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Hepatocellular carcinoma after direct-acting antivirals for hepatitis C is associated with KIR-HLA types predicting weak NK cell-mediated immunity

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Second-generation direct-acting antivirals (2G DAA) to cure HCV have led to dramatic clinical improvements. HCV-associated hepatocellular carcinoma (HCC), however, remains common. Impaired immune tumor surveillance may play a role in HCC development. Our cohort evaluated the effects of innate immune types and clinical variables on outcomes including HCC. Participants underwent full HLA class I/KIR typing and long-term HCV follow-up. A total of 353 HCV+ participants were followed for a mean of 7 years. Cirrhosis: 25% at baseline, developed in 12% during follow-up. 158 participants received 2G DAA therapy. HCC developed without HCV therapy in 20 subjects, 24 HCC after HCV therapy, and 10 of these after 2G DAA. Two predictors of HCC among 2G DAA-treated patients: cirrhosis (OR, 10.0, $p = 0.002$) and HLA/KIR profiles predicting weak natural killer (NK) cell-mediated immunity (NK cell complementation groups 6, 9, 11, 12, OR of 5.1, $p = 0.02$). Without 2G DAA therapy: cirrhosis was the main clinical predictor of HCC (OR, 30.8, $p < 0.0001$), and weak NK-cell-mediated immunity did not predict HCC. Cirrhosis is the main risk state predisposing to HCC, but weak NK-cell-mediated immunity may predispose to post-2G DAA HCC more than intermediate or strong NK-cell-mediated immunity.

Keywords: Antiviral Agents · Carcinoma · Hepatocellular · Hepatitis C · Immunity · Innate · Killer Cells · Natural

Introduction

Highly efficacious second-generation direct-acting antivirals (2G DAA) against HCV produce a sustained virological response (SVR) or cure in more than 90% of infected individuals who complete a course of therapy. SVR is associated with a lower risk of cirrhosis, hepatocellular carcinoma (HCC), and death [1–4]. HCC risk persists, however, over many years after SVR particularly in cirrhotics [5]; all major societal guidelines recommend continued HCC screening after SVR in cirrhotics [6, 7]. The overall risk of HCC declines following 2G DAA-induced SVR [8]. Several studies, however, have described HCCs developing shortly after the initiation of 2G DAA treatment [9, 10].

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There are several theoretical reasons why DAA therapy could be associated with HCC development. Initially, 2G DAA therapies were targeted to patients with advanced cirrhosis, who were already at high risk for HCC, so incipient early HCC may coincidentally be identified post-SVR. In addition, following viral cure, the healing liver expresses high levels of specific growth factors, including vascular endothelial growth factor and EGF, which may be associated with enhanced tumorigenesis [11]. We also postulate that some patients have enhanced HCC risk based on changes in immune surveillance that are associated with viral clearance. HCC tumor cells often express HCV-encoded antigens during chronic infection, and the loss of HCV-encoded tumor antigens following viral cure might result in diminished tumor surveillance by HCV-specific T cells [12, 13]. This altered T-cell surveillance could result in the rapid growth of microscopic/inapparent HCC shortly after 2G DAA treatment. In addition to T cells, tumor surveillance can be performed in part by natural killer (NK) cells. Differences in NK cell killing capacity can be determined by interactions of killer cell immunoglobulin-like receptors (KIR) on NK cells with specific human leukocyte antigen (HLA) class I ligands. KIR have extensive variability in the type and number of genes and alleles, and can either activate or inhibit NK cell activity [14]. Together, HLA class I molecules and KIR form a system of ligands and receptors that diversify NK-cell responses, which can alter clinical disease in individuals and distinct populations.

In chronic HCV infection, host immune responses contribute to viral control and participate in immunosurveillance against HCC, but robust antiviral immunity also promotes the progression to HCV cirrhosis, a strong risk factor for HCC. In order to more thoroughly evaluate immune effects on HCC incidence, we enrolled a longitudinal cohort to study the effect of innate immune parameters on HCV outcomes. Patients underwent full HLA class I and KIR typing and long-term clinical follow-up. The aim of the current study is to describe the association between innate immune types and other factors on the development of HCC in the cohort, including in 2G DAA-treated patients.

Methods

Study inclusion

BASIC-HepC is a longitudinal cohort of HCV seropositive adult participants enrolled between January 2008 and July 2014 from the Liver Research Program at the San Francisco Department of Veterans Affairs (VA) Medical Center, a tertiary care hospital in Northern California. Enrollment was conducted within a protocol approved by the University of California, San Francisco Institutional Review Board, and all patients signed written, informed consent to participation. Consecutive patients with chronic hepatitis C were enrolled in a convenient manner. Excluded: chronic hepatitis B, solid organ transplant, or other concomitant liver disease. Clinical, demographic, and immunogenetic characteristics were compiled at enrollment, which began in 2008 and was completed in July 2014. The current report describes follow-up through June 2019. Questionnaires at enrollment captured: route, timing of likely first HCV exposure, comprehensive medical histories, and self-identified race (grouped as Black, White, Latino, Asian). Eleven participants self-identified as "mixed Black:" categorized for analysis as Black. Longitudinal clinical data: obtained through the VA computerized patient record system and VISTA-Web system, including comprehensive antiviral treatment data. Anti-HCV therapy: provided based on standard clinical management criteria during follow-up, including 2G DAA therapies for viremic patients when they became available in late 2013 and thereafter. Liver biopsies or liver imaging were performed when indicated as a part of standard clinical care, including cirrhotic HCC surveillance approximately every 6 months. Participants who left the VA system, died, or underwent a liver transplant had their follow-up censored at that time.

