was decided on the basis of liver function (Child–Pugh score), comorbidities, age, and location of hepatocellular carcinoma nodules. Patients with early hepatocellular carcinoma were evaluated by a multidisciplinary group (interventional radiologist, hepatologist, and surgeon) to decide treatment allocation based on liver function, portal hypertension, number, site, and size of hepatocellular carcinoma 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 hepatocellular carcinoma.

All patients undergoing liver resection were within Child-A score. Hepatitis B surface antigen (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 ultrasonography every 4 months and dynamic computed tomography or contrast-enhanced 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 clinicopathologic 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, 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). KIR genotypes were determined by duplex real-time PCR (18) on 5 ng DNA in 10 μL containing 1 × SsoFas EvaGreen Supermix (Bio-Rad Laboratories), 0.3 μmol/L of each KIR-specic primer, 0.1 μmol/L 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 Fig. S1.

HLA typing was conducted 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 C1, C2, Bw4, Bw6, and Bw4 I80/T80 variants,
high-resolution typing was conducted by PCR Sequence Specific Oligonucleotide Probes (Proimmune Co.).

**Immunostaining of NK cells**

In 38 of 61 patients, an aliquot of frozen PBMCs obtained on the same day or the day before treatment was available for phenotypic analysis. PBMCs were resuspended in RPMI-1640 and 8% human serum. PBMCs (3 × 10⁶) were stained with CD3–PerCP (BD Biosciences–Pharmingen) and CD56–APC (Miltenyi Biotec). Cells were analyzed on FACS Canto II flow cytometer (BD Biosciences) by the FACSDiva Software. Frequency of CD56\(^{\text{dim}}\) and CD56\(^{\text{bright}}\) NK cells was defined for each patient evaluating different fluorescence intensity of CD3\(^{-}\)CD56\(^{+}\) cells.

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

In 31 of 61 patients, an aliquot of frozen PBMCs obtained on the same day or the day before treatment was available for functional analysis. For the evaluation of the cytotoxic function, PBMCs (1 × 10⁶) were incubated for 14 hours at 37°C in the presence or absence of 1 ng/mL rhIL-15 (R&D Systems). PE/Cy5.5–conjugated CD107a (BD Biosciences) was then added with or without K562-target cells at an effector:target ratio of 5:1. After 1 hour, 10 μg/mL of brefeldin A (Sigma-Aldrich) was added and cells were incubated further 3 hours 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.

PBMCs (1 × 10⁶) were incubated for 14 hours 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 hours of incubation. After staining with CD56–APC (Miltenyi Biotec) and CD3–APC/Cy7 (BioLegend), cells were fixed and permobilized using Fix and Perm medium A and B (CalTag Laboratories) 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 interleukin (IL)-12/IL-18–stimulated samples.

**Statistical analysis**

The differences between groups of continuous variables were analyzed by Student \(t\) test for unpaired data. Categorical variables were compared by the \(\chi^2\) 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, receiver operating characteristic (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 conducted 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. \(P < 0.05\) (two-tailed) was considered significant.

**Results**

**Clinical outcome**

The study was carried out on 61 consecutive Caucasian patients with diagnosis of HCV-related hepatocellular carcinoma allocated to treatment by either surgical resection or RTA (Table 1). The median overall survival (OS) was 43 months and the median time to the first hepatocellular carcinoma recurrence (TTR) was 17 months. By dividing patients according to the treatment received, it was found that age was significantly higher and liver function worse in RTA-treated patients, whereas hepatocellular carcinoma nodules were on average larger in patients treated by surgical resection. Subjects that carried out surgical resection showed significantly shorter OS and TTR compared with 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 hepatocellular carcinoma 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, as 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.

**Table 1. Clinical characteristics of patients**

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Surgical resection</th>
<th>RTA</th>
<th>(P) (surgical resection vs. RTA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients #</td>
<td>61</td>
<td>28</td>
<td>33</td>
<td>NA</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>33/28</td>
<td>19/9</td>
<td>14/19</td>
<td>NS</td>
</tr>
<tr>
<td>Mean age at diagnosis ± SD (y)</td>
<td>70.2 ± 7.99</td>
<td>67.9 ± 8.97</td>
<td>72.15 ± 6.57</td>
<td>0.037</td>
</tr>
<tr>
<td>Child A/B</td>
<td>55/6</td>
<td>28/0</td>
<td>27/6</td>
<td>0.027</td>
</tr>
<tr>
<td>Median nodules # (range)</td>
<td>1 (1–5)</td>
<td>1 (1–5)</td>
<td>1 (1–3)</td>
<td>NS</td>
</tr>
<tr>
<td>Mean nodules size ± SD, mm</td>
<td>42.3 ± 23.6</td>
<td>58 ± 25.7</td>
<td>29.9 ± 11.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Median TTR, mo</td>
<td>17</td>
<td>34</td>
<td>9</td>
<td>0.0002</td>
</tr>
<tr>
<td>Median OS, mo</td>
<td>43</td>
<td>84</td>
<td>41</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Abbreviations: F, females; M, males; NA, not applicable; NS, not significant.
same ligand HLA-C1. Activating KIR2DS2 is in strong link-
progression and OS

