·Clinical Research·

# Prognostic significance of natural killer cell infiltration in hepatocellular carcinoma

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**[Abstract] Background and Objective:** Several studies have Shown correlation of high numbers of tumor-infiltrating natural killer (NK) cells with a good prognosis for cancer patients. This study was to investigate the impact of NK cell infiltration on the survival and prognosis of patients with hepatocellular carcinoma (HCC) after resection. **Methods:** The proportion of infiltrating NK cells of HCC patients was measured using flow cytometry, and the expression of $CD56^+$ (NK) cells was investigated using immunohistochemistry. Prognostic values of intratumoral and peritumoral NK cell densities were evaluated by Kaplan-Meier method and Cox regression. **Results:** The level of NK cells was significantly lower in tumor-infiltrating lymphocytes (TIL) of HCC patients than in nontumor-infiltrating lymphocytes (NIL) [(11.8 ± 8.1)% vs. (18.0±7.9)%, $P$=0.002]. The density of NK cells was also significantly lower in cancer nests than in peritumoral lesions (2.3 ± 2.6 vs. 8.5 ± 4.5 cells per field, $P$<0.001). Patients with low intratumoral NK cells had shorter disease-free survival ($P$=0.027) and overall survival ($P$=0.005) than patients with high intratumoral NK cells. In contrast, NK cells in the peritumoral area showed no prognostic significance for either disease-free survival or overall survival. Multivariate Cox proportional hazards analysis showed that intratumoral NK cell density was an independent prognostic fator of prolonged overall survival (hazard ratio = 2.658, $P$=0.019). **Conclusions:** Low NK cells infiltration could predict poor prognosis in patients with HCC.

**Key words:** liver neoplasm, natural killer cell, infiltration, prognosis

Natural killer (NK) cells are lymphocytes that make important contributions to the protective response of the body to many infections and cancers. Normal hepatic tissue has a large number of NK cells, which exert stronger cytotoxicity against tumor cells than NK cells in peripheral blood. These NK cells are also more cytotoxic than hepatic NK cells in patients with cancer.[1] Our earlier laboratory studies found varied kinds of immune cells in hepatocellular carcinoma (HCC) tissue, such as tumor-related macrophages,[2] Th17 cells,[3] and regulatory T cells;[4] their distributions in tumor tissue differed significantly and the amount of infiltration was related to prognosis. However, NK cells, despite being important immune system killer cells, have not been adequately studied. Although impaired NK cell function has been shown in some patients with cancers, and some studies on solid tumors have suggested that a high density of NK cell infiltration in tumors is related to good prognosis,[5-8] currently the correlation between NK cell infiltration and prognosis in HCC remains unclear. Therefore, we attempted to examine NK cell infiltration in HCC tissue using flow cytometry and immunohistochemistry and to investigate its correlation with prognosis.

## Materials and Methods

### Study subjects

Resected hepatic tissue was collected from October 2006 to October 2007 from 19 patients with HCC. Tumor-infiltrating lymphocytes (TIL) were extracted from tumor tissues and non-tumor-infiltrating lymphocytes (NIL) from cancer-adjacent tissues. Extracted cells were detected using flow cytometry. Thirteen of the patients were male and 6 female, with a median age of 55 years (range 34-75 years). Sixteen patients had hepatitis B, 16 had hepatic cirrhosis, and 13 had positive alpha-fetoprotein (AFP) (≥25 ng/mL). In the TNM staging system, the disease was rated as stage I-II in nine patients and

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stage Ⅲ-Ⅳ in 10 patients.

In addition, a total of 62 HCC specimens were collected from patients who underwent surgical resection during 2002–2004 and who had complete followup data. Of these patients, 53 were male and 9 female, with a median age of 43.5 years (range 27–78 years). Among them, 54 had hepatitis B, 56 had hepatic cirrhosis, and 42 had positive AFP; 38 were at stage Ⅰ–Ⅱ and 24 at stage Ⅲ–Ⅳ.

All of the HCC specimens were harvested in the Sun Yat-sen Cancer Center and were pathologically confirmed. All of the patients were treatment-naive before surgery and had no previous history of autoimmune diseases or immunotherapy.

### Reagents

The antibodies and reagents used in our study and their sources are as follows: fluorescently labeled CD3 and CD56 antibodies (BD PharMingen), CD56 antibody and immunohistochemical staining system (DakoCytomation), lymphocyte separation medium (Tianjin Jin Hao Yang Biological Manufacture Co., Ltd), collagenase IV (Sigma), and DNase I (Roche).

### Methods

**Flow cytometric detection** The tissue was first cut into pieces of 1–2 mm in diameter and was then added to tissue digestion buffer (RPMI 1640 medium with 0.05% collagenase IV, 0.002% DNase I, and 20% fetal bovine serum (FBS) where it was digested for 20 min at 37℃ in an oscillator. Subsequently, Hank's buffered salt solution (HBSS) was added to terminate the digestion reaction; undigested pieces were removed using a 300-mesh filter, and the filtered solution was collected. Finally, the cell suspension was added to lymphocyte separation medium and subjected to density gradient centrifugation; the middle layer of cells (the lymphocytes) were our target cells. Antibody staining was performed in accordance with the protocol of the reagent kit. Flow cytometry was conducted using a FACSVantage SE flow cytometer (BD Medical Device; United States), and the results were analyzed using Cellquest software.

**Immunohistochemical staining** Staining was conducted as follows: the tissue was deparaffinized with dimethylbenzene and rehydrated, endogenous peroxidase was blocked with 3% hydrogen peroxide, and then the antigen was recovered by microwaving the tissue in citrate (pH 8.0). The tissue was incubated with the primary antibody at 4℃ overnight and with the secondary antibody for 30 min. Finally, the tissue was treated with DAB, counterstained with hematoxylin, and then sealed with neutral gum. Two specialists independently counted each specimen at 400 magnification (in an area of 0.15 $mm^2$). The numbers of $CD56^+$ cells were counted in 5 visual fields for each specimen, and the mean was regarded as the infiltration density.

**Statistical analyses** All of the data were analyzed by using SPSS 13.0; the results are shown as mean ± standard deviation. Comparisons between mean values were performed with the paired-samples *t* test; survival curves were generated using the Kaplan-Meier method, and survival was tested with the log-rank method; and the Cox proportional hazard model was used in the multivariate analysis. $P < 0.05$ indicated a significant difference.

## Results

### Fewer infiltrating NK cells in cancer lesions than in adjacent tissues

TIL and NIL were extracted from the resected hepatic tissues of 19 patients with HCC and used for flow cytometric detection. The percentage of NK cells among the TIL in patients with HCC was (11.8 ±8.1)%, which was significantly lower than the percentage of NK cells in matched NIL samples [(18%±7.9)%, $P = 0.002$] (Fig. 1), indicating decreased NK cell infiltration in the tumoral environment.

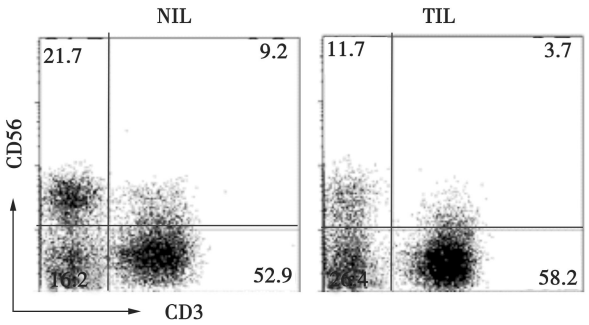


Figure 1 Proportion of natural killer (NK) cells in nontumor-infiltrating lymphocytes (NIL) and tumor-infiltrating lymphocytes (TIL) of hepatocellular carcinoma (HCC) patients measured by flow cytometry

To further confirm this finding, in-situ observation of CD56+ cells (NK cells) in tumor tissue was performed using immunohistochemistry. Similar to the result obtained with flow cytometry, we found significantly fewer NK cells in cancer lesions than in cancer-adjacent tissues (Fig. 2). The calculated cell numbers were (2.3±2.6) cells/visual field and (8.5±4.5) cells/visual field, respectively ($P < 0.001$, $n = 62$).

### Correlation of NK cell infiltration density in cancer lesions to prognosis

On the basis of the infiltration density of $CD56^+$ cells in cancer lesions, we assigned patients to a higher-density group and a lower-density group, with different percentiles as the cut point. The log-rank method was used to reveal the difference in disease-free survival (DFS) and overall survival (OS) between two groups; the corresponding *P* values are shown in Figure 3. Within a wide range of cut points (30%–40% as cut points for the comparison of DFS and 10%–40% as cut points for the comparison of OS), the *P* values indicated significance, suggesting good reproducibility.

Considering the cut point that produced the lowest *P* value,[3,9] the integer cell count, and a goal of a similar number of patients in the two groups, we selected the 40th percentile as the cut point for assigning patients into a group with higher NK cell infiltration density (≥1 cell/visual field) and a group with lower NK cell infiltration density (<1 cell/visual field). The analysis suggested that NK infiltration density in cancer lesions was

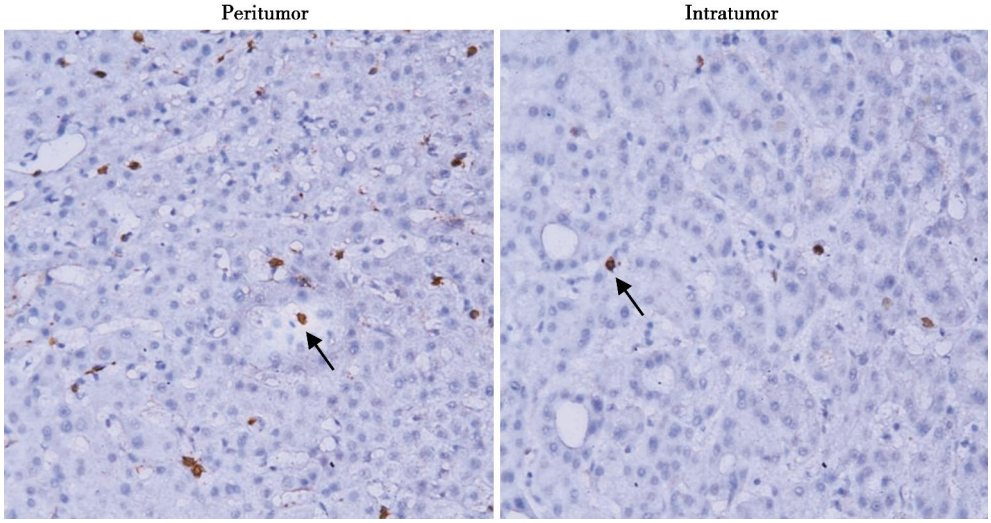


Figure 2 $CD56^+$ (NK) cell infiltration in different HCC tissues (immunohistochemistoy ×400)
The arrow denotes a representative $CD56^+$ cell.

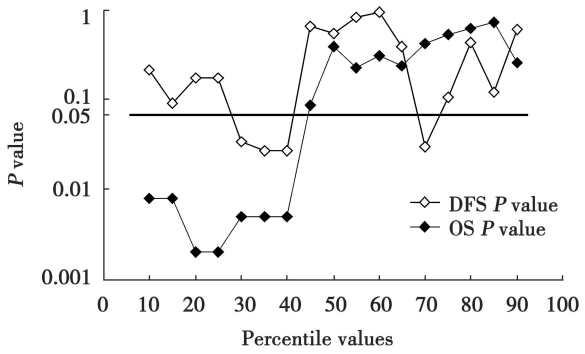


Figure 3 Survival analyses using different percentile values as cut-offs

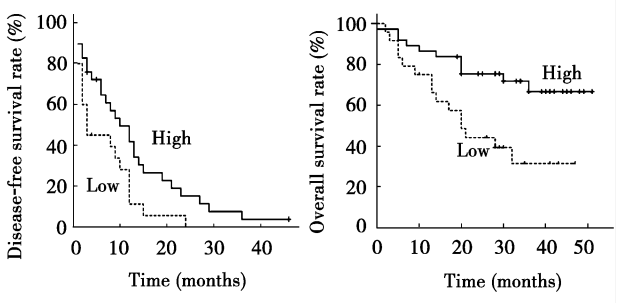


Figure 4 Survival curves of the HCC patients with high or low density of NK cells
DFS,disease-free survival; OS,overall survival.

positively related to DFS and OS ($P$ = 0.027 and $P$ = 0.005) (Fig. 4). The 3-year DFS and OS in the lower-infiltration-density group were 0% and 32%, respectively, which were lower than those in the higher-infiltration-density group (3% and 64%, respectively). When compared to patients with lower NK cell infiltration density in the cancer lesion, those with higher NK cell infiltration density enjoyed longer DFS (median: 3 months vs. 9 months) and OS (median: 20 months vs. 30 months). However, NK cell infiltration density in cancer-adjacent tissue was not significantly related to DFS or OS, regardless of the percentile selected as the cut point (data not shown).

### NK cell infiltration density in cancer lesions as an independent prognostic factor

We introduced all the variables with a $P$ value <0.05, including TNM staging, tumor capsule, cancer embolus, hepatitis B, and NK cell infiltration density, into a Cox regression model for multivariate analysis. The results revealed that, in addition to TNM staging and cancer embolus, NK cell infiltration density in cancer lesions was an independent prognostic factor for OS rate ($P$ = 0.019); however, unlike TNM staging and cancer embolus, it was not an independent influential factor for DFS rate.

## Discussion

In this study, we selected HCC patients as study subjects to investigate the infiltration distribution of natural killer cells (NK cells) in cancer tissue and to analyze the impact of this distribution on the survival and prognosis of these patients. The percentage of NK cells in TIL was significantly lower than that in NIL. In-situ observation also revealed significantly fewer NK cells in cancer lesions than in cancer-adjacent tissues. The NK cell infiltration density in cancer lesions was positively related to DFS and OS, whereas NK cell infiltration density in adjacent tissue was not. Further analysis with a multivariate model revealed that NK cell infiltration density in the cancer lesion was an independent prognostic factor for OS. Therefore, NK cells infiltrating the cancer lesion provided important protection for patients with HCC, but the tumor influenced the infiltration and distribution of NK cells.

We found that HCC patients with lower NK cell infiltration

Table 1 Cox regression prognosis analysis for the hepatocellular carcinoma patients

| Varible | Disease-free survival | | | | Overall survival | | | |
|---|---|---|---|---|---|---|---|---|
| | Univariate | Multivariate | | | Univariate | Multivariate | | |
| | *P* | HR | 95% CI of HR | *P* | *P* | HR | 95% CI of HR | *P* |
| HbsAg (positive/negative) | 0.031 | 5.055 | 0.665-38.445 | 0.118 | | | | |
| Fibrous capsule (present/absent) | | | | | 0.017 | 0.773 | 0.284-2.100 | 0.613 |
| TNM stage (Ⅰ+Ⅱ/Ⅲ+Ⅳ) | <0.001 | 0.354 | 0.182-0.687 | 0.002 | 0.001 | 0.341 | 0.145-0.801 | 0.014 |
| Vascular invasion (present/absent) | <0.001 | 0.328 | 0.144-0.746 | 0.008 | <0.001 | 0.259 | 0.098-0.685 | 0.007 |
| Intratumoral $CD56^+$ cells (high/low) | 0.027 | 1.313 | 0.686-2.512 | 0.411 | 0.005 | 2.658 | 1.172-6.027 | 0.019 |

density in the cancer lesion had significantly lower 3-year survival rates, and that both DFS and OS were remarkably lower in the group with lower NK cell infiltration density than in the group with higher NK cell infiltration density. These findings indicate that lower NK cell infiltration density is an independent negative prognostic factor. These findings are consistent with the results from studies on various solid tumors, including colon cancer,[5] lung adenocarcinoma,[6] gastric cancer,[7] and squamous cell lung cancer,[8] which also showed that higher NK cell infiltration into tumors is related to better prognosis and higher survival rates in patients. Although no study has reported a correlation between NK cell density in HCC tissue and prognosis, evidence from a study on peripheral NK cells in HCC supports our results: Taketomi et al.[10] found that peripheral NK cell activity could help predict tumor recurrence and prognosis after hepatic resection. These findings clearly show that the natural cytotoxicity of NK cells has an important role in the host anticancer mechanism. Immune surveillance of tumors by NK cells has been shown in various types of studies.[11] For example, NK cells killed a number of tumor cell lines in an in vitro study, and numerous studies with animal models have shown that NK cells provide resistance against tumors. Direct evidence that NK cells target human cancer cells came from studies on the mechanism of NK cells' cytocidal effect on tumor cells in primary culture; some clinical studies with NK cell-based immunotherapies, including stem cell transplantation and adoptive transfer of NK cells into patients with cancer, also showed that NK cells can target tumors.

Our study also found a significant differential distribution of NK cell infiltration in primary HCC tissue, with a lower percentage and density of infiltrating NK cells in cancer lesions than in adjacent tissues. This is consistent with results reported by other investigators,[12] indicating that, instead of contacting target cells directly, NK cells aggregate in the tumor mesenchyme. Although the reason for the insufficient numbers of NK cells in cancer tissue is still not clear, more and more evidence suggests that the tumor microenvironment affects the infiltration and function of NK cells. When circulating NK cells are activated to flow out of the bloodstream and infiltrate tissue containing malignant cells, they have to go through the endothelium and its basement membrane. Some scientists believe that extracellular matrix proteins might prevent the direct contact of NK cells and tumor cells.[13] In the extravascular space, NK cells might encounter a complicated cytokine microenvironment and interact with numerous kinds of immune cells;[14] on the other hand, biological and chemical conditions in solid tumors, such as hypoxia, low pH level, and low glucose, could downregulate the activity of NK cells.[15] Oxidative radicals produced by monocytes in a respiratory burst can even induce apoptosis in NK cells.16 From these results, we deduce that tumors have developed multiple mechanisms to escape the cytotoxicity of NK cells.

In conclusion, our study found that a low infiltration density of NK cells in cancer lesions is related to poor prognosis in patients with HCC, indicating that NK cells have important roles in fighting tumors and prolonging survival. We also found that far fewer NK cells infiltrated cancer lesions than cancer-adjacent tissues, suggesting that tumors counteract NK cells by changing the local microenvironment and thus escaping their cytotoxicity. How to increase the number of infiltrating NK cells in cancer lesions should be explored further in future studies in order to develop possible NK cell-based immunotherapies.

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