

## Priority Brief

**Quantitative Effect of Natural Killer–Cell Licensing on Hepatocellular Carcinoma Recurrence after Curative Hepatectomy**Naoki Tanimine<sup>1,2</sup>, Yuka Tanaka<sup>1,2</sup>, Tsuyoshi Kobayashi<sup>1,2</sup>, Hiroataka Tashiro<sup>1,2</sup>, Daiki Miki<sup>2,3</sup>, Michio Imamura<sup>2,4</sup>, Hiroshi Aikata<sup>2,4</sup>, Junko Tanaka<sup>2,5</sup>, Kazuaki Chayama<sup>2,3,4</sup>, and Hideki Ohdan<sup>1,2</sup>**Abstract**

Natural killer (NK) cells have a potential role in immune surveillance of hepatocellular carcinoma (HCC). Self-recognition of human leukocyte antigens (HLA) through killer immunoglobulin-like receptors (KIR) confers competence to NK cells—a process termed "licensing." We investigated the effect of NK-cell licensing on the susceptibility of patients to HCC recurrence. A total of 170 Japanese patients with HCC who underwent primary curative hepatectomy between 1996 and 2010 were enrolled in this study. The median follow-up period was 5.4 years. We analyzed their KIR-HLA genotypes with sequence-specific polymorphism-based typing and estimated their susceptibility to HCC recurrence by performing propensity score–matching analyses. The presence of KIR2DL1-C2, KIR2DL2-C1, KIR3DL1-BW4, or KIR3DL2-A3/11, functional compound genotypes that intrinsically license NK cells, did not markedly affect HCC recurrence. However, the multiplicity of those compound KIR-HLA genotypes was significantly associated with the HCC recurrence rate, i.e., the cumulative risk of recurrence in patients with at least three compound genotypes was significantly lower than that in patients with one or two compound genotypes, suggesting that the effect of NK-cell licensing on HCC recurrence is quantitative. Patients at high risk of HCC recurrence after curative hepatectomy could be identified by KIR-HLA genotyping. *Cancer Immunol Res*; 2(12): 1142–7. ©2014 AACR.

**Introduction**

Hepatocellular carcinoma (HCC) frequently recurs despite curative resection (1). Because augmented cytolytic activities of natural killer (NK) cells in the liver are thought to be critical for HCC immune surveillance (2, 3), functional NK-cell competence potentially affects HCC recurrence and prognosis.

NK-cell activation is dependent upon inhibitory-activating receptor equilibrium, among which killer immunoglobulin-like receptors (KIR) are the most polymorphic. KIRs contribute to receptor–ligand interactions that determine NK-cell responses by recognizing specific human leukocyte antigen (HLA) class I allotype ligands (4). Self-specific inhibitory KIR and cognate HLA ligand interactions are fundamental to "licensing" (5), a process in which NK cells expressing inhibitory KIRs for self-

HLA have a higher resting response capacity (6). Ligand specificities for five inhibitory KIRs have been defined: KIR2DL1 for the HLA-C Lys80 (C2) group of alleles, KIR2DL2 and KIR2DL3 for the HLA-C Asn80 (C1) group, KIR3DL1 for the Bw4 group of HLA-B (and some A) alleles, and KIR3DL2 for the HLA-A3/11 alleles (7). The genes for KIR and their cognate HLA ligands display extensive polymorphism and generate diverse immune responses to neoplastic cells. Here, we show that the multiplicity of functional compound KIR-HLA genotypes influences posthepatectomy recurrence.

