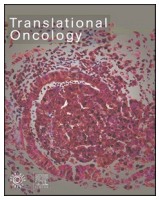
Translational Oncology 52 (2025) 102198

Contents lists available at ScienceDirect

Translational Oncology

journal homepage: www.elsevier.com/locate/tranon



Original Research

Prognostic significance of perioperative circulating CD56^{bright} NK cell and recovery of NK cell activity in patients with colorectal cancer undergoing radical surgery

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ARTICLE INFO

Keywords:
CD^{56bright} natural killer cell
Colorectal cancer
Prognosis
NK cell activity
surgery

ABSTRACT

Introduction: Natural killer (NK) cell activity (NKA) is downregulated in patients with colorectal cancer (CRC), and its dysfunction is possibly associated with increased risk of recurrence. However, its role in prognosis of CRC remains unclear. Prior research has shown that surgical stress can suppress NKA. This study explores the relationship between NK cell/NKA and clinicopathological factors during the perioperative period in patients with CRC.

Methods: We prospectively enrolled 45 patients with CRC. Venous blood samples were collected preoperatively and on postoperative day 3 (POD3) and 30 (POD30). NKA was assessed by measuring the plasma levels of NK cell-secreted IFN-γ.

Results: NKA was significantly reduced on POD3 compared with baseline levels before surgery but showed significant recovery by POD30. NKA on POD30 was considerably higher in patients with advanced disease stages or one or more high-risk preoperative factors. Additionally, a higher NKA recovery in patients with advanced stage exhibited improved recurrence-free survival (RFS) and progression-free survival (PFS) (hazards ratio (HR): 0.2442). Furthermore, an increased percentage of CD56^{bright} NK cells and a higher CD56^{bright}/CD56^{dim} NK cell ratio postoperatively on POD30 were associated with better RFS/PFS (HR: 0.2732, $P = 0.0433$ and HR: 0.2193, $P = 0.024$, respectively).

Conclusions: Our findings indicate that a notable postoperative increase in CD56^{bright} NK cells on POD30, both in percentage and ratio, correlates with a more favorable prognosis in CRC patients. Additionally, higher recovery rates of NKA in patients with advanced stages may offer potential applications in risk stratification and the development of treatment strategies for CRC.

Introduction

Colorectal cancer (CRC) is the third-leading cause of cancer-related deaths worldwide [1]. According to the American Cancer Society's Cancer Facts & Figures 2021, the five-year relative survival rate for CRC is 65 %, though this rate varies depending on cancer stage. Surgical resection remains the primary treatment approach. Several factors are associated with poor CRC prognosis post-surgery, including poorly differentiated histology, lymphovascular and perineural invasion, T4 stage, and elevated levels of carcinoembryonic antigen (CEA) and carbohydrate antigen 19–9 (CA19–9). These factors are closely linked to increased risks of recurrence, metastasis, and lower survival rates in patients with CRC [2–5]. Recent studies have also emphasized the profound influence of patients' inflammatory and nutritional statuses on cancer outcomes, with relevant biomarkers including the Royal Marsden Hospital (RMH) score, pan-immune-inflammation value (PIV), and

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https://doi.org/10.1016/j.tranon.2024.102198
Received 5 July 2024; Received in revised form 13 October 2024; Accepted 10 November 2024

neutrophil-to-lymphocyte ratio (NLR) [6–8]. Moreover, emerging therapies in CRC have led to the identification of predictive and prognostic markers for therapy response and adverse effects, such as upfront chemotherapy in liver transplant patients with liver metastases of CRC and various side effects (e.g., peripheral neuropathy, headache, hypertransaminasemia, hearing loss) in patients undergoing immune checkpoint inhibitor therapy [9–12]. Additionally, immunosuppression has been associated with poor outcomes in cancer patients [13,14]. Notably, natural killer (NK) cell activity (NKA) is generally lower in CRC patients than in healthy individuals and tends to decrease further during the postoperative period [15]. However, the relationship between postoperative NKA downregulation, high-risk factors, and prognosis in CRC remains unclear.

NK cells are critical components of the innate immune system, characterized by their cytotoxic abilities. They play a dual role: directly eliminating infected or malignant cells and modulating adaptive immune responses through cytokine secretion [16,17]. The two major subsets of human NK cells, $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{-}$, are distinguished by the relative expression levels of CD56 and CD16 [18,19]. $CD56^{dim}CD16^{+}$ NK cells are the major circulating subset, accounting for approximately 90 % of all NK cells in the peripheral blood, whereas $CD56^{bright}CD16^{-}$ NK cells predominate in tissues and secondary lymphoid organs [20]. Studies have demonstrated that $CD56^{dim}CD16^{+}$ NK cells exert cytolytic activity via perforin and granzyme production, while $CD56^{brigh}CD16^{-}$ NK cells are responsible for secreting cytokines, such as interferon-γ (IFN-γ) and tumor necrosis factor-α [20–22].

NK cells produce IFN-γ to regulate immune responses [23], with studies showing that IFN-γ from NK cells can inhibit tumor growth and enhance cytolysis and apoptosis of tumor cells [24–27]. Additionally, IFN-γ has been implicated in suppressing metastasis [28,29]. Beyond their direct cytotoxic effects, NK cells also indirectly suppress cancer progression through IFN-γ-mediated regulation of other innate and adaptive immune cells, such as macrophages and T cells [30,31]. Consequently, measuring IFN-γ secretion offers valuable insights into the immune system's response during tumor progression.

In this study, we assessed the levels of IFN-γ secreted by NK cells (as NKA) using the NK VUE ELISA kit. We also analyzed NK cell subsets in patients with CRC during the preoperative and postoperative periods to evaluate the impacts of surgical intervention and preoperative risk factors on NKA and NK cell profiles following tumor resection.

Materials and methods

Patient selection

This study was conducted in accordance with the Declaration of Helsinki and received approval from the Institutional Review Board of Chang Gung Memorial Hospital, Linkou, Taiwan (Approval Number: 201900981B0). The inclusion criteria were as follows: (1) histologically confirmed CRC; (2) undergoing bowel resection for primary colorectal adenocarcinoma; (3) diagnosed with stage I–IV CRC. Patients with autoimmune diseases, inflammatory conditions, severe hematological disorders, or major organ failure were excluded. We prospectively enrolled 45 patients from the referral center, all of whom underwent bowel resection for primary colorectal adenocarcinoma. Data collected included clinicopathological characteristics and measurement variables such as preoperative parameters (age, sex, CEA, and CA19–9, NLR [8]) and postoperative pathological features (histological grade, angiolymphatic invasion, perineural invasion, and final clinical staging). Additionally, healthy subjects were recruited to compare NKA between patients with CRC and healthy controls.

Whole blood processing

Blood was drawn from each patient at three distinct times: before surgery (baseline, n = 45), 3 days after surgery (postoperative day (POD) 3, n = 43), and approximately 30 days post-surgery (POD30, n = 45).

Whole blood was collected in heparinized vacuum blood collection tubes (10 mL per tube). An aliquot of 1 mL of whole blood was transferred to a vacutainer tube containing Promoca™ for NK cell stimulation. The remaining blood was used to isolate peripheral blood mononuclear cells (PBMCs) via Ficoll density gradient centrifugation. The isolated PBMCs were then stored at -80 °C for further analysis.

