Anti-PD-1 Therapy Reverses TIGIT + CD226 + NK Depletion in Immunotherapy Resistance of Hepatocellular Carcinoma through PVR/TIGIT Pathway

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Research Article

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Abstract

**Background:** Immunotherapy combined with targeted therapy significantly improved the prognosis of patients with hepatocellular carcinoma (HCC). Immunotherapy resistance conducts the main reason for the failure of PD-1-based immune checkpoint inhibitors (ICIs). The gist of this study was to clarify the mechanism of Nature kill cells (NK) depletion in immunotherapy resistance of HCC.

**Methods:** Cancerous /paracancerous tissues and peripheral blood (PB) of 55 patients with HCC treated in our hospital from Sep. 2019 to Sep. 2021 were collected. Patients were grouped according to the degree of differentiation, FCM, IHC, and in vitro lymphocyte culture drug intervention experiments were used to determine the degree of NK cell depletion. Furthermore, a novel mouse model of HCC in situ was constructed and divided into different groups according to the various intervention measures of ICIs. Immunofluorescence thermography was used to observe the changes in tumor burden for survival analysis.

**Results:** NK cells in cancerous tissues significantly up-regulated the expression of TIGIT compared with paracancerous tissues (P < 0.001). FCM for PB indicated a more severe depletion of NK cells and higher expression of TIGIT and PD-1 in poorly differentiated HCC (P < 0.001). Intervention experiments in vitro revealed that expression of TIGIT and PD-1 decreased gradually with the increase of PD-1 inhibitor dose in moderately-highly differentiated patients (P < 0.05), while there was no change in poorly differentiated patients. Animal experiment showed that the proliferation of tumors in the experimental group was significantly inhibited compared with the control group after being blocked by PD-1 through immunofluorescence thermography, meanwhile, WB results of tumor tissues indicated that ICIs effectively decreased the expression of TIGIT, increased the expression of CD226, decreased the expression of PVRL1 protein and increased the expression level of PVRL3 protein.

**Conclusion:** TIGIT\(^+\)NK cells competitively bind to PVR with activator receptor CD226, thus promoting NK cell depletion. Anti-PD-1 efficiently decreases the expression of PVRL1 through the PD-1/PD-L1 pathway, promoting the independent endocytosis of PVRL3 and PVR binding, reducing the PVR/TIGIT inhibitory signal pathway, and enhancing the function of PVR/CD226 activation signal, which provides a theoretical basis for improving the immunotherapy resistance of HCC.

**Introduction**

The incidence of primary hepatocellular carcinoma (HCC) represents steadily increasing on a global scale, emerging as a significant threat to public health and safety. Projections from relevant research indicate that by 2025, there will be over one million new cases of HCC annually \(^{(1)}\). In China, a country with a high burden of liver disease, situation remains even more critical. HCC ranks fifth in terms of incidence among all malignant tumors in China, and most patients are often diagnosed at an advanced stage of the disease. The median survival period for these patients is less than 2 years, resulting in HCC being the second leading cause of cancer-related mortality \(^{(2)}\).
Immune cell depletion represents an important part of immune escape in tumorigenesis and development. Immune checkpoint blocking has become the most promising treatment for reversing immune cell depletion (3). Antibodies against cytotoxic T lymphocyte associated antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1) have achieved amazing success in the treatment of a variety of cancers (4). However, in patients with advanced HCC, the objective response rate (ORR) to PD-1/PD-L1 immunotherapy is only around 16%-20%. Moreover, it does not extend the survival of patients who previously received sorafenib treatment. The PD-L1 combined with bevacizumab (T + A regimen) has significantly improved the survival benefits for advanced, unresectable HCC patients recently. However, Asian HCC patients do not exhibit an ideal ORR to immune checkpoint inhibitors (ICIs) therapy (5). Immune therapy resistance and the lack of effective predictive clinical efficacy biomarkers are the main reasons for the failure of PD-1-based immune checkpoint blockade therapy (6, 7).

Natural killer (NK) cells are cytotoxic lymphocytes in the innate immune system that can kill viral infections and/or cancer cells. They are a highly heterogeneous cell type and play a crucial role in cancer immunity, which possess cytotoxic activity similar to CD8⁺ T cells (8). Unlike CD8⁺ T cells, NK cell activity does not depend on antigen processing and presentation. Immature NK cells exhibit high proliferation rates but are functionally impaired. In contrast, mature NK cells are highly functional effector cells, indicating that NK cells must be in a mature state to exert their cytotoxic functions (9). At present, the field of cancer therapy based on NK cells is growing exponentially, and it has become the main field of tumor immunotherapy innovation (10). However, the specific mechanism remains unclear. Studies indicate that NK cell reactivity is regulated through a balance between co-stimulatory and co-inhibitory signals. T cell immunoglobulin and ITIM domain T-cell immuno-receptor with Ig and ITIM domains (TIGIT) are coinhibitory receptors which expressed specifically in T cells and NK cells, is a co-inhibitory receptor (11). It is currently unclear whether TIGIT is suitable as a novel target for immunotherapy in HCC.