Clinical measures

Data were obtained by chart review and were cross-validated by two independent teams of investigators. Chronic HCV infection: HCV viremia on at least two timepoints >6 months apart. Cirrhosis: stage 4 scarring on liver biopsy; or by liver imaging (CT liver protocol, MRI liver protocol, abdominal ultrasound) showing 2 or more of the following: (1) nodular liver contour, (2) splenomegaly, (3) portal hypertension: enlarged portal vein and/or varices, or (4) ascites. HCV therapeutic histories and outcomes were obtained by chart review, with confirmation of SVR by negative viral load 12 or more weeks after therapy completion using the following therapeutic regimens: IFN-α, IFN-α/RBV, or pegylated IFN-α/RBV, 1st gen DAA: PEG-IFN-α/RBV/BOC or TVR; 2nd gen DAA: SOF/PEG/RBV, SOF/RBV, SOF/SIM ± RBV, SOF/LED ± RBV, PrOD ± RBV, EBV/GRZ ± RBV, SOF/VEL ± RBV).

HCC was defined by an accepted liver imaging system as a "LIRADS-5 lesion" (>98% likelihood of HCC diagnosis) on CT liver protocol or MRI liver protocol ± AFP, or by biopsied specimens.

KIR typing methodology

Biospecimen processing and KIR typing: performed on blood samples obtained at enrollment, de-identified, and processed as described previously [15]. Genomic DNA was isolated from frozen peripheral blood mononuclear cells using the Qiagen genomic DNA isolation kit. KIR genotyping was performed using PCR sequence-specific priming with paired primers for each *KIR* gene, as described previously, as well as with a set of primers for an internal control gene, GALC [16]. The presence or absence of the following *KIR* genes was assessed: *KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL4, KIR2DL5, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5, KIR3DL1, KIR3DL2, KIR3DL3*, and *KIR3DS1*. Melt curve analysis was performed after cycling and could discriminate between galactosylceramidase (Tm = 74.89 C) and each KIR amplicon (Tm = 78.5–85.9 C). An internal control amplicon ensured that the absence of a KIR amplicon was a true result.

KIR haplotype categorization

Distinct KIR haplotypes differ in their KIR gene content and are associated with subtle affinity differences of specific KIR alleles for common HLA ligands. The common KIR haplotype A is characterized by the weakest inhibitory KIR2DL3 receptor (at the *KIR2DL2/3* locus) with a paucity of stimulatory "S" KIR, whereas KIR haplotype B includes numerous genetic types characterized by the intermediate inhibitory KIR2DL2 receptor (at the *KIR2DL2/3* locus) and/or by the presence of one or more stimulatory KIR. KIR haplotype assignments in this cohort were based upon total gene content encoded by both chromosomes, so only individuals homozygous for the A type at the KIR locus could be categorized as "KIR haplotype A", whereas any haplotype B gene led to categorization as "KIR haplotype B" [17].

HLA class I typing

Genotyping of the HLA class I loci to four-digit resolution was performed using the sequence-base typing method [17]. Amplicons of ∼1 kb, which included exon 2 and 3 genomic regions, were amplified with locus-specific primers. Amplicons were cleaned with AMPure XP (Agencourt Biosource Corporation) in a Biomek FX Laboratory Automation Workstation (Beckman Coulter) and sequenced with ABI Prism BigDye Terminator v1.1 Cycle Sequencing Kits (Applied Biosystems). Sequence reactions were cleaned with CleanSeq (Agencourt Biosource Corporation) in a Biomek FX Laboratory Automation Workstation and run on an ABI 3730XL DNA analyzer (Applied Biosystems). Sequences were analyzed with the "Assign 400 ATF" software (Conexio Genomics) to group the HLA alleles. All *HLA-C* were categorized as either HLA-C1 or HLA-C2 alleles based on α_1-domain polymorphisms [18]. *HLA-B* alleles were similarly categorized as either HLA-Bw4 or HLA-Bw6 supratype alleles. The polymorphism at residue 80 within each HLA-Bw4 allele determined its categorization as a weak (80-T) or strong (80-I) inhibitory ligand for KIR3DL1.

Flow cytometry

To quantify KIR expression on NK cells and identify common nonexpressing (null) KIR3DL1 alleles, NK cells ($CD56^+CD3^-$ in the lymphocyte gate) were phenotyped by multicolor flow cytometry using a Becton–Dickinson FACSAria. The reagent panel was comprised of fluorescein-conjugated anti-KIR3DL1 (R&D Systems clone DX9), allophycocyanin-conjugated anti-KIR2DL2/2DL3 (R&D Systems, clone 180701), phycoerythrin-conjugated anti-KIR2DL1 (R&D Systems, clone 143211), Pacific orange-conjugated anti-CD3 (Invitrogen, clone UCHT1), and PerCP-Cy5.5-conjugated anti-CD56 (BioLegend, clone HCD56). The Dead Red 488 stain (molecular probes) was used to exclude dead cells.

Formation of NK complementation groups

Although $KIR2DL^+$ NK cell subset expression (as defined by the percentage of the overall NK-cell population) was variable in each individual, $KIR2DL1^+$ NK cell subsets were identifiable by flow cytometry in all individuals expressing the KIR2DL1 ligand HLA-C2, and $KIR2DL2^+$ or $KIR2DL3^+$ NK cell subsets could be identified in all individuals expressing HLA-C1 (not shown). In a reductionist approach to examine the effects of KIR/HLA on NK cell functions, we stratified inhibitory KIR receptor-ligand pairs by their avidities. HLA-C mediated inhibitory NK cell effects were logically classified as weak ($KIR2DL2/3^+$ HLA-C1), or strong ($KIR2DL1^+$ HLA-C2) based upon available composite avidity data [19], although HLA-C2 proteins have classically been described as potent inhibitory ligands for KIR2DL1. The *HLA-C*04* allele is present at high frequencies in most ancestrally distinct human populations and is represented in the vast majority of cases by a single allele (*HLA-C*04:01*), suggesting that unique properties of *HLA-C*04* may confer a selective evolutionary advantage [20, 21]. Among HLA-C2 proteins, Hilton et al. [19], have quantified the binding affinities for numerous HLA-C2 proteins for KIR2DL1. Although there is significant variability in KIR2DL1 binding characteristics among HLA-C2 (nonC*04) proteins, they all appear to bind KIR2DL1 with higher avidities compared with *HLA-C*04*, and were grouped together as "strong" HLA-C2S inhibitory ligands for our complex analyses [16]. As such, combined HLA-C types were initially used to classify NK inhibitory strengths as weak/weak (HLA-C1C1); weak/intermediate [HLA-C1C2(C*04)]; weak/strong (HLA-C1C2S); or strong/strong (HLA-C2C2) inhibition [16]. Notably, among HLA-C2C2 cohort individuals, all patients expressed at least one strong inhibitory HLA-C2S ligand (no patient was homozygous for HLA-C*04).