NKA assay

The level of IFN-γ secretion by NK cells was quantified using the NK VUE ELISA kit (NKMAX, Korea) following the manufacturer's instructions. Briefly, 1 mL of whole blood was incubated in a vacutainer tube containing Promoca™ at 37 °C for 22 h After incubation, the samples were centrifuged, and the plasma was collected and stored at -80 °C. For the assay, cryopreserved plasma samples were thawed and centrifuged at 11,500 × g for 1 min at room temperature. The supernatants were then transferred to ELISA wells and incubated for 1 h at room temperature. The wells were washed, and 100 μl of detection solution containing biotin conjugate and streptavidin horseradish peroxidase was added, followed by an incubation for 1.5 h at room temperature. Tetramethylbenzidine solution was then added and incubated for 30 min, followed by a final wash. A stop solution was added, and the absorbance was measured at 450 nm to determine the concentration of IFN-γ secreted by the NK cells.

Flow cytometry analysis

PBMCs were isolated from whole blood and analyzed using flow cytometry. Briefly, cryopreserved PBMCs were thawed in a 37 °C water bath and transferred to a 15 mL centrifuge tube containing 5 mL of PBS. Then, PBMCs were incubated at 37 °C for 5 min, followed by centrifugation at 300 × g for 10 min. The supernatant was discarded, and the PBMCs were resuspended in 1 mL of PBS. After incubating for an additional hour at 37 °C, the cells were centrifuged again at 300 × g for 10 min. PBMCs were then resuspended and stained with directly conjugated monoclonal antibodies (mAbs) including CD45, CD3, CD56, and CD16 (BD Biosciences) for 1 h according to the manufacturer's instructions. Stained PBMCs were analyzed on a BD Fortessa flow cytometer (BD Biosciences, Becton Dickinson, Franklin Lakes, NJ, USA). NK cells were identified using the phenotype $CD45^{+}CD3^{-}CD16^{+/-}CD56^{+}$. Flow cytometry data were processed using BD FACSDiva and FlowJo™ software. The gating strategy was consistent with our prior studies on NK cell phenotypes in post-surgical glioblastoma patients [32].

Clinical outcome assessment

The primary clinical endpoints were recurrence-free survival (RFS) for stage I–III patients and progression-free survival (PFS) for stage IV patients. RFS was defined as the time interval between the date of surgery and the occurrence of disease recurrence or death, whichever came first. PFS was defined as the period from surgery to disease progression or death, whichever occurred first.

Statistical analysis

Comparisons between two distinct groups were performed using the two-tailed Mann–Whitney U test. For paired samples, comparisons at specified time points were conducted using the two-tailed Wilcoxon matched-pairs signed-rank test. Correlations between perioperative changes in NKA and NK cell subsets were assessed using Pearson's product-moment correlation coefficient (r). Changes in the percentage of each NK cell subset during the perioperative period were measured

for their association with RFS and PFS. Prognostic cutoff points for associating NK cell subset/NKA values with survival were established to optimize the significance of splits in Kaplan-Meier plots [33].

Kaplan–Meier analyses and log-rank tests were used to investigate and compare survival rates within patient subgroups. Hazard ratios (HRs) were calculated using Cox regression analysis and are presented with their 95 % confidence intervals (CIs). Univariate Cox regression analysis evaluated the relationship between baseline factors and survival rate. Variables with *P* values < 0.05 were included in the multivariable Cox regression model. All statistical tests were two-sided, and a *P* value of <0.05 was considered statistically significant. Notations for significance are as follows: * indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.001$.

Results

Demographic characteristics

The demographic variables of the patients—including sex, age, histologic grade, angiolymphatic invasion, perineural invasion, CEA and CA19–9 levels, and TNM stage—are summarized in Table 1. Patients with advanced stages showed higher pathologic N stages and increased angiolymphatic invasion. We divided our patient population into two groups based on the presence (high risk) or absence (low risk) of specific clinicopathological risk factors, that included poorly differentiated histology, lymphovascular invasion, perineural invasion, T4 stage, and preoperative CEA levels >5 ng/mL and CA19–9 levels >37 U/mL.

NKA at the preoperative period, POD3, and POD30

As illustrated in Supplementary Figure 1, CRC patients exhibited significantly lower NKA than healthy subjects (21.8 pg/mL vs. 874.0 pg/mL, $p = 0.0013$), indicating a severe impairment of NKA in CRC patients. To assess the impact of surgery on NKA, we measured NKA levels before surgery (baseline) and on POD3 and POD30, as shown in Fig. 1A. NKA was significantly reduced on POD3 (median: 20.9 pg/mL, 95 % CI: 16.1–30.2) compared to the baseline (median: 125.8 pg/mL, 95 % CI: 75.23–163.4), with a median difference of -92.1 pg/mL (95 % CI: -163.4 to -40.24). Conversely, NKA showed a marked recovery on POD30 (median: 190.1 pg/mL, 95 % CI: 126.5–503.4), significantly higher than the baseline and POD3 levels. The median differences in NKA on POD30 compared to POD3 and the baseline were 160.7 pg/mL (95 % CI: 72.83–491.1) and 40.96 pg/mL (95 % CI: -1.4 to 238.4), respectively. The average follow-up duration was 27.5 months (standard deviation:

Table 1
Demographic and baseline clinicopathologic characteristics.

	Total N(%)	Stage I/II N(%)	Stage III/IV N(%)	p-value	Low risk group N(%)	High risk group N(%)	p-value
Total	45	23	22		15	30	
Age(median years, 64; 95 % CI, 61–67)				0.051			0.205
< 65 y/o	24(53)	9(39)	15(68)		6(40)	18(60)	
> = 65 y/o	21(47)	14(61)	7(32)		9(60)	12(40)	
Gender				0.1			0.673
Female	22(49)	14(61)	8(36)		8(53)	14(47)	
male	23(51)	9(39	14(64)		7(47)	16(53)	
Histology grade				0.274			0.138
low	41(91)	22(96)	19(86)		15(100)	26(87)	
High	4(9)	1(4)	3(14)		0(0)	4(13)	
pathologic T stage*				0.515			0.001
T1	4(9)	3(13)	1(5)		3(20)	1(3)	
T2	4(9)	3(13)	1(5)		4(27)	0(0)	
T3	29(64)	13(57)	16(72)		8(53)	21(72)	
T4	8(18)	4(17)	4(18)		0(0)	8(27)	
pathologic N stage*				< 0.001			0.105
N0	23(51)	23(100)	0(0)		11(73)	12(40)	
N1	15(33)	0(0)	15(68)		3(20)	12(40)	
N2	7(16)	0(0)	7(32)		1(7)	6(20)	
M stage*				<0.015			0.094
M0	40(89)	23(100)	17(77)		15(100)	25(83)	
M1	5(11)	0(0)	5(23)		0(0)	5(17)	
Angiolymphatic invasion				< 0.001			0.001
absent	31(69)	22(96)	9(41)		15(100)	16(53)	
present	14(31)	1(4)	13(59)		0(0)	14(47)	
Perineural invasion(PNI)				0.425			0.011
absent	35(78)	19(83)	16(73)		15(100)	20(67)	
present	10(22)	4(17)	6(27)		0(0)	10(33)	
Preop CEA				0.053			<0.001
<= 5 ng/ml	25(56)	16(70)	9(41)		15(100)	10(33)	
> 5 ng/ml	20(44)	7(30)	13(59)		0(0)	20(67)	
Preop CA199				0.053			0.018
<=37U/ML	36(80)	21(91)	15(68)		15(100)	21(70)	
>37U/ML	9(20)	2(9)	8(32)		0(0)	9(30)	
Preop N/L ratio				0.666	12(80)	22(73)	0.624
<3	34(76)	18(78)	16(73)		3(20)	8(27)	
>=3	11(24)	5(22)	6(27)				
Blood drawing							
Baseline	45	23	22		15	30	
POD3	43	22	21		15	28	
POD30	45	23	22		15	30	