**Patients and Methods****Patients and outcomes**

A total of 170 Japanese patients with HCC who underwent primary hepatectomy at Hiroshima University between 1996 and 2010 were enrolled in this study based on the following inclusion criteria: Presence of histologically confirmed HCC by an expert pathologist; preserved preoperative liver function, i.e., Child-Pugh grade A; no residual tumor after surgery; no evidence of comorbid malignant tumor; and written informed consent. None of the patients received adjuvant HCC therapy. This study was approved by the Hiroshima University Research Ethics Committee, and informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

Clinicopathologic and follow-up data were collected for 5 years after primary hepatectomy. After hepatectomy, patients were followed up by using ultrasound sonography, contrast-enhanced computed tomography, or magnetic resonance,

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combined with evaluation of serum  $\alpha$ -fetoprotein and Des- $\gamma$  carboxyprothrombin levels at 3-month intervals for up to 3 years. Thereafter, follow-up was performed at 6-month intervals for up to 5 years. HCC recurrence was defined as the appearance of a new focal liver lesion with typical characteristics: lymph node enlargement in the liver hilum or suspected extrahepatic lesions. The diagnosis was histologically confirmed if necessary. Cumulative risk of recurrence was defined as the time from the surgery date to the first tumor recurrence date. Overall survival (OS) was defined as the time from the surgery date to the date of death from any cause.

### KIR and HLA genotyping

Genomic DNA was extracted from peripheral blood mononuclear cells derived from patients by using a QIAamp DNA Blood Mini Kit (Qiagen). KIR allele genotyping for KIR2DL1/2DL2/2DL3/3DL1/3DL2 was performed by sequencing KIR transcripts and detected by the reverse sequence-specific polymorphism-polymerase chain reaction (SSP-PCR)-Luminex typing method using a KIR genotyping SSO kit (One Lambda). HLA-A, HLA-B, and HLA-C alleles were identified by SSP-PCR using a WAKFlow HLA typing kit (Wakunaga). The presence of the HLA ligand for KIR was determined according to HLA genotypes, as previously described (8, 9).

### Statistical analysis

A comparison of categorical and continuous variables was performed using the  $\chi^2$  test and the Wilcoxon test, respectively.

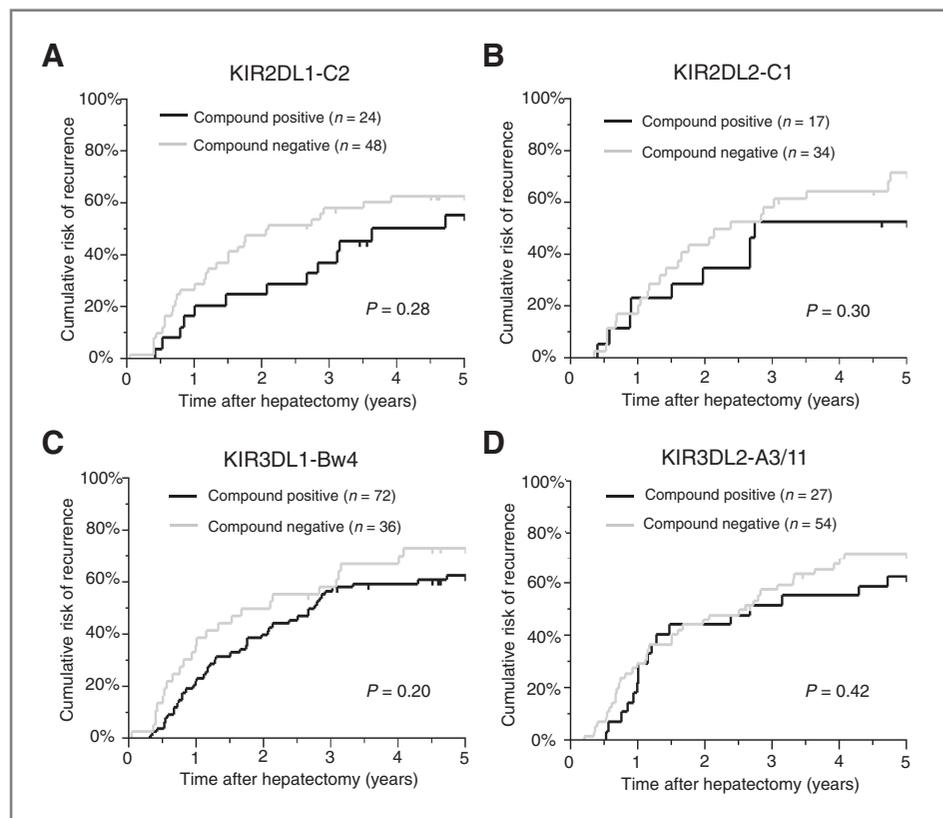
To adjust for differences in baseline characteristics, one-to-two or two-to-one propensity score models were constructed on the basis of each patient's estimated propensity score. Variables used included age, sex, etiology [hepatitis B virus/hepatitis C virus (HCV)/others], number of tumors (1, 2, 3, or  $\geq 4$ ), maximum tumor diameter ( $\leq 20$  mm,  $>20$  mm and  $\leq 50$  mm, or  $>50$  mm), histologic type (G1 or G2/G3), vascular invasion (negative/positive), satellite lesions (negative/positive), and surgical margin ( $<5$  mm/ $\geq 5$  mm). Propensity score matching was performed using IBM SPSS Statistics 18 (SPSS Inc.) and R statistical software version R2.10.0 (R Foundation for Statistical Computing; ref. 10). One-to-two or two-to-one nearest-neighbor matching were performed using a noncaliper.

