in vivo *38*: 174-183 (2024)
doi:10.21873/invivo.13423

Altered NCR3 Splice Variants May Result in Deficient NK Cell Function in Renal Cell Carcinoma Patients

XUELEI WANG[1], LIQUN HUANG[1], XIAOFEI WEN[1], DONGYANG LI[1], GUOSHENG YANG[1] and JUNHUA ZHENG[2]

[1]*Department of Urology, Shanghai East Hospital, Tongji University School of Medicine, Shanghai, P.R. China;*
[2]*Department of Urology, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, P.R. China*

Abstract. *Background/Aim: The natural killer (NK) cell function of patients with malignant tumours may be suppressed by deficiency, and the poor prognosis of renal cell carcinoma (RCC) patients may be due to escape from NK cell cytotoxicity, especially with respect to natural cytotoxicity receptors (NCRs) on the NK cell surface. However, the specific mechanism remains unclear. Therefore, in this study, we sought to explore the role of NCR, especially NCR3 splice variants, in the process of NK cell deficiency in RCC patients. Materials and Methods: We used flow cytometry to analyse the phenotype of NK cells from the peripheral blood and kidney tumour tissue of RCC patients. The NKp30-mediated NK cell killing function was measured by antibody-dependent cell-mediated cytotoxicity (ADCC) in NK and RCC cell coincubation. We extracted RNA from the peripheral blood mononuclear cells (PBMCs) of RCC patients and renal carcinoma tissue and carried out real-time quantitative PCR to detect the mRNA levels of NKp30a, NKp30b and NKp30c. mRNA expression levels of cytokines (IL-6, IL-8, IL-10, IL-18 and TGF-β) based on RNA extracted from renal carcinoma tissue and adjacent normal kidney tissues were also measured by real-time quantitative PCR. Results: Regarding the phenotype of NK cells in RCC patients, the proportion of NK cells in tumour tissue was significantly reduced, with changes in the NK cell proportion being most obvious in NKp30$^+$ NK cells. Furthermore, the results of the ADCC function assay showed limited NKp30$^+$ NK cell-mediated cytotoxicity in RCC patients. Through real-time quantitative PCR, we found lower expression of NKp30a and NKp30b, the immunostimulatory splice variants of NCR3 encoding NKp30, in RCC patients. Moreover, expression of activating cytokines (IL-6 and IL-8) in renal cancer tissue was decreased, though inhibitory cytokine (TGF-β) expression remained unchanged, which may result in an immunosuppressive cytokine microenvironment. Conclusion: Decreased expression of immunostimulatory NCR3 splice variants and the inhibitory cytokine microenvironment in RCC patients may contribute to deficient NK cell cytotoxicity and renal carcinoma cell immune escape from NK cell killing, which may provide a theoretical basis for finding new immunotherapeutic targets for RCC.*

Correspondence to: Junhua Zheng, Department of Urology, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200125, P.R. China. Tel: +86 13901722408, e-mail: zhengjh0471@sina.com

Key Words: Renal cell carcinoma, NK cell, NCR3, splice variants, cytokine microenvironment.

Natural killer (NK) cells play an important role in human antitumour innate immunity by interacting with receptors on the surface of NK cells and ligands on the surface of tumour cells (1-3). NK cells can be activated to kill tumour cells through induction of positive signalling pathways and inhibition of negative signalling pathways (4, 5). Activating receptors on the surface of NK cells include DNAM-1, natural killer group 2 member D (NKG2D) and natural cytotoxicity receptors (NCRs) (6). The NCR family consists of NKp30, NKp44 and NKp46. NKp30 and NKp44 are type I transmembrane glycoproteins composed of one immunoglobulin-like extracellular structural domain, with NKp46 containing two domains. The three NCR family members (NKp30, NKp44 and NKp46) are encoded by the genes NCR3, NCR2 and NCR1 (7, 8). The NCR3 gene is transcribed by alternative splicing into three splice variants of NKp30 (NKP30a, NKp30b and NKp30c) that have different biological functions (9). NKp30a and NKp30b exert immunostimulatory effects by inducing cytotoxic effects and Th1 cytokine secretion, respectively, whereas the NKp30c isoform has immunosuppressive effects by triggering release of interleukin (IL)-10 (10-12).

The function of NK cells may be suppressed to a relatively low level in patients with malignant tumours (13, 14). For example, it has been found that NK cell activity is markedly reduced in hepatocellular carcinoma patients and associated with poor prognosis (15-17). Renal cell carcinoma (RCC) is one of the most common malignant tumours in the urinary system, and its poor prognosis may result from escape from NK cell cytotoxicity by kidney cancer cells (18, 19). There have also been some studies investigating the correlation of NK cell receptors and RCC. For instance, as an inhibitor of vascular endothelial growth factor (VEGF) and a targeted therapy for renal cell cancer, axitinib increases expression of the ligand for NKG2D on the surface of cancer cells and enhances NK cell recognition and killing effects towards renal carcinoma cells (20). Another study found that valproic acid upregulates NKG2D ligand expression on the surface of renal cancer cells, leading to increased susceptibility of these cells to cytotoxicity mediated by NK cells (21). However, NCRs, as the most important activating receptors on the surface of NK cells, have not been systematically investigated for specific roles in the NK cell phenotype and function in RCC. To date, there is no research focusing on how NCRs participate in the process of RCC escape from NK cell cytotoxicity.

In this study, we analysed the phenotype of NK cells in RCC patients and preliminarily found that their proportion in kidney tumour tissue was significantly reduced. Such changes in the proportion of NK cells were most obvious in NKp30$^+$ NK cells. We also used antibody-dependent cell-mediated cytotoxicity (ADCC) to measure NK cell function, with the results showing that the cytotoxicity mediated by NKp30$^+$ NK cells is limited in renal cancer patients. Accordingly, NKp30 may play an important role in the mechanism of deficient NK cell function and that of renal cancer cell escape from immune killing by NK cells. Therefore, in the further study, we sought to explore the role of NKp30 in the above process by analysing the changes of different splice variants of NCR3 and the variation of the cytokine microenvironment in RCC patients, which may provide a theoretical basis for finding new targets for renal cancer immunotherapy.

Materials and Methods

Specimens and peripheral blood samples. Surgically resected kidney tumour specimens along with matched non-neoplastic surrounding kidney tissue were obtained from RCC patients of the Shanghai East Hospital from January 2020 to December 2022. Peripheral blood was obtained from the above patients and healthy controls (HCs). A written informed consent was obtained from each individual. The study protocol was approved by our institutional ethical committee. Tissue samples were stored in tissue storage solution (Miltenyi Biotec, Bergisch Gladbach, Germany) or RNA later (Sigma-Aldrich, St. Louis, MO, USA). Clinical characteristics of patients and HCs are listed in Table I.

Table I. *Clinical characteristics of renal cell carcinoma (RCC) patients and healthy controls (HCs).*

	RCCs	HCs
Number of subjects	20	20
Male/Female	15/5	15/5
Median age (years) – range	56 (44-69)	52 (39-62)
Median tumour size (mm) – range	51 (21-87)	None
Pathology		
Clear cell RCC	17	
Papillary RCC	3	
Grade		
I	2	
II	14	
III	4	
IV	0	
Stage		
I	3	
II	15	
III	2	
IV	0	

PBMC/TIL/KIL isolation. Peripheral blood mononuclear cells (PBMCs) were obtained from the peripheral blood of RCC patients (n=20) and healthy controls (n=20) by standard methods. Tumour-infiltrating lymphocytes (TILs) were obtained from renal tumour specimens removed by surgery (n=20), and the control group cells were kidney-infiltrating lymphocytes (KILs) derived from normal kidney tissue adjacent to the renal carcinoma (n=20). Tissue samples were treated by enzymatic and mechanical dissociation with the human Tumour Dissociation Kit by gentle MACS Dissociator (Miltenyi Biotec), according to the manufacturer's instructions. The cell suspension was filtered in a 70 μm cell strainer (Miltenyi Biotec) and centrifuged twice at 50 g for 2 min. The supernatant containing lymphocytes was processed for flow cytometry or cryopreserved in liquid N_2.

