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Failure recovery of circulating NKG2D^{+}CD56^{dim}NK cells in HBV-associated hepatocellular carcinoma after hepatectomy predicts early recurrence

Jian Gao[1,#], Zhaojun Duan[1,#], Ling Zhang[2], Xiangbo Huang[1], Lu Long[1], Jing Tu[1], Hua Liang[3], Yu Zhang[4], Tao Shen[1,*], and Fengmin Lu[1,*]

[1]State Key Laboratory of Natural and Biomimetic Drugs; The Department of Microbiology & Infectious Disease Center; School of Basic Medicine; Peking University Health Science Center; Beijing, China; [2]Department of Hepatobiliary and Pancreatic Surgery; Affiliated Tumor Hospital of Zhengzhou University; Zhengzhou, China; [3]State Key Laboratory for Infectious Disease Prevention and Control; National Center for AIDS/STD Control and Prevention; Chinese Center for Disease Control and Prevention; Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases; Beijing, China; [4]Department of Immunology; Peking University Health Science Center; Beijing, China

[#]These authors made equal contributions to this manuscript.

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Abbreviations: aCGH, array-based Comparative Genomic Hybridization; ADAM9, ADAM metallopeptidase domain 9; ALT, alanine aminotransaminase; ATCC, American Type Culture Collection; Ct, threshold cycle; CTBP, C-terminal-binding protein; DMEM, Dulbecco's modified Eagle's medium; FRE, recurrence-free; GGT, gamma-glutamyl transferase; HD, healthy donors; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; KIR, killer immunoglobulin receptor; MICA, major histocompatibility complex class I polypeptide-related sequence A; MICB, major histocompatibility complex class I polypeptide-related sequence B; NK, natural killer; OS, overall survival; PBMCs, peripheral blood mononuclear cells; RE, recurrence; RFA, radiofrequency thermal ablation; RFS, recurrence-free survival; sMICA, soluble MICA; sMICB, soluble MICB; TACE, transcatheter arterial cheoembolization; Treg, regulatory T cells; TILs, tumor infiltrated lymphocytes; TNM, tumor-node-metastasis; ULBPs, UL-16 protein-ligand family

Dysfunction of natural killer (NK) cells has been implicated in the failure of antitumor immune responses in hepatocellular carcinoma (HCC) patients. However, the changes of NK profile in peripheral blood after surgery and tumor tissues of HCC patients, as well as the underlying reason and the significance are vague. Here, we observed that the frequencies of circulating NKG2D^{+}CD56^{dim}NK cells decreased significantly in HBV-related HCC and were negatively correlated with the levels of serum TGF-β and soluble MICA (sMICA). *In vitro* experiments confirmed that the TGF-β and sMICA in tumor tissue homogenates, as well as sMICA in HCC cells culture supernatants could reduce the frequency of NKG2D^{+}CD56^{dim}NK cells. In addition, in HCC patients the lower frequency of circulating NKG2D^{+}CD56^{dim}NK cells was associated with larger tumor size and/or higher serum GGT. Noticeably, the frequency of NKG2D^{+}CD56^{dim}NK cells at one month after surgery usually failed to restore in early recurrent patients, and that frequency was negatively associated with early recurrence and shorter overall survival. These results suggest that declined frequency of NKG2D^{+}CD56^{dim}NK cells in HCC was associated with higher TGF-β and sMICA production, and low frequency of circulating NKG2D^{+}CD56^{dim}NK cells at one month after surgery may predict poor prognosis of HBV-related HCC patients accepting hepatectomy.

Introduction

HCC is the fifth most common cancer and the third leading cause of cancer related death worldwide.[1] Currently, HCC therapies include surgical resection, radiofrequency thermal ablation (RFA) and transcatheter arterial cheoembolization (TACE). Despite these options, the prognosis of HCC remains poor due to the high frequency of early recurrence, which was at least partially ascribed to the sustained dysfunction of antitumor immune responses, including impaired immune response of NK cells.[2]

NK cells-mediated innate immunity is proposed to play a pivotal role in host defense against cell malignancy transformation.[3,4] NK

*Correspondence to: Tao Shen; Email: taoshen@hsc.pku.edu.cn; Fengmin Lu; Email: lu.fengmin@hsc.pku.edu.cn;
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cells recognition is triggered by a broad range of activating and inhibitory receptors, which act as sensors of abnormal cells.[5,6] NKG2D is an activating receptor of NK cells and could induce target cells lysis through recognizing ligands major histocompatibility complex class I polypeptide-related sequence A and B (MICA, MICB) and the UL-16 protein-ligand family (ULBPs), which often over-expressed or expressed *de novo* as a consequence of tumor transformation, viral infection and cell stress. In parallel, NK cells also express inhibitory receptors such as NKG2A, an important killer immunoglobulin receptor (KIR) specifically recognizing HLA-class I molecules. [7] NK cells can kill target cells losing or expressing low levels of HLA-class I molecules, which was often seen in tumor cells including HCC cells. Tumor cells also have the capacity to impair cytotoxicity of peripheral NK cells through modulating the expression of activating and/or inhibitory receptors.[8-10] Despite the fact that NK cells are dramatically enriched in liver and the significance of NK cells-mediated cytotoxic and immunoregulatory role in antitumor immunity are widely recognized, the alterations occurred in the frequency and phenotypical characteristics of NK cells in the context of hepatitis B virus (HBV) related HCC have not been well elucidated.

In the present study, the distributional and phenotypical pattern of pre-operative and post-operative $CD56^+$ NK cells were investigated in pre-operative and post-operative HBV related HCC patients. The clinical significance and the prognosis predicting value of $NKG2D^+CD56^{dim}$ subset on tumor recurrence and overall survival (OS) were evaluated. In addition, the influence of TGF-β and sMICA on $NKG2D^+CD56^{dim}$NK cells was analyzed. This study provided further evidences for immune-escape from impaired NK cells in HCC and suggested that NKG2D on $CD56^{dim}$NK was a potential biomarker for predicting the prognosis of HCC patients receiving surgical resection.

Results

Failure restoration of circulating $NKG2D^+CD56^{dim}$NK cells after curative surgery was associated with early recurrence of HCC

The frequencies of peripheral NK cells in HCC patients and HD were determined using the panel shown in **Fig. 1A**. In accordance with the previous report, [11] our results confirmed that circulating NK cells, primarily the $CD56^{dim}$NK subset were reduced in HCC patients with tumors at stages either I or II/III (**Fig. 1B**). In order to address if the tumor-harboring status contributed to the redistributions and subset alterations of NK cell in HCC patients, NK cells in peripheral lymphocytes before and one month after surgery were measured. Though no significant difference was observed between pre-operative and post-operative frequencies and subset distributions of NK cells (data not shown), according to the status of HCC recurrence during a two-year follow-up, the post-operative frequencies of NK cells and its major $CD56^{dim}$NK subset were significantly increased in the recurrence-free (FRE) group compared with recurrence (RE) group (**Fig. 1C**).