P values were derived by using Chi-square test.
CI, confidence interval; CEA, carcinoembryonic antigen; CA19–9, carbohydrate antigen 19–9; AJCC, the American Joint Committee Cancer; POD, postoperative day; N/L ration, Neutrophil/Lymphocyte ration
* AJCC (American Joint Committee Cancer) TNM stage, 7th edition

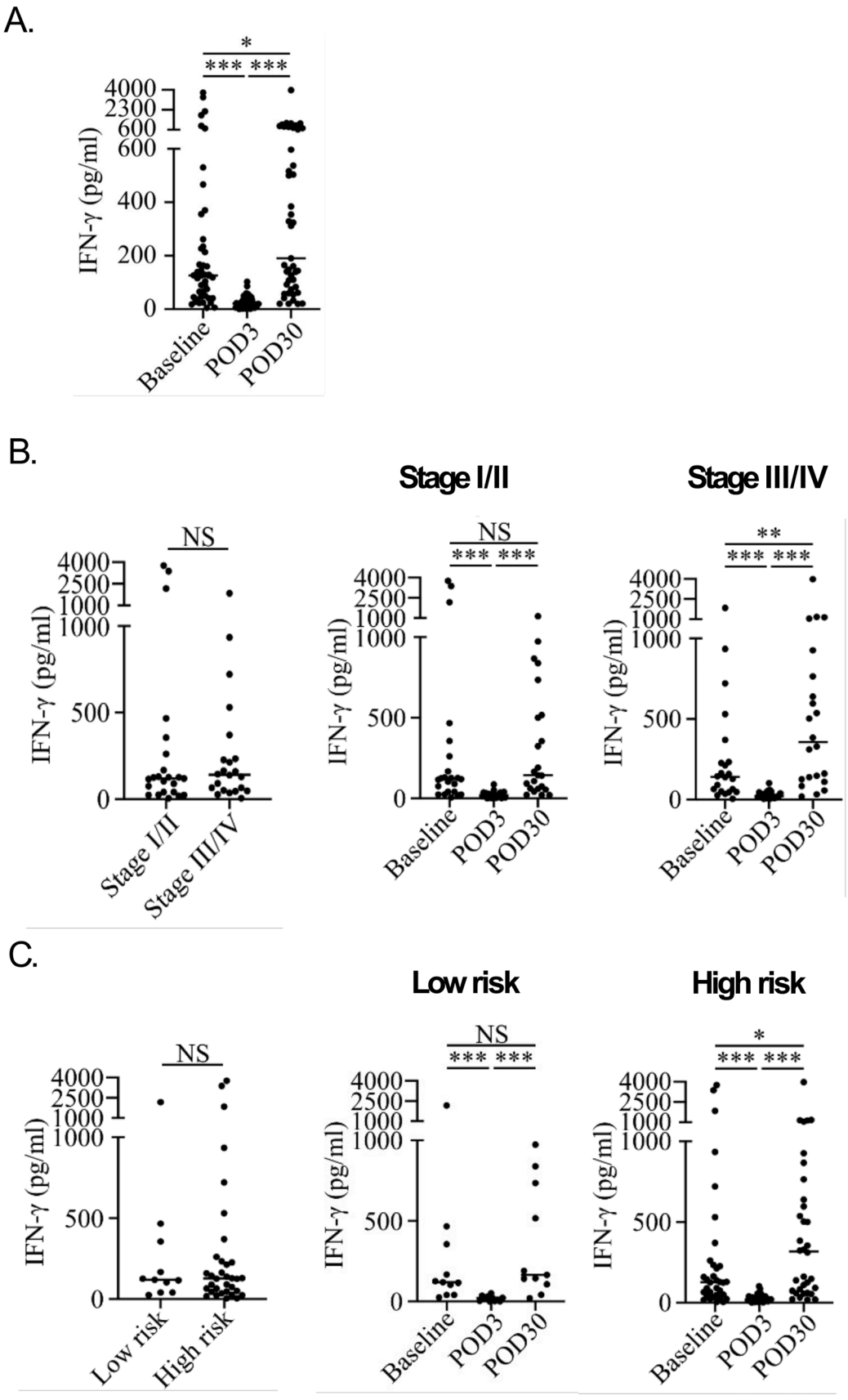

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Fig. 1. NKA expression in the perioperative period based on stage and risk factors. (A) NKA was downregulated after surgery on POD3 and recovered on POD30. The NK VUE ELISA assay was used to measure NKA levels in the peripheral blood of patients with CRC. (B) NKA was upregulated at the recovery stage (POD30) when compared to baseline in patients with stage III/IV CRC. (C) NKA was significantly upregulated on POD30 in patients with high-risk factors. (Data were statistically analyzed using the two-tailed Mann–Whitney U test and two-tailed Wilcoxon matched-pairs signed-rank test and are presented as a scatter plot with the median. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$); NKA: natural killer cell activity; CRC: colorectal cancer.

6.5 months, range: 9.6–34.8 months).

Higher recovery of NKA in patients with advanced tumor stages and preoperative high-risk factors

Patients were classified into early (stage I/II) and advanced (stage III/IV) stages according to the American Joint Committee on Cancer (AJCC) staging system. At baseline, no significant differences in NKA were observed between the two groups (stage I/II, median: 118.9 pg/mL, 95 % CI: 41.1–167.2; stage III/IV, median: 141.5 pg/mL, 95 % CI: 50.4–234) as shown in Fig. 2A. However, NKA was significantly higher on POD30 (median: 356.5 pg/mL, 95 % CI: 126.5–764.1) than at baseline (median: 141.5 pg/mL, 95 % CI: 50.4–234) in patients with stage III/IV CRC (baseline, median: 118.9 pg/mL, 95 % CI: 41.1–167.2; POD30, median: 143.7 pg/mL, 95 % CI: 61.6–500.3) (Fig. 2B and C, Supplementary Table 1). NKA was dramatically decreased on POD3 in patients with either stage I/II (median: 18.3 pg/mL, 95 % CI: 3.9–33.1) or stage III/IV (median: 21.01 pg/mL, 95 % CI: 17–44.9) CRC (Fig. 1B, Supplementary Table 1).