Flow cytometry. Flow cytometry analysis of *ex vivo* isolated PBMC, KIL and TIL cells after co-culture with the A498 renal cancer cell line, was performed using CyAn (Beckman Coulter, Brea, CA, USA) and FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA). The following mouse anti-human fluorescent antibodies were used: CD3-FITC (76-228, ImmunoTools, Friesoythe, Germany), CD56-Pc5.5 (IM2659U, Beckman Coulter), CD56-Pc5 (IM2654U, Beckman Coulter), NKp30-Alexa Fluor®647 (325212, BioLegend, San Diego, CA, USA), NKp44-PE (558563, Becton Dickinson), NKp46-Brilliant Violet 421™ (331913, Becton Dickinson) and CD3-PacBlue (520026, Becton Dickinson). Briefly, 2×10^5 PBMC were stained with mAb for 30 min at 4˚C, washed, immediately fixed in CellFix solution (Becton Dickinson) and analysed. Lymphocytes were identified by the characteristic forward scatter (FSC) and side scatter (SSC) parameters. Total NK cells and NK cell subsets were identified as a CD56$^+$ population within the CD3 negative gate. The proportions of receptor positive cells were expressed as the frequency of cells of the CD3$^-$CD56$^+$ gated population.

Antibody-dependent cellular cytotoxicity (ADCC) assay. Functional redirecting assay, also named reverse antibody-dependent cellular

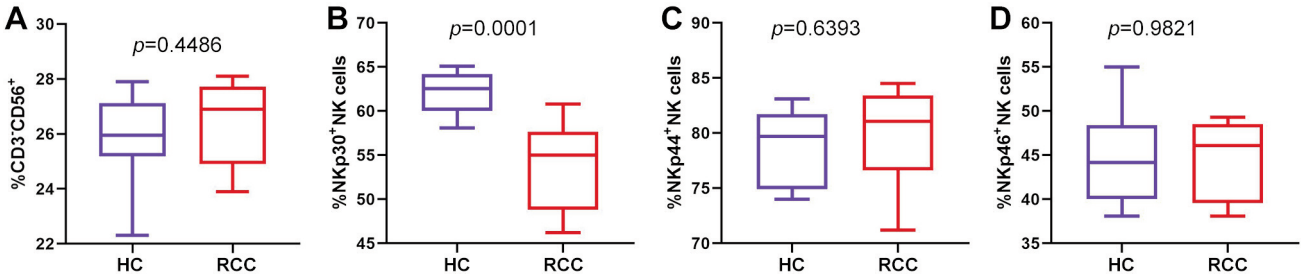

Figure 1. *The proportion of total natural killer (NK) cells (CD3⁻CD56⁺ cells), NKp30⁺, NKp44⁺ and NKp46⁺ subsets in peripheral blood mononuclear cells (PBMCs) of renal cancer carcinoma patients (RCC) (n=20) and healthy controls (HCs) (n=20). There was no significant difference in the proportions of total NK cells (A), NKp44⁺ (C) and NKp46⁺ (D) between the groups. The proportion of NKp30⁺ NK cells (B) among PBMCs of RCC patients was significantly lower than that of HCs. Statistical analysis was performed with unpaired t-tests.*

cytotoxicity (ADCC) assay, was performed after cross-linking of NKp30 and FcγR+ P815 murine cell line, as previously described (22). Briefly, PBMC, TIL and LIL were incubated overnight with or without IL-15 (20 ng/ml, PeproTech EC, London, UK) and subsequently washed and incubated for 4 hours at 37˚C with target cells (E:T=1:1) in the presence of anti-NKp30 specific mAb (R&D), anti-CD107a-PE (Becton Dickinson) and the Protein Transport Inhibitor GolgiStop (Becton Dickinson). After washing, lymphocytes were stained for surface NK cell markers using CD3-Brilliant Violet 421™ (Becton Dickinson) and CD56-Pc5.5 (Beckman Coulter). Cells were fixed with BD Cytofix/Cytoperm and permeabilized with the BD Perm/Wash buffer (Becton Dickinson) according to the manufacturer's instructions, in the presence of IFNγ-Alexa Fluor®647 (557729, Becton Dickinson) and TNFα-Alexa Fluor®488 (560242, Becton Dickinson). Data analysis was performed with the Kaluza 1.3 software (Beckman Coulter).

RNA extraction and real-time PCR. Expression of major NKp30 isoforms was quantified by real-time PCR (qPCR) in PBMC of RCC patients, HC and in TIL and KIL as described (23). IL-6, IL-8, IL-10, IL-18 and TGF-b mRNA expression were evaluated in RCC specimens and surrounding matched non-neoplastic kidney tissue. Tissue RNA extraction was performed with TRIzol reagent (Thermo Fisher Scientific, St. Louis, MO, USA) using a gentleMACS Dissociator (MiltenyiBiotec) and with RNeasy Plus Mini kit and DNAse treatment on column (Qiagen), following the manufacturer's instructions. First-strand cDNA was synthesized from 5 μg of total RNA using SuperScript III reverse transcriptase and random primers, following the manufacturer's instructions (Thermo Fisher Scientific). The Fast SYBR® Green Master Mix (Thermo Fisher Scientific) and the SsoAdvanced Universal SYBR Green Supermix (BioRad, Hercules, CA, USA) were used. All reactions were performed using the CFX96 Real-Time machine detection system (BioRad). Each sample was amplified in triplicate and the qPCR data were analysed using the $2^{-\Delta Ct}$ method.

Statistical analysis. Data for PBMCs obtained from the kidney cancer group and healthy control group were analysed using unpaired *t*-tests. The paired *t*-test was performed to analyse the paired data of tumour tissue and normal kidney tissue. Statistical analysis was performed with GraphPad 9.0 (GraphPad Software Inc., San Diego, CA, USA) statistical software. A *p*-Value ≤0.05 was considered statistically significant.

Results

Phenotype of NK cells in the peripheral blood and tumour tissue of RCC patients. We used flow cytometry to detect and compare the proportions of total NK cells ($CD3^-$ $CD56^+$ cells) and NK cell subsets, including $NKp30^+$, $NKp44^+$ and $NKp46^+$ NK cells, in PBMCs from renal carcinoma patients and healthy controls. There was no significant difference between the two groups in the proportions of total NK cells, $NKp44^+$ and $NKp46^+$ NK cells; however, the $NKp30^+$ NK cell percentage among PBMCs of RCC patients was significantly lower than that of healthy controls (Figure 1).

We found no significant difference in proportions of $NKp44^+$ and $NKp46^+$ NK cells between TILs in renal carcinoma specimens and KILs in the adjacent normal kidney tissue, similarly to the results for the peripheral blood. However, the total NK cell ($CD3^-CD56^+$ cell) percentage in kidney cancer tissue was significantly lower than that in the normal kidney tissue adjacent to the renal cancer, and the proportion of $NKp30^+$ NK cells in kidney cancer tissue was significantly higher than that in the adjacent normal kidney tissue, showing the opposite results to peripheral blood (Figure 2).