NK cells activity was tightly regulated by activating and inhibitory receptors, therefore, we further analyzed the expressions of activating receptors, including CD69, HLR-DR, CD38, NKG2D and NKG2C, as well as the inhibitory receptor NKG2A on NK cells. [12] As shown in **Fig. 1D**, compared with HD, the frequencies of $NKG2D^+$NK cells and $NKG2D^+CD56^{dim}$NK subset were significantly decreased in either stage I or stage II/III HCC patients, while the frequencies of $NKG2A^+$NK cells and $NKG2A^+CD56^{dim}$NK subset were increased. The proportions of $NKG2C^+$, $CD69^+$, $HLA\text{-}DR^+$, $CD38^+$ NK cells and $CD56^{dim}$NK subset showed no significant difference between HCC patients and HD (**Fig. 1D** and **Fig. S1**). In addition, the post-operative frequencies of NK cells, $CD56^{dim}$NK, $NKG2D^+$NK and $NKG2D^+CD56^{dim}$NK subsets in FRE HCC patients were significantly higher than that in recurrence patients (**Fig. 1E**). Also, we found that the post-operative frequencies of $NKG2D^+$NK cells and $NKG2D^+CD56^{dim}$NK subset were significantly recovered compared with the pre-operative frequencies in FRE HCC patients but not in recurrence patients (**Fig. 1E**). These phenomena were not seen in $NKG2A^+$, $NKG2C^+$, $CD69^+$, $HLA\text{-}DR^+$ and $CD38^+$ NK cells (**Fig. 1E** and **Fig. S1**). The observations above demonstrated that the activation of circulating NK cells was suppressed in HCC patients, and the frequency of circulating $NKG2D^+CD56^{dim}$NK subset was recovered in FRE HCC patients and could be used as predictive marker for HCC recurrence after curative surgery.

Frequency of intrahepatic $NKG2D^+CD56^{dim}$NK cells was decreased in tumor tissue

To further investigate the status of liver-resident NK cells in HCC patients, the characteristics of NK cells and subsets in tumor and para-tumor tissues were evaluated (**Fig. 2A**). We found that the frequencies of NK cells, $CD56^{dim}$NK subset and $CD56^{bright}$NK subset were significantly lower in tumor, as compared to the corresponding para-tumor tissues (**Fig. 2B**). The ratio of $CD56^{bright}$NK/$CD56^{dim}$NK subset was also lower in tumor, indicating that the $CD56^{bright}$NK subset reduced more obviously than $CD56^{dim}$NK subset (**Fig. 2C**). In addition, the frequencies of $CD38^+CD56^{dim}$NK cells (**Fig. 2D**), $NKG2A^+CD56^{dim}$ and $NKG2D^+CD56^{dim}$ NK subsets (**Fig. 2E**) were also lower in tumor-infiltrated lymphocytes (TILs). In contrast, the frequencies of $CD69^+CD56^{dim}$, $HLA\text{-}DR^+CD56^{dim}$ and $NKG2C^+CD56^{dim}$ NK cells showed a declined trend in TILs but did not reach to a significant difference (**Fig. 2D and E**). The downregulation of CD38 and NKG2D on $CD56^{dim}$NK cells suggested that the activation of liver-resident NK cells was suppressed in tumor compared to para-tumor tissues in HCC patients.

TGF-β and sMICA may attribute to the low frequency of $NKG2D^+CD56^{dim}$NK cells in HCC patients

Based on the observation that decreased frequency of $NKG2D^+CD56^{dim}$NK cells in HCC patients might be tumor-bearing status related, the underlying mechanism was further investigated. It has been shown that TGF-β, [13,14] sMICA[15] and IL-12[16] could downregulate the surface expression of NKG2D on NK cells and IFN-α could upregulate it, [17] while IL-10, [18] IL-17, [19] IFNr, [20] IL-4, [21] sMICB[22] were also reported to be