The purpose of our study is to determine the effect of PD-L1 antibody on the immune mechanism of HCC mediated by TIGIT⁺NK cells through PD-1 / PD-L1 signal pathway and whether it has additional value in reversing the depletion of hepatocellular carcinoma tumor NK cells and restoring their immune response against HCC.

**Methods**

**Ethics approval**

The study was performed under a project license (No. 2019-D.-304) granted by the Ethics Committee of Beijing Chaoyang Hospital and complied with the institutional guidelines for the care and use of animals. All procedures in this study involving human participants were performed in accordance with the ethical standards of the institutional research committee (No. 2020-D. -301) and the 1964 Helsinki Declaration.

**Patients’ selection**
Peripheral blood and fresh hepatocellular carcinoma and adjacent tissue specimens were collected from 55 patients with HCC between Sep. 2019 and Sep. 2021 in our hospital. Patients were informed about the experimental procedures and protocols before specimen collection, and their information was included in the candidate database after obtaining signed informed consent.

**Inclusion and Exclusion Criteria**

**Inclusion criteria:** (1) Patients with HCC underwent surgery from Sep. 2019 to Sep. 2021. (2) No restriction was imposed on age and gender. (3) No distant metastasis. (4) Underwent en bloc resection during surgery. (5) Postoperative pathology confirming primary hepatocellular carcinoma. (6) Clinical and follow-up information was complete.

**Exclusion criteria:** (1) Unabled to resect the tumor during surgery. (2) Patients with HCV or alcohol-related liver disease (ALD). (3) Patients who received radiotherapy, chemotherapy, or immunotherapy before or after surgery. (4) Lost follow-up after surgery.

**Experimental animals**

Male BALB/c mice aged 6–8 weeks (weighing 20-22g) were selected for the study. These mice were purchased from Beijing Weitonglihua Experimental Animal Co., Ltd. They were housed in a specific pathogen-free (SPF) animal facility within the Medical Research Center of Beijing Chaoyang Hospital, Capital Medical University. The housing conditions were controlled as follows: fresh air, clean environment, humidity maintained at 50%-60%, constant temperature, noise level below 85 decibels, and provided with standard rodent feed and drinking water. The H22 hepatocellular carcinoma cell line used in this experiment was obtained from the Peking Union Medical College Cell Bank. The experiment strictly adhered to the ethical guidelines of the Institutional Animal Care and Use Committee of Capital Medical University.

**Establishment of the in situ HCC mouse model and perioperative management**

Prior to each modeling session, a total of 20 healthy male BALB/c mice were selected and fasted for 10 hours while deprived of water for 4 hours. Anesthetization was achieved by administering 0.1 ml/10g of 4% chloral hydrate via intraperitoneal injection, supplemented with gas anesthesia. Each mouse intended for modeling was fitted with a marked ear tag. During surgery, the mice were placed in a supine position, and the abdomen was routinely sterilized and draped. A 1cm incision was made just below the xiphoid process in the upper abdomen. The skin and peritoneum were incised layer by layer. Sterile gauze soaked in physiological saline was placed above and below the incision, and gentle pressure was applied to both sides of the ribcage to expose the liver lobes on the damp gauze. A 50 µl microsyringe was used to inject H22 tumor cells co-labeled with CFSE parallel to the long axis of the liver and at a 45-degree angle to the liver surface. The needle was inserted approximately 0.5 cm parallel to the liver's surface, depending on the fullness and thickness of the mouse liver lobes. The tumor cells were injected slowly until the liver lobes turned pale. The needle was quickly withdrawn, and mild pressure was applied to the needle site.
with a cotton swab for 5 minutes. After confirming no active bleeding, the abdominal wall was sutured.
Postoperatively, the mice were placed on a warming pad for observation until they could move freely. They were deprived of food and water for 4 hours, after which 0.3% glucose water was provided for rehydration. Normal feeding and watering resumed after 12 hours. This modeling process was repeated for 20 mice, and ear tag numbers were recorded post-modeling. Euthanasia was performed by anesthesia, followed by abdominal exploration to obtain tissue, and then exsanguination through the inferior vena cava.

laboratory detection

1) Peripheral Blood and Tumor Tissue Flow Cytometry Analysis

For flow cytometry analysis of peripheral blood in hepatocellular carcinoma patients, the following anti-human antibodies were used to analyze the expression of surface and intracellular markers in peripheral blood mononuclear cells (Table 1), while the flow cytometry analysis of peripheral blood, liver, and spleen lymphocytes in mice used the following anti-mouse antibodies to analyze the expression (Table 2). Samples were taken into flow cytometry tubes, labeled with respective identifiers, and then mixed with antibodies and samples. After adding 1 ml of red blood cell lysis solution, the samples were gently shaken and incubated in the dark for 15 minutes. Following incubation, the samples were centrifuged, and 750 µl of PBS was added before being prepared for analysis.
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2) Immunohistochemical Staining of Tumor Tissues