Further investigation into whether clinicopathological risk factors were correlated with the postoperative recovery of NKA on POD30 revealed that although there was no statistical difference in NKA between low-risk and high-risk groups at baseline (low risk, median: 118.9 pg/mL, 95 % CI: 40.6–466.8; high risk, median: 127.8 pg/mL, 95 % CI: 65.4–213.5), NKA on POD30 was significantly higher (median: 317.7 pg/mL, 95 % CI: 92.2–537.2) compared to baseline in high-risk patients (Fig. 1C, Supplementary Table 1). In contrast, in low-risk patients, NKA showed no significant change from baseline to POD30 (median: 165 pg/mL, 95 % CI: 41.4–838.4) (Fig. 1C, Supplementary Table 1). Regardless of risk status, NKA was markedly decreased on POD3 compared with baseline (low-risk, median: 22.46 pg/mL, 95 % CI: 2.3–36.4; high-risk, median: 20.3 pg/mL, 95 % CI: 16.1–35.6) (Fig. 1A and B, Supplementary Table 1). During the perioperative period, postoperative increments in circulating $CD56^{dim}$ NK cells were significantly positively correlated with increments in $CD56^{+}$ NK cells (r: 0.75, $P = 0.00$) but negatively with increments in $CD56^{bright}$ NK cells (r: -0.304, $P = 0.043$). However, postoperative recovery of NKA was not associated with postoperative increments in circulating NK cell subsets.

Association between clinicopathologic factors and perioperative change of NKA and circulating NK cell subset

Patients with a higher postoperative increase in the percentage of $CD56^{bright}$ NK cells were younger. Additionally, those with a greater postoperative increase in the percentage of $CD56^{bright}/CD16^{-}$ NK cells had lower preoperative CA19–9 levels. Although patients with a higher histological grade exhibited a higher postoperative circulating $CD56^{bright}$ NK cell/$CD56^{dim}$ NK cell ratio, the sample size is too small to draw definitive conclusions; thus, further patient enrollment is necessary to confirm these findings (Table 2).

Association between RFS/PFS and perioperative change of NKA and circulating NK cell subset

In the entire group, postoperative NKA recovery was not significantly associated with RFS or PFS in patients with CRC (Fig. 2A). However, in patients with stage III/IV disease, those with higher postoperative NKA recovery on POD30 demonstrated improved RFS and PFS (Fig. 2B, HR: 0.2442). Functional NK cell subsets contribute to NKA, including $CD56^{bright}/CD16^{-}$ and $CD56^{dim}/CD16^{+}$ cells. Therefore, we analyzed the association between perioperative changes in the percentage or postoperative ratio of circulating functional NK cell subsets and RFS/PFS. A higher postoperative increase in the percentage of $CD56^{bright}$ NK cells on POD30 was associated with better RFS/PFS (Fig. 2C, HR: 0.2732, $P = 0.0433$). Conversely, a higher postoperative increase in the percentage of CD56 $^{dim}/CD16^{+}$ NK cells was linked to worse survival outcomes (Fig. 2D, HR: 7.752, $P = 0.0209$). Patients with a higher postoperative circulating $CD56^{bright}$ NK cell/$CD56^{dim}$ NK cell ratio on POD30 also experienced better RFS/PFS (Fig. 2E, HR: 0.2193, $P = 0.024$), similar to the $CD56^{bright}\_CD16^{-}$ NK cells / CD56 $^{dim}\_$ $CD16^{+}$ NK cells ratio group (Fig. 2F, HR: 0.233, $P = 0.0338$).

Compared with the entire group, higher postoperative increments in the percentage of $CD56^{dim}$ NK and $CD56^{+}$ NK cells on POD30 were significantly associated with poorer RFS/PFS in patients with advanced-stage CRC (Fig. 3A, HR: 8.945, $P = 0.0129$ and Fig. 3B, HR: 6.868, $P = 0.0363$) and in those with preoperative high-risk factors (Fig. 3C, HR: 5.897, $P = 0.0093$ and Fig. 3D, HR: 5.192, $P = 0.0191$). Further multivariable analysis of clinicopathological and NK cell subset parameters identified no independent prognostic markers for RFS/PFS (Supplementary Table 2).

Subgroup analysis of RFS/PFS in the entire group and groups stratified by preoperative risk and stage

A higher postoperative circulating $CD56^{bright}$ NK cell/$CD56^{dim}$ NK cell ratio on POD30 was associated with improved RFS and PFS across the entire group (Fig. 4), as well as in patients with stage III/IV disease and those classified as preoperative high-risk (Fig. 5A and B). This trend was similarly observed in the $CD56^{bright}\_CD16^{-}$ NK cell/$CD56^{dim}\_CD16^{+}$ cell group. Additionally, higher postoperative NKA recovery was linked to better RFS/PFS in the stage III/IV group (Fig. 5A). Other clinical factors such as T stage, N stage, preoperative levels of CEA and CA19–9, angiolymphatic invasion, perineural invasion, and preoperative NLR also showed significant associations with RFS/PFS in the whole group (Fig. 4).

Discussion

This study assessed changes in NKA before and after tumor resection and examined the association between preoperative risk factors and NKA in patients with CRC. We measured the levels of IFN-γ secreted by NK cells in patients with CRC preoperatively and postoperatively. We found that NKA was significantly reduced on POD3 and recovered to at least baseline levels by POD30. Notably, on POD30, NKA was significantly higher compared to preoperative levels, particularly in patients diagnosed with advanced-stage disease or those with one or more preoperative high-risk factors. Additionally, a greater recovery of NKA in these patients was associated with improved RFS and PFS. A higher $CD56^{bright}$ NK cell/$CD56^{dim}$ NK cell ratio on POD30 was also significantly correlated with better RFS/PFS in patients with CRC. These findings suggest that the degree of NKA upregulation following surgery-induced suppression is linked to tumor stage and preoperative high-risk characteristics, highlighting the importance of postoperative $CD56^{bright}$ NK cells and NKA in the clinical outcomes of patients with CRC after curative surgery and providing a comprehensive overview of the immune response.

Numerous preclinical and clinical studies have reported that surgical manipulation can enhance metastatic disease and shorten overall