Effect of the compound KIR–HLA genotype on disease
homozygous for HLA-C2 (Fig. 2A).

patients, and both longer OS and TTR compared with those
significantly longer OS compared with HLA-C1 heterozygous
detected that HLA-C1 homozygous patients showed signif-
analyzing the effect of HLA-C1 copy number on survival, we
HLA-C1 for TTR, and only KIR2DS5 for OS (Table 3). By
come the type of treatment (surgical resection vs. RTA) and
identified as independent parameters associated with out-
OS, whereas HLA-C2 and HLA-Bw4T80 were associated with significantly better
Bw4, I80 variant, were associated with significantly better
lated to clinical outcome. Homozygous HLA-C1 and HLA-
KIR2DS2-C1 with a better TTR and for KIR3DS1-Bw4T80
KIR2DL2/C1+ vs. C1−
KIR2DL3/C1+ vs. C1−
KIR2DS2/C1+ vs. C1−
KIR3DS1/I80+ vs. I80−
KIR3DS1/T80+ vs. T80−

**Individual KIRs and HLA expression and their association with disease progression and OS**

KIRs frequencies identified in our patients cohort were comparable with the ones previously reported in Caucasian patients from Northern Italy (19, 20), without any significant difference in the prevalence of each reported KIR (Supplementary Fig. 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 (Fig. 1). KIR2DS5 is an activating receptor in linkage disequilibrium with KIR2DS1, that also showed a trend toward 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, whereas HLA-C2 and HLA-Bw4T80 were associated with a worse OS (Fig. 1 and Table 2). Multivariate analysis identified as independent parameters associated with outcome the type of treatment (surgical resection 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 with HLA-C1 heterozygous patients, and both longer OS and TTR compared with those homozygous for HLA-C2 (Fig. 2A).

**Effect of the compound KIR–HLA genotype on disease progression and OS**

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 to HLA-C1 is not available at present. KIR2DL1 and KIR2DS1 bind to HLA-C2, whereas KIR3DL1 binds to HLA-Bw4, with higher and lower affinity for I80 and T80 variants, respectively (21). Because 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.

Because almost all our patients were positive for both KIR2DL1 and KIR3DL1 (Supplementary Fig. S1), the effect of compound KIR–HLA genotype on disease progression was not evaluated for these molecules. Altogether, combined survival analysis was conducted 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 (Fig. 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. Cutoff 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 CD56dim phenotype higher and lower affinity for I80 and T80 variants, respectively (21). Because 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.

Because almost all our patients were positive for both KIR2DL1 and KIR3DL1 (Supplementary Fig. S1), the effect of compound KIR–HLA genotype on disease progression was not evaluated for these molecules. Altogether, combined survival analysis was conducted 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 (Fig. 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. Cutoff 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 CD56dim phenotype higher and lower affinity for I80 and T80 variants, respectively (21). Because 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.

Because almost all our patients were positive for both KIR2DL1 and KIR3DL1 (Supplementary Fig. S1), the effect of compound KIR–HLA genotype on disease progression was not evaluated for these molecules. Altogether, combined survival analysis was conducted 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 (Fig. 2B and Table 2).

**Table 2. TTR and OS of patients with hepatocellular carcinoma according to clinical characteristics, KIRs, HLA genotypes, and compound KIR–HLA genotypes**