NKp30-mediated NK cell killing function assay using the peripheral blood and tumour tissues of RCC patients. Studies of NK cell phenotype have found that the phenotype of NKp30 cells is significantly different from that of NKp44 and NKp46 cells. Therefore, NKp30 may play an important role in renal cancer cell escape from NK cell killing. We further used an NKp30-mediated ADCC assay to examine the killing function of NK cells collected from the peripheral blood and renal cancer tissues of RCC patients. The NK cell degranulation function was reflected by the percentage of $CD107a^+$ NK cells detected by flow cytometry, and the ability to release cytokines was reflected by the percentage of $IFN\gamma^+$ NK cells. We evaluated the NKp30-mediated NK killing function of PBMCs and found no significant

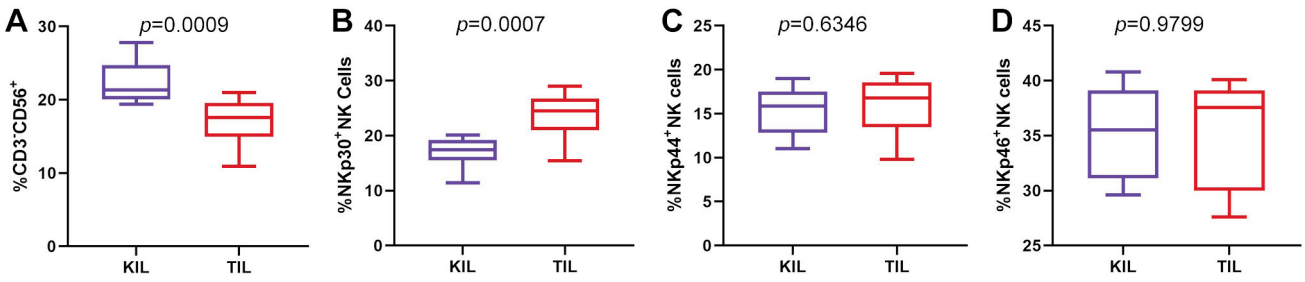

Figure 2. *The proportion of total natural killer (NK) cells ($CD3^-CD56^+$ cells) and $NKp30^+$, $NKp44^+$ and $NKp46^+$ subsets among tumour-infiltrating lymphocytes (TILs) of renal carcinoma tissue (n=20) and kidney-infiltrating lymphocytes (KILs) from adjacent normal kidney tissue (n=20). The proportion of NK cells ($CD3^-CD56^+$ cells) among TILs was significantly lower than that among KILs (A). The proportion of $NKp30^+$ NK cells among TILs was significantly higher than that among KILs (B). There was no significant difference in $NKp44^+$ and $NKp46^+$ NK cell percentages between the TIL and the KIL groups (C and D). Statistical analysis was performed with paired t-tests.*

difference in NK cell degranulation or cytokine release between the RCC group and the healthy control group in the absence of IL-15 stimulation. However, with IL-15 stimulation, both the degranulation function and cytokine release of NK cells of renal cancer patients were significantly lower in comparison with those of healthy participants. Then, we further determined the killing function of NK cells extracted from renal cancer tissue and adjacent normal kidney tissue, and our results revealed that with or without IL-15 stimulation, the level of cytokine release between the groups was not remarkably different but that the NK cell degranulation effect of the renal cancer group was lower than that of the normal kidney tissue group (Figure 3).

NCR3 splice variant mRNA expression in peripheral blood and tumour tissues of RCC patients. mRNA levels of the three NCR3 splice variants, NKp30a, NKp30b and NKp30c, were evaluated by real-time quantitative PCR in PBMCs of renal cancer patients. mRNA expression of NKp30a and NKp30b was remarkably lower in PBMCs of renal cancer patients compared to healthy controls, with no significant difference for NKp30c between the two groups (Figure 4). The same method was applied to examine mRNA levels of NKp30a, NKp30b and NKp30c among tumour-infiltrating lymphocytes (TILs) of renal cancer tissue and kidney-infiltrating lymphocytes (KILs) in adjacent normal kidney. There were no significant differences in mRNA levels of NKp30a and NKp30c between the TIL and the KIL group. However, compared with normal kidney tissue, mRNA expression of NKp30b in renal cancer tissue was remarkably lower (Figure 5).

Measurement of cytokine levels in the tumour tissue of RCC patients. Real-time quantitative PCR was performed to evaluate mRNA levels of several common cytokines (IL-6, IL-8, IL-10, IL-18 and TGF-β), which can reflect the status of the body's cytokine microenvironment, in both renal tumour tissue and surrounding normal kidney tissue. The results showed that in tumour tissue, mRNA levels of IL-6, IL-8 and IL-10 were significantly lower than those in normal kidney tissues. However, mRNA levels of IL-18 and TGF-β in tumour tissue were not significantly different from those in the surrounding normal kidney tissues (Figure 6).

Discussion

It has been found that in patients with various kinds of malignant tumours, such as liver cancer and oral squamous carcinoma, the NK cell proportion is reduced and the NK cell killing function is suppressed (13, 14). NK cell cytotoxicity is remarkably downregulated in hepatocellular carcinoma patients, and a lower level of NK cell activity is associated with worse prognosis (15-17). In oral squamous carcinoma tumour tissues, both the biological function and antitumour activity of NK cells are reduced (24, 25).

RCC is one of the most aggressive malignancies of the genito-urinary tract, having a poor prognosis especially in patients with metastasis (26). For advanced renal cancer, surgical resection combined with the currently available immunotherapy is still of limited efficacy (27). Finding new immunotherapy targets may be the key breakthrough to improve the efficacy of renal cancer treatment. Although most immune therapies have focused on enhancing T cell functions, there is a growing interest in developing therapies that can target other immune cell subsets, such as natural killer cells. For example, one study focusing on a functional screen to rapidly identify antibodies that can activate NK cells, found that bispecific antibodies targeting NCR3 can potently activate NK cells (28). In terms of studies focused on the NK cell phenotype in RCC, researchers have investigated the phenotype of NK cells extracted from peripheral blood lymphocytes and TILs in renal cancer patients, as early as 1997 (29). They found that tumour grading correlated inversely with the proportion of NK cells,