NK cell inhibition by HLA-B ligands was similarly categorized as null (no surface expression of KIR3DL1 by flow cytometry, no 3DL1 gene by typing, or no HLA-Bw4 supratype alleles), weak (expressed $KIR3DL1^+$ HLA-Bw4-80 T), or strong (expressed $KIR3DL1^+$ HLA-Bw4-80 I). The HLA-A*23, -A*24, and –A*32 molecules contain the HLA-Bw4 supratype motif and were thus categorized as additional KIR3DL1 ligands [22]. NK-cell KIR3DL1-mediated inhibitory strength in our cohort was therefore classified according to the strongest $KIR3DL1^+$ HLA-Bw4 ligand identifiable in each patient — null, weak, or strong. Using these classifications, KIR2DL/HLA-C and KIR3DL1/HLA-B inhibitory avidities were used to assemble cohort patients into 12 substratified NK-cell complementation groups, shown in Table 1. In order to prevent confusion, these groups were categorized by the predicted strength of NK-cell-mediated immune function as defined by composite KIR/HLA inhibitory profile.

Statistical methods

Chi-square or Fisher's exact tests were performed for categorical data analysis, and ANOVA was used to evaluate differences in the distribution of continuous predictors. Data are expressed

Table 1. Natural killer cell complementation groups were constructed for this analysis, based on HLA-B and -C alleles.

KIR/HLA grouping	HLA-C alleles	HLA-B alleles	NK cell immunity
1	HLA-C1-C1	HLA non-Bw4	Strong
2	HLA-C1-C1	HLA-Bw4-80T	Strong
3	HLA-C1-C1	HLA-Bw4-80I	Intermediate
4	HLA-C1-C04	HLA non-Bw4	Strong
5	HLA-C1-C04	HLA-Bw4-80T	Intermediate
6	HLA-C1-C04	HLA-Bw4-80I	Weak
7	HLA-C1-C2S	HLA non-Bw4	Strong
8	HLA-C1-C2S	HLA-Bw4-80T	Intermediate
9	HLA-C1-C2S	HLA-Bw4-80I	Weak
10	HLA-C2-C2	HLA non-Bw4	Intermediate
11	HLA-C2-C2	HLA-Bw4-80T	Weak
12	HLA-C2-C2	HLA-Bw4-80I	Weakest

as means with SD or medians with range and interquartile range (IQR). Pairwise differences in the distribution of continuous variables were evaluated using the Kruskal–Wallis test. In the subgroup of participants treated with 2G DAAs, logistic regression was used to estimate odds ratios and 95% confidence intervals for factors associated with developing HCC, using continuity-adjusted *p*-values for categorical factors. Bivariable models assessed the association between HCC development and the immunity group after adjusting for clinical variables as noted. Measurements of linkage disequilibrium were determined using Haploview software (Broad Institute). All other analyses were performed using SAS version 9.2 (SAS Institute Inc.).

Results

Cohort characteristics

The demographic, clinical, and genetic characteristics of the 353 chronically HCV-infected subjects in BASIC-HepC are presented in Table 2, broken down by race/ethnicity due to NK immunity differing significantly between racial/ethnic groups. Median age at enrollment was 59 years (IQR, 55–63; range, 30–83), and was similar in each race/ethnicity. HIV–HCV coinfection was present in 10 patients. The liver biopsy had been performed on 216 subjects (61%), and 341 patients (97%) had undergone liver imaging; patients without biopsy or liver imaging were excluded from this analysis, as liver disease severity could not be estimated. The mean duration of follow-up was 7 years. A total of 89/353 subjects (25%) developed cirrhosis prior to their baseline visit, and 43 (12%) were diagnosed with cirrhosis during study follow-up. Eight patients underwent a liver transplant during their follow-up, and 107 patients (30%) died by the end of the follow-up. Complete KIR/HLA typing was available for all included subjects. Black patients had a lower overall prevalence of cirrhosis (24%) compared with non-Black patients (42%, $p = 0.006$).

Twenty subjects (6%) developed HCC prior to any anti-HCV therapy, and an additional 24 patients developed HCC after some form of HCV therapy. Table 3 shows cohort HCC development stratified by treatment status and regimen. An IFN-α-based treatment course was used in 119 participants, with 52 (44%) achieving SVR. Twelve subjects who received only IFN-α-based therapy developed HCC (7 non-SVR, 5 SVR). Of patients who remained viremic after 2011, 25 were treated with a first-generation DAA regimen (1G DAA) (PEG-IFN-α/ribavirin plus telaprevir or boceprevir); 8 (32%) achieved SVR, and 2 SVR patients later developed HCC. A total of 158 viremic patients were treated with 2G DAA with 149 (94%) achieving SVR. Of 2G DAA treated subjects 8/149 (5%) SVR subjects developed HCC as did 2/9 (22%) who did not achieve SVR. The mean time to HCC diagnosis among patients treated only with IFN-α was 79 months (IQR, 34–119) after IFN-α initiation; among the two 1G DAA-treated patients: 48 months (IQR, 43–53), and among 2G DAA-treated patients: 9 months after DAA initiation (IQR, 0.5–28), $p = 0.001$ for difference between months to HCC for IFN only vs. 2G DAA. Overall, 7/44 patients who developed HCC (16%) did not have evidence of cirrhosis at the time of tumor diagnosis, and this included 2/10 (20%) among post-2G DAA-treated patients.

