Failure recovery of circulating NKG2D<sup>+</sup>CD56<sup>dim</sup>NK cells in HBV-associated hepatocellular carcinoma after hepectectomy predicts early recurrence

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

<sup>1</sup>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; <sup>2</sup>Department of Hepatobiliary and Pancreatic Surgery; Affiliated Tumor Hospital of Zhengzhou University; Zhengzhou, China; <sup>3</sup>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; <sup>4</sup>Department of Immunology; Peking University Health Science Center; Beijing, China

#These authors made equal contributions to this manuscript.

**Keywords:** HCC, NK cells, NKG2D, sMICA, TGF-β

**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 Ipolypeptide-related sequence A; MICB, major histocompatibility complex class Ipolypeptide-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 chemoembolization; 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<sup>+</sup>CD56<sup>dim</sup>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<sup>+</sup>CD56<sup>dim</sup>NK cells. In addition, in HCC patients the lower frequency of circulating NKG2D<sup>+</sup>CD56<sup>dim</sup>NK cells was associated with larger tumor size and/or higher serum GGT. Noticeably, the frequency of NKG2D<sup>+</sup>CD56<sup>dim</sup>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<sup>+</sup>CD56<sup>dim</sup>NK cells in HCC was associated with higher TGF-β and sMICA production, and low frequency of circulating NKG2D<sup>+</sup>CD56<sup>dim</sup>NK cells at one month after surgery may predict poor prognosis of HBV-related HCC patients accepting hepectectomy.

**Introduction**

HCC is the fifth most common cancer and the third leading cause of cancer related death worldwide. Currently, HCC therapies include surgical resection, radiofrequency thermal ablation (RFA) and transcatheter arterial chemoembolization (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.

NK cells-mediated innate immunity is proposed to play a pivotal role in host defense against cell malignancy transformation. NK...
cells recognition is triggered by a broad range of activating and inhibitory receptors, which act as sensors of abnormal cells. 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 UL16 protein-ligand family (ULBP), 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. 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. 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+CD56dim subset on tumor recurrence and overall survival (OS) were evaluated. In addition, the influence of TGF-β and sMICA on NKG2D+CD56dimNK cells was analyzed. This study provided further evidences for immune-escape from impaired NK cells in HCC and suggested that NKG2D+CD56dimNK was a potential biomarker for predicting the prognosis of HCC patients receiving surgical resection.

Results

Failure restoration of circulating NKG2D+CD56dimNK 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, our results confirmed that circulating NK cells, primarily the CD56dimNK 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 CD56dimNK 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. As shown in Fig. 1D, compared with HD, the frequencies of NKG2D+ NK cells and NKG2D+CD56dimNK subset were significantly decreased in either stage I or stage II/III HCC patients, while the frequencies of NKG2A+NK cells and NKG2A+CD56dimNK subset were increased. The proportions of NKG2C+, CD69+, HLA-DR+, CD38+ NK cells and CD56dimNK subset showed no significant difference between HCC patients and HD (Fig. 1D and S1). In addition, the post-operative frequencies of NK cells, CD56dimNK, NKG2D+NK and NKG2D+CD56dimNK 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+CD56dimNK 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-DR+ and CD38+ NK cells (Fig. 1E and S1). The observations above demonstrated that the activation of circulating NK cells was suppressed in HCC patients, and the frequency of circulating NKG2D+CD56dimNK subset was recovered in FRE HCC patients and could be used as predictive marker for HCC recurrence after curative surgery.

Frequency of intrahepatic NKG2D+CD56dimNK 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, CD56dimNK subset and CD56brightNK subset were significantly lower in tumor, as compared to the corresponding para-tumor tissues (Fig. 2B). The ratio of CD56brightNK/CD56dimNK subset was also lower in tumor, indicating that the CD56bright NK subset reduced more obviously than CD56dimNK subset (Fig. 2C). In addition, the frequencies of CD38+CD56dimNK cells (Fig. 2D), NKG2A+CD56dim and NKG2D+CD56dim NK subsets (Fig. 2E) were also lower in tumor-infiltrated lymphocytes (TILs). In contrast, the frequencies of CD69+CD56dim, HLA-DR+CD56dim and NKG2C+CD56dim 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 CD56dimNK 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+CD56dimNK cells in HCC patients

Based on the observation that decreased frequency of NKG2D+CD56dimNK cells in HCC patients might be tumor-bearing status related, the underlying mechanism was further investigated. It has been shown that TGF-β, sMICA and IL-12 could downregulate the surface expression of NKG2D on NK cells and IFN-α could upregulate it, while IL-10, IL-17, IFNγ, IL-4, sMICB 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<sup>+</sup>CD56<sup>dim</sup>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<sup>bright</sup> and CD56<sup>dim</sup> NK cell subsets in tumor and para-tumor tissues. (D)(E) Pool data showed the frequency of CD38<sup>+</sup>, CD69<sup>+</sup>, HLA-DR<sup>+</sup>, NKG2A<sup>+</sup>, NKG2C<sup>+</sup> and NKG2D<sup>+</sup> 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 ((×10^12/L))           | 4.9(3.8–5.9) | 5.3(4.2–5.7) | 0.09 |
| WBC ((×10^9/L))            | 6.3(2.8–8.1) | 6.8(2.1–9.2) | 0.25 |
| PLT ((×10^9/L))            | 145.5(59.0–268.0) | 152.0(94.0–220.0) | 0.02 |
| HBs(g/L)                   | 313.5(64.6–176.8) | 134.6(121.9–152.2) | 0.32 |
| HBV DNA (log10 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)                 | 124.1(815–1210.0) | 1.10(3.4–10.3) | 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(22.5–51.5) | 26.5(23.4–32.3) | 0.23 |
| TBI (µmol/L)               | 16.2(53.3–59.5) | 6.2(4.1–17.6) | 0.04 |
| DBIL (µmol/L)              | 8.3(3.31–18.4) | 3.30(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: alpha-fetoprotein; TBIL: total bilirubin; DBIL: direct bilirubin; 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 NK2G^+ 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 sMICB were significantly higher in HCC patients and were negatively correlated with the frequency of circulating NK2G^+CD56^dim NK cells (Fig. 3B), indicating that lower frequency of NK2G^+ NK cells in HCC patients might ascribe to increased serum TGF-β and sMICB.

To investigate if tumor microenvironment has an effect on the frequency of NK2G^+CD56^dim NK cells, PBMCs from healthy donors (HD) were co-cultured with tumor, para-tumor or hemangioma tissue homogenates and frequency of NK2G^+CD56^dim NK cells was evaluated by flow cytometry. As shown in Fig. 3C, the proportion of NK2G^+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 sMICB 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 metalloproteinase domain 9 (ADAM9), which can recognize MICA cleavage site in HCC cells, 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 of 10 HCC cell lines were added to PBMCs to identify the mechanism of tumor cells on reduction of NK2G^+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 NK2G^+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 NK2G^+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 NK2G^+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 NK2G^+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 NK2G^+ positive cells in total NK and CD56^dim NK cells, confirming the negative regulation of TGF-β and sMICA on NK2G^+.

The frequency of post-operative NK2G^+CD56^dim NK cells was a valuable prognosis marker for curative surgery recipients of HCC

To investigate the clinical significance of NK2G^+ NK cells in HCC, association of the frequency of NK2G^+ NK cells with clinical pathological features was evaluated. As shown in Table 2, HCC patients with higher frequency of NK2G^+ NK cells...
Table 2. Association analysis of the frequencies of NKG2D+ CD56+ NK cells with clinical pathologic features

| Variables               | NKG2D+ / CD56+/NK (%) | P   | NKG2D+ / CD56dimNK (%) | P   |
|-------------------------|-----------------------|-----|------------------------|-----|
| Age(years)              |                       |     |                        |     |
| ≤55                     | 55.0(39.5–93.0)       | 0.343 | 44.7(30.5–90.4)        | 0.262 |
| >55                     | 67.1(28.8–90.3)       | 0.748 | 58.5(20.3–87.6)        | 0.636 |
| Sex                     |                       |     |                        |     |
| Female                  | 67.8(28.8–90.3)       | 0.820 | 57.8(20.3–87.6)        | 0.145 |
| Male                    | 56.8(34.7–73.0)       | 0.820 | 49.0(27.0–90.4)        | 0.016 |
| Liver cirrhosis         |                       |     |                        |     |
| Absent                  | 52.2(34.7–93.0)       | 0.018 | 40.6(27.0–74.2)        | 0.258 |
| Present                 | 67.8(28.8–90.3)       | 0.018 | 59.1(20.3–90.4)        | 0.058 |
| Tumor size(cm)          |                       |     |                        |     |
| ≤5                      | 62.2(39.5–89.7)       | 0.174 | 59.1(31.1–88.5)        | 0.016 |
| >5                      | 52.2(28.8–93.0)       | 0.174 | 42.9(20.3–90.4)        | 0.258 |
| No. of tumors           |                       |     |                        |     |
| 1                       | 58.0(28.8–93.0)       | 0.258 | 49.0(20.2–90.4)        | 0.226 |
| >1                      | 77.5(49.8–90.3)       | 0.258 | 71.1(30.5–71.1)        | 0.226 |
| Tumor encapsulation     |                       |     |                        |     |
| Complete                | 57.9(28.8–93.0)       | 0.757 | 46.5(20.3–90.4)        | 0.796 |
| Incomplete              | 76.1(39.5–89.7)       | 0.757 | 66.0(31.1–88.5)        | 0.796 |
| AFP (ng/mL)             |                       |     |                        |     |
| ≤800                    | 64.6(28.8–89.7)       | 0.391 | 56.3(20.3–88.5)        | 0.572 |
| >800                    | 58.0(34.7–93.0)       | 0.391 | 46.3(28.2–90.4)        | 0.572 |
| Total Bilirubin (μmol/L)|                       |     |                        |     |
| ≤17.1                   | 61.6(28.8–93.0)       | 0.162 | 55.9(20.3–90.4)        | 0.221 |
| >17.1                   | 58.0(34.7–93.0)       | 0.162 | 49.1(27.0–87.6)        | 0.221 |
| ALB(μL)                 |                       |     |                        |     |
| ≤50                     | 60.5(28.8–89.7)       | 0.654 | 51.2(20.3–88.5)        | 0.654 |
| >50                     | 52.2(28.8–93.0)       | 0.654 | 63.3(30.5–90.4)        | 0.654 |
| ALT(μL)                 |                       |     |                        |     |
| ≤40                     | 66.3(39.5–89.7)       | 0.761 | 54.1(20.3–88.5)        | 0.761 |
| >40                     | 52.2(28.8–93.0)       | 0.761 | 42.4(20.3–90.4)        | 0.761 |
| AST(μL)                 |                       |     |                        |     |
| ≤40                     | 61.9(28.8–89.7)       | 0.032 | 54.1(20.3–88.5)        | 0.027 |
| >40                     | 56.8(34.7–93.0)       | 0.032 | 49.4(20.3–90.4)        | 0.027 |
| ALP(μL)                 |                       |     |                        |     |
| ≤150                    | 61.6(28.8–93.0)       | 0.032 | 57.9(20.3–88.5)        | 0.027 |
| >150                    | 56.8(34.7–90.3)       | 0.032 | 45.3(27.0–90.4)        | 0.027 |
| GGT(μL)                 |                       |     |                        |     |
| ≤50                     | 72.3(28.8–89.7)       | 0.032 | 68.1(20.3–88.5)        | 0.027 |
| >50                     | 52.2(34.7–93.0)       | 0.032 | 46.0(27.0–90.4)        | 0.027 |

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

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. 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 NKGD2 on peripheral CD56dimNK 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 NKGD2+CD56dimNK 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.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 NKGD2+CD56dimNK 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 NKGD2+CD56dimNK 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, and the mortality of early recurrent patients during the initial five-year period after resection was 95.5%. The finding in the current study that the correlation between NKGD2 and early recurrence may be of great clinical significance offering a reference for further therapies, such as chemotherapy and immunotherapy, to eliminate the existing tumor completely. Since the number of HCC patients we followed was limited, we must acknowledge that the threshold frequency of NKGD2+CD56dimNK cells at one-month after operation for clinical application might be confirmed in larger sample size. Meanwhile, we noticed that the frequency of NKGD2 expression on CD56dimNK subset before operation was lower in patients with tumor greater than 5cm, indicating that larger tumor could reduce NKGD2 expression in HCC patients to a greater degree. In human, the interaction of NKGD2 with its ligands on tumor cells surface plays an important role in the immune response to...
CD38 expressions on CD56 dim NK subset was decreased in malignancies. NKG2D played a critical role in the immunosurveillance of tumors. 

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. 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 expression on CD56 dim NK cells is the synthetic action of activating and inhibitory receptors, there is no inhibitory counterpart known for NK cells. 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. 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. 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. 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 Histopaque-1077 (Sigma, 100771–500ML) according to the manufacturer'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<sup>-</sup> CD56<sup>+</sup> 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 (Immun-cytes, 335791), APC-NKG2D (BD Biosciences, 558071), 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/PRF5 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% CO₂. Cell culture supernatants were collected from 1×10<sup>7</sup> 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<sup>+</sup>CD56<sup>dim</sup>NK cells was detected by flow cytometry.

**In vitro cells culture and stimulation**

1×10<sup>6</sup> NK cells were incubated overnight with (1) medium alone, (2) TGF-β (1 ng/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<sup>+</sup>CD56<sup>+</sup>NK cells and IFNγ detection**

Purified NKG2D<sup>+</sup>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<sup>®</sup> 450-CD3 (eBioscience, 48-0038-42), PE-CD14 (BD Biosciences, 555802), GolgiStop (BD Biosciences, 554715) and Brefedlin A (Santa Cruz Biotechnology, sc-200861) 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-phycocerythin for 30 min. Samples were analyzed using Luminex 200<sup>TM</sup> (Luminex, Austin, TX) and Statia 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’-GTGAAACCCCAACGGAAA-3’), TGF-β-R (5’-TAAGGCAGAACCTCAAT-3’), MICA-F (5’-CAAGATCCATGGTCTGC-3’), MICA-R (5’-AGGCCCTCAGGACC-3’), ADAM9-F (5’-GGGATTAATGTTGTGGA-3’), ADAM9-R (5’-AAAGTTTCTGGACCCCCATGC-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^-ΔΔCt) 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).

References

1. Jermal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA: a cancer journal for clinicians 2011; 61:69-90; PMID:21296859; http://dx.doi.org/10.3322/caac.20107
2. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet 2003; 362:1907-17; PMID:14667750; http://dx.doi.org/10.1016/S0140-6736(03)14964-1
3. Chew Y, Tow C, Teo M, Wong HL, Chan J, Gehring A, Loi M, Bolte A, Quik R, Lee VK et al. Inflammatory tumor microenvironment is associated with superior survival in hepatocellular carcinoma patients. J Hepatol 2010; 52:378-9; PMID:19720422; http://dx.doi.org/10.1016/j.jhep.2009.07.013
4. Sui Q, Zhang J, Sun X, Zhang C, Han Q, Tian Z. NK cells are the crucial antitumor mediators when STAT3-associated tumour microenvironment is associated with superior survival in hepatocellular carcinoma patients. J Hepatol 2010; 52:378-9; PMID:19720422; http://dx.doi.org/10.1016/j.jhep.2009.07.013
5. Moretta L, Botinotto C, Pende D, Castriconi R, Mingari MC, Moretta A. Surface NK receptors and their ligands on tumor cells. Semin Immunol 2006; 18:151-8; PMID:16730454; http://dx.doi.org/10.1016/j.smim.2006.03.002
6. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. Nat Immunol 2008; 9:593-10; PMID:18425107; http://dx.doi.org/10.1038/nri1582
7. Moretta A, Botinotto C, Virale M, Pende D, Biasioni R, Mingari MC, Moretta L. Receptors for HLA class-I molecules in human natural killer cells. Ann Rev Immunol 1996; 14:619-48; PMID:8717527; http://dx.doi.org/10.1146/annurev.immunol.14.1.619
8. Jarahian M, Ward C, Issa Y, Alevy P, Mombaum F. Blockade of natural killer cell-mediated lysis by NCM141 expressed on tumor cells. Int J Cancer 2007; 120:6265-34; PMID:17294447; http://dx.doi.org/10.1002/ijc.22579
9. McGivney RW, Eagle RA, Watson NF, Al-Attar A, Ball G, Jeffery J, Trowdale J, Durrant LG. NKG2D ligand expression in human colorectal cancer reveals associations with prognosis and evidence for immunoeediting. Clin Cancer Res 2009; 15:6993-7002; PMID:19861434; http://dx.doi.org/10.1158/1078-0432.CCR-09-0991
10. Orr MT, Lianer LL. Natural killer cell education and tolerance. Cell 2010; 142:875-96; PMID:20850008; http://dx.doi.org/10.1016/j.cell.2010.08.031
11. Cai L, Zhang Z, Zhou L, Wang H, Fu J, Zhang S, Shi M, Zhang H, Yang Y, Wu H et al. Functional impairment in circulating and intrahepatic NK cells and relative mechanism in hepatocellular carcinoma patients. Clin Immunol 2008; 129:426-37; PMID:18824414; http://dx.doi.org/10.1016/j.cellimm.2008.08.012
12. Zhang Z, Zhang S, Zou Z, Shi J, Zhao J, Fan R, Qin E, Li B, Li Z, Xu X et al. Hypoerythroid activity of hepatic natural killer cell correlates with liver injury in chronic hepatitis B patients. Hepatology 2011; 53:73-85; PMID:21254163; http://dx.doi.org/10.1002/hep.23977

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank all patients and volunteers who participated in the study and all staff in Affiliated Tumor Hospital of Zhengzhou University for their help in collection of samples.

Funding

This work was supported by the 973 Programme (2015CB554000), the National Science and Technology Major Project for Infectious Diseases (2012ZX10002005 and 2012ZX10004094), 863 Programme (2012AA022605) and 111 project (B07001).

Supplemental Material

Supplemental data for this article can be accessed on the publisher’s website.
13. Allan DS, Rybalov B, Awong G, Zuniga-Pflucker JC, Kopcow HD, Carlyle JR, Strominger JL. TGF-beta affects development and differentiation of human natural killer cell subsets. Eur J Immunol 2010; 40:2289-99; PMID:20700109; http://dx.doi.org/10.1002/eji.200939910

14. Sun C, Fu B, Gao Y, Liao X, Sun R, Tian Z, Wei H. TGF-beta1 down-regulation of NKGD2/DAP10 and 2B4/SAP expression on human NK cells contributes to HBV persistence. PLoS pathogens 2012; 8:e1002594; PMID:22438812; http://dx.doi.org/10.1371/journal.ppat.1002594

15. Jinushi M, Takehara T, Tatsutomi T, Hiranuma N, Sakamori R, Yamaguchi S, Hayashi N. Impairment of natural killer cell and dendritic cell functions by the soluble form of MHC class I-related chain A in advanced human hepatocellular carcinomas. J Hepatol 2005; 43:1013-20; PMID:16168521; http://dx.doi.org/10.1016/j.jpeden.2005.06.026

16. Munsael MJ, Magri G, Pende D, Angelo A, Lopez-Boter M. Inhibition of NKGD2 expression in NK cells by cytokines secreted in response to human cytomegalovirus infection. Blood 2010; 115:5710-9; PMID:20697399; http://dx.doi.org/10.1182/blood.2010-1152679

17. Konjevic G, Jrnicic Martinovic K, Vuletic A, Radenovic S. Novel aspects of in vitro IL-2 or IFN-alpha enhanced NK cytotoxicity of healthy individuals based on NKGD2 and CD161 NK cell receptor induction. Biomed Pharmacother 2010; 64:663-71; PMID:20800424; http://dx.doi.org/10.1016/j.biopha.2010.06.013

18. Paramo KG, Kumar A, Bailey AD, Sanchez-Darden JL, Chambers KA, Young CD, Lim WT, Kravcik S, Cameron DW, Angel JB. Normalization of natural killer cell function and phenotype with effective anti-HIV therapy and the role of IL-10. AIDS 2002; 16:1251-6; PMID:12045490; http://dx.doi.org/10.1097/00002030-200206140-00007

19. Reves RK, Rajakumar PA, Evans TI, Conole M, Gills J, Wong FE, Kuzmichev YV, Carville A, Johnson RP. Gut inflammation and indoleamine 2,3-dioxygenase inhibit IL-17 production and promote cytotoxic potential in NKp44+ mucosal NK cells during SIV infection. Blood 2011; 118:3321-36; PMID:21791421; http://dx.doi.org/10.1182/blood-2011-04-347260

20. Li ZY, Chao HH, Liu HY, Song ZH, Li LL, Zhang YJ, Yang Y, Peng JP. IFN-gamma induces aberrant CD49b (+) NK cell recruitment through regulating CX3CL1: implications for mucosal NK cells. J Proteome Res 2014; 13:4847-58; PMID:24967658; http://dx.doi.org/10.1021/pr500262p

21. Kohga K, Takehara T, Tatsutomi T, Ishida H, Miyagi T, Hosui A, Hayashi N. Soraenfib inhibits the shedding of major histocompatibility complex class I-related chain A on hepatocellular carcinoma cells by down-regulating a disintegrin and metalloproteinase 9. Hepatol 2010; 51:1264-73; PMID:20099300; http://dx.doi.org/10.1002/hep.23456

22. Zerbini A, Pilli M, Lacabce D, Pelo G, Molinari A, Negri E, Cerion S, Fagion F, Soliani P, Ferranti C et al. Radiofrequency thermal ablation for hepatocellular carcinoma stimulates autologous NK-cell response. Gastroenterology 2010; 138:1931-42; PMID:20608229; http://dx.doi.org/10.1053/j.gastro.2009.12.051

23. Aratlah AM, Talbi AA, El-Sadany M, Ibrahim TA, El-Dessicy I. Dysregulation of lymphoproliferative subsets and natural killer cells in schistosomal liver cirrhosis and hepatocellular carcinoma. Clin Exp Med 2003; 3:181-5; PMID:14646824; http://dx.doi.org/10.1007/s10147-003-0032-y

24. Tian Z, Chen Y, Gao B. Natural killer cells in liver disease. Hepatology 2013; 57:1654-62; PMID:22311152; http://dx.doi.org/10.1002/hep.26215

25. Ichihara Y, Ohdan H, Ohita M, Mitsuta H, Arihko K, Asahara T. Differences in cytotoxicity against hepatocellular carcinoma between liver and periphery natural killer cells in humans. Hepatology 2006; 43:362-72; PMID:16444347; http://dx.doi.org/10.1002/hep.21005

26. Hoehn AN, Goelglaender T, Ormanny L, Garekeshvili L, Zhao F, Wedemeyer H, Lehner F, Manns MP, Gerot TF, Korangy F. Myeloid derived suppressor cells inhibit natural killer cells in patients with hepatocellular carcinoma via the Nkp30 receptor. Hepatology 2009; 49:790-9; PMID:19551844; http://dx.doi.org/10.1002/hep.23054

27. Kobayashi A, Miyagawa S, Miwa S, Nakata T. prognostic impact of anatomical resection on early and late intrahepatic recurrence in patients with hepatocellular carcinoma. Cancer 2008; 112:515-21; PMID:18836080; http://dx.doi.org/10.1007/s00534-007-1293-7

28. Imamura H, Matsuyama Y, Tanaka E, Ohkubo T, Hatai Y, Watanabe Y, Ohba Y, Ono Y, Miyayama T et al. Prognostic impact of anatomical resection on early and late intrahepatic recurrence after liver resection for hepatocellular carcinoma: results of a multicenter study. J Surg Oncol 2004; 85:36-41; PMID:14606858; http://dx.doi.org/10.1002/jso.10284

29. Nauoch N, Cerovsek A. NKGD2 ligands in tumor immunology. Oncotarget 2014; 5:2794-9; PMID:24859230; http://dx.doi.org/10.18632/oncotarget.2872

30. Raulet DH. Roles of the NKGD2 immunoreceptor and its ligands. Nat Rev Immunol 2003; 3:781-90; PMID:12822846; http://dx.doi.org/10.1038/nri1118

31. Chang YH, Connolly J, Shimasaki N, Mimura K, Kono K, Campana D. A chimeric receptor with NKGD2 specificity enhances natural killer cell activation and killing of tumor cells. Cancer Res 2013; 73:4876-86; PMID:23802228; http://dx.doi.org/10.1158/0008-5472.CAN-12-3558

32. Regimbeau JM, Abdalla EK, Vauthey JN, Lauwers GY, Durand F, Nagorney DM, Ikai I, Yamaoka Y, Belghiti J. Risk factors for early death due to recurrence after liver resection for hepatocellular carcinoma: results of a multicenter study. J Hepatol Oncol 2004; 5:36-41; PMID:14696805; http://dx.doi.org/10.1016/j.jho.10284

33. Morsy MA, Norman PJ, Mitry R, Rela M, Heaton MM, Hoogduijn MJ, Fibbe WE, Roelofs H. Multipo-
tic intervention. Oncoimmunology 2014; 3:e28497; PMID:25050215; http://dx.doi.org/10.1080/2014.70

34. Chamspaur M, Lanier LL. Effect of NKGD2 ligand expression on human immune responses. Immunological Rev 2010; 235:267-85; PMID:20536569; http://dx.doi.org/10.1111/j.0105-2896.2009.00893.x

35. Baragano Raineros A, Suarez-Alavez B, Lopez-Larrea C. Secretory pathways generating immuno-
suppressive NKGD2 ligands: new targets for thera-
petic intervention. Oncoimmunology 2014; 3:e28497; http://dx.doi.org/10.1080/2014.70

36. Melsef SM, Schrama E, Brugman MH, Tiemessen MM, Hoogduijn MJ, Fiehe WS, Roodbol F. Multipo-
tist T cells induce human regulatory T cells through a novel pathway involving skewing of mono-
cytes toward anti-inflammatory macrophages. Stem Cells 2013; 31:1980-91; PMID:23712682; http://dx.doi.org/10.1002/stem.1432

37. Moity MA, Norman PJ, Misty R, Rela M, Heaton ND, Vaughan RW. Isolation, purification and flow cytometric analysis of human intrahepatic lymphocytes using an improved technique. Lab Invest 2005; 85:285-96; PMID:15664335; http://dx.doi.org/10.1038/labinvest.0702019

www.tandfonline.com
Oncoimmunology

e1048061-11