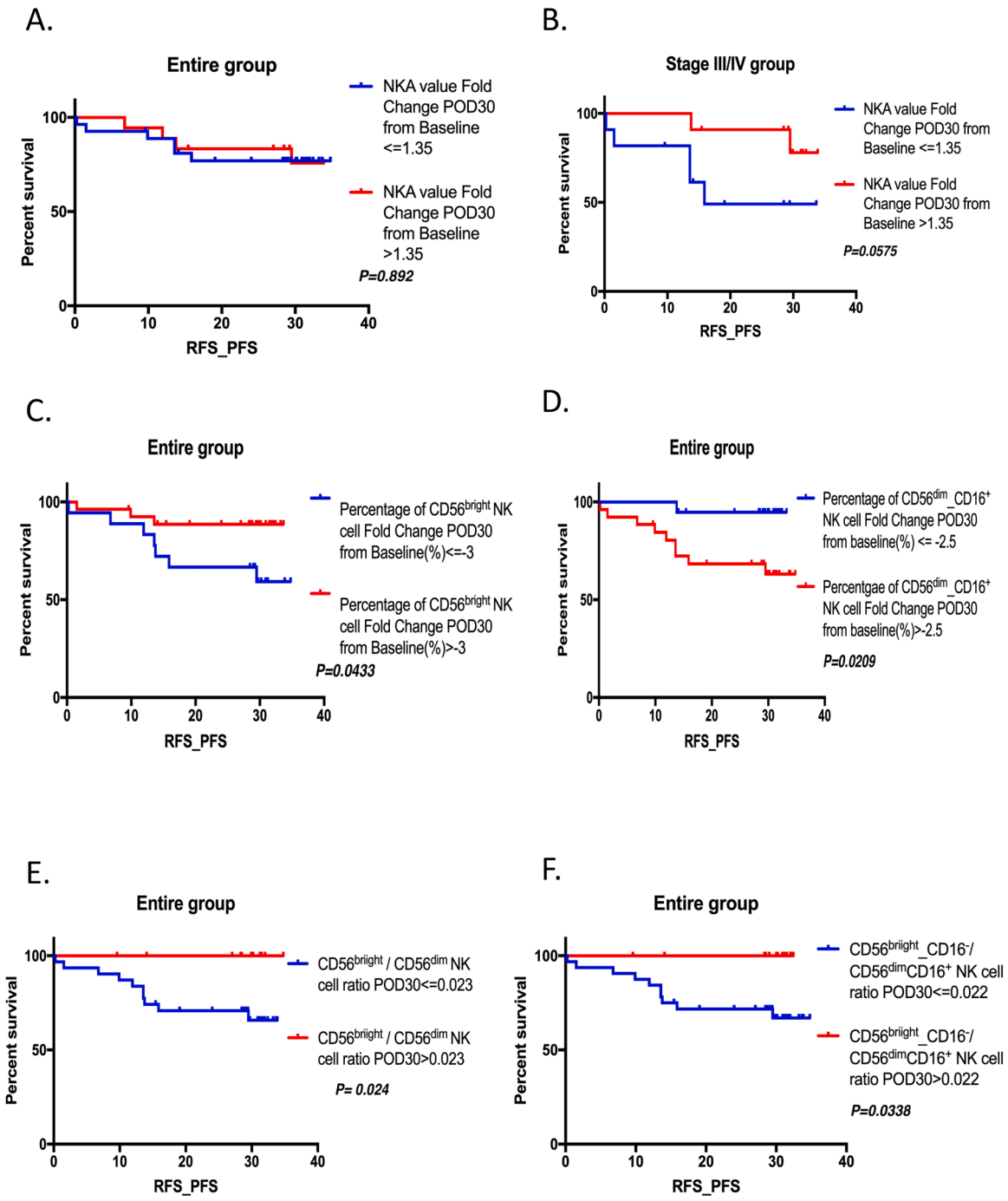

Fig. 2. Relationship between perioperative change of circulating lymphocyte subsets with varying immunophenotypes and recurrence-free survival (RFS)/progression-free survival (PFS) in the entire group. (A) RFS/PFS for NKA recovery on POD30 in the entire group. (B) RFS/PFS for NKA recovery on POD30 in the stage III/IV group. (C) RFS/PFS for postoperative increment of percentage of $CD56^{bright}$ NK cell on POD30 in the entire group. (D) RFS/PFS for postoperative increment of percentage of $CD56^{dim}\_CD16^{+}$ NK cell on POD30 in the entire group. (E) RFS/PFS for postoperative circulating $CD56^{bright}$ NK cell/$CD56^{dim}$ NK cell ratio in the entire group. (F) RFS/PFS for postoperative circulating $CD56^{bright}\_CD16^{-}$ NK cell/$CD56^{dim}\_CD16^{+}$ cell ratio in the entire group.

survival [34–38]. In contrast, NK cell-secreted IFN-γ has been shown to play a crucial role in anti-metastasis [39–41], promoting the accumulation, activation, and cytotoxic ability of NK cells to eradicate metastatic cancer cells. Additionally, depletion of IFN-γ significantly enhances metastasis in a mouse model [39,41]. NK cells are also essential for eliminating circulating tumor cells [42]. Furthermore, evidence suggests that surgery-induced NK cell dysfunction is strongly linked to postoperative metastasis and recurrence [43,44]. Perioperative measures to prevent surgery-suppressed NKA can reduce postoperative metastatic disease and recurrence [43,45]. These findings underscore the indispensable roles of IFN-γ and NK cells in preventing postoperative metastasis. In our study, we confirmed that NKA was markedly suppressed in patients with CRC three days after surgical resection, likely due to surgical stress. However, NKA recovered by POD30 and even increased significantly compared to baseline levels. The NK VUE ELISA, an alternative assay, proved to be a powerful and convenient tool for measuring NKA in peripheral blood [46]. Unlike traditional methods such as tumor killing and degranulation assays, its high-throughput, time-efficient, and reproducible characteristics make it well-suited for clinical practice.

Table 2
Association between clinicopathologic factors and Perioperative change of NKA and Circulating NK Cell subset.