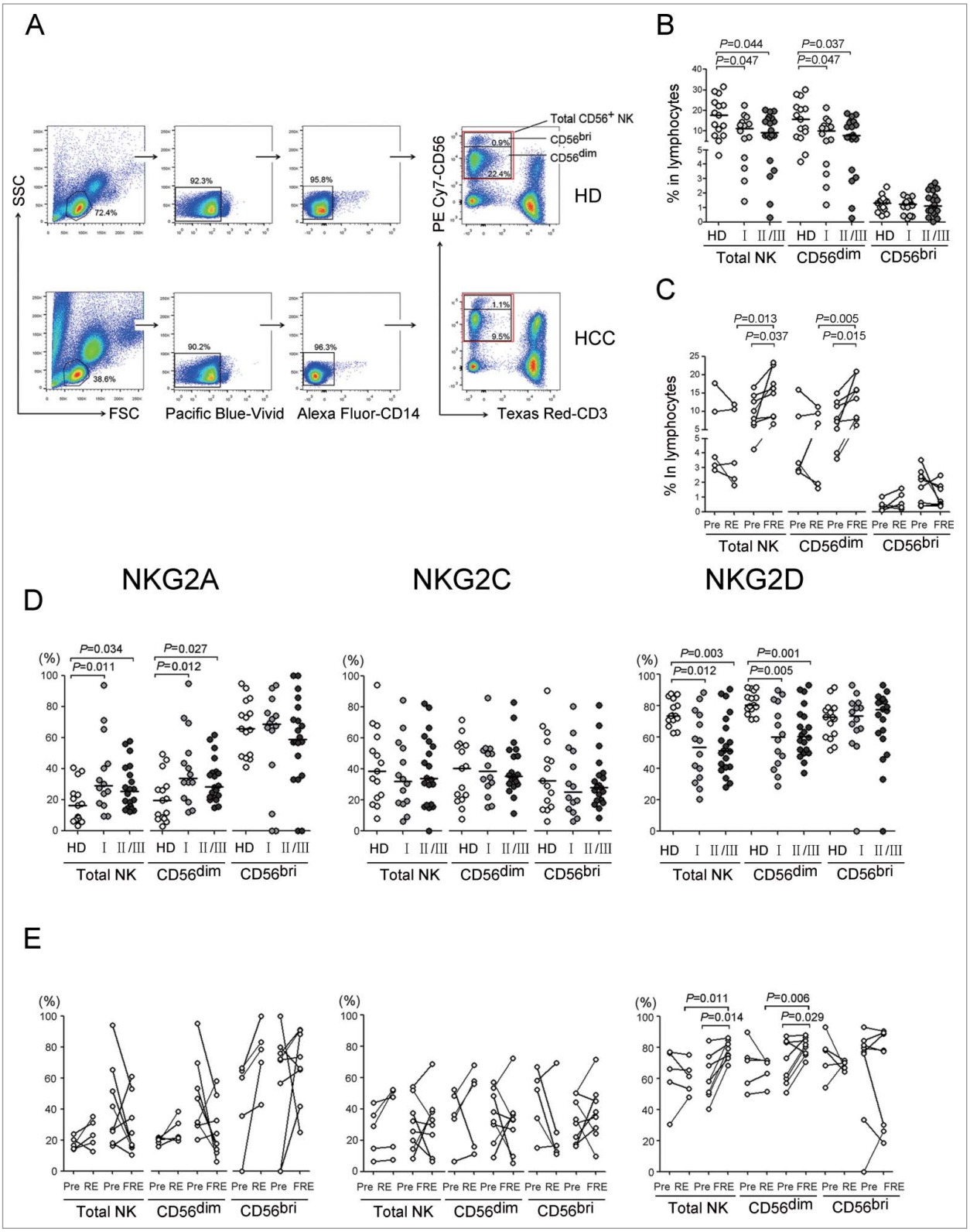

Figure 1. Circulating NK cells stained in HCC patients. (**A**) Representative dot plots of NK cells from HD and HCC patients. (**B**) Frequencies of NK cells among lymphocytes in pre-operative (Pre) HCC patients and HD. (**C**) Frequencies of NK cells among lymphocytes in pre- and post-operative HCC patients, and the post-operative patients were divided into recurrence (RE) and recurrence-free (FRE) groups. (**D**) Frequencies of NK subsets (from left to right: NKG2A^{+}, NKG2C^{+}, NKG2D^{+}) among lymphocytes in pre-operative HCC patients and HD. (**E**) Frequencies of NK subsets (from left to right: NKG2A^{+}, NKG2C^{+}, NKG2D^{+}) among lymphocytes in Pre, RE and FRE groups. Each dot in (**B–E**) represents one subject. Horizontal lines illustrate the median percentiles. I, II, III stand for different stages of HCC.

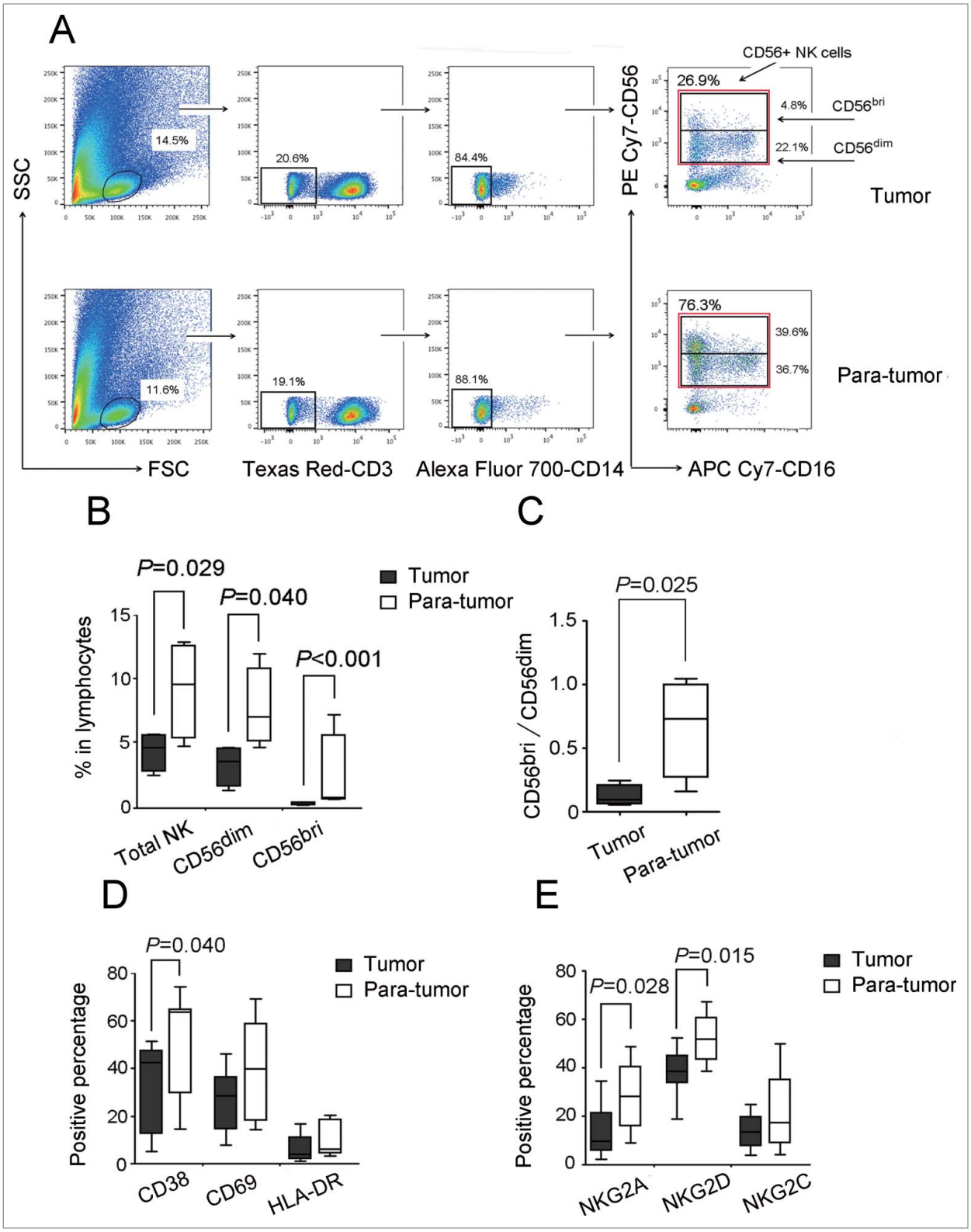

Figure 2. Liver resident NK and NKG2D^{+}CD56^{dim}NK cells decreased significantly in HCC tumor infiltrated lymphocytes. (**A**) Representative dot plots of NK cell subsets from tumor and para-tumor tissues were shown respectively. (**B, C**) Pool data showed the frequency of NK cells among lymphocytes and the ratio of CD56^{bright} and CD56^{dim} NK cell subsets in tumor and para-tumor tissues. (**D**)(**E**) Pool data showed the frequency of CD38^{+}, CD69^{+}, HLA-DR^{+}, NKG2A^{+}, NKG2C^{+} and NKG2D^{+} NK cells in tumor and para-tumor tissues.