Characteristics of patients treated with 2G DAA

Of the 158 patients treated with 2G DAAs, 152 were analyzed separately to evaluate factors associated with HCC development in this group; 6 were excluded due to 2G DAA only being used in the post-HCC therapy setting. All 152 had longstanding HCV, with a mean estimated infection duration of 42 (range 10–60) years at the time of 2G DAA therapy. Ninety-three patients (61%) were White, 37 (24%) were Black, 22 (14%) were Latino, and none were Asian. Thirty-nine (26%) had previously not achieved SVR to IFN-α-based therapy, which was similar to the cohort overall (32%), and three were HIV–HCV coinfected. Of the 10 patients who developed post-2G DAA HCC, several were racial/ethnic minorities: three were Black and three were Latino (Table 4), but racial/ethnic distribution of patients with post-2G DAA HCC did not differ significantly from post-2G DAA-treated patients overall. Weak NK cell immunity/strong inhibition KIR/HLA types such as KIR2DL1/HLA-C2 (often with KIR3DL1/HLA-Bw4) were seen among 6/10 post-2G DAA HCC patients, however, suggesting

Table 2. Cohort characteristics (*n* = 353).

Variable	Total	White (*N* = 210, 59%)	Black (*N* = 86, 24%)	Latino (*N* = 53, 15%)	Asian (*N* = 4, 1%)
Age (mean ± SD)	59 ± 7	59 ± 6	59 ± 7	59 ± 8	57 ± 6
Male gender (*n*, %)	341 (97%)	202 (96%)	83 (97%)	52 (98%)	4 (100%)
BMI (mean ± SD)	27.5 ± 5	27.1 ± 5	28.3 ± 5	27.8 ± 5	27.4 ± 5
Diabetes mellitus (*n*, %)	81 (23%)	39 (19%)	24 (28%)	15 (28%)	3 (75%)
HIV	10 (3%)	6 (3%)	3 (3%)	1 (2%)	0
HCV genotype (*n*, %): 1	252 (71%)	136 (65%)	75 (87%)	38 (72%)	3 (75%)
2	49 (14%)	39 (19%)	4 (5%)	6 (11%)	0 (0%)
3	42 (12%)	27 (13%)	6 (7%)	8 (15%)	1 (25%)
4	2 (1%)	1 (0.5%)	1 (1%)	0 (0%)	0 (0%)
Unavailable	8	7	0	1	0
Strong NK immunity (groups 1, 2, 4, 7) (*n*, %)	146 (41%)	98 (47%)	23 (27%)	22 (42%)	3 (75%)
Intermediate NK immunity (groups 3, 5, 8, 10) (*n*, %)	104 (29%)	66 (31%)	22 (26%)	16 (30%)	0 (0%)
Weak NK immunity (groups 6, 9, 11) (*n*, %)	78 (22%)	37 (18%)	30 (35%)	10 (19%)	1 (25%)
Weakest NK immunity (group 12) (*n*, %)	25 (7%)	9 (4%)	11 (13%)	5 (9%)	0 (0%)
Liver biopsy (*n*, %)	216 (61%)	122 (58%)	53 (62%)	40 (75%)	1 (25%)
Treated IFN-RBV (n, %)	119 (34%)	80 (38%)	17 (20%)	22 (42%)	0 (0%)
Treated IFN-RBV, achieved SVR (*n*, %)	52 (44%)	38 (48%)	5 (29%)	9 (41%)	0 (0%)
Treated 1st Gen DAA (*n*, %)	25 (6%)	16 (8%)	3 (3%)	5 (9%)	1 (25%)
Treated 1st Gen DAA, achieved SVR (*n*, %)	8 (32%)	6 (38%)	0 (0%)	1 (20%)	1 (100%)
Treated 2nd Gen DAA (*n*, %)	158 (45%)	95 (45%)	41 (48%)	22 (42%)	0 (0%)
Treated 2nd Gen DAA, achieved SVR (n, %)	149 (94%)	90 (95%)	39 (95%)	20 (91%)	0 (0%)
Cirrhosis (*n*, %)					
Cirr dx at time of enrollment	89 (25%)	59 (28%)	10 (12%)	19 (36%)	1 (25%)
Cirr dx after enrollment	43 (12%)	21 (10%)	11 (13%)	10 (19%)	1 (25%)
Cirr not present	221 (63%)	130 (62%)	65 (76%)	24 (45%)	2 (50%)
HCC (*n*, %)	44 (12%)	24 (11%)	12 (14%)	7 (13%)	1 (25%)
Liver transplant (*n*, %)	8 (2%)	5 (2%)	2 (2%)	1 (2%)	0 (0%)
Death (*n*, %)	107 (30%)	56 (27%)	26 (30%)	22 (42%)	3 (75%)
Liver-related death (*n*, %)	51 (48%)	26 (46%)	11 (42%)	11 (50%)	3 (100%)

diminished tumor immunosurveillance. At the time of 2G DAA therapy, cirrhosis was present in 52/152 (34%) patients. Eight of the 52 cirrhotics (15%) developed HCC during the post-2G DAA period, as did 2/100 non-cirrhotics (2%). Cirrhosis was the principal risk factor for HCC development during post-DAA follow-up (OR, 10.2; 95% CI,2.21-–71.71, $p = 0.002$, Table 5). HCV genotype, patient race/ethnicity, age at enrollment, SVR status, and KIR haplotype A vs. B were not significantly associated

Table 3. Table of relationship and timing of hepatitis C therapy to HCC diagnosis.