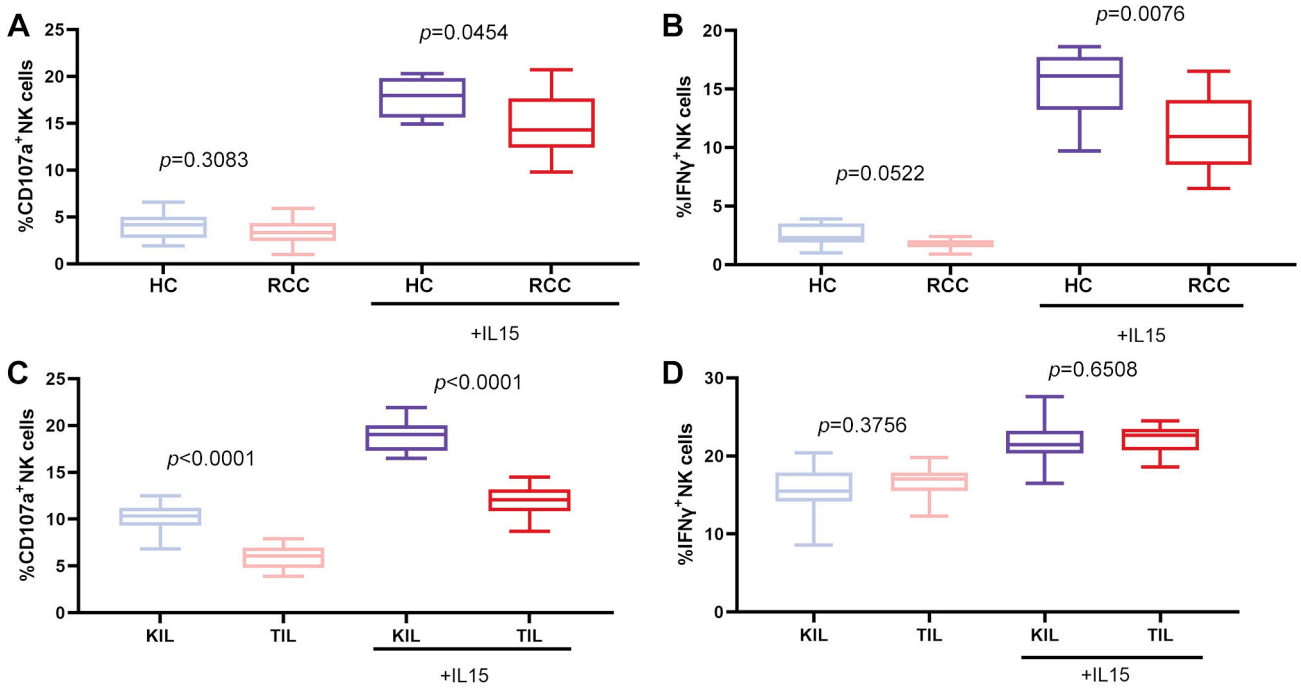

Figure 3. *NKp30-mediated natural killer (NK) cell killing function in the peripheral blood and tumour tissues of kidney cancer patients. In the antibody-dependent cellular cytotoxicity (ADCC) assay of peripheral blood NK cell killing function (A, B), there was no significant difference in NK cell degranulation or cytokine release between the renal cell carcinoma (RCC) group (n=20) and the healthy control (HC) group (n=20) in the absence of IL-15 stimulation. Under IL-15 stimulation, both degranulation function and cytokine release were significantly lower in RCC patient cells (n=20) than in those of the HC group (n=20). In samples of renal cancer tissue and adjacent normal kidney tissue (C, D), there was no significant difference in the cytokine release level between tumour-infiltrating lymphocytes (TIL) of renal carcinoma tissue (n=20) and kidney-infiltrating lymphocytes (KILs) from adjacent normal kidney tissue groups (n=20) with or without IL-15 stimulation, but the NK cell degranulation effect in the TIL group (n=20) was lower than that in the KIL group (n=20). Statistical analysis was performed using the t-test.*

indicating lower NK cell cytotoxicity in more aggressive renal carcinoma (29). Since then, only a few studies have focused on the correlation of RCC and NK cells, especially with regard to the association between receptors on the surface of NK cells and immune escape by kidney cancer cells. We found two articles about NKG2D, one of which reported that the VEGF inhibitor axitinib, a targeted therapeutic agent for kidney cancer, increases expression of the ligand NKG2D on the surface of renal cancer cells to enhance the ability of NK cells to recognize and further kill cancer cells (20). Another study found that valproic acid increases NKG2D ligand expression on the surface of renal cancer cells, thereby enhancing the vulnerability of cancer cells to NK cell-mediated immune effects (21). Nevertheless, the NCR receptor system, involving an important activating receptor on the surface of NK cells, has not been explored in renal cancer.

Our results showed that the proportion of NK cells in the tumour tissue of RCC patients was remarkably lower than that in adjacent normal kidney tissue, consistent with previous reports and suggesting that the reduced NK cell percentage in renal cancer tissue may be one of the mechanisms by which RCC cells escape from the cytotoxicity mediated by NK cells. However, in our research, the percentage of NK cells obtained from the peripheral blood of RCC patients was not significantly different compared to the healthy control group. This may be because we included more early-stage to mid-stage renal carcinoma cases, in which the NK cell number in peripheral blood was not significantly different from that in healthy individuals. That can be explained by the lower clinical benefit of surgical treatment in patients with advanced renal cancer, and the difficulty to obtain histological specimens of advanced renal cancer. Further studies including a larger number of patients with all clinical stages are needed.

Natural cytotoxic receptors (NCR) engage in an important activating receptor system on the surface of NK cells, mainly NKp30, NKp44 and NKp46. We first examined and compared the NCR phenotype and function of NK cells in RCC patients simultaneously and found that the percentage of $NKp30^+$ NK cells in peripheral blood lymphocytes was significantly lower in the renal cancer group than that in the healthy control group. However, proportions of $NKp44^+$ and $NKp46^+$ NK cells in RCC patients were not significantly

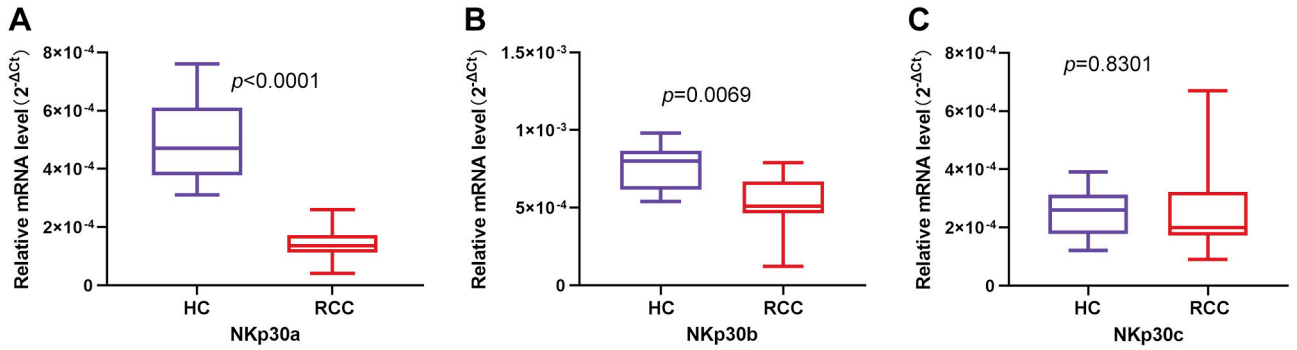

Figure 4. *Measurement of mRNA levels of the three NCR3 splice variants (NKp30a, NKp30b and NKp30c) in peripheral blood mononuclear cells (PBMCs). mRNA levels of NKp30a and NKp30b in PBMCs of patients with renal cell carcinoma (RCC) (n=20) were significantly lower than those in healthy controls (HCs) (n=20) (A, B). There was no significant difference in NKp30c mRNA level between the two groups (C). Statistical analysis was performed with the unpaired t-test.*