	Total	Age<=65	Age>=65	p-value	Female	Male	p-value	Stage I/II	Stage III/IV	p-value	Low preop risk group	High preop risk group	p-value	T1/2	T3/4	p-value	N0	N1/2	p-value	Low histological grade	High histological grade	p-value	No Angiosymphatic invasion	Angiosymphatic invasion	p-value	No perineural invasion	perineural invasion	p-value	Preop N/L ratio baseline <3	Preop N/L ratio baseline >=3	p-value	Preop CEA<=5	Preop CEA>5	p-value	Preop CA199<=37	Preop CA199>37	p-value
	N(%)	N(%)	N(%)		N(%)	N(%)		N(%)	N(%)		N(%)	N(%)		N(%)	N(%)		N(%)	N(%)		N(%)	N(%)		N(%)	N(%)		N(%)	N(%)		N(%)	N(%)		N(%)	N(%)		N(%)	N(%)	
Total	45	24(53)	21(47)		22(49)	23(51)		23(51)	22(49)		15	30		8(18)	37(82)		23(51)	22(49)		41(91)	4(9)		31(69)	14(31)		35(78)	10(22)		34(76)	11(24)		25(56)	20(44)		36(80)	9(20)	
NKA value Fold Change POD30 from Baseline				0.807			0.465			0.181			0.519			0.08			0.181			0.669			0.793			0.464			0.777			0.54			0.648
<=1.35	27(60)	14(58)	13(62)		12(54)	15(65)		16(70)	11(50)		10(67)	17(57)		7(88)	20(54)		16(70)	11(50)		25(61)	2(50)		19(61)	8(57)		22(63)	5(50)		20(59)	7(64)		16(64)	11(55)		21(58)	6(67)	
>1.35	18(40)	10(42)	8(38)		10(46)	8(35)		7(30)	11(50)		5(33)	30(43)		1(12)	17(46)		7(30)	11(50)		16(39)	2(50)		12(39)	6(43)		13(37)	5(50)		14(41)	4(36)		9(36)	9(45)		15(42)	3(33)	
Percentage of $CD56^{high}$ NK cell Fold Change POD30 from Baseline(%)				0.905			0.873			0.661			1			0.322			0.661			0.889			0.731			0.632			0.431			0.841			0.1
<=3	24(53)	13(54)	11(52)		12(54)	12(52)		13(57)	11(50)		8(53)	16(53)		3(37)	21(57)		13(57)	11(50)		22(54)	2(50)		16(52)	8(57)		18(51)	6(60)		17(50)	7(64)		13(52)	11(55)		17(47)	7(78)	
>3	21(47)	11(46)	10(48)		10(46)	11(48)		10(43)	11(50)		7(47)	14(47)		5(63)	16(43)		10(43)	11(50)		19(46)	2(50)		15(48)	6(43)		17(49)	4(40)		17(50)	4(36)		12(48)	9(45)		19(53)	2(22)	
Percentage of $CD56^{dim}$ NK cell Fold Change POD30 from Baseline(%)				0.661			0.652			0.458			0.399			0.136			0.458			0.963			0.235			0.524			0.666			0.641			0.766
<=-0.5	22(49)	11(46)	11(52)		10(46)	12(52)		10(43)	12(55)		6(40)	16(53)		2(25)	20(54)		10(43)	12(55)		20(49)	2(50)		17(55)	5(36)		18(51)	4(40)		16(47)	6(55)		13(52)	9(45)		18(50)	4(44)	
>-0.5	23(51)	13(54)	10(48)		12(54)	11(48)		13(57)	10(45)		9(60)	14(47)		6(75)	17(46)		13(57)	10(45)		21(51)	2(50)		14(45)	9(64)		17(49)	6(60)		18(53)	5(45)		12(48)	11(55)		18(50)	5(56)	
Percentage of $CD56^{+}$ NK cell Fold Change POD30 from Baseline(%)				0.012			0.661			0.661			0.526			0.176			0.661			0.889			0.322			0.632			0.431			0.423			0.37
<=-0.6	21(47)	7(29)	14(67)		13(50)	10(43)		10(43)	11(50)		6(40)	15(50)		2(25)	19(51)		10(43)	11(50)		19(46)	2(50)		16(52)	5(36)		17(49)	4(40)		17(50)	4(36)		13(52)	8(40)		18(50)	3(33)	
>0.6	24(53)	17(71)	7(33)		11(50)	13(57)		13(57)	11(50)		9(60)	15(50)		6(75)	18(49)		13(57)	11(50)		22(54)	2(50)		15(48)	9(64)		18(51)	6(60)		17(50)	7(64)		12(48)	12(60)		18(50)	6(67)	
$CD56^{bright}$ / $CD56^{dim}$ NK cell ratio POD30				0.763			0.457			0.92			0.82			0.681			0.92			0.047			0.805			0.389			0.752			0.428			0.147
<=0.023	31(69)	17(71)	14(67)		14(64)	17(74)		16(70)	15(68)		10(67)	21(70)		6(75)	25(68)		16(70)	15(68)		30(73)	1(25)		21(68)	10(71)		23(66)	8(80)		23(68)	8(73)		16(64)	15(75)		23(64)	8(89)	
>0.023	14(31)	7(29)	7(33)		8(36)	6(26)		7(30)	7(32)		5(33)	9(30)		2(25)	12(32)		7(30)	7(32)		11(27)	3(75)		10(32)	4(28)		12(34)	2(20)		11(32)	3(27)		9(36)	5(25)		13(36)	1(11)	
Percentage of $CD56^{bright}$ _CD16^{-} NK cell Fold Change POD30 from baseline(%)				0.905			0.051			0.873			1			0.835			0.873			0.363			0.322			0.632			0.547			0.316			0.017
<=16	24(53)	13(54)	11(52)		15(68)	9(39)		12(52)	12(55)		8(53)	16(53)		4(50)	20(54)		12(52)	12(55)		21(51)	3(75)		15(48)	9(64)		18(51)	6(60)		19(56)	5(45)		15(60)	9(45)		16(44)	8(89)	
>16	21(47)	11(46)	10(48)		7(32)	14(61)		11(48)	10(45)		7(47)	14(47)		4(50)	17(46)		11(48)	10(45)		20(49)	1(25)		16(52)	5(23)		17(49)	4		15(44)	6(55)		10(40)	11(55)		20(56)	7(11)	
Percentage of $CD56^{dim}$ _CD16^{+} NK cell Fold Change POD30 from baseline(%)				0.079			0.445			0.928			1			0.318			0.928			0.937			0.356			0.28			0.129			0.651			0.178
<= 2.5	33(73)	15(63)	18(86)		15(68)	18(78)		17(74)	16(73)		11(73)	22(73)		7(88)	26(70)		17(74)	16(73)		30(73)	3(75)		24(77)	9(64)		27(77)	6(60)		23(68)	10(91)		19(76)	14(70)		28(78)	5(56)	
>2.5	12(27)	9(37)	3(14)		7(32)	5(22)		6(26)	6(27)		4(27)	8(27)		1(12)	11(30)		6(26)	6(27)		11(27)	1(25)		7(23)	5(36)		8(23)	4(40)		11(32)	1(9)		6(24)	6(30)		8(22)	4(44)	
$CD56^{bright}$ _CD16 / $CD56^{dim}$CD16^{+} NK cell ratio POD30				0.538			0.279			0.815			0.816			0.789			0.815			0.033			0.458			0.93			0.892			0.066			0.188
<=0.022	32(71)	18(75)	14(67)		14(64)	18(78)		16(70)	16(73)		11(73)	21(70)		6(75)	26(70)		16(70)	16(73)		31(76)	1(25)		21(68)	11(79)		25(71)	7(70)		24(71)	8(73)		15(60)	17(85)		24(67)	8(89)	
>0.022	13(29)	6(25)	7(33)		8(36)	5(22)		7(30)	6(27)		4(27)	9(30)		2(25)	11(30)		7(30)	6(27)		10(24)	3(75)		10(32)	3(23)		10(29)	3(30)		10(29)	3(27)		10(40)	3(15)		12(33)	1(11)	

P values were derived by using Chi-square test; NK cell, Nature Killer cell; NKA, Nk cell activity; POD, postoperaive day