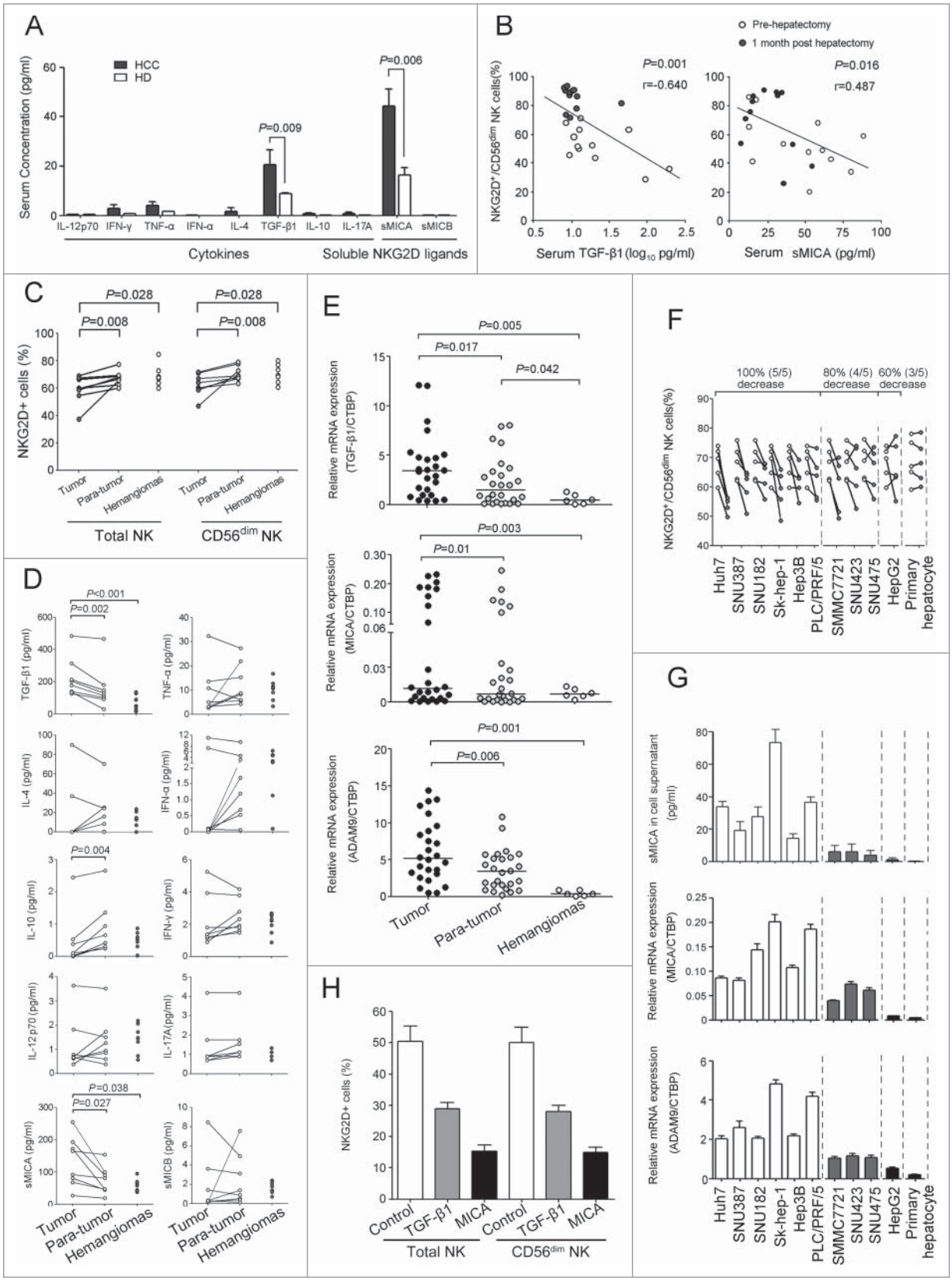

Figure 3. For figure legend, see next page.

Table 1. Clinical and biochemical characteristics of participants enrolled in the study

Variables	HCC (n = 34)	HD (n = 15)	*P*
Age(years)	55(41–72)	51(38–74)	0.34
Sex, n(%)			0.53
Female	13(38.2%)	5(33.3%)	
Male	21(61.8%)	10(66.7%)	
BMI	22.1(20.2–23.5)	23.0(21.3–25.3)	0.03
Blood routine			
RBC($\times 10^{12}$/L)	4.9(3.8–5.9)	5.3(4.2–5.7)	0.09
WBC(10^9/L)	6.3(2.0–8.1)	6.8(2.1–9.2)	0.25
PLT($\times$109/L)	145.5(59.0–268.0)	152.0(94.0–220.0)	0.02
Hb(g/L)	133.5(64.6–176.8)	134.6(121.9–152.2)	0.32
HBV DNA($\log_{10}$ IU/mL)	6.0(3.4–7.9)	N.A.	<0.001
Liver function index			
ALT(IU/mL)	40.5(14.7–248.3)	12.6(7.6–20.3)	0.02
AST(IU/mL)	41.6(13.2–150.7)	22.7(10.1–3.62)	0.05
ALP(IU/mL)	106.9(53–106.5)	81.5(51.3–119.7)	0.13
GGT (IU/mL)	88.5(14.4–678.8)	23.5(13.4–39.6)	0.02
AFP(μg/L)	212.4(1.85–1210.0)	1.1(0–3.4)	0.01
Total protein(g/L)	67.8(38.40–79.2)	74.1(70.9–85.5)	0.06
Albumin(g/L)	32.1(21.90–51.0)	45.3(42.0–48.8)	0.034
Globulin(g/L)	32.2(22.5–51)	26.5(23.4–32.3)	0.23
TBI(μmol/L)	16.2(5.3–35.9)	6.2(4.1–17.6)	0.04
DBIL(μmol/L)	8.3(3.31–18.4)	3.3(0–6.8)	0.03

Data are median (minimum–maximum); BMI: body mass index, calculated as the weight in kilograms divided by the square of height in meters; RBC: red blood cell; WBC: white blood cell; PLT: platelet; Hb: hemoglobin; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP:alkaline phosphatase; GGT: gamma-glutamyl transferase; AFP: alphafetalprotein; TBIL: Total bilirubin; DBIL: Directbilirubin; N.A: not applicable. *P* value refers to comparison between HCC patients with HD.

associated with NK cells. To identify factors contributing to the lower frequency of NKG2D$^+$NK cells in HCC patients, serum concentrations of certain cytokines, as well as sMICA and sMICB, were quantified. As shown in **Fig. 3A**, the concentrations of serum TGF-β and sMICA were significantly higher in HCC patients and were negatively correlated with the frequency of circulating NKG2D$^+$CD56^{dim}NK cells (**Fig. 3B**), indicating that lower frequency of NKG2D$^+$NK cells in HCC patients might ascribe to increased serum TGF-β and sMICA.