Pathological tissue specimens of hepatocellular carcinoma and adjacent non-cancerous tissues from patients were collected and prepared as slides. The staining process involved binding of the primary antibody to the target protein antigen in the tissue, followed by binding of the secondary antibody labeled with markers like HRP. Subsequently, a reaction with DAB chromogen was carried out, followed by slide sealing. The stained slides were observed under a microscope, and photographs were taken. The evaluation of staining was performed in a blinded manner, with two experienced researchers independently scoring the immunohistochemical staining.

Specific scoring method: Under high magnification (400X), three representative areas of hepatocellular carcinoma tumor tissue and adjacent non-cancerous tissue were randomly selected, and 100 cells were scored for staining: no staining, scored as 0; weak staining, scored as 1; moderate staining, scored as 2; strong staining, scored as 3. Overall staining assessment: 0–30 points were considered negative; 30–100 points were considered weakly positive; 100–300 points were considered strongly positive.

3) Western Blotting for Protein Expression Analysis

Western blotting was employed to detect the protein expression levels. In this method, proteins were transferred onto a membrane, and their expression of the target protein was detected by reacting with
known antibodies. Denatured proteins were subjected to polyacrylamide gel electrophoresis for separation and then transferred to the membrane. This membrane forms a covalent bond with the proteins without disrupting their peptide types and biological activities. The proteins or peptides on the membrane, acting as antigens, were firmly bound to known primary antibodies (Anti-PVR Rabbit Antibody, Anti-PVRL1 Rabbit Antibody, Anti-PVRL2 Rabbit Antibody, Anti-PVRL3 Rabbit Antibody, Anti-PVRL4 Rabbit Antibody, Anti-TIGIT Rabbit Antibody, Anti-PD-1 Rabbit Antibody, Anti-CD96 Rabbit Antibody, Anti-CD226 Rabbit Antibody, Anti-CD16 Rabbit Antibody) through immune reactions. Subsequently, they were reacted with secondary antibodies targeting the primary antibodies, and the protein levels were detected through substrate chromogenic reactions.

4) Peripheral Blood Cultures and Intervention Experiments in HCC Patients

2ml of peripheral blood samples were collected from HCC patients and placed in anticoagulant tubes containing EDTA. The samples were labeled with the patient's name, gender, age, differentiation degree, and other basic information. In a sterile workbench, 4ml of Ficoll lymphocyte separation solution was added to a 15ml centrifuge tube. Then, at a ratio of 1:2, the patient's 2ml peripheral blood was slowly added to the 15 ml centrifuge tube. This tube was placed in a low-temperature, high-speed centrifuge with the following parameters: 1800 rpm, 4°C, acceleration 6, and deceleration 0. After centrifugation for 38 minutes, the middle-layer lymphocytes were aspirated, and PBS solution was added to reach a volume of 10 ml. The tube was then centrifuged at 1000 rpm for 5 minutes, and the supernatant was discarded. Next, 10% FBS RPMI-1640 was added to reach a volume of 10 ml, and the tube was centrifuged at 1000 rpm for 5 minutes. Cell counts were performed, and 1 ml of cell suspension and 1 ml of 10% FBS RPMI-1640 were added to three wells in a 6-well plate. The cells were counted, and each well was labeled as the control group, experimental group 1, and experimental group 2, respectively. Then, 5µl of PBS, 5µl of PD-1 antibody, and 50µl of PD-1 antibody were added to their respective wells. The plate was gently shaken in a figure-eight pattern and kept for subsequent analysis.

5) Immunofluorescence Imaging of H22 Orthotopic Tumor-Implanted Mice Stained with CFSE

Mice with orthotopic H22 tumors stained with CFSE from the same batch were randomly grouped according to their ear-tag numbers seven days after tumor implantation. Intervention was carried out, and subsequently, at intervals of seven days (i.e., 7, 14, 21, and 28 days after inoculation), an In-Vivo FX PRO small animal live imaging system was used to observe tumor burden and morphological changes in mice from different groups. In the initial step, white light calibration was performed to determine parameters such as f-stop, focus, FOV (Field of View), binning, and other settings. These parameters were adjusted to achieve the optimal mode. White light wavelength was set at (2.000 Sec_120.00mm_Excitation Filter:0_Emission Filter:0), and it was used to capture the overall morphology of the mice. Afterward, while maintaining the mouse's position, fluorescence imaging was initiated. Fluorescence wavelength was set at (2.000 Sec_120.00mm_Excitation Filter:530_Emission Filter:600), which allowed capturing the size and
activity of tumors within the mouse. Subsequently, image overlay mode was applied to generate fluorescent images. This facilitated the comparison of changes in fluorescence values representing tumor burden within the same group of mice, as well as differences in tumor size and fluorescence peak values among different groups. Statistical analysis was performed to interpret the results.