Variable	Total (*N* = 44)	Mean time post-HCV therapy initiation to HCC diagnosis (months)	Range (months)
Prior to HCV therapy	20		
IFN-α therapy alone (SVR or not)	12	78.8	(2–236)
IFN-α therapy alone (no SVR)	7	99.4	(17–236)
IFN-α therapy alone (SVR)	5	50.0	(2–76)
1G DAA therapy (SVR or not)	2	48.0	(43–53)
Eventual failure of 1st gen. DAA	0		
Eventual SVR to 1st gen. DAA	2	48.0	(43–53)
2G DAA therapy (SVR or not)	10	8.6	(0.5–28)
Eventual failure of 2st gen. DAA	2	2.8	(0.5–5)
Eventual SVR to 2nd gen DAA	8	10.0	(1–28)

Note: Twenty-four cohort patients had been treated with hepatitis C therapy prior to HCC diagnosis, which included: twelve who developed HCC after receiving IFN-α therapy alone, with or without sustained virological response (SVR) or viral cure. Similar data for the IFN-α-ribavirin-1G DAA group (1G DAA) and 2G DAA groups are also shown. HCC developed a much shorter time after 2G DAA than after the two earlier therapies.

Table 4. Clinical and immunological characteristics of the cohort patients ($n = 10$) who developed HCC after being treated with 2G DAA against hepatitis C.

Race/ Ethnicity	Time DAA start to Liver Tumor (mos)	2G DAA response	HCV duration at 2G DAA treatment (yrs)	Cirrhosis at tumor Dx	Prior IFN non-SVR	HCV duration at IFN non-SVR (yrs)	MELD at 2G DAA Tx Start	MELD after 2G DAA Tx	KIR 3DL1 expression	HLA-B	HLA-C	HLA-B inhibitory type	HLA-C inhibitory type	Modified NK group	NK immunity	KIR haplotype
White	3	SVR	52	No	Yes	34	6	11	Present	Bw6 Bw6	C1-C04	Null	Interm.	4	Strong	B
Latino	10	SVR	50	Yes	Yes	36	7	7	Present	Bw4 80-I	C1-C2S	Stongest	Interm.	9	Weak	B
Latino	28	SVR	35	Yes	Yes	21	11	10	Present	Bw4 80-T	C2-C2	Interm.	Strongest	11	Weak	B
Black	5	SVR	47	Yes	Yes	30	8	6	Present	Bw4 80-I	C2-C2	Strongest	Strongest	12	Weakest	B
White	17	SVR	37	Yes	No		9	7	Present	Bw4 80-I	C1-C1	Strongest	Weakest	1	Interm.	B
Latino	0.5	nonSVR	45	Yes	No		15	Died	Present	Bw4 80-T	C1-C04	Interm.	Interm.	5	Interm.	A
White	6	SVR	51	Yes	No		16	16	Absent	Bw4 80-I	C1-C04	Null	Interm.	4	Strong	B
White	10	SVR	48	Yes	No		8	7	Present	Bw4 80-I	C1-C2S	Strongest	Interm.	9	Weak	B
Black	1	SVR	44	Yes	No		22	14	Present	Bw4 80-I	C1-C2S	Strongest	Interm.	9	Weak	A
Black	5	nonSVR	41	No	No		7	7	Present	Bw4 80-I	C2-C2	Strongest	Strongest	12	Weakest	A

Note: Whether patients had previously failed to have their hepatitis C cured by interferon therapy (IFN), and estimated duration of hepatitis C infection at the time of interferon therapy are also shown. Model of end-stage liver disease (MELD) score estimating cirrhosis severity at the time of 2G DAA therapy and after therapy are also shown. Immunological characteristics by patients are shown, including whether KIR 3DL1 expression was present; which HLA-B and -C alleles a patient possessed, with simplified strength of inhibitory type; summary HLA/KIR group and simplified NK grouping (1–12, as shown in Table 1); and KIR haplotype.

with HCC development (Table 5). Specific NK complementation groups, however, were associated with post-2G DAA HCC development (Table 5), with weak immunity (groups 6, 9, 11, 12, $n = 6/38$ patients developed HCC) having an OR of 5.09, 95% CI, 1.31–21.58, $p = 0.03$ for HCC compared with patients with intermediate or strong immunity (NK groups 1–5, 7, 8, 10, n = 4/114 patients developed HCC). Dichotomizing NK complementation groups into two categories showed that group 12 (the weakest immune group, $n = 2/8$ patients developed HCC) also had a trend toward more HCC than the strong, intermediate, and weak immunity groups combined (OR, 5.51; 95% CI, 0.68–31.45, $p = 0.10$), but this was not statistically significant. In bivariable analyses of HCC in 2G DAA-treated patients, weak immunity (groups 6, 9, 11, 12) and cirrhosis retained their independent associations with HCC (OR, 4.13; 95% CI, 1.01–18.35, $p = 0.048$ for weak immunity, OR 8.6 95% CI 1.85–62.27, p = 0.004 for cirrhosis, Table 5). The association with weak NK-cell immunity was independent of KIR haplotype (A or B), HCV genotype 3a infection, and age. A sensitivity analysis in which HIV+ patients were excluded showed similar results.

Predictors of HCC development in patients not treated with 2G DAA

In the evaluation of the 195 KIR-typed BASIC-HepC patients who were not treated with 2G DAAs, cirrhosis was again the main clinical factor associated with HCC (OR, 30.8; 95% CI, 8.14–198.95, $p < 0.0001$, $n = 26$ HCC's developed). An association between weak NK cell immunity and HCC could not be demonstrated in this group, however (e.g. NK cell immunity in 3 complementation groups: strong immunity [groups 1, 2, 4, 7], intermediate immunity [groups 3, 5, 8, 10], and weak immunity [groups 6, 9, 11, 12]: the OR for weak immunity was 0.86, 95% CI, 0.31–2.26, $p = 0.76$). Baseline GT 2 infection ($n = 27$) was associated with a reduced incidence of HCC (OR, 0.14; 95% CI, 0–0.50, $p = 0.007$). Neither KIR haplotype, HCV genotype 3a, nor age was associated with HCC risk among patients not treated with 2G DAA.