<table>
<thead>
<tr>
<th></th>
<th>TTR</th>
<th></th>
<th>OS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P</td>
<td>HR (95% CI)</td>
<td>P</td>
<td>HR (95% CI)</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>0.15</td>
<td>0.65 (0.36–1.17)</td>
<td>0.014</td>
<td>0.43 (0.22–0.84)</td>
</tr>
<tr>
<td>Age at diagnosis*</td>
<td>0.077</td>
<td>1.70 (0.94–3.05)</td>
<td>0.064</td>
<td>1.86 (0.96–3.61)</td>
</tr>
<tr>
<td>Child (A vs. B)</td>
<td>0.048</td>
<td>0.28 (0.08–0.99)</td>
<td>0.38</td>
<td>0.51 (0.11–2.31)</td>
</tr>
<tr>
<td>Treatment (surgical resection vs. RTA)</td>
<td>&lt;0.001</td>
<td>0.496 (0.246–1.00)</td>
<td>0.0493</td>
<td>0.312 (1.00–4.06)</td>
</tr>
<tr>
<td>KIR2DS5</td>
<td>0.024</td>
<td>0.5 (0.27–0.91)</td>
<td>0.029</td>
<td>0.47 (0.24–0.93)</td>
</tr>
<tr>
<td>KIR2DS1</td>
<td>0.06</td>
<td>0.57 (0.32–1.02)</td>
<td>0.11</td>
<td>0.58 (0.29–1.14)</td>
</tr>
<tr>
<td>HLA-Bw4I80</td>
<td>0.72</td>
<td>0.90 (0.51–1.59)</td>
<td>0.0499</td>
<td>0.43 (0.18–1)</td>
</tr>
<tr>
<td>HLA-Bw4T80</td>
<td>0.57</td>
<td>1.20 (0.64–2.23)</td>
<td>0.008</td>
<td>2.93 (1.33–6.43)</td>
</tr>
<tr>
<td>HLA-C1</td>
<td>0.057</td>
<td>0.44 (0.19–1.03)</td>
<td>0.13</td>
<td>0.50 (0.2–1.24)</td>
</tr>
<tr>
<td>HLA-C2</td>
<td>0.093</td>
<td>1.65 (0.92–2.98)</td>
<td>0.011</td>
<td>2.36 (1.22–4.54)</td>
</tr>
<tr>
<td>KIR2DL2/C1+ vs. C1−</td>
<td>0.027</td>
<td>0.29 (0.09–0.86)</td>
<td>0.56</td>
<td>0.72 (0.24–1.19)</td>
</tr>
<tr>
<td>KIR2DL3/C1+ vs. C1−</td>
<td>0.073</td>
<td>0.42 (0.16–1.08)</td>
<td>0.310</td>
<td>0.61 (0.23–1.59)</td>
</tr>
<tr>
<td>KIR2DS2/C1+ vs. C1−</td>
<td>0.026</td>
<td>0.28 (0.09–0.86)</td>
<td>0.5897</td>
<td>0.7371 (0.24–2.23)</td>
</tr>
<tr>
<td>KIR3DS1/I80+ vs. I80−</td>
<td>0.91</td>
<td>1.08 (0.27–4.36)</td>
<td>0.079</td>
<td>0.17 (0.02–1.23)</td>
</tr>
<tr>
<td>KIR3DS1/T80+ vs. T80−</td>
<td>0.81</td>
<td>1.17 (0.32–4.30)</td>
<td>0.020</td>
<td>5.64 (1.32–24.16)</td>
</tr>
</tbody>
</table>

**Abbreviation:** CI: confidence interval.

Only parameters presenting P < 0.1 in at least one analysis (TTR and/or OS) are shown.

*Age ≥ vs. < median (71 years).
HLA and KIR genotypes in the subgroup of patients with samples available for functional analysis was comparable with 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 (Fig. 3 top right and bottom). No significant differences could be found for IFN-γ production (data not shown).

**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 KIR2DS5 gene and 19 were homozygous for HLA-C1. 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, whereas opposite behavior was shown by the T80 variant.

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

<table>
<thead>
<tr>
<th></th>
<th>TTR</th>
<th>OS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P</strong></td>
<td><strong>HR (95% CI)</strong></td>
<td><strong>P</strong></td>
</tr>
<tr>
<td>Gender (M vs. F)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Age at diagnosis (≥71 vs. &lt;71 years)</td>
<td>0.106</td>
<td>1.67 (0.89–3.12)</td>
</tr>
<tr>
<td>Child (A vs. B)</td>
<td>0.106</td>
<td>0.43 (0.160–1.19)</td>
</tr>
<tr>
<td>Treatment (surgical resection vs. RTA)</td>
<td>0.021</td>
<td>0.43 (0.21–0.88)</td>
</tr>
<tr>
<td>KIR2DS5 (positive vs. negative)</td>
<td>0.664</td>
<td>0.74 (0.19–2.91)</td>
</tr>
<tr>
<td>KIR2DS1 (positive vs. negative)</td>
<td>0.698</td>
<td>0.78 (0.22–2.79)</td>
</tr>
<tr>
<td>HLA-C1 (positive vs. negative)</td>
<td>0.023</td>
<td>0.40 (0.18–0.88)</td>
</tr>
<tr>
<td>HLA-C2 (positive vs. negative)</td>
<td>0.961</td>
<td>1.02 (0.50–2.09)</td>
</tr>
</tbody>
</table>

Abbreviation: CI: confidence interval.
NA: not applicable (P > 0.10 in the univariate analysis).
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 hepatocellular carcinoma 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 hepatocellular carcinoma pathogenesis.