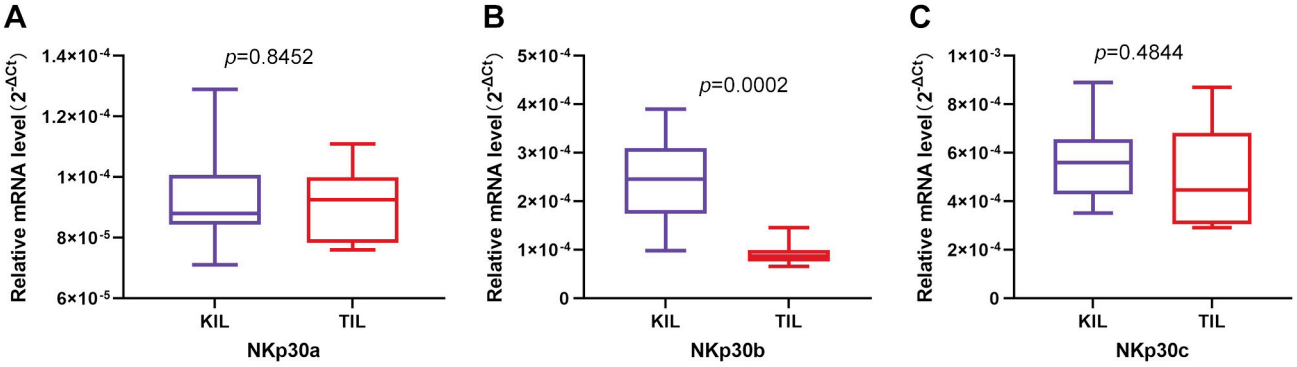

Figure 5. *Measurement of mRNA levels of NKp30a, NKp30b and NKp30c in tumour-infiltrating lymphocytes (TILs) in renal carcinoma tissue and kidney-infiltrating lymphocytes (KILs) in adjacent normal kidney tissue. There were no significant differences in the mRNA levels of NKp30a and NKp30c between the TIL (n=20) and KIL (n=20) groups (A, C), but mRNA expression of NKp30b in TILs (n=20) was lower than that in KILs (n=20) (B). Statistical analysis was performed with the paired t-test.*

different from those in healthy participants when comparing not only peripheral blood lymphocytes but also samples from kidney cancer tissues. The results highlighted the specificity of NKp30 among the receptors of NK cells and indicated that it may have an important effect in the escape of renal cancer cells from the immune killing by NK cells. Therefore, we further investigated the cytotoxic effect of NKp30$^+$ NK cells using the ADCC method. The results showed that the NKp30-mediated degranulation function of NK cells in RCC patients was significantly lower than that in healthy controls, not only in peripheral blood but also in kidney tumour tissue. These results are also compatible with the decreased proportion of NK cells in patients with renal carcinoma, suggesting that the proportion and function of NK cells are suppressed in renal cancer in association with NKp30, one member of the NCR family.

The ADCC assay is an *in vitro* killing test. However, the cytokine microenvironment within the tumour tissue is extremely complex *in vivo*, which explains why the percentage of IFNγ^+ NK cells in the tumour tissue was not significantly different from that in normal kidney tissue for the process of NK cell killing reflected by ADCC. Additionally, lymphocytes in tumour tissue, which are influenced by the internal environment of the tumour tissue, showed remarkably higher NKp30$^+$ NK cell levels than those in normal kidney tissue, which is opposite to the results for peripheral blood lymphocytes. This is a seemingly paradoxical phenomenon. Although NKp30$^+$ NK cells were enriched in tumour tissue, the NKp30-mediated ADCC effect was relatively low, which may be due to NKp30 isoform expression, the cytokine microenvironment of the tumour and the interaction of NKp30 with its ligands.

NKp30 is encoded by the NCR3 gene, and the transcript can be processed by alternative splicing into three main splice variants: NKp30a, NKp30b and NKp30c. These three isoforms have different biological functions. NKp30a and

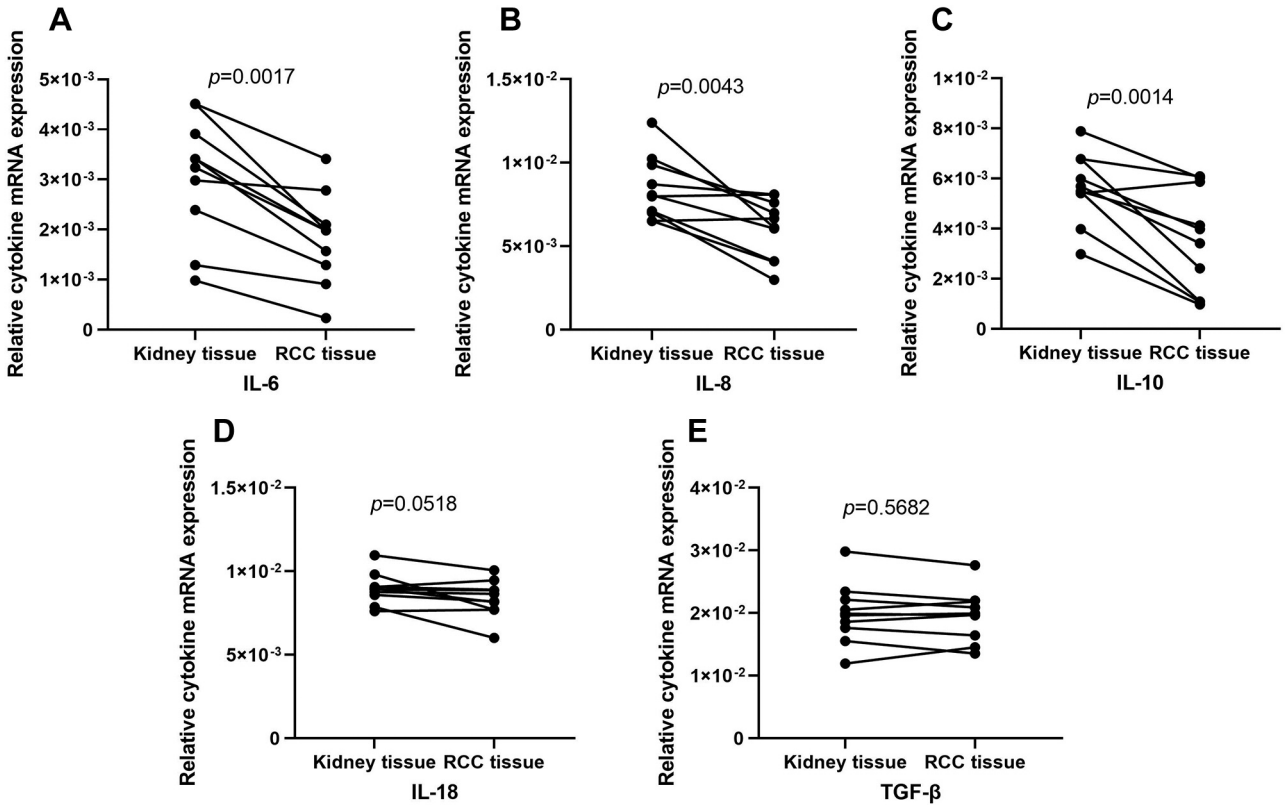

Figure 6. *Determination of mRNA levels of common cytokines (IL-6, IL-8, IL-10, IL-18 and TGF-β). mRNA levels of cytokines IL-6, IL-8 and IL-10 in RCC tissue were significantly lower than those in normal kidney tissue (A, B, C), whereas mRNA levels of IL-18 and TGF-β in RCC tissue were not significantly different from those in surrounding normal kidney tissues (C, D). Statistical analysis was performed with the paired t-test.*

NKp30b are immunostimulatory because they induce cytotoxicity and promote Th1 cytokine secretion, respectively, whereas NKp30c has an immunosuppressive effect by inducing the release of IL-10. An increase in mRNA encoding NKp30c, as opposed to the NKp30a and NKp30b isoforms, indicates poor prognosis, tumour progression and metastasis (23, 30, 31). For instance, in gastrointestinal sarcoma, elevated expression of the NKp30c isoform correlates with a reduced survival rate and reduced release of NKp30-dependent TNF-α and CD107a (32). In cervical cancer patients, decreased expression of the NKp30c isoform was found when compared against healthy subjects, which describes a unique down-modulation or non-fitness status of the immune response (33). The diverse expression of NKp30 isoforms is also influenced by the cytokine microenvironment. Indeed, NK cells can be affected by different cytokine microenvironments to generate different splice variants of NCR3, which have distinct functions. A group of researchers have reported that cytokines, including TGF-β/IL-15/IL-18, promote the expression of NKp30b and NKp30c to a large extent (34). In the placental tissues of pregnant women undergoing miscarriage, expression of NKp30a and NKp30b, the activating isoforms of NKp30, are highly upregulated and associated with increased secretion of cytokines, such as TNFα, IL-10 and VEGF-A (35).