To investigate if tumor microenvironment has effect on the frequency of NKG2D$^+$CD56^{dim}NK cells, PBMCs from healthy donors (HD) were co-cultured with tumor, para-tumor or hemangioma tissue homogenates and frequency of NKG2D$^+$CD56^{dim}NK cells was evaluated by flow cytometry. As shown in **Fig. 3C**, the proportion of NKG2D$^+$CD56^{dim}NK cells was significantly decreased when PBMCs were co-cultured with tumor tissue homogenates compared with homogenates of para-tumor or hemangioma tissues.

In line with the hypothesis above that TGF-β or sMICA might be an influence factor, the concentrations of TGF-β and sMICA were also higher in tumor tissue homogenates (**Fig. 3D**). In addition, mRNA levels of *TGF*-β and *MICA* in tumor tissues were higher than in para-tumor and hemangioma tissues (**Fig. 3E**). Similarly, mRNA level of intracellular ADAM metallopeptidase domain 9 (*ADAM9*), which can recognize MICA cleavage site in HCC cells, [23] was also increased in tumor tissues (**Fig. 3E**). The data of aCGH from 25 tumor and paired non-tumor tissues further proved that both *TGF*-β and *MICA* were amplified in 28% tumor tissues (**Table S1**).

Since the main components of tumor tissue were tumor cells, culture supernatants from 10 HCC cell lines were added to PBMCs to identify the mechanism of tumor cells on reduction of NKG2D$^+$CD56^{dim}NK cells. As shown in **Fig. 3F**, supernatants from six HCC cell lines (Huh7, SNU387, SNU182, Sk-hep-1, Hep3B and PLC/PRF/5) resulted in the reduction of NKG2D$^+$CD56^{dim}NK cell frequency in all five HD, and supernatants from four other HCC cell lines (SMMC7721, SNU423, SNU475 and HepG2) reduced the frequency of NKG2D$^+$CD56^{dim}NK cells to different extents. In contrast, culture supernatants of primary hepatocytes failed to influence NK cell subsets obviously. Strikingly, the reduction of NKG2D$^+$CD56^{dim}NK cells was sMICA associated, since sMICA in supernatants and mRNA levels of *MICA* and *ADAM9* were denser in the six cell lines mentioned above (**Fig. 3G**). However, no significant difference in TGF-β was detected in various HCC cell lines (data not shown).

To verify the effects of TGF-β and sMICA on the frequency of NKG2D$^+$CD56^{dim}NK subset, NK cells from healthy control were purified and incubated with recombinant human TGF-β or sMICA. As shown in **Fig. 3H**, both TGF-β and sMICA could reduce the frequency of NKG2D positive cells in total NK and CD56^{dim}NK cells, confirming the negative regulation of TGF-β and sMICA on NKG2D.

The frequency of post-operative NKG2D$^+$CD56^{dim}NK cells was a valuable prognosis marker for curative surgery recipients of HCC

To investigate the clinical significance of NKG2D$^+$NK cells in HCC, association of the frequency of NKG2D$^+$NK cells with clinical pathological features was evaluated. As shown in **Table 2**, HCC patients with higher frequency of NKG2D$^+$NK

Figure 3 (See previous page). Possible effects of TGF-β/sMICA on NKG2D$^+$CD56^{dim}NK cells in sera, tissue homogenates and cell line supernatants. (**A**) Concentrations of serum cytokines, sMICA and sMICB. (**B**) Inverse linear relationship between serum TGF-β/sMICA and circulating NKG2D$^+$CD56^{dim}NK cells. (**C**) Influences of tissue homogenates on NKG2D$^+$CD56^{dim}NK cells. PBMCs from HD were co-cultured with homogenates from central tumor or para-tumor tissues of HCC patients or from hemongioma subjects and NKG2D$^+$ NK and NKG2D$^+$CD56^{dim}NK cells were gated by flow cytometry. (**D**) Concentrations of cytokines and sMICA/B in tissue homogenates. (**E**) mRNA expression of TGF-β, MICA and ADAM9 in tissues. (**F**) Influences of supernatants from HCC cell line on NKG2D$^+$CD56^{dim}NK cells. PBMCs from HD were co-cultured with supernatants from 10 different HCC cell lines or primary hepatocytes respectively and NKG2D$^+$CD56^{dim}NK cells were gated by flow cytometry. (**G**) Concentration of sMICA in supernatants of HCC cell lines and mRNA expressions of MICA/ADAM9 in HCC cell lines. (**H**) Effects of TGF-β/sMICA on NKG2D$^+$CD56^{dim}NK cells. Purified NK cells were cultured in the presence or absence of TGF-β or sMICA, and NKG2D$^+$ NK and NKG2D$^+$CD56^{dim}NK cells were gated by flow cytometry.

Table 2. Association analysis of the frequencies of NKG2D$^+$ CD56$^+$NK cells with clinical pathologic features

Variables	NKG2D$^+$/ NK(%)	*P*	NKG2D$^+$/ CD56^{dim} NK(%)	*P*
Age(years)		0.343		0.262
≤55	55.0(39.5–93.0)		44.7(30.5–90.4)	
>55	67.1(28.8–90.3)		58.5(20.3–87.6)	
Sex		0.748		0.636
Female	67.8(28.8–90.3)		57.8(20.3–87.6)	
Male	56.8(34.7–93.0)		49.4(27.0–90.4)	
Liver cirrhosis		0.820		0.145
Absent	52.2(34.7–93.0)		40.6(27.0–74.2)	
Present	67.8(28.8–90.3)		59.1(20.3–90.4)	
Tumor size(cm)		0.018		0.016
≤5	62.2(39.5–89.7)		59.1(31.1–88.5)	
>5	52.2(28.8–93.0)		42.9(20.3–90.4)	
No. of tumors		0.174		0.187
1	58.0(28.8–93.0)		49.4(20.2–90.4)	
>1	77.5(49.8–90.3)		71.1(30.5–71.1)	
Tumor encapsulation		0.258		0.226
Complete	57.9(28.8–93.0)		46.5(20.3–90.4)	
Incomplete	71.6(39.5–89.7)		66.0(31.1–88.5)	
AFP (ng/mL)		0.757		0.687
≤800	64.6(28.8–89.7)		56.3(20.3–88.5)	
>800	58.0(34.7–93.0)		46.3(28.2–90.4)	
Total Bilirubin (μmol/L)		0.783		0.796
≤17.1	61.6(28.8–93.0)		55.9(20.3–90.4)	
>17.1	58.0(34.7–90.3)		49.1(27.0–87.6)	
ALB(U/L)		0.391		0.572
≤50	60.5(28.8–89.7)		51.2(20.3–88.5)	
>50	52.2(28.8–93.0)		63.3(30.5–90.4)	
ALT(U/L)		0.162		0.221
≤40	66.3%(39.5–89.7)		54.1(20.3–88.5)	
>40	52.2%(28.8–93.0)		42.4(20.3–90.4)	
AST(U/L)		0.654		0.654
≤40	61.9%(28.8–89.7)		54.1(20.3–88.5)	
>40	56.8%(34.7–93.0)		49.4(20.3–90.4)	
ALP(U/L)		0.761		0.761
≤150	61.6%(28.8–93.0)		57.9(20.3–88.5)	
>150	56.8%(34.7–90.3)		45.5(27.0–90.4)	
GGT(U/L)		0.032		0.027
≤50	72.3%(28.8–89.7)		68.1(20.3–88.5)	
>50	52.2%(34.7–93.0)		46.0(27.0–90.4)	