In the present study, we evaluated the impact of the immunogenetic profile on the clinical outcome of patients with hepatocellular carcinoma undergoing curative treatment by either surgical resection 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 hepatocellular carcinoma may develop in the pretumorous environment of liver cirrhosis. The direct clinical consequences of the underlying liver disease have also a major impact on OS after treatment. Because the biologic characteristics of the tumor and the host immune response are both involved in hepatocellular carcinoma 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 patients with hepatocellular carcinoma.

**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 with heterozygous HLA-C1 (HLA-C1C2; 1 copy, detected in 29 patients) and with significantly longer TTR and OS compared with 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), whereas 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 were homozygous and 26 also positive for HLA-C1 (homozygous or heterozygous); KIR2DS2 was detected in 33 patients, 25 were also positive for HLA-C1 (homozygous or heterozygous); KIR3DS1 (homozygous or heterozygous) was detected in 18 patients, 9 of whom were also positive for HLA-Bw4T80 (all heterozygous, either Bw4T80/BwI80 or Bw4T80/Bw6).
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 as the ligand of KIR2DS5 has not been identified yet. Whether KIR2DS5 is a marker for a haplotype involved in hepatocellular carcinoma 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-

Previous studies mainly analyzed the implications of the coexpression 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 coexpressed 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 the reduced functional competence of NK cells. In patients with hepatocellular carcinoma, 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 toward the development of HCV-linked hepatocellular carcinoma (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 shown so far, these results suggest that a less
effective interaction of KIR3DS1 and HLA-Bw4T80 in patients with hepatocellular carcinoma 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 seem 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 hepatocellular carcinoma was also reported in patients with chronic HBV infection carrying HLA-C1C1, HLA-Bw4I80, and 22 bp-deleted form of KIR2DS4 (KIR2DS4/1D; ref. 35), thus suggesting that increased NK-cell function might represent a risk factor for HBV-related hepatocellular carcinoma. The apparent discrepancy between these observations and those reported in HCV-linked hepatocellular carcinoma (17) might derive from differences in the specific immunopathogenic mechanisms. Because NK cells exert both antiviral and antitumor functions, an enhanced effector phenotype might be protective against tumor development altogether increasing the proinflammatory antiviral environment and the consequent tissue damage, both favoring a preneoplastic condition. However, in our study, the tumor had already developed and immunosurveillance by NK cells would represent an immunologic 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 hepatocellular carcinoma after curative treatment. Results suggest a central role of NK cells in the immunoresponse against hepatocellular carcinoma, 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 hepatocellular carcinoma treatment. Thus our results, if confirmed on larger series of patients, will be relevant for the implementation of individualized posttreatment monitoring schemes and novel therapeutic strategies in patients with hepatocellular carcinoma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: E. Cariani, C. Ferrari, G. Missale
Development of methodology: E. Cariani, M. Pilli, G. Missale
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Pilli, C. Rota, A. Oliviani, P. Zanelli, A. Zanetti
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Cariani, M. Pilli, A. Zerbini, P. Zanelli, C. Ferrari, G. Missale
Writing, review, and/or revision of the manuscript: E. Cariani, A. Zerbini, C. Rota, T. Trenti, C. Ferrari, G. Missale
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Rota, G. Missale
Study supervision: E. Cariani, T. Trenti, C. Ferrari

Grant Support
This work was supported by a grant from Fondazione CARIPARMA (Parma, Italy) and by the grant RBA10TP5X from Fondo per gli Investimenti della Ricerca di Base (Ministry of Education, University and Research, Italy).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 12, 2013; revised August 4, 2013; accepted August 7, 2013; published OnlineFirst August 12, 2013.

References


HLA and Killer Immunoglobulin-like Receptor Genes as Outcome Predictors of Hepatitis C Virus–Related Hepatocellular Carcinoma

Elisabetta Cariani, Massimo Pilli, Alessandro Zerbini, et al.

Clin Cancer Res Published OnlineFirst August 12, 2013.

Updated version Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-13-0986

Supplementary Material Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2013/08/12/1078-0432.CCR-13-0986.DC1

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.