Our results showed that although the mRNA levels of NKp30c were not significantly different in PBMCs and TILs, those of NKp30a and NKp30b in PBMCs of the kidney cancer group were reduced compared with those in the healthy group, and the mRNA level of NKp30b in renal cancer tissue was decreased compared with that in normal kidney tissue. In other words, in renal carcinoma, although there was no remarkable change in the inhibitory isoforms of NKp30, its activating isoform was suppressed, which may be involved in the immune escape of renal cancer cells mediated by NKp30. To some extent, this may also offer an explanation for a seemingly contradictory phenomenon: although $NKp30^+$ NK cells were enriched in renal cancer tissue, the NKp30-mediated ADCC of RCC patients was relatively low. Overall, $NKp30^+$ NK cells were enriched in renal carcinoma, but the inhibitory effect due to the altered NCR3 splice variants was predominant, which resulted in a NKp30-mediated NK cell cytotoxicity effect that was also deficient.

Impaired NK effector function may be related to the potent immunosuppressive activity of the tumour microenvironment (TME) (36-39). Such distinct expression of NCR3 splice variants is influenced by the cytokine microenvironment, which is extremely complex *in vivo*, especially in tumour tissues (40, 41). In acute myeloid leukemia, the previously reported NCR3 encoding NKp30, which purportedly influence mRNA splicing into isoforms with discrete functions, did not affect the immunotherapy outcome in a study, which may also be due to the complex microenvironment *in vivo* (42). In our study, we found that although the expression of cytokines in inhibitory signalling pathways (*e.g.*, TGF-β) remained unchanged, that of activating cytokines (*e.g.*, IL-6, IL-8) was decreased in renal cancer tissue (43-45), creating an inhibitory microenvironment that may suppress the activating NCR3 splice variants NKp30a and NKp30b and accordingly inhibit the NK cell killing function. Therefore, to address the role of NKp30 in the mechanism of kidney cancer cell escape from NK cell immune killing, we carried out analyses from two perspectives: expression of different isoforms of NKp30 and changes in the cytokine microenvironment. We found that both decreased NCR3 activating splice variants expression and the inhibitory cytokine microenvironment may contribute to restrict the killing function of NK cells and promote escape of renal cancer cells from NK cell-mediated cytotoxicity.

RCC is a highly angiogenic tumour, and the discovery of vascular endothelial growth factor (VEGF) and mammalian/mechanistic target of rapamycin (mTOR) pathways in the development of RCC has led researchers to study therapeutic agents targeting these pathways, resulting in multiple Food and Drug Administration (FDA) approvals of drugs in this category (46). These drugs were associated with improved response and decreased risk of toxicities; however, a durable complete response is uncommon (3, 47-49). Hence, there is a need for a continued search for novel targets and drugs in addition to already existing targeting agents. According to our results, splice variants of NCR3 and tumour cytokine microenvironment may become new therapeutic targets for RCC. Potential novel immuno-modulatory agents, which focus on the splice variants of NCR3 and cytokine microenvironment, may modulate the anti-tumour function of NK cells and further improve the prognosis of RCC patients.

Conclusion

In conclusion, our study found that both the proportion and function of NK cells are decreased in renal carcinoma tissue. NKp30, a member of the NCR family on the surface of NK cells, may play an important role in the mechanism of restricted NK cell function. In addition, decreased expression of immunostimulatory NCR3 splice variants and the inhibitory cytokine microenvironment in RCC patients may be involved in the mechanism of restricted NK cell function and kidney cancer cell escape from NK cell cytotoxicity, providing a theoretical basis for finding new targets for renal cancer immunotherapy.

Conflicts of Interest

The Authors declare that they have no financial or commercial conflicts of interest related to this study.

Authors' Contributions

Xuelei Wang and Liqun Huang: experimental design, flow cytometry, real-time quantitative PCR. Dongyang Li: specimen processing. Xiaofei Wen and Guosheng Yang: data analysis. Junhua Zheng: manuscript editing. All Authors have seen and approved the final draft before submission.

Acknowledgements

This work was supported by the Youth National Science Foundation of Jiangxi Province (20192BAB215014)

References

1 Morvan MG, Lanier LL: NK cells and cancer: you can teach innate cells new tricks. Nat Rev Cancer 16(1): 7-19, 2016. DOI: 10.1038/nrc.2015.5

2 Terrén I, Borrego F: Role of NK cells in tumor progression. Exp Suppl 113: 169-187, 2022. DOI: 10.1007/978-3-030-91311-3_6

3 Yu Y: The function of NK cells in tumor metastasis and NK cell-based immunotherapy. Cancers (Basel) 15(8): 2323, 2023. DOI: 10.3390/cancers15082323

4 Lanier LL: Up on the tightrope: natural killer cell activation and inhibition. Nat Immunol 9(5): 495-502, 2008. DOI: 10.1038/ni1581

5 Toledo E, Le Saux G, Edri A, Li L, Rosenberg M, Keidar Y, Bhingardive V, Radinsky O, Hadad U, Di Primo C, Buffeteau T, Smith AS, Porgador A, Schvartzman M: Molecular-scale spatio-chemical control of the activating-inhibitory signal integration in NK cells. Sci Adv 7(24): eabc1640, 2021. DOI: 10.1126/sciadv.abc1640

6 Quatrini L, Della Chiesa M, Sivori S, Mingari MC, Pende D, Moretta L: Human NK cells, their receptors and function. Eur J Immunol 51(7): 1566-1579, 2021. DOI: 10.1002/eji.202049028

7 Kruse PH, Matta J, Ugolini S, Vivier E: Natural cytotoxicity receptors and their ligands. Immunol Cell Biol 92(3): 221-229, 2014. DOI: 10.1038/icb.2013.98

8 Barrow AD, Martin CJ, Colonna M: The natural cytotoxicity receptors in health and disease. Front Immunol 10: 909, 2019. DOI: 10.3389/fimmu.2019.00909

9 Pinheiro PF, Justino GC, Marques MM: NKp30 - A prospective target for new cancer immunotherapy strategies. Br J Pharmacol 177(20): 4563-4580, 2020. DOI: 10.1111/bph.15222

10 Kinlein A, Janes ME, Kincer J, Almeida T, Matz H, Sui J, Criscitiello MF, Flajnik MF, Ohta Y: Analysis of shark NCR3 family genes reveals primordial features of vertebrate NKp30.