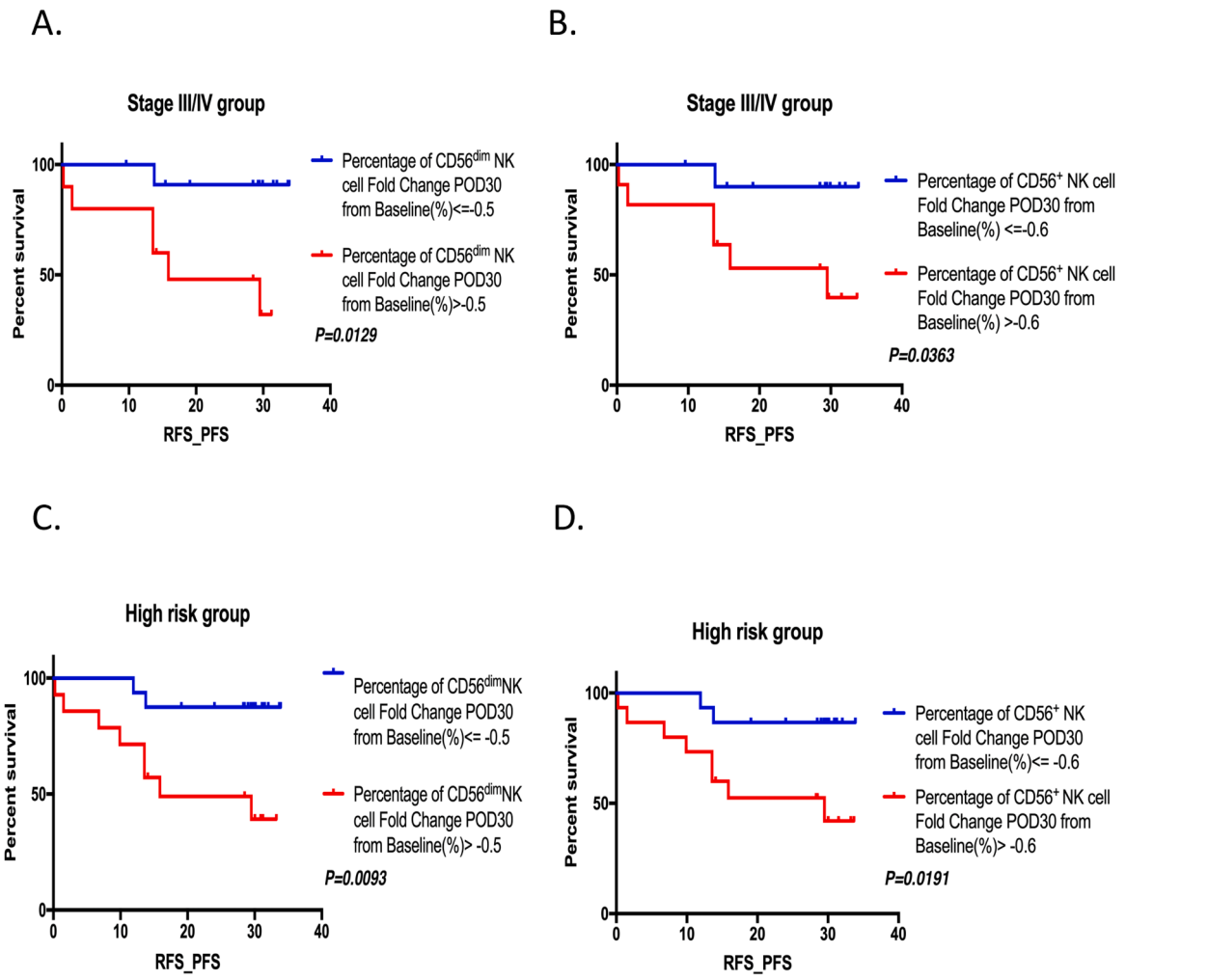

Fig. 3. Relationship between perioperative change of circulating lymphocyte subsets with varying immunophenotypes and recurrence-free survival (RFS)/progression-free survival (PFS) in the stage II/IV group and high-risk group. (A) RFS/PFS for postoperative increments of percentage of $CD56^{dim}$ NK cell on POD30 in stage III/IV group. (B) RFS/PFS for postoperative increments of percentage of $CD56^{+}$ NK cell on POD30 in stage III/IV group. (C) RFS/PFS for postoperative increments of percentage of $CD56^{dim}$ NK cell on POD30 in preoperative high-risk group (D) RFS/PFS for postoperative increments of percentage of $CD56^{+}$ NK cell on POD30 in preoperative high-risk group.

Some clinicopathological risk factors are associated with poor prognosis, recurrence, metastasis, and decreased survival in cancer patients. Clinicians often consider these risk factors when making treatment decisions. In this study, we evaluated commonly used risk factors, such as poorly differentiated histology, lymphovascular invasion, perineural invasion, T4 stage, and elevated tumor markers (CEA and CA19–9), to explore their relationship with NKA during the perioperative period of tumor resection. Our results indicated that preoperative

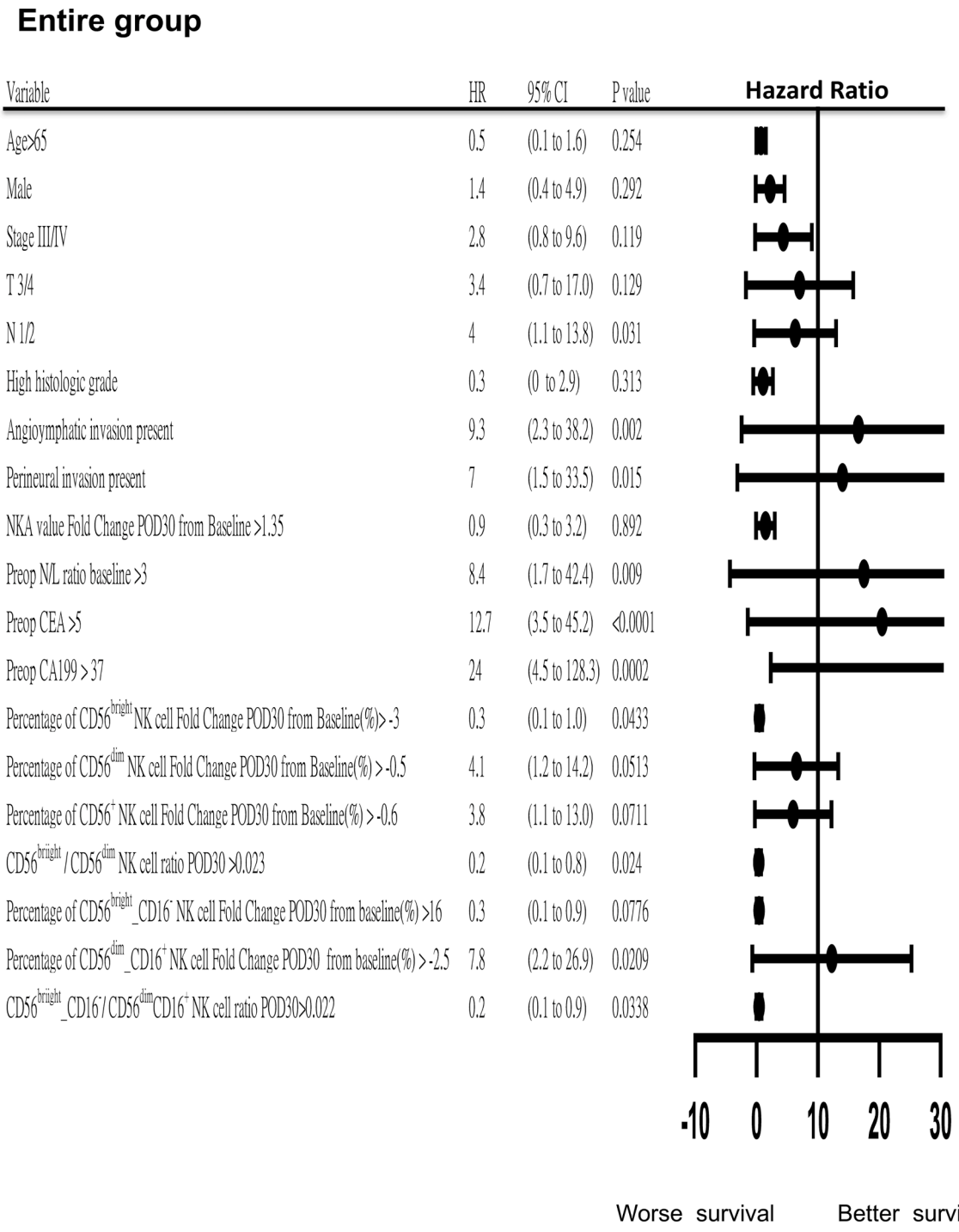

Fig. 4. Forest plot of hazard ratios (HRs) with 95 % confidence intervals (CI) for recurrence-free survival (RFS)/ progression-free survival (PFS) in the entire group.

high-risk factors correlated with NKA recovery on POD30, with NKA increasing on POD30 compared to baseline in patients with high-risk factors. Similarly, tumor stage—whether early or advanced—was associated with NKA recovery on POD30, with a significant increase in NKA on POD30 compared to baseline observed in patients with advanced tumor stages. Consequently, NKA may serve as an adjunct marker for determining postoperative therapy in clinical practice, though this requires further evaluation.