Data are median (minimum–maximum). *P* value refers to comparison between HCC patients with HD.

or NKG2D$^+$CD56^{dim}NK cells were prone to have normal level of gamma-glutamyl transferase (GGT) and smaller tumors. In addition, we found that NKG2D$^+$CD56^{dim}NK cells from HCC patients displayed an impaired function for degranulation and IFNγ production, as shown by significantly declined CD107a and IFNγ production and a declined trend of TNF-α production compared with healthy individuals (**Fig. S2**). The results were consistent with previous reports that NK cell-mediated cytotoxicity was significantly impaired in HCC compared to healthy individuals, including total CD56$^+$NK cells[24] and NKG2D$^+$NK cells.[15] Considering that the post-operative frequency of NKG2D$^+$CD56^{dim}NK cells was significantly restored in FRE HCC patients (within two years follow-up), we further explored whether the post-operative frequency of NKG2D$^+$CD56^{dim}NK cells could predict the prognosis of HCC patients accepting curative surgery.

By Kaplan–Meier survival curves analysis, patients with above-median frequency of NKG2D$^+$CD56^{dim}NK cells at one month-post surgery had significantly higher two-year recurrence-free survival (RFS) (**Fig. 4A**) and OS (**Fig. 4B**). Multivariate analyses revealed that the association of higher frequency of NKG2D$^+$CD56^{dim}NK subset with better prognosis was independent of AFP level, tumor encapsulation, tumor size and vascular invasion.

Discussion

Redistribution and functional impairment of NK cells and their subsets, as well as the expression levels of activating and inhibitory receptors have been identified in liver diseases, including HCC.[25-28] However, little information is currently available about the changes of NK cells in post-operative HCC patients with different prognosis and even the underlying mechanisms.

In this study, we first found that the expression of NKG2D on peripheral CD56^{dim}NK cells was significantly restored one month after surgery in patients without recurrence within two years, as compared to those patients with HCC recurrence. In accordance with this, we also noticed that patients with relatively low post-operative frequency of NKG2D$^+$CD56^{dim}NK cells had poor two-year FRE survival and OS. It is widely recognized that recurrence within two years is regarded as early recurrence, which mainly results from intrahepatic metastasis.[29-32] HCC patients with early recurrence might have residual tumor cells expanding rapidly *in vivo* after surgery, resulting to a persistent suppression of NK cells, particularly NKG2D$^+$CD56^{dim}NK cells. However, post-operative patients without early recurrence might clear tumor cells or restrict residual tumor cells to a resting state, facilitating the restoration of NKG2D$^+$CD56^{dim}NK cells after surgery. As we know, early recurrence after liver resection for HCC still severely influences the prognosis of HCC patients. It was reported that early recurrence rate after anatomical resection and non-anatomical resection were 22.6% and 46.3%, respectively, [29] and the mortality of early recurrent patients during the initial five-year period after resection was 95.5%. [33] The finding in the current study that the correlation between NKG2D and early recurrence may be of great clinical significance offering a reference for further therapies, such as chemotherapy [34] and immunotherapy, [35–37] to eliminate the existing tumor completely. Since the number of HCC patients we followed up was limited, we must acknowledge that the threshold frequency of NKG2D$^+$CD56^{dim}NK cells at one-month after operation for clinical application might be confirmed in larger sample size. Meanwhile, we noticed that the frequency of NKG2D expression on CD56^{dim}NK subset before operation was lower in patients with tumor greater than 5cm, indicating that larger tumor could reduce NKG2D expression in HCC patients to a greater degree. In human, the interaction of NKG2D with its ligands on tumor cells surface plays an important role in the immune response to

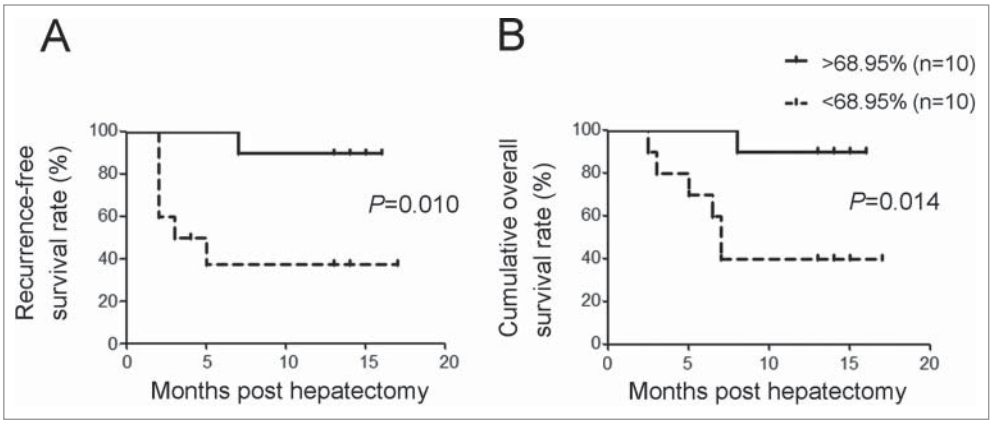

Figure 4. Kaplan–Meier survival curves of 20 HCC patients after hepatectomy with different frequency of NKG2D^{+}CD56^{dim}NK cells. HCC patients were divided into two groups according to the media of the frequency of NKG2D^{+}CD56^{dim}NK cells at one month-post surgery in each relative analysis. Above-median frequency of NKG2D^{+}CD56^{dim}NK cells corresponds to higher (**C**) recurrence-free survival rate and (**D**)overall survival rate.

tumors.[34,38,39] Therefore, the detection of NKG2D^{+}CD56^{dim}NK cells before operation may be useful in judging the severity of HCC. Also, it is widely accepted that tumors greater than 5 cm are likely to have more micrometastases.[40] This further supports the discovery mentioned above that the change of NKG2D expression on CD56^{dim}NK subset is influenced by the existence of micrometastases after resection. Interestingly, we also found in the current study that HCC patients with higher level of sera GGT, an important tumor specific antigen, were more likely to have lower NKG2D expression level on NK cells. Although the underlying reason needed to be studied further, this correlation indicated that NKG2D played a critical role in the immunosurveillance of malignancies.