Immunogenetics 73(4): 333-348, 2021. DOI: 10.1007/s00251-021-01209-6

11 Shemesh A, Brusilovsky M, Kundu K, Ottolenghi A, Campbell KS, Porgador A: Splice variants of human natural cytotoxicity receptors: novel innate immune checkpoints. Cancer Immunol Immunother 67(12): 1871-1883, 2018. DOI: 10.1007/s00262-017-2104-x

12 Mantovani S, Oliviero B, Lombardi A, Varchetta S, Mele D, Sangiovanni A, Rossi G, Donadon M, Torzilli G, Soldani C, Porta C, Pedrazzoli P, Chiellino S, Santambrogio R, Opocher E, Maestri M, Bernuzzi S, Rossello A, Clément S, De Vito C, Rubbia-Brandt L, Negro F, Mondelli MU: Deficient natural killer cell NKp30-mediated function and altered NCR3 splice variants in hepatocellular carcinoma. Hepatology 69(3): 1165-1179, 2019. DOI: 10.1002/hep.30235

13 Cai L, Zhang Z, Zhou L, Wang H, Fu J, Zhang S, Shi M, Zhang H, Yang Y, Wu H, Tien P, Wang FS: Functional impairment in circulating and intrahepatic NK cells and relative mechanism in hepatocellular carcinoma patients. Clin Immunol 129(3): 428-437, 2008. DOI: 10.1016/j.clim.2008.08.012

14 Rennert C, Tauber C, Fehrenbach P, Heim K, Bettinger D, Sogukpinar Ö, Schuch A, Zecher BF, Bengsch B, Lang SA, Bronsert P, Björkström NK, Fichtner-Feigl S, Schultheiss M, Thimme R, Hofmann M: Adaptive subsets limit the anti-tumoral NK-cell activity in hepatocellular carcinoma. Cells 10(6): 1369, 2021. DOI: 10.3390/cells10061369

15 Yoshida Y, Yoshio S, Yamazoe T, Mori T, Tsustui Y, Kawai H, Yoshikawa S, Fukuhara T, Okamoto T, Ono Y, Takahashi Y, Hashida R, Kawaguchi T, Taketomi A, Kanto T: Phenotypic characterization by single-cell mass cytometry of human intrahepatic and peripheral NK cells in patients with hepatocellular carcinoma. Cells 10(6): 1495, 2021. DOI: 10.3390/cells10061495

16 Sun C, Xu J, Huang Q, Huang M, Wen H, Zhang C, Wang J, Song J, Zheng M, Sun H, Wei H, Xiao W, Sun R, Tian Z: High NKG2A expression contributes to NK cell exhaustion and predicts a poor prognosis of patients with liver cancer. Oncoimmunology 6(1): e1264562, 2016. DOI: 10.1080/2162402X.2016.1264562

17 Zhang QF, Yin WW, Xia Y, Yi YY, He QF, Wang X, Ren H, Zhang DZ: Liver-infiltrating CD11b(-)CD27(-) NK subsets account for NK-cell dysfunction in patients with hepatocellular carcinoma and are associated with tumor progression. Cell Mol Immunol 14(10): 819-829, 2017. DOI: 10.1038/cmi.2016.28

18 Bukavina L, Bensalah K, Bray F, Carlo M, Challacombe B, Karam JA, Kassouf W, Mitchell T, Montironi R, O'Brien T, Panebianco V, Scelo G, Shuch B, van Poppel H, Blosser CD, Psutka SP: Epidemiology of renal cell carcinoma: 2022 update. Eur Urol 82(5): 529-542, 2022. DOI: 10.1016/j.eururo.2022.08.019

19 Chen J, Lou J, He Y, Zhu Z, Zhu S: A comprehensive analysis of renal cell carcinoma as first and second primary cancers. World J Surg Oncol 20(1): 57, 2022. DOI: 10.1186/s12957-022-02493-6

20 Morelli MB, Amantini C, Santoni M, Soriani A, Nabissi M, Cardinali C, Santoni A, Santoni G: Axitinib induces DNA damage response leading to senescence, mitotic catastrophe, and increased NK cell recognition in human renal carcinoma cells. Oncotarget 6(34): 36245-36259, 2015. DOI: 10.18632/oncotarget.5768

21 Yang F, Shao Y, Yang F, Liu M, Huang J, Zhu K, Guo C, Luo J, Li W, Yang B, Shi J, Zheng J: Valproic acid upregulates NKG2D ligand expression and enhances susceptibility of human renal carcinoma cells to NK cell-mediated cytotoxicity. Arch Med Sci 9(2): 323-331, 2013. DOI: 10.5114/aoms.2013.34413

22 Oliviero B, Varchetta S, Paudice E, Michelone G, Zaramella M, Mavilio D, De Filippi F, Bruno S, Mondelli MU: Natural killer cell functional dichotomy in chronic hepatitis B and chronic hepatitis C virus infections. Gastroenterology 137(3): 1151-1160.e7, 2009. DOI: 10.1053/j.gastro.2009.05.047

23 Mantovani S, Mele D, Oliviero B, Barbarini G, Varchetta S, Mondelli MU: NKp30 isoforms in patients with chronic hepatitis C virus infection. Immunology 146(2): 234-242, 2015. DOI: 10.1111/imm.12495

24 Cantoni C, Huergo-Zapico L, Parodi M, Pedrazzi M, Mingari MC, Moretta A, Sparatore B, Gonzalez S, Olive D, Bottino C, Castriconi R, Vitale M: NK cells, tumor cell transition, and tumor progression in solid malignancies: New hints for NK-based immunotherapy? J Immunol Res 2016: 4684268, 2016. DOI: 10.1155/2016/4684268

25 Dutta A, Banerjee A, Saikia N, Phookan J, Baruah MN, Baruah S: Negative regulation of natural killer cell in tumor tissue and peripheral blood of oral squamous cell carcinoma. Cytokine 76(2): 123-130, 2015. DOI: 10.1016/j.cyto.2015.09.006

26 Spyropoulou D, Tsiganos P, Dimitrakopoulos FI, Tolia M, Koutras A, Velissaris D, Lagadinou M, Papathanasiou N, Gkantaifi A, Kalofonos H, Kardamakis D: Radiotherapy and renal cell carcinoma: a continuing saga. In Vivo 35(3): 1365-1377, 2021. DOI: 10.21873/invivo.12389

27 Shirotake S, Miyama YU, Baba Y, Tajima H, Okada Y, Nakazawa K, Usami Y, Yasuda M, Igarashi D, Kaneko GO, Kanao K, Oyama M, Nishimoto K: Impact of cytoreductive nephrectomy following nivolumab plus ipilimumab therapy for patients with advanced renal cell carcinoma. Anticancer Res 42(5): 2727-2735, 2022. DOI: 10.21873/anticanres.15751

28 Kang E, Kadoch C, Rubenstein JL, Lanier LL, Wells JA: A functional mammalian display screen identifies rare antibodies that stimulate NK cell-mediated cytotoxicity. Proc Natl Acad Sci U.S.A. 118(31): e2104099118, 2021. DOI: 10.1073/pnas.2104099118

29 Kowalczyk D, Skorupski W, Kwias Z, Nowak J: Flow cytometric analysis of tumour-infiltrating lymphocytes in patients with renal cell carcinoma. Br J Urol 80(4): 543-547, 1997. DOI: 10.1046/j.1464-410x.1997.00408.x

30 Messaoudene M, Fregni G, Enot D, Jacquelot N, Neves E, Germaud N, Garchon HJ, Boukouaci W, Tamouza R, Chanal J, Avril MF, Toubert A, Zitvogel L, Rusakiewicz S, Caignard A: NKp30 isoforms and NKp46 transcripts in metastatic melanoma patients: Unique NKp30 pattern in rare melanoma patients with favorable evolution. Oncoimmunology 5(12): e1154251, 2016. DOI: 10.1080/2162402X.2016.1154251