6) Statistical Analysis

For normally distributed continuous data, mean ± standard deviation is presented, while non-normally distributed data is expressed as median (interquartile range). The comparison of continuous data between two groups, when normally distributed, was performed using the t-test, and when non-normally distributed, was conducted using the rank-sum test. Comparison of categorical data between two groups was carried out using the chi-square test, and when the expected frequency was less than 1, Fisher's exact probability test was applied. For three or more independent samples, one-way analysis of variance (ANOVA), LSD-t method, and SNK method were used for multiple comparisons. Image acquisition was performed using confocal and fluorescence microscopy, with a significance level set at P < 0.05, indicating statistical significance. All data were analyzed using SPSS 24.0 software.

Results

In order to elucidate the immunosuppressive role of NK cells during the development of hepatocellular carcinoma and the impact of the interaction between activating and inhibitory receptors on NK cell exhaustion, we first collected peripheral blood samples and fresh tissue samples (tumor and adjacent non-tumor tissues) from 55 HCC patients treated at our hospital from Sep. 2019 to Sep. 2021. We analyzed the changes in the expression levels of PD-1 and TIGIT signaling molecules on the surface of NK cells during the progression of hepatocellular carcinoma.

1. Patients with poorly differentiated exhibited higher levels of positive TIGIT expression in HCC tumor tissues.

In comparison to adjacent non-cancerous tissues, tumor tissues had a higher number of patients with strong positive expression of TIGIT through immunohistochemistry analysis. In contrast, TIGIT expression in adjacent non-cancerous tissues was either absent or weak. Moreover, when examining staining results in HCC tumor tissues and adjacent non-cancerous tissues based on different degrees of differentiation, it was evident that patients in the poorly differentiated group exhibited higher levels of TIGIT expression in tumor tissues compared to those with moderate to high differentiation, with no significant differences observed in adjacent non-cancerous tissues (Fig. 1).


Flow cytometry results showed that in HCC patients, NK cells in peripheral blood significantly expressed PD-1 and TIGIT on their surfaces (Fig. 2). After grouping the patients based on differentiation levels, it was observed that in comparison to moderately to highly differentiated patients, poorly differentiated
HCC patients had lower expression levels of the activation receptor CD226 on the surface of NK cells. Moreover, as the differentiation grade worsened, the expression levels of CD226^PD-1^ NK cells decreased (P < 0.01). In contrast, concerning the inhibitory receptor TIGIT, poorly differentiated HCC patients had higher expression levels of CD226^TIGIT^ NK cells compared to moderately to highly differentiated patients (P < 0.001).

3. The trend of changes in CD8^+^ T cells and NK cells was consistent across different levels of differentiation.

We determined the absolute lymphocyte count in the peripheral blood of HCC patients, which indicated that the absolute count of NK cells was lower in poorly differentiated patients compared to moderately/highly differentiated patients (P < 0.001). Furthermore, when comparing patients with different levels of differentiation, we observed a consistent trend in the changes of CD8^+^ T cells and NK cells across different levels of differentiation (P < 0.0001). However, there was no significant statistical difference in the expression levels of CD4^+^ T cells (P > 0.05) (Fig. 3).

4. The expression level of TIGIT in poorly differentiated patients does not decrease with an increase in drug dosage.

Furthermore, to investigate potential methods for reversing the state of NK cell exhaustion in liver cancer patients and to enhance the intrinsic anti-tumor immune effect and secretion function of NK cells, we cultured PBLCs extracted from peripheral blood of HCC patients with different degrees of differentiation, establishing control and experimental groups. The experimental groups were treated with 5μL and 50μL of PD-1 inhibitors, respectively, for intervention experiments, and observed the expression of NK cell surface activating/inhibitory receptors. Our study found that in moderately/highly differentiated patients, the application of PD-1 inhibitors resulted in a significant decrease in the expression of the inhibitory receptors PD-1 and TIGIT on the surface of NK cells compared to the control group (P = 0.0286, P = 0.0253). In moderately differentiated groups, with increasing drug dosage, the expression level of the inhibitory receptor PD-1 decreased more significantly than TIGIT (P = 0.0015, P = 0.0224). In poorly differentiated groups, with increasing drug dosage, the expression level of the inhibitory receptor PD-1 further decreased (P = 0.0436), while the expression level of TIGIT showed no significant change in the low-dose group (P > 0.05) (Fig. 4). These results may suggest that in patients with poorly differentiated advanced liver cancer, the degree of NK cell exhaustion is more severe. Furthermore, the inhibitory receptor TIGIT, compared to PD-1, may be a better indicator of NK cell exhaustion in poorly differentiated HCC patients.