We considered the 5-year cumulative risk of recurrence as a primary outcome. Cumulative risk of recurrence and OS were estimated and compared using Kaplan–Meier and log-rank statistics. The Cox proportional hazards model was used to calculate the hazard ratio (HR) and 95% confidence intervals (CI). Statistical analyses, except propensity score matching, were performed using JMP10 for Windows (SAS Institute). *P* values of  $<0.05$  were considered statistically significant.

### Results

In this study, 170 patients with HCC who underwent curative hepatectomy were enrolled. Because preoperative liver dysfunction is a risk factor for postoperative HCC recurrence (11, 12), patients with Child-Pugh grade A were included

**Figure 1.** The presence of a single functional compound of KIR-HLA genotype did not affect HCC recurrence. The effect of each compound genotype (KIR2DL1-C2, KIR2DL2-C1, KIR3DL1-BW4, KIR3DL2-A3/11) on HCC recurrence was analyzed by propensity score-matched studies using nine variables (age, sex, etiology, tumor number, maximum diameter, histologic differentiation, vascular invasion, satellite lesion, and surgical margin). The 5-year cumulative risk of recurrence was compared between one-to-two or two-to-one patient pairs with or without each compound KIR-HLA genotype. A, KIR2DL1-C2; B, KIR2DL2-C1; C, KIR3DL1-Bw4; and D, KIR3DL2-A3/11.



(Supplementary Table S1). The median follow-up period and median OS were 5.4 and 9.1 years, respectively.