Additionally, we found that the frequency of NKG2D and CD38 expressions on CD56^{dim}NK subset was decreased in HCC tumor tissues. As we know, although the activation of NK cells is the synthetic action of activating and inhibitory receptors, there is no inhibitory counterpart known for NKG2D and it could override signals provided by inhibitory receptors on NK cells. [41,42] Therefore, the decreased frequency of NKG2D expression on CD56^{dim}NK subset would lead to dramatic suppression of tumor resident NK cells, and the decline of CD38^{+}CD56^{dim}NK subset was possibly one of the results. What's more, the frequencies of CD56^{dim}NK and NKG2D^{+}CD56^{dim}NK cells in TIL presented the same tendency as in peripheral blood, indicating that the circulating NK cells may be a reflection of that in tumor microenvironment in HCC.

As for the exploration of influencing factors on NKG2D in HCC, we discovered that the concentrations of TGF-β and sMICA in serum were negatively correlated with the frequency of NKG2D expression on CD56^{dim}NK cells respectively. Their relationship was confirmed by our finding of higher TGF-β and sMICA in tumor tissues derived homogenates, experiments with stimulation from cell lines supernatant *in vitro*, and also supported by other publications. [14,15,43] The possible underlying mechanism was that the interaction between NKG2D and sMICA lead to the endocytosis of NKG2D, following by the deactivation of NK cells for the loss of intracellular contacts. However, we did not detect high level of TGF-β in cell lines supernatants those obviously reduced the NKG2D on CD56^{dim}NK cells. This could possibly be explained by the evidence that TGF-β was mainly secreted by regulatory T cells (Treg) and macrophages but not tumor cells or HCC cells lines. [44] What is worth mentioning, NKG2D^{+}CD56^{dim}NK cells were significantly decreased under the stimulation of tumor homogenates, purified TGF-β and sMICA, while their percentages failed to recover after the receptor of TGF-β and monoclonal antibody of sMICA were added into homogenates to block the function of TGF-β and sMICA (data not shown). Thus, it could be inferred that TGF-β and sMICA did have the inhibitory influences on NKG2D, however, some other inhibitors still exist.

In conclusion, this study demonstrated that the frequency of peripheral and tumor-infiltrating NKG2D^{+}CD56^{dim}NK cell subset was declined in HCC patients and at least partially ascribed to higher TGF-β and sMICA production. The recovery of NKG2D^{+}CD56^{dim}NK cells was related to the prognosis of HCC patients. Our findings provided an insight into the mechanisms of impairment of NKG2D on NK cells in HCC patients. The frequency of NKG2D^{+}CD56^{dim}NK subset could be a considerable biomarker for predicting the prognosis of HCC patients with hepatectomy.

Materials and Methods

Study subjects

34 patients with advanced HCC and 15 HD were enrolled at Affiliated Tumor Hospital of Zhengzhou University during October 2012 and May 2013 and followed up until October 2014. All patients were positive for serum hepatitis B surface antigen (HBsAg) and negative for HIV and hepatitis C. Clinical and biochemical characteristics of all the participants were shown in **Table 1**. Histologically hepatic hemangiomas specimens were obtained from seven patients. All hemangiomas patients had normal level of serum alanine aminotransaminase (ALT) levels and were negative for HBsAg, anti-HCV and anti-HIV. The diagnosis of HCC and tumor-node-metastasis (TNM) stage were determined according to the standard of the Union for International Cancer Control, UICC 2010. The clinical diagnosis was based on several factors, including HBV infection, elevated serum AFP,

imaging of liver space-occupying lesions, vascular and bile duct invasion and hepatic or distant metastatic lesions. Central tumor and adjacent tumor tissues from HCC patients were collected during resection surgery. Peripheral blood mononuclear cells (PBMCs) were also collected from patients before and one month after surgery, stored in liquid nitrogen. This study was approved by the Ethics Committees of Peking University Health Science Center. Informed consent was obtained from each patient prior to participation.

PBMC and tissue-infiltrating lymphocytes isolation

PBMCs were isolated from heparinized blood by the density gradient centrifugation technique using HISTOPAQque-1077 (Sigma, 100771–500ML) according to the manufacture's instruction. Tumor, para-tumor and hemangioma infiltrating lymphocytes were isolated from fresh tissue samples as previously reported. [45]

NK cells purification

NK cells were purified from PBMC by negative selection using the MACS NK cell Isolation Kit (Miltenyi, 130-092-657), according to the manufacturer's instructions. The purity of $CD3^{-}CD56^{+}$ NK cells was greater than 95% as measured by flow cytometry.

Flow Cytometry

PBMCs or tissue-infiltrating lymphocytes were stained as follows. Frozen cells were thawed and resuspended in complete RPMI1640 medium (Corning, 10–040-CVR) containing 10% fetal bovine serum (Gibco, 12662029), 1% glutamine (Immundiagnostic, K7732) and 1% penicillin and streptomycin (Gbico, 15140–122). 1×10^{6} cells were used for each panel and stained in dark at room temperature (20–25 °C) for 30 min. The following monoclonal antibodies (Abs) were used for NK cell phenotypic characterization: Pacific Blue-Vivid (Invitrogen, CA), PE-CF594-CD3 (BD Biosciences, 562280), Alexa Fluor® 700-CD14 (BD Biosciences, 557923), PE-Cy7-CD56 (BD Biosciences, 335791), APC-NKG2D (BD Biosciences, 558071), PE-NKG2C (R&D, FAB138C), PE-NKG2A (R&D, FAB1059C), APC-CD69 (BD Biosciences, 340560), PerCP-Cy5-HLA-DR (BD Biosciences, 347364) and FITC-CD38 (BD Biosciences, 555459). After staining, the PBMCs were washed twice with phosphate buffer saline and detected using a BD LSR II Fortessa flow cytometer (BD Biosciences, NJ). The data were analyzed using the FlowJo software (TreeStar, San Carlos, CA).