Meanwhile, in our in vitro experiments, the application of a PD-1 inhibitor resulted in a reduction in the expression levels of the inhibitory receptor TIGIT on the cell surface and an increase in the expression of the activation receptor CD226. This effectively reversed the state of NK cell exhaustion. To further investigate the underlying causes and molecular signaling mechanisms responsible for this
phenomenon, we established an in vivo liver cancer orthotopic implantation mouse model for subsequent in vivo experiments and molecular mechanism exploration.

5. The experimental group of mice showed no significant variation in fluorescence peak values of tumor burden over time, whereas the control group of mice exhibited a poorer survival prognosis.

After modeling orthotopic liver cancer in mice, we administered PBS and a PD-1 inhibitor separately on the 7th day. We conducted live immunofluorescence imaging every 7 days to monitor changes in tumor burden and peak values in thermal tumor imaging. Additionally, we defined a tumor size of $\geq 1000 \text{ mm}^3$ as an indicator of death in the imaging assessment. Survival statistics were conducted for mice in different treatment groups. Our results demonstrated that mice in the control group exhibited poor tumor growth and proliferation, with a significant increase in fluorescence peak values over time ($P < 0.0001$). In contrast, mice in the experimental group showed minimal changes in tumor burden, and the variation in fluorescence peak values over time was not statistically significant ($P > 0.05$). This suggests that the application of a PD-1 inhibitor effectively inhibited the progression of liver cancer. Furthermore, survival analysis revealed that the control group mice had a worse prognosis, reaching the criteria for death around 2 weeks post-surgery, while the survival status of the experimental group mice significantly improved (Fig. 5).

6. Expression of CD107a and CD155 in peripheral blood and tissue NK cells in multiple groups of mice.

We conducted flow cytometry analysis on peripheral blood, liver, and spleen tissues collected from the experimental and control group mice. The results indicated that, compared to the control group, the experimental group exhibited higher levels of the activation signal molecule CD107a on peripheral blood NK cells ($P < 0.01$). Conversely, the content of the inhibitory signal molecule CD155 in tumor cells was significantly reduced, even approaching non-expression ($P < 0.0001$). Similar results were observed in the liver tissue. In the experimental group, the expression of CD107a in liver tumor tissues was significantly higher compared to the control group, while CD155 content showed a significant decrease ($P < 0.01$, $P < 0.0001$). In the spleen tissue analysis of both experimental and control groups, there were no significant differences observed in either the CD107a signal molecule, representing NK cell activation, or the CD155 receptor, which inhibits NK cell activity ($P > 0.05$) (Fig. 6).

7. The experimental group showed increased expression of CD226, while PD-1, TIGIT, and CD96 exhibited decreased expression compared to the control group.

We conducted WB assays on PBMC from HCC patients and liver cancer tissues from in situ mice model. In the PBMC, the WB results showed that the expression of the activating receptor CD226 was significantly higher in the experimental group compared to the control group ($P < 0.001$). On the other hand, the inhibitory receptors PD-1, TIGIT, and CD96 were all lower in the experimental group compared to the control group ($P < 0.05$). With increasing dosage of ICIs, the inhibitory receptors PD-1, TIGIT, and CD96 in the moderately to highly differentiated group showed a further decrease ($P < 0.05$). However, in the
poorly differentiated group, the expression of the inhibitory receptor TIGIT did not significantly decrease with increasing dosage (P > 0.05) (Fig. 7).

8. PD-1 inhibitors effectively reduce the expression levels of PVRL1 and PVR.

In the mouse model of HCC in situ, WB analysis of PVR, PVRL1, PVRL2, PVRL3, and PVRL4 protein expression was conducted. The results revealed high expression of PVRL1, PVRL2, and PVR in the mouse liver cancer tissue, while the expression of PVRL3 and PVRL4 was absent. Upon intervention with PD-1 inhibitors in the experimental group of mice, we observed a significant decrease in the expression levels of PVRL1 and PVR (P < 0.001), while the expression of PVRL3 increased (P < 0.05). This suggests that through a certain mechanism, the intervention of PD-1 inhibitors may reduce the expression of PVRL1 and PVR (Fig. 7).