We considered the possibility that influences of NK cell immunity on HCC developing after 2G DAA therapy might be largely due to possible confounding influences of KIR-mediated inhibition on the development of cirrhosis, a known strong risk factor for HCC in its own right. This did not appear to be the case, however. Figure 1 graphically depicts a temporally restricted subanalysis of the 353 BASIC-HepC patients prior to any 2G DAA therapy (naïve or pretreatment, panels B, D), and the 152 patients treated with 2G DAA prior to HCC development (panels A, C). The development of both cirrhosis (panel D) and HCC (panel B) were similarly distributed in each NK-cell immunity group in subjects not treated with 2G DAA. In 152 subjects who were eventually treated with 2G DAA, the distribution of cirrhosis in each NK cell immunity group was similar to that seen in the overall cohort (panel C), but the development of post-2G DAA HCC was more prevalent in patients in the weak or weakest NK cell immune groups (panel A), suggesting that weak NK cell immune types might exhibit unique

Table 5. Multivariable analysis of clinical and immunological predictors of hepatocellular carcinoma (HCC) development among cohort patients treated with 2nd generation direct-acting antivirals against hepatitis C.

Variable	Univariate OR (95% CI)	*p*-value	Bivariate OR (95% CI)	*p*-value
Female gender	1.93 (0–11.05)	1		
Age at 2G DAA	1.07 (0.96–1.20)	0.22		
Diabetes mellitus	1.82 (0.29–8.57)	0.61		
BMI	1.06 (0.94–1.21)	0.32		
Black race (compared with White race)	1.83 (0.26–11.41)	0.68		
Latino ethnicity (compared with White race)	3.09 (0.42–19.70)	0.31		
HCV GT 3 (compared with others)	3.60 (0.33–21.89)	0.31		
Cirrhosis pre-2G DAA	10.2 (2.21–71.71)	0.002	OR 8.6 (1.85–62.27)	0.004
SVR to post-2G DAA	0.36 (0.04–18.14)	0.71		
KIR haplotype A vs. B	2.71 (0.42–13.09)	0.32		
Weakest Immunity (groups 6, 9, 11, 12) compared with Int/strong immunity	5.09 (1.31–21.58)	0.03	OR 4.13 (1.01–18.35)	0.048

Note: Logistic regression was used to estimate odds ratios and 95% confidence intervals for factors associated with developing HCC, using continuity-adjusted *p*-values for categorical factors. A bivariable model assessed the association between HCC development and immunity group after adjusting for cirrhosis.

effects on HCC surveillance, independent of any associations of KIR immunotypes upon cirrhosis development.

Discussion

Hepatocellular carcinoma is a common and lethal complication of longstanding infection with the hepatitis C virus, particularly when it has led to cirrhosis in an infected patient. To attempt to understand whether immune polymorphisms may play a role in HCC development, we enrolled an observational cohort of 353 patients with chronic hepatitis C and followed their clinical outcomes over 7 years. In the main analysis of immunological characteristics of patients, we included patients in 12 NK cell complementation groups as defined by their complex KIR/HLA genotypes and phenotypes (Table 1). HCC risk in the 152 participants treated with second-generation direct-acting antivirals against hepatitis C (2G DAA) was associated with the NK cell complementation groups 6, 9, 11, and 12. These groups are predicted to have the weakest overall NK cell immunity, as each group has at least one strong inhibitory HLA-C2 ligand for KIR2DL1$^+$ NK cells and an inhibitory ligand for KIR3DL1$^+$ NK cells. Specifically, HCC was associated with the weak immunity NK cell receptor:ligand combination KIR2DL1/HLA-C1C2 plus KIR3DL1/Bw4 80-I or the weakest immunity types KIR2DL1/C2C2 plus any KIR3DL1/Bw4. Patients treated with 2G DAA who had these weak NK-cell immunity groups experienced 4.1-fold odds of developing posttreatment HCC in the bivariable analysis compared with treated patients with intermediate or strong immunity. Although cirrhosis itself was associated with risk for HCC (8.6-fold increased risk compared with patients without cirrhosis), the influences of weak NK cell immunotypes after 2G DAA treatment appeared to be independent of cirrhosis in our bivariable analyses. Our interpretation of these results is that cirrhosis is the main risk state that predisposes to HCC development, and those with predicted weak NK cell immunity have an independent risk for HCC in the setting of 2G DAA therapy. The rapidity of HCC development in some patients after 2G DAA therapy also contrasts with the longer interval for HCC emergence following IFN-α-based SVR.

Cohort follow-up spanned three sequential hepatitis C treatment eras: IFNα/RBV, 1G DAA + IFNα/RBV, and 2G DAA. HCC was diagnosed in 44 patients, 20 (45%) prior to receipt of any HCV therapy, and 10 (23%) after 2G DAAs. In treated subjects who successfully cleared HCV, the overall prevalence of HCC was similar in those receiving IFN-α-based and IFNα-free 2G DAA regimens, but incident HCC was diagnosed very early after 2G DAA initiation, a mean of 8.6 months, compared with a mean of 4 years after 1G DAA$^+$ IFNα/RBV or 9.8 years after IFNα/RBV therapy alone ($p = 0.001$, data shown in Table 3). Cirrhosis was present in 89 patients (25%) at study enrollment and developed in a further 43 patients (12%) during follow-up. HCC in chronic HCV patients develops largely in patients with cirrhosis, so cirrhosis and HCC were expected to share many common risk factors. This was not the case for Black patients in this study, however, as Black patients were at lower risk for cirrhosis but had a proportionally higher risk of HCC.