Our findings demonstrated that NKA was suppressed on POD3 and returned to at least baseline levels by POD30, regardless of tumor stage or risk factors. Patients with advanced tumor stages showed notable NKA recovery on POD30 compared to the preoperative baseline levels. Additionally, patients who exhibited higher NKA recovery on POD30 had better RFS and PFS. This suggests that NKA is crucial for managing relatively advanced CRC tumors. The P-value was not significant, possibly due to inadequate follow-up time and a limited number of patients. Another factor is that IFN-γ, while a key contributor to NKA, is not the sole mediator; other cytokines such as TNF-α, IL-2, IL-12, IL-15, and IL-18 also play roles in inducing NK 'cell cytotoxicity [47]. Therefore, it is necessary to utilize a convenient method such as the whole blood NK cytotoxicity assay for clinical laboratory research, such as the flow cytometry-based overnight whole blood NK cytotoxicity assay [48].

$CD56^{bright}$ NK cells are now recognized as more than just a minor subpopulation among total NK cells. Due to their ability to produce

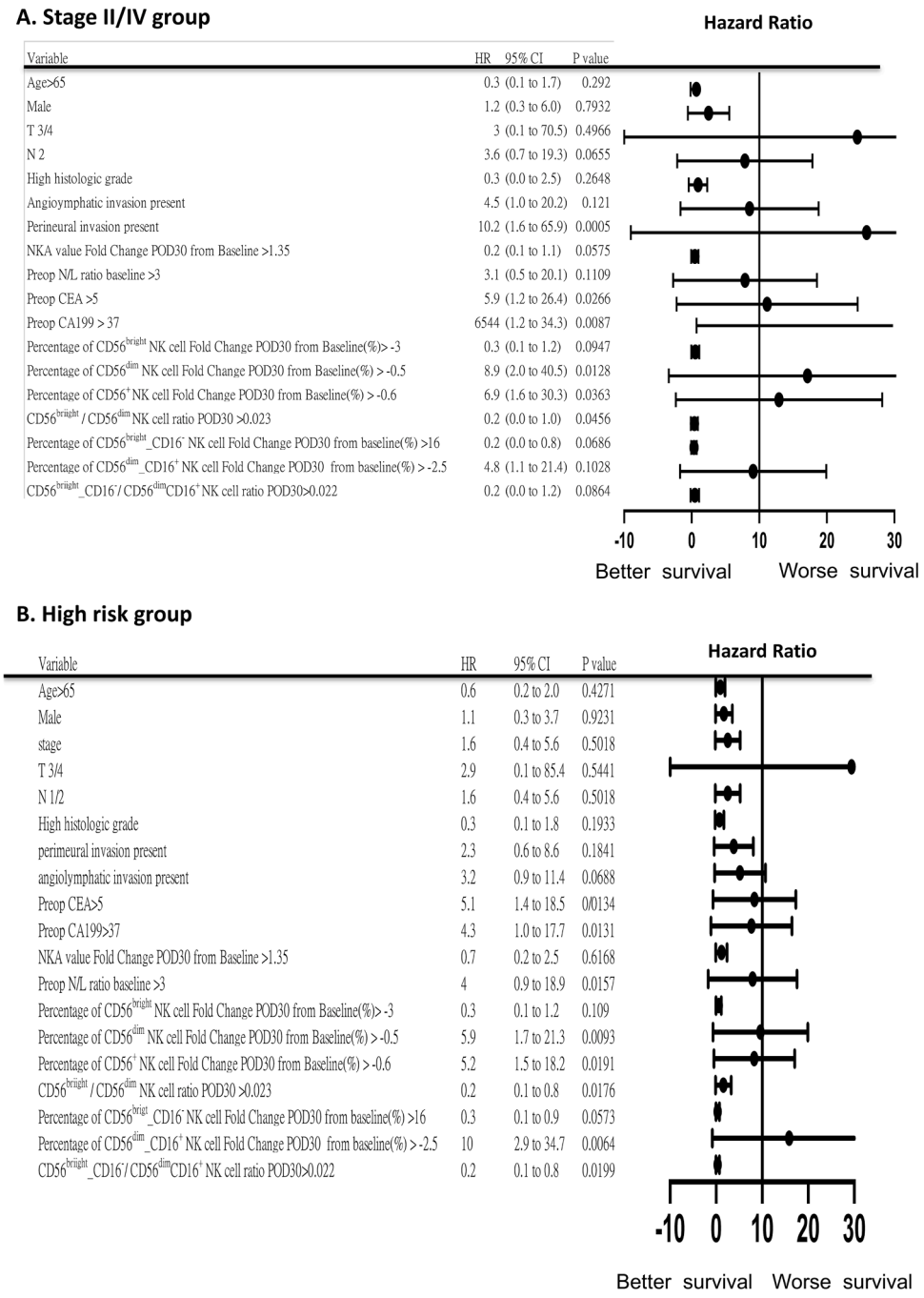

Fig. 5. Forest plot of hazard ratios (HRs) with 95 % confidence intervals (CI) for recurrence-free survival (RFS)/ progression-free survival (PFS) in the stage III/IV group and high-risk group, (A) Stage III/IV groups. (B) high-risk group.

various cytokines, they play a critical role in early immune responses and in shaping the adaptive immune response through IFN-γ and act as regulatory NK cells through interleukin-10 (IL-10) [49]. In comparison with $CD56^{dim}$ NK cells, human $CD56^{bright}$ NK cells uniquely contribute to the innate immune response as the primary producers of immunoregulatory cytokines such as IFN-γ, thereby regulating NKA [50]. Our findings indicate that a higher $CD56^{bright}$ NK cell/$CD56^{dim}$ NK cell ratio and increased percentages of circulating $CD56^{dim}$ NK cells on POD30 were significantly associated with improved RFS and PFS in patients with CRC. Conversely, higher postoperative increments of circulating $CD56^{dim}$ NK and $CD56^{+}$ NK cells on POD30 were significantly associated with poorer RFS/PFS. These results underscore the prognostic importance of $CD56^{bright}$ NK cells and their role in regulating NKA.

This study has several limitations. First, the small number of cases limited further exploration of potential confounders associated with NKA recovery after CRC surgery. Second, despite the clinical significance of NKA recovery on POD30 in patients with advanced tumor stages and high-risk factors, the underlying mechanisms remain poorly understood. Therefore, prospective studies with larger sample sizes and the inclusion of healthy controls are warranted to elucidate these findings further.

Conclusion

Our results reveal that a higher postoperative increase in the percentage/ratio of $CD56^