Discussion

Hepatocellular carcinoma (HCC) most commonly arises from chronic viral inflammation, such as hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, excessive alcohol consumption, exposure to aflatoxin B1, obesity-related non-alcoholic fatty liver disease, type II diabetes, and exposure to environmental toxins. HCC ranks as the fourth leading cause of cancer-related deaths globally (12, 13). Prolonged chronic HBV infection can lead to cirrhosis and advanced-stage HCC (14). Treatments such as liver transplantation, surgical resection, or radiofrequency ablation can be used for early-stage HCC, but these therapies often come with high recurrence rates. For mid-stage cases, transarterial chemoembolization or radioembolization may be utilized, yet the overall survival remains less than 20 months. Moreover, HCC is often diagnosed at advanced stages when treatment options are limited, and any of the above-mentioned options are associated with poor prognosis (15). Due to tumor heterogeneity and the lack of suitable treatment strategies, advanced-stage HCC remains difficult to cure (16). The molecular mechanisms driving HCC development are complex and not fully understood (17). Therefore, immunotherapy has gradually become an important field in the treatment and research of HCC.

Cancer immunotherapy typically involves harnessing the patient's own immune system to eradicate tumors. CTLA-4 and PD-1 were the first two inhibitory immune checkpoints discovered, with their function being to block the anti-tumor immune response of T-cells. Encouragingly, monoclonal antibodies blocking these inhibitory immune checkpoints can reactivate the anti-tumor immune response of tumor-infiltrating T-cells (18–20). The liver, as a naturally immune-tolerant organ, has a unique immune anatomical structure that contributes to the establishment of an immunosuppressive microenvironment (21). However, the immunobiology of liver cancer, its impact on the molecular mechanisms of the immune system, and tumor-associated immune checkpoint signals exert a profound inhibitory effect on this microenvironment. Liver cancer is an inflammation-driven disease and may result from virus infection-related inflammation, liver fibrosis, and cirrhosis. Integration of HBV-DNA frequently occurs in HBV-related liver cancer patients (22).
Currently, the U.S. Food and Drug Administration (FDA) has approved only two drugs for advanced-stage HCC: multikinase inhibitors and immune checkpoint inhibitors. While oral multikinase inhibitors (sorafenib, regorafenib, and lenvatinib) can extend the median overall survival by less than 4 months (23, 24), PD-1 immune checkpoint inhibitors (nivolumab and pembrolizumab) offer promising clinical benefits in various malignancies, including liver cancer. However, only a subset of HCC patients derives clear and sustained clinical survival benefits from PD-1 blockade therapy (25). While the true potential of immunotherapy is being actively explored, better understanding of the mechanisms underlying its ineffectiveness in most HCC patients is crucial for the development of effective interventions for liver cancer.

Pathways responsible for anti-tumor immune responses tend to activate immune inhibitory mechanisms to counterbalance excessive immune activity. Effector T cells and NK cells released by anti-PD-1/CTLA-4 tend to elevate interferon-gamma (IFN-γ) levels within the tumor, contributing to anti-tumor activity. However, high concentrations of IFN-γ can further promote the exhaustion of NK cells and T cells, and upregulate the expression of their inhibitory immune checkpoints. For example, IFN-γ induces the expression of galectin-9 (GAL-9) in tumor cells and GAL-9 receptors, T-cell immunoglobulin and mucin domain 3 (TIM-3), in effector T cells. The binding of GAL-9 to TIM-3 results in cell cycle arrest and defective cytokine production in effector T cells (26, 27). Currently, there is a lack of research and reports on how the blockade effect after applying PD-1 inhibitors affects the exhaustion status of NK cells and T cells in HCC. Understanding the mechanisms of the effects of PD-1 blockade on reshaping the HCC tumor immune microenvironment, T cell trafficking, and the degree of HCC differentiation may provide a theoretical basis for better designing combination therapies to enhance the efficacy of anti-PD-1 treatment.

The density of NK cells in tumor tissue is positively correlated with prognosis. This was first described in colorectal cancer(28) and has since been reported in many other types of cancer, including hepatocellular carcinoma, lung adenocarcinoma, gastric cancer, and renal cancer, among others (29–34). NK cells are major effector cells of innate immunity. They can eliminate tumor cells and virus-infected cells, primarily due to their two essential effector functions: cytotoxicity and cytokine secretion. While direct killing of tumor cells by NK cells may help reduce the tumor burden and improve prognosis, the density of NK cells in solid tumor tissues is generally low (fewer than 100 cells per square millimeter). The liver contains abundant NK cells that participate in immune surveillance and attack tumor cells through cytotoxic actions. However, the number of NK cells in tumor tissue is lower than in normal tissue, and tumor-infiltrating NK cells exhibit a low-activation state, severely impairing their tumor cell-killing capacity and cytokine-producing ability (35). Unlike CD8⁺ T cells, NK cells do not undergo somatic DNA rearrangement during development to generate receptor diversity but instead detect missing or altered "self" via a series of germline-encoded inhibitory and activating receptors (36). This unique capability allows NK cells to directly target cancer cells with low or absent HLA class I expression, which are no longer recognized by T cells and thus are refractory to checkpoint inhibitors. NK cells recruit stimulatory dendritic cells (DCs) at the tumor site through the release of the cytokine Flt3 ligand, which, in turn, stimulates cytotoxic T cells to
promote anti-tumor responses (37). NK cells, in addition to their cytotoxic actions, produce various cytokines, with interferon-gamma (IFN-γ) being the most important. IFN-γ plays a role in immune responses by regulating the activation of other immune cells. Studies have shown that NK cells have vital physiological roles in host defense against pathogenic infections through IFN-γ secretion. Furthermore, NK cells within tumors can produce chemokines CCL5 and XCL1 to recruit conventional type 1 dendritic cells (cDC1) subpopulations, which can effectively capture tumor cell antigens and activate CD8⁺ T cells. The presence of high cDC1 and NK cell gene signals is positively correlated with the survival of cancer patients. Therefore, NK cells play a crucial role in both direct cytotoxicity against tumors and in recruiting and priming other cell subpopulations to enhance immune attacks (38).