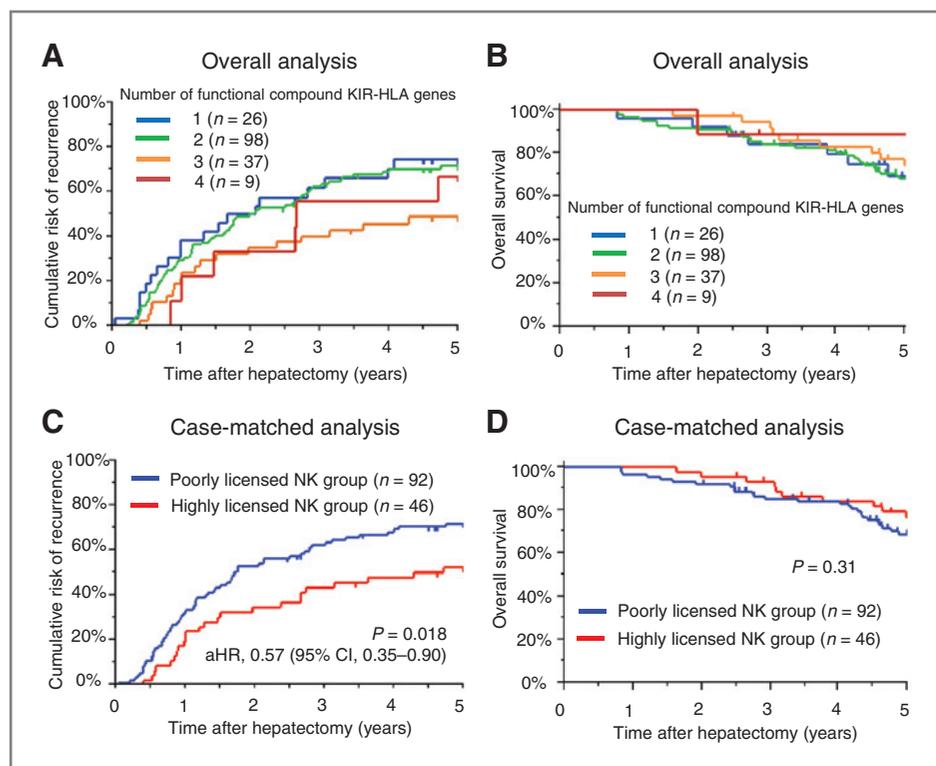
The functional compound KIR-HLA genotypes KIR2DL1-C2, KIR2DL2-C1, KIR2DL3-C1, KIR3DL1-Bw4, and KIR3DL2-HLA A3/11, which intrinsically license NK cells, were found in 14.1%, 10.0%, 98.2%, 80.0%, and 15.9% of the cohort, respectively (Supplementary Table S2). The relatively low KIR and HLA genotype heterogeneity agreed with that previously reported (13).

We analyzed the effect of each compound genotype (KIR2DL1-C2, KIR2DL2-C1, KIR3DL1-BW4, and KIR3DL2-A3/11) on HCC recurrence. The KIR2DL3-C1 was present in nearly all patients. Propensity score-matched studies using nine variables (age, sex, etiology, tumor number, maximum diameter, histologic differentiation, vascular invasion, satellite lesion, and surgical margin), in which one-to-two or two-to-one patient pairs with or without each compound KIR-HLA genotype were created, minimized baseline characteristics bias (Supplementary Table S3). Those propensity score-matched analyses revealed that none of the compound KIR-HLA genotypes had a statistically significant effect on post-operative HCC recurrence and OS, although the recurrence rate in the group with each functional compound KIR-HLA genotype was lower than that in the group without that particular genotype (Fig. 1 and Supplementary Fig. S1).