2G DAA-treated patients overall experience significantly lower rates of cirrhosis and HCC, and have significantly better survival, than untreated patients [1, 3, 23, 24]. It is also clear, however, that HCC incidence persists at a fairly steady level after SVR, for at least 2–3 years and possibly up to 10 years or beyond [5]. 2G DAAs are unlikely to cause HCC directly, as some tumors in this and other studies [9, 10] were diagnosed very early after the initiation of therapy. In these cases, early posttreatment HCC may represent small subclinical tumors that were present prior to treatment but only diagnosed during close clinical follow-up. Alternatively, the rapid loss of virus-specific antigens on tumor cells might abrogate longstanding antitumor immune surveillance by virus-specific T cells that have kept subclinical or early HCC in check. HCV antigens are expressed in many HCC tumors where

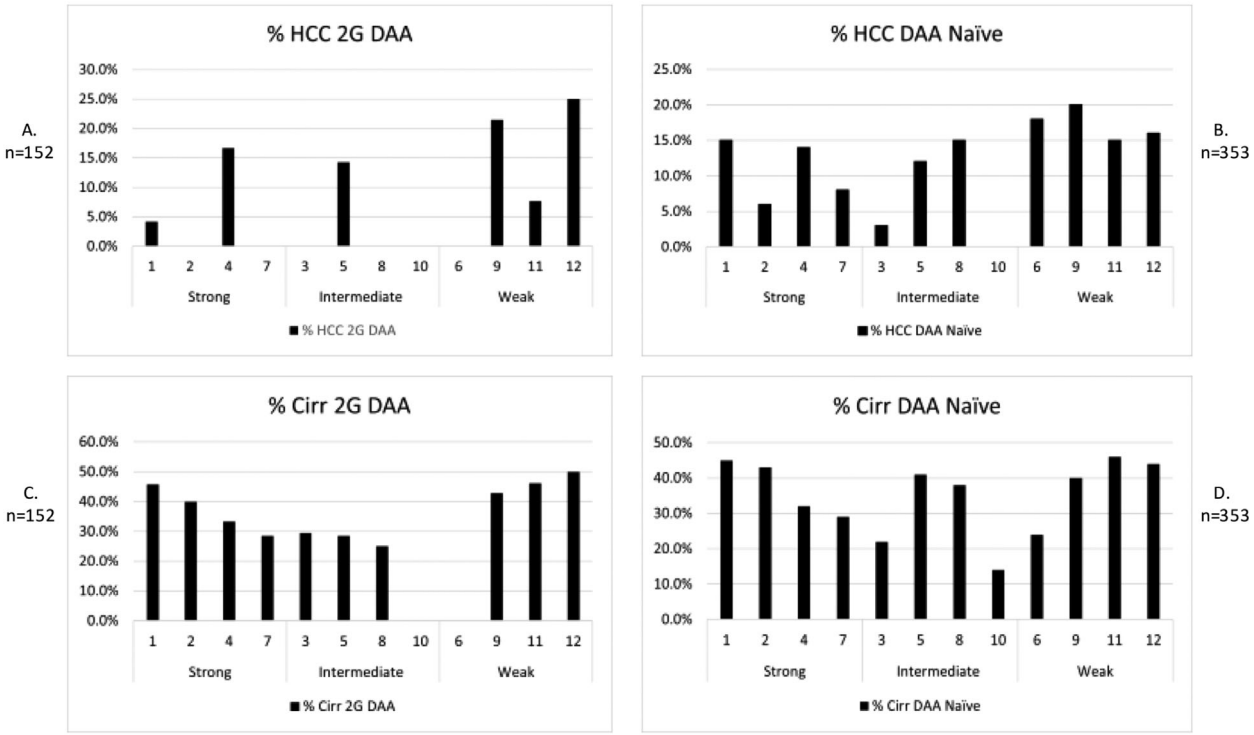

Figure 1. Associations of HCC and Cirrhosis with putative KIR-mediated immunity are discordant in 2G DAA-treated individuals compared with HCC and Cirrhosis in 2G DAA-naïve HCV patients. The development of posttreatment HCC and the incidence of cirrhosis in 152 patients treated with 2G DAA (panels A, C), is compared with the treatment naïve (or pretreatment) incidence of HCC and cirrhosis in 353 BASIC-HepC patients (naïve or pretreatment, panels B, D). The development of both cirrhosis (panel D) and HCC (panel B) was similarly distributed in each NK cell immunity group (strong, intermediate, or weak) in DAA naïve subjects. In 2G DAA-treated subjects, the development of post-2G DAA HCC was uniquely skewed toward patients with the weak NK cell immune groups (panel A), but not in cirrhotic subjects with predicted strong immunity (panel C).

they may serve as target epitopes of HCV-specific T cells [12, 13]. Treatment with 2G DAA could lead to an abrupt loss of T-cell-mediated immune surveillance, but unlike SVR using IFN-α/RBV with or without 1G DAA, viral cure would not be accompanied by the intercurrent pharmacologic IFN-α immune stimulation of T-cell immunity. Tumor immunosurveillance would thus depend primarily on innate immune NK cells.

The relative contributions of T cells and NK cells in HCC tumor surveillance are not entirely clear. A recent dissection of tumor immunity in primary and recurrent HCC suggests a complex interplay of T cells, NK cells, and antigen-presenting cells, with cytotoxic T cells predicted to have a predominant antitumor effect within the tumor microenvironment [25]. This is not unexpected, as HCV, unlike some viruses, fails to downregulate HLA class I encoded proteins on infected cells and does not lead to "missing self" NK-cell cytotoxicity or T-cell escape of virally infected cells [26]. Consistent with this paradigm, individuals with the highest HCC risk after DAA-induced clearance of HCV-encoded T cell epitopes would be those with weaker NK cell-mediated immunity as defined by stronger inhibitory KIR/HLA combinations such as KIR2DL1/HLA-C2 and KIR3DL1/Bw4, as we found in this study.