The activation of NK cells is tightly regulated by a range of activating and inhibitory receptors expressed on their surface. Activating receptors on NK cells include NKG2D, 2B4, NKP30, NKP44, NKP46, and CD226, among others. NK cells also express various inhibitory receptors that primarily recognize major histocompatibility complex class I (MHC I) molecules to protect the host from NK cell attack. NK cells also express non-MHC class I molecule inhibitory receptors, the functions of which are still not well understood. Recently, T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif (ITIM) domain (TIGIT) with Ig and ITIM domains has emerged as another important inhibitory immune checkpoint. TIGIT is a surface protein with a cytoplasmic domain that contains typical ITIM and immunoglobulin tyrosine tail motif (ITTM). TIGIT expression is restricted to specific lymphocytes, primarily NK cells and CD4⁺ and CD8⁺ T cells. Poliovirus receptor (PVR), also known as CD155, is mainly expressed on the surface of DCs, fibroblasts, endothelial cells, and some tumor cells. The PVR family primarily includes PVRL1 (Nectin1 or CD111), PVRL2 (Nectin2 or CD112), PVRL3 (Nectin3 or CD113), and PVRL4 (Nectin3). Among them, PVR has the highest affinity for TIGIT, and TIGIT can also bind to some ligands with weaker affinity, such as PVRL2 (Nectin2 or CD112), PVRL3 (Nectin3 or CD113), and PVRL4 (Nectin3). Previous studies have suggested that TIGIT/PVR binding can inhibit the activation of NK cells and anti-tumor immune responses competitively by phosphorylating the ITIM of TIGIT's intracellular domain or by interfering with the binding of the activating receptor CD226 on the surface of NK cells to PVR.

Previous research has reported that, to elucidate the expression levels of the PVR family on tumor cells in hepatocellular carcinoma (HCC), studies were conducted using techniques such as qRT-PCR and immunohistochemistry. It was found that, compared to healthy individuals, PVRL1 is the only member of the PVR family that is overexpressed in human hepatocellular carcinoma. The overexpression of PVRL1 was further correlated with a poorer overall survival and disease-free survival (39). However, the underlying mechanisms and how it acts on the TIGIT/PVR axis, thereby affecting the anti-tumor immune efficacy of immune cells such as NK, CD4⁺, and CD8⁺ T cells, have not been deeply investigated. Nevertheless, our preliminary in vivo experiments have shown that TIGIT is highly expressed on the surface of NK cells in poorly differentiated hepatocellular carcinoma patients. The reversal of NK cell exhaustion after the application of PD-1 inhibitors and the changes in the expression levels of PD-1,
TIGIT, and CD226 on the surface of NK cells may be related to the TIGIT/PVR signaling pathway, and the alteration in the expression level of PVRL1 protein may play a role in this process.

We found in our study that, after the application of PD-1 inhibitors, the expression level of TIGIT on the surface of hepatocellular carcinoma NK cells decreased relative to the control group, while the expression level of CD226 increased. This suggests that the expression levels of activating receptors on the surface of NK cells and inhibitory receptors exhibit completely opposite changes after the application of PD-1 blockade, further indicating that the activating receptor CD226 gained an advantage in binding to the ligand PVR, reversing the exhaustion of NK cells, enhancing the direct cytotoxicity of NK cells against hepatocellular carcinoma tumor cells, and stimulating the activation of cytotoxic CD8+ T cells by recruiting stimulatory DCs. Although research on immune checkpoint inhibitors in anti-tumor immunity is rapidly growing, and many immune checkpoints have been proposed, understanding the mechanisms that guide the combination of different immune checkpoint inhibitors requires a theoretical foundation. Our study addresses this gap by highlighting the significant role of the TIGIT/PVRL1 pathway in the progression of hepatocellular carcinoma. Additionally, TIGIT represents a promising target against PD-1 inhibitor resistance, potentially enhancing the effectiveness of immunotherapy for hepatocellular carcinoma.