31 Semeraro M, Rusakiewicz S, Minard-Colin V, Delahaye NF, Enot D, Vély F, Marabelle A, Papoular B, Piperoglou C, Ponzoni M, Perri P, Tchirkov A, Matta J, Lapierre V, Shekarian T, Valsesia-Wittmann S, Commo F, Prada N, Poirier-Colame V, Bressac B, Cotteret S, Brugieres L, Farace F, Chaput N, Kroemer G, Valteau-Couanet D, Zitvogel L: Clinical impact of the NKp30/B7-H6 axis in high-risk neuroblastoma patients. Sci Transl Med 7(283): 283ra55, 2015. DOI: 10.1126/scitranslmed.aaa2327

32 Delahaye NF, Rusakiewicz S, Martins I, Ménard C, Roux S, Lyonnet L, Paul P, Sarabi M, Chaput N, Semeraro M, Minard-Colin V, Poirier-Colame V, Chaba K, Flament C, Baud V, Authier H, Kerdine-Römer S, Pallardy M, Cremer I, Peaudecerf L, Rocha B, Valteau-Couanet D, Gutierrez JC, Nunès JA, Commo F, Bonvalot S, Ibrahim N, Terrier P, Opolon P, Bottino C, Moretta A, Tavernier J, Rihet P, Coindre J, Blay J, Isambert N, Emile J, Vivier E, Lecesne A, Kroemer G, Zitvogel L: Alternatively spliced NKp30 isoforms affect the prognosis of gastrointestinal stromal tumors. Nat Med 17(6): 700-707, 2011. DOI: 10.1038/nm.2366

33 Gutierrez-Silerio GY, Bueno-Topete MR, Vega-Magana AN, Bastidas-Ramirez BE, Gutierrez-Franco J, Escarra-Senmarti M, Pedraza-Brindis EJ, Pena-Rodriguez M, Ramos-Marquez ME, Delgado-Rizo V, Banu N, Alejandre-Gonzalez AG, Fafutis-Morris M, Haramati J, Del Toro-Arreola S: Non-fitness status of peripheral NK cells defined by decreased NKp30 and perforin, and increased soluble B7H6, in cervical cancer patients. Immunology 168(3): 538-553, 2023. DOI: 10.1111/imm.13593

34 Siewiera J, Gouilly J, Hocine HR, Cartron G, Levy C, Al-Daccak R, Jabrane-Ferrat N: Natural cytotoxicity receptor splice variants orchestrate the distinct functions of human natural killer cell subtypes. Nat Commun 6: 10183, 2015. DOI: 10.1038/ncomms10183

35 Shemesh A, Tirosh D, Sheiner E, Tirosh NB, Brusilovsky M, Segev R, Rosental B, Porgador A: First trimester pregnancy loss and the expression of alternatively spliced NKp30 isoforms in maternal blood and placental tissue. Front Immunol 6: 189, 2015. DOI: 10.3389/fimmu.2015.00189

36 Miura Y, Anami T, Yatsuda J, Motoshima T, Oka S, Suyama K, Inoshita N, Kinowaki K, Urakami S, Kamba T, Komohara Y: HLA-DR and CD74 expression and the immune microenvironment in renal cell carcinoma. Anticancer Res 41(6): 2841-2848, 2021. DOI: 10.21873/anticanres.15065

37 Tumino N, Nava Lauson CB, Tiberti S, Besi F, Martini S, Fiore PF, Scodamaglia F, Mingari MC, Moretta L, Manzo T, Vacca P: The tumor microenvironment drives NK cell metabolic dysfunction leading to impaired antitumor activity. Int J Cancer 152(8): 1698-1706, 2023. DOI: 10.1002/ijc.34389

38 Hu Z, Xu X, Wei H: The adverse impact of tumor microenvironment on NK-cell. Front Immunol 12: 633361, 2021. DOI: 10.3389/fimmu.2021.633361

39 Balzasch BM, Cerwenka A: Microenvironmental signals shaping NK-cell reactivity in cancer. Eur J Immunol: e2250103, 2023. DOI: 10.1002/eji.202250103

40 Hernandez-Gea V, Toffanin S, Friedman SL, Llovet JM: Role of the microenvironment in the pathogenesis and treatment of hepatocellular carcinoma. Gastroenterology 144(3): 512-527, 2013. DOI: 10.1053/j.gastro.2013.01.002

41 Anft M, Netter P, Urlaub D, Prager I, Schaffner S, Watzl C: NK cell detachment from target cells is regulated by successful cytotoxicity and influences cytokine production. Cell Mol Immunol 17(4): 347-355, 2020. DOI: 10.1038/s41423-019-0277-2

42 Hussein BA, Hallner A, Wennström L, Brune M, Martner A, Hellstrand K, Bernson E, Thorén FB: Impact of NK cell activating receptor gene variants on receptor expression and outcome of immunotherapy in acute myeloid leukemia. Front Immunol 12: 796072, 2021. DOI: 10.3389/fimmu.2021.796072

43 Chia CS, Ban K, Ithnin H, Singh H, Krishnan R, Mokhtar S, Malihan N, Seow HF: Expression of interleukin-18, interferon-γ and interleukin-10 in hepatocellular carcinoma. Immunol Lett 84(3): 163-172, 2002. DOI: 10.1016/s0165-2478(02)00176-1

44 Markowitz GJ, Yang P, Fu J, Michelotti GA, Chen R, Sui J, Yang B, Qin WH, Zhang Z, Wang FS, Diehl AM, Li QJ, Wang H, Wang XF: Inflammation-dependent IL18 signaling restricts hepatocellular carcinoma growth by enhancing the accumulation and activity of tumor-infiltrating lymphocytes. Cancer Res 76(8): 2394-2405, 2016. DOI: 10.1158/0008-5472.CAN-15-1548

45 Klausz K, Pekar L, Boje AS, Gehlert CL, Krohn S, Gupta T, Xiao Y, Krah S, Zaynagetdinov R, Lipinski B, Toleikis L, Poetzsch S, Rabinovich B, Peipp M, Zielonka S: Multifunctional NK cell-engaging antibodies targeting EGFR and NKp30 elicit efficient tumor cell killing and proinflammatory cytokine release. J Immunol 209(9): 1724-1735, 2022. DOI: 10.4049/jimmunol.2100970

46 Capitanio U, Montorsi F: Renal cancer. Lancet 387(10021): 894-906, 2016. DOI: 10.1016/S0140-6736(15)00046-x

47 Della Chiesa M, Setti C, Giordano C, Obino V, Greppi M, Pesce S, Marcenaro E, Rutigliani M, Provinciali N, Paleari L, DeCensi A, Sivori S, Carlomagno S: NK cell-based immunotherapy in colorectal cancer. Vaccines (Basel) 10(7): 1033, 2022. DOI: 10.3390/vaccines10071033

48 Lv D, Khawar MB, Liang Z, Gao Y, Sun H: Neoantigens and NK cells: "Trick or treat" the cancers? Front Immunol 13: 931862, 2022. DOI: 10.3389/fimmu.2022.931862

49 Tong L, Jiménez-Cortegana C, Tay AHM, Wickström S, Galluzzi L, Lundqvist A: NK cells and solid tumors: therapeutic potential and persisting obstacles. Mol Cancer 21(1): 206, 2022. DOI: 10.1186/s12943-022-01672-z

Received August 28, 2023
Revised October 18, 2023
Accepted October 25, 2023