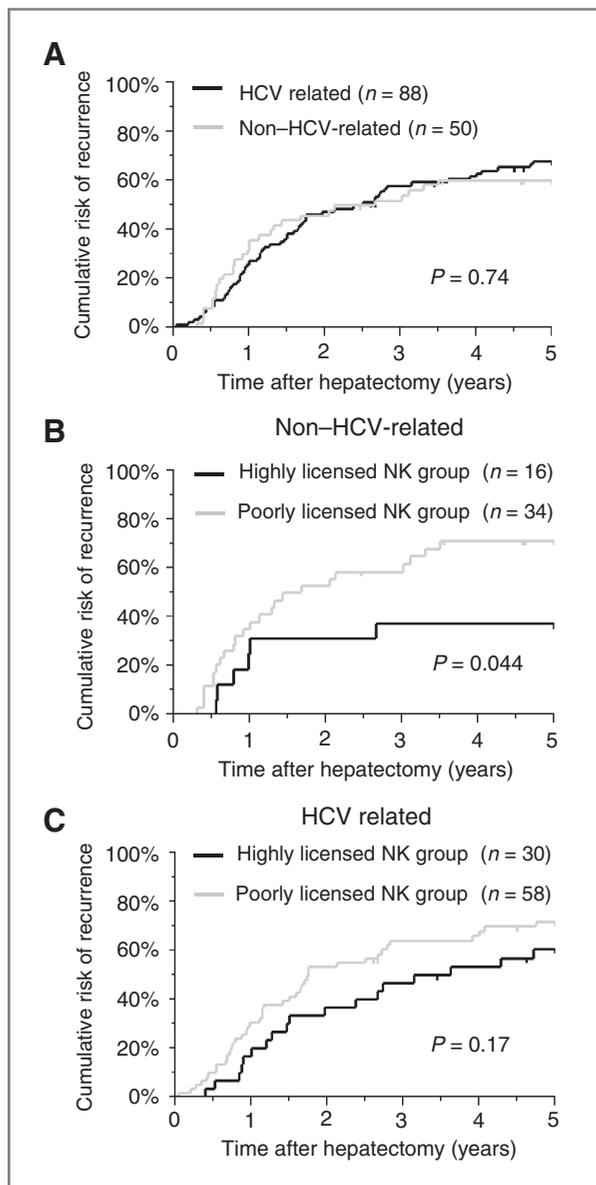
Because NK cells expressing greater numbers of self-reactive inhibitory receptors have increased responsive potential (14), we questioned whether functional compound KIR-HLA genotype multiplicity influenced HCC recurrence. All patients had between one and four of the five functional

compound KIR-HLA genotypes. Accordingly, the patients were divided into four groups for risk recurrence comparison. Compound KIR-HLA genotype multiplicity tended to be associated with the HCC recurrence rate and OS (Fig. 2A and B). In the propensity score-matched study using the same nine variables, in which one to two pairs of patients with at least three compound genotypes (the highly licensed NK group;  $n = 46$ ) and patients with one or two compound genotypes (the poorly licensed NK group;  $n = 92$ ) were created (Supplementary Table S4), it revealed that the cumulative recurrence risk in the highly licensed NK group was significantly lower than that in the poorly licensed NK group ( $P = 0.018$ ; adjusted HR, 0.57; Fig. 2C). Likely because treatments against recurring HCC were persistently maintained, no statistical difference was found in OS between the two groups (Fig. 2D).

Subgroup analysis based on tumor-node-metastasis (TNM) classification (7th edition of Union for International Cancer Control) demonstrated that the difference in the cumulative risk of recurrence between the highly and poorly licensed NK groups was consistently recognized in stages I and II (Supplementary Fig. S2A–S2C). No difference was observed between the two groups in stage IIIA (Supplementary Fig. S2D), indicating that the surveillance function of NK cells is most critical in the early stages of HCC. Considering the possible effect of HCV infection on NK-cell activity, additional subgroup analyses were performed among patients with or without HCV. The lower cumulative risk of recurrence in the highly licensed NK group was statistically significant in the non-HCV-related cohort, but not in the HCV-related cohort ( $P = 0.044$  and 0.17,



**Figure 2.** The multiplicity of compound KIR-HLA genotypes stratified the recurrence risk of HCC. Kaplan-Meier analyses of the 5-year cumulative risk of recurrence (A) and OS (B) for 170 patients were performed according to the number of functional compound KIR-HLA genotypes. Propensity score-matched analyses of the 5-year cumulative risk of recurrence (C) and OS (D) were also performed for patients with at least three compound genotypes (highly licensed NK group) and patients with one or two compound genotypes (poorly licensed NK group). The cumulative risk of recurrence in the highly licensed NK group was significantly lower than in the poorly licensed NK group. aHR, adjusted HR.