Our data predict clinical associations of NK-cell immunity based on the segregation of our cohort into logically designated subgroups, but our model is most likely imperfect. Indeed, although HCC after 2G DAA treatment skews toward weaker NK-cell immune groups, some exceptions were seen, as a higher percentage of posttreatment HCC was seen in group 4 (strong immunity) compared with group 11 (weak immunity, see Fig. 1), although the subgroups were small. Marked allelic diversity in KIR and their HLA ligands were likely evolutionarily selected for diverse repertoires of affinity, gene dosage, and NK-cell expression, which were not included in our reductionist model. Similarly, clinical effects mediated by combinations of KIR2DL1, 2, 3, and KIR3DL1 receptors might be complex, rather than simply additive, as we assumed in our analyses, and our model does not include complex effects due to activating KIR. A more precise evaluation of rheostatic inhibitory influences of KIR would agnostically examine the binding affinity of each allelic KIR variant for each allelic variant of its cognate HLA ligand. A complete compilation of affinity data for each KIR receptor:ligand pair has yet to be defined, and *KIR* gene variants were not allelically typed with any precision in our cohort. KIR^+ subset size, as defined by the percentage of NK cells expressing any specific KIR, was highly variable in our cohort, ranging from 2% to 78% of NK cells for any specific KIR in different individuals, as was the percentage of NK cells present within the Ficoll-purified peripheral blood lymphocytes. Attempts to correlate NK cell subset percentage with clinical outcome failed to reveal any coherent results, however

(not shown). It is possible that this was due to power limitations inherent in a reductionist approach to continuous variables within the 12 patient subgroups in our cohort. Alternatively, NK cell subset KIR^+ percentages might have minimal, if any influence on clinical outcomes. A possible null or minimal effect subset size might not be surprising. Similar to minority populations of cytotoxic T cells, even small subsets of KIR-defined NK cells might be adequately "armed and licensed" to efficiently lyse and survey tumor targets in vivo. In the absence of a similar large replication cohort, our study is a hypothesis-generating analysis. HCC diagnosis fairly soon following 2G DAA treatment has been observed in some, but not all treatment cohorts. The relatively high incidence (∼6%) of early HCC after treatment (i.e. within 12 months) in our cohort may be related in part to our patients' characteristics. Our Northern California cohort is ethnically diverse and comprised of older patients with longstanding HCV and a high burden of cirrhosis. Weak NK cell immune types such as KIR2DL1/HLA-C2 and KIR3DL1/HLA-Bw4 80-I are enriched in ancestrally distinct populations such as Africans and some Latinos [27]. HCC developing shortly after 2G DAA was first reported in 2016 by separate groups in Spain and Italy, although these treatment cohorts were not typed for KIR and HLA [9, 10]. Spaniards and Italians, compared with other Europeans, however, are notably enriched in genes encoding strong inhibitory HLA-Bw4 80-I ligands for KIR3DL1 [28–31]. These classically African HLA immunotypes may derive in part from the historical Trans-Mediterranean genetic introgressions during the Moorish period in Spain and during the era of the expanded Roman Empire centered in Italy. A recent large Japanese study also linked KIR2DL1/HLA-C2 and KIR3DL1/HLA-Bw4 to increased HCC risk in a cohort of patients with HCV cirrhosis [32]. The role of 2G DAA therapy on HCC development in that study could not be considered, as study enrollment ended in 2013, prior to 2G DAA availability. The fact that similar allelic combinations were linked to HCV-associated HCC in such a disparate Asian patient population supports the notion that genetic factors controlling NK cell immune potential might play a key role in HCC tumor surveillance in chronic HCV.

Although our findings remain preliminary due to the retrospective nature of our cohort and the fact that detailed NK cell immune typing assays have not been widely evaluated in this setting, our data suggest that specific NK cell immune polymorphisms in KIR and HLA may identify those at greatest risk for early HCC in the post-2G DAA treatment period. 2G DAA treatment lowers HCC risk overall in chronic HCV, but our study and others have the potential to lend new insights into HCC risks in unique patient populations. If confirmed by additional studies, KIR/HLA testing could be used to identify patients who may benefit from heightened surveillance for HCC. Checkpoint blockade of PD-1 on T cells and NK cells is now employed in HCC chemotherapy, and our data may suggest that blockade of inhibitory NK cell KIR might have added efficacy in specific cases of HCC.

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Abbreviations: **AFP**: alpha-fetoprotein · **ANOVA**: analysis of variants statistical method · **CT**: computerized tomography imaging · **EBZ/GRZ**: elbasvir/grazoprevir HCV therapy · **HCC**: hepatocellular carcinoma · **HLA/KIR**: human leukocyte antigen/killer cell immunoglobulin-like receptor · **IFN-α/PEG-IFN-α**: interferon or pegylated interferon-alpha therapy · **IQR**: inter-quartile range · **LED**: ledipasvir HCV therapy · **LIRADS-5**: Liver Imaging Reporting and Data System Category 5 · **MRI**: magnetic resonance imaging · **NK**: natural killer · **OR**: odds ratio statistical measure · **PrOD**: ritonavir-boosted paritaprevir-ombitasvir-dasabuvir HCV therapy · **RBV**: ribavirin HCV therapy · **SIM**: simeprevir HCV therapy · **SOF**: sofosbuvir HCV therapy · **SVR**: sustained virological response (to hepatitis C therapy) · **2G DAA**: second-generation direct-acting antiviral therapy · **VA**: United States Department of Veterans Affairs · **VEL**: velpatasvir HCV therapy

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