HCC cell lines

All the human HCC-derived cell lines were obtained from the American Type Culture Collection (ATCC), with exception of SMMC7721, which was obtained from China Infrastructure of Cell Line Resource. Huh7, Sk-hep-1, Hep3B, HepG2, PLC/PRF/5 and SMMC7721 were cultured in Dulbecco's modified Eagle's medium (DMEM) (Corning,10–013-CVR) while SNU387, SNU182, SNU423 and SNU475 were cultured in RPMI1640(Corning,10-040-CVR), both of which were supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were maintained at 37°C in a humid atmosphere containing 5% CO_2. Cell culture supernatants were collected from 1×10^{7} cells and added into PBMCs cultural system at a volume ratio of 1:4 and cultured overnight.

Tissue homogenate preparation

Central tumor, para-tumor and hemangioma homogenates from HCC and hemangioma patients were suspended in 1 mL RPMI1640/100 mg tissue before being homogenized. The mixture was grinded up into homogenate followed by centrifugation (4°C, 15,000 rpm, 30 min). Then the supernatant was filtered using a 0.22 μm membrane and stored at −80°C. In *in vitro* experiments, homogenates were added into PBMCs cultural system at a volume ratio of 1 : 4 and cultured overnight. The frequency of $NKG2D^{+}CD56^{dim}$NK cells was detected by flow cytometry.

***In vitro* cells culture and stimulation**

1×10^{6} NK cells were incubated overnight with (1) medium alone, (2) TGF-β (1ng/mL; PeproTech,100–21), (3) sMICA (1 ng/mL, Prospec, pro-367-a), or with 20% HCC tissue homogenate in the presence or absence of TGF-βRII(10 ng/mL; Prospec, PKA-26) or anti-MICA-neutralizing Ab (10 ng/mL; Biolegend, 320909). IL-15 (10 ng/mL, PeproTech, 200-5-2) and IL-2 (100 U/mL; R&D, SXR028-48) were also added to the culture system above.

Degranulation of $NKG2D^{+}CD56^{+}$NK cells and IFNγ detection

Purified $NKG2D^{+}$NK cells from eight HCC patients and eight HD were sorted by flow cytometry (BD, FACSAriaII) and stimulated with PMA (25 ng/mL) (Sigma-Aldrich, P1585) and Ionomycin (1 μg/mL) (Santa Cruz Biotechnology, 56092-82-1) at 37°C for 1 h. Then, Pecy5-CD107a (BD Biosciences, 555802), GolgiStop (BD Biosciences, 554715) and Brefedlin A (Santa Cruz Biotechnology, sc-200861) were added into the medium. After 5 h, cells were collected and stained with the following monoclonal surface Abs, eFluor®450-CD3 (eBioscience, 48-0038-42), PE-CD14 (BD Biosciences, 555398), Pecy7-CD56 (BD Biosciences, 335791), APC-NKG2D (BD Biosciences, 558071). Then, cells were intracellularly stained with Alexa Fluor® 700-IFNγ (BD Biosciences, 557995) and FITC-TNF-α (BD Biosciences, 554512) and detected by flow cytometry.

Cytokines, sMICA and sMICB detection

The levels of IL-12p70, IFNγ, TNF-α, IL-4, IL-10 and IL-17A in sera, tissue homogenates and cell culture supernatants were analyzed using multiplex luminex inflammation kits (eBioscience, EPX010-10420-901) according to the manufacturer's instructions. Briefly, 50 μL samples or standard recombinant protein dilution were added to a mixture of capture beads coated with related mAbs to a group of cytokines, washed beads were further incubated with biotin-labeled anti-human cytokine Abs for 1 h at room temperature followed by incubation with streptavidin–phycoerythrin for 30 min. Samples were analyzed using Luminex 200™ (Luminex, Austin, TX) and Statlia software

(Brendan, Carlsbad, CA). Standard curves of known concentrations of recombinant human cytokines were used to convert median fluorescence intensity (MFI) to cytokine concentration in pg/mL. Only the linear portions of the standard curves were used to quantify cytokine concentrations. TGF-β in the sera, tissue homogenates and cell culture supernatants were analyzed by standard sandwich ELISA kits (eBioscience, BMS24914) according to the manufacturer's high sensitivity protocol. sMICA (Abcam, ab100592) and soluble major histocompatibility complex class I polypeptide-related sequence B (sMICB) (Abcam, ab100593)were analyzed by standard sandwich ELISA kits according to the manufacturer's protocols.

Analysis of mRNA expression by real-time quantitative RT-PCR

Total RNA was prepared from hemangioma, para-tumor and tumor tissues using TRIzol Reagent (Invitrogen, 15596026) according to the manufacturer's instructions and was used for cDNA synthesis. Real-time qPCR was performed using LightCycler 480 thermo cycler (Roche, USA). The primers were as follows: *TGF*-β-F (5′-GTGGAAACCCACAACGAAA-3′), *TGF*-β-R (5′-TAAGGCGAAAGCCCTCAAT- 3′), *MICA*-F (5′-CAGACATTCCATGTTTCTGC-3′), *MICA*-R (5′-AGGCTCACGAGCTCTGG ACC-3′), *ADAM9*-F (5′-GCGGGATTAATGTGTTTGGA-3′), *ADAM9*-R (5′-AAAGTTTCTGG AACCCGATGC-3′). The endogenous C-terminal-binding protein (*CTBP*) gene was used for normalization. Each gene was amplified in triplicate and the average threshold cycle (Ct) was used for calculation. The relative folder changes in gene expression were calculated using the comparative Ct ($2^{-\Delta Ct}$) method.

Array-based Comparative Genomic Hybridization (aCGH)

The procedures for DNA digestion, labeling, and hybridization for the oligo arrays were performed according to the standard Agilent protocol v7.1. Data were extracted using Agilent Feature Extraction software (version 11.0.1.1) using the CGH_1100_Jul11 protocol, then analyzed for copy-number changes using Agilent Genomic Workbench 7.0 software package (Agilent Technologies, CA) and/or BioDiscovery Nexus 6.1 (BioDiscovery, CA).

Statistical analysis

All of the statistical analyses were performed using SAS version 9.1. Characteristics of the studied population and the different immune or biochemical parameters were recorded as median and inter-quartile range. Comparison between different groups was performed using the Mann–Whitney *U* test. Comparison among the same individual was performed using the Wilcoxon-matched pairs *T* test (Two-tailed). The relationships between NKG2D expression and clinical pathological features were analyzed by the Chi-square test. The Kaplan–Meier method was applied for survival analysis and Multivariate Cox regression analysis for independent prognostic indicators. Spearman correlation coefficients were used to estimate the correlations among continuous variables. Two-tailed P values < 0.05 was considered to be significant.

This study was approved by the Ethics Committees of Peking University Health Science Center. Informed consent was obtained from each patient prior to participation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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