**Figure 3.** The lower cumulative risk of recurrence in the highly licensed NK group was significant among the non-HCV-related cohort. A, according to the presence or absence of HCV infection, Kaplan-Meier analyses of the 5-year cumulative risk of recurrence were performed for 138 matched patients belonging to the highly and poorly licensed NK groups. Further subgroup analyses were performed in non-HCV-related patients (B) and HCV-related patients (C).

respectively; Fig. 3). Consistently, on the log-rank and the Cox proportional hazards model analyses, the number of KIR-HLA genotype ( $\leq 2$ ) was defined as a significant risk factor for HCC recurrence in an unadjusted overall cohort, but was not so in the HCV-related cohort (Table 1).

## Discussion

Postoperative recurrent HCC can be monocentric, leading to intrahepatic metastasis, or multicentric as a *de novo* carcino-

genesis. To study the role of NK cells in intrahepatic metastasis, we previously investigated the effect of decreasing NK-cell functions on the engraftment susceptibility of intraportally injected HCC cells in a mouse model (15, 16). The anti-HCC activity of hepatic NK cells significantly decreased after partial hepatectomy, allowing intrahepatic metastasis growth in mice receiving HCC cells (16). Intravenous adoptive immunotherapy performed using activated NK cells extracted from normal livers markedly inhibited intrahepatic metastasis. NK-cell competence and ability to survey and eliminate *de novo* neoplastic cells may provide defense against both monocentric and multicentric recurrence.

The human liver contains an unusually high number of infiltrating immune cells; 30%–50% of lymphocytes are NK cells (2). Liver NK cells have unique properties, including TNF-related apoptosis-inducing ligand (TRAIL)-dependent cytotoxicity, high NKp46 and CD122 expression, and specific cytokine profiles (2, 3). TRAIL on NK cells binds to four receptors, including death-inducing receptors (DR4 and DR5) that signal apoptosis and decoy receptors (DcR1 and DcR2; refs. 17). Moderately/poorly differentiated HCC remarkably expresses DR4/DR5 but not DcR1/DcR2, increasing TRAIL-expressing NK cell-mediated cell killing susceptibility (2, 18). On the basis of those findings, we proposed a novel immunotherapy of intravenously injecting activated liver allograft-derived NK cells into liver transplant recipients to control HCC recurrence (19).

In addition to TRAIL, hepatic NK-cell roles in immune tumor surveillance are likely mediated by perforin, granzyme, and interferon- $\gamma$  (20). Gene polymorphisms for KIR and its HLA ligands possibly contribute to the heterogeneous tumor-surveillance functions of NK cells and likely affect clinical HCC outcomes. Recently, a small cohort study of patients with HCV-related HCC who underwent curative treatment by either surgical resection or radiofrequency thermal ablation (RTA) showed that the compound KIR2DL2-C1 and KIR3DS1-Bw4T80 genotypes are associated with longer time to recurrence and worse OS, respectively (21). We also analyzed the impact of these genotypes in the present study, but did not observe consistent results (Table 1 and Supplementary Table S5). This discrepancy might be related to the fact that the time to recurrence was markedly longer in our study than that in the previous study (median time to recurrence = 29.7 vs. 17 months, respectively), which is likely due to the heterogeneity of the therapeutic modality used in the previous study (i.e., time to recurrence in patients treated with RTA was significantly shorter than that in patients treated by resection; ref. 21). Our propensity score-matched studies demonstrated that the presence of a single functional compound KIR-HLA genotype did not markedly affect HCC recurrence, but that compound KIR-HLA genotype multiplicity was associated with the HCC recurrence rate. Taken together, with this finding and the fact that the number and type of host MHC class I alleles quantitatively tune the responsiveness of individual NK-cell subsets expressing the corresponding KIR (14, 22), the effect of NK-cell licensing on HCC recurrence should be quantitative. This effect of NK-cell licensing on HCC recurrence reached statistical significance in the non-HCV-related cohort but not in the

**Table 1.** Cumulative risk of recurrence and overall survival of patients with HCC according to clinicopathologic characteristics and compound KIR-HLA genotypes

	Total patients (N = 170)				HCV-related patients (n = 97)			
	Cumulative risk of recurrence		OS		Cumulative risk of recurrence		OS	
	P	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)
Age ( $\leq 65$ vs. $>65$ years)	0.745	NA	0.362	NA	0.626	NA	0.592	NA
Sex (male vs. female)	0.078	1.54 (0.99–2.53)	0.966	NA	0.554	NA	0.216	NA
Etiology (HBV vs. HCV vs. others)	0.448	NA	0.117	NA	NA	NA	NA	NA
Tumor number ( $\geq 2$ vs. 1)	0.010	1.57 (1.06–2.31)	0.039	1.64 (1.11–2.39)	0.088	1.56 (0.92–2.51)	0.604	NA
Maximum diameter ( $\leq 50$ mm vs. $>50$ mm)	0.222	NA	0.511	NA	0.749	NA	0.313	NA
Histologic differentiation (G1+G2 vs. G3)	0.253	NA	0.498	NA	0.866	NA	0.909	NA
Vascular invasion	0.733	NA	0.851	NA	0.612	NA	0.454	NA
Satellite lesion	0.657	NA	0.743	NA	0.451	NA	0.486	NA
Surgical margin ( $<5$ mm vs. $\geq 5$ mm)	0.126	NA	0.162	NA	0.039	1.88 (0.98–3.36)	0.003	1.88 (0.97 – 3.37)
KIR2DL1-C2	0.113	NA	0.754	NA	0.189	NA	0.964	NA
KIR2DL2-C1	0.253	NA	0.945	NA	0.456	NA	0.745	NA
KIR2DL3-C1	0.793	NA	0.283	NA	0.694	NA	0.105	NA
KIR3DL1-Bw4	0.269	NA	0.359	NA	0.572	NA	0.935	NA
KIR3DL2-A3/11	0.585	NA	0.570	NA	0.845	NA	0.986	NA
Number of KIR-HLA genotypes ( $\geq 3$ vs. $\leq 2$ )	0.016	0.61 (0.38–0.94)	0.224	NA	0.130	NA	0.735	NA

NOTE: Cumulative risk of recurrence and OS were compared by log-rank statistics for univariate analysis. Cox proportional hazards model was conducted for multivariate survival analysis. Only variables presenting  $P < 0.1$  in the univariate analysis were included in the multivariate model.  $P < 0.05$  was considered statistically significant.

Abbreviation: NA, not assessed.

HCV-related cohort, which might be explained by the fact that hepatic NK cells exhibited reduced cytotoxicity and TRAIL expression in patients with chronic HCV infection (23).

We demonstrated that patients at high risk of HCC recurrence after curative hepatectomy could be identified by KIR-HLA genotyping. Licensed NK cells generally have higher resting capacity for responses including interferon- $\gamma$  production and cytotoxicity than unlicensed NK cells, but both NK-cell types are highly activated by *in vitro* stimuli (24). Therefore, therapeutic strategies manipulating NK-cell activity either *in vivo* or *in vitro* could compensate for genetic susceptibility to HCC recurrence. This concept might also be supported by a previous randomized trial demonstrating that adoptive immunotherapy with autologous lymphocytes activated *in vitro* with recombinant IL2 and anti-CD3 Abs decreased the frequency of recurrence after HCC curative resection (25).

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Authors' Contributions

**Conception and design:** N. Tanimine, Y. Tanaka, H. Ohdan

**Development of methodology:** N. Tanimine, Y. Tanaka

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** N. Tanimine, T. Kobayashi, M. Imamura, H. Aikata, H. Ohdan

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** N. Tanimine, J. Tanaka, H. Ohdan

**Writing, review, and/or revision of the manuscript:** N. Tanimine, K. Chayama, H. Ohdan

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** N. Tanimine, D. Miki

**Study supervision:** H. Tashiro, J. Tanaka, H. Ohdan

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# Cancer Immunology Research

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