**Conclusion**

TIGIT+ NK cells competitively bind to PVR with activator receptor CD226, thus promoting NK cell depletion. Anti-PD-1 efficiently decreases the expression of PVRL1 through the PD-1/PD-L1 pathway, promoting the independent endocytosis of PVRL3 and PVR binding, reducing the PVR/TIGIT inhibitory signal pathway, and enhancing the function of PVR/CD226 activation signal, which provides a theoretical basis for improving the immunotherapy resistance of HCC.

**Declarations**

**Ethics approval and consent to participate**

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was performed under a project license (No. 2019-D.-304) granted by the Ethics Committee of Beijing Chaoyang Hospital and complied with institutional guidelines for animals’ care and use.

**Consent for publication**

All authors.

**Availability of data and materials**
The datasets used and/or analysed during the current study available from the corresponding author (Shao-cheng Lyu, Address: No. 8 Gongtinan Road, Chaoyang District, Beijing, PR China, 100020, E-mail: shaocheng0502@163.com Fax: 86-010-85231503;) on reasonable request.

Competing interests

Not applicable.

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Authors’ contributions

(I) Conception and design: JW, LZ; (II) Administrative support: RL, LZ; (III) Provision of study materials: BH, JW, YZ; (IV) Establishment of rat model: LY, JW; (V) Collection and assembly of data: JW, YZ, LZ, YG; (VI) Data analysis and interpretation: JW, LY, SP-C; (VII) Manuscript writing: All authors; (VIII) Final approval of manuscript: All authors

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work

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None.

Footnote

None.

References


Figures

Figure 1

(A) Immunohistochemical detection of TIGIT expression in HCC cancer and paracancerous tissues of different differentiation levels; (B) Statistical analysis of TIGIT expression levels in HCC cancer and paracancerous tissues of different differentiation levels.
Figure 2

(A) Flow cytometry analysis of PD-1 and TIGIT expression on peripheral blood NK cells from HCC patients with different degrees of differentiation; (B) Proportion of CD226^+PD-1^+/TIGIT^+ expression on peripheral blood NK cells from HCC patients with different degrees of differentiation.

ns: P > 0.05, no significant difference; ***P < 0.001; ****P < 0.0001.
Figure 3

(A) Absolute counts of peripheral blood NK cells in HCC patients with different degrees of differentiation; (B) Absolute counts of peripheral blood CD4$^+$ and CD8$^+$ T cells in HCC patients with different degrees of differentiation; (C) Statistical results of absolute counts of peripheral blood NK, CD4$^+$, and CD8$^+$ T cells in HCC patients with different degrees of differentiation.

ns: $P > 0.05$, no significant difference; ****$P < 0.0001$. 
Figure 4

(A) Flow cytometric analysis of PD-1/TIGIT expression levels on NK cells in the peripheral blood of HCC patients with different degrees of differentiation in control and experimental groups; (B) Statistical results of PD-1-positive expression levels on NK cells in the peripheral blood of HCC patients with different degrees of differentiation in control and experimental groups; (C) Statistical results of TIGIT-positive expression levels on NK cells in the peripheral blood of HCC patients with different degrees of differentiation in control and experimental groups.

ns: P > 0.05, no significant difference; *P < 0.05, **P < 0.01.
Figure 5

(A) Schematic representation of the treatment model for the H22 orthotopic hepatocellular carcinoma mouse model; (B) In vivo immune fluorescence detection images of mice from different groups; (C) Statistical graph of immune fluorescence peak values in mice from different groups; (D) Macroscopic images of tumor tissue in mice from different groups; (E) Survival curves of mice from different groups, with a tumor size $\geq 1000\text{mm}^3$ considered as death, Mantel-Cox test.

ns: $P > 0.05$, no significant difference; ****$P < 0.0001$
Figure 6

(A) Flow cytometric analysis of CD107a expression on tumor-infiltrating NK cells in the peripheral blood, liver, and spleen of mice from different groups; (B) Proportional results of CD107a expression on tumor-infiltrating NK cells in the peripheral blood, liver, and spleen of mice in control and experimental groups; (C) Flow cytometric analysis of CD155 expression on tumor cells in the peripheral blood, liver, and spleen of mice from different groups; (D) Proportional results of CD155 expression on tumor cells in the peripheral blood, liver, and spleen of mice in control and experimental groups.

ns: P > 0.05, no significant difference; **P < 0.01; ****P < 0.0001
Figure 7

(A) Results of Western blotting protein band detection for tissues from different groups of mice and lymphocytes from the peripheral blood of HCC patients with varying degrees of differentiation; (B) Statistical analysis of protein quantification in tissues from different groups of mice and lymphocytes from the peripheral blood of HCC patients with varying degrees of differentiation.

ns: P > 0.05, indicating no significant difference; *P < 0.05, ***P < 0.001, ****P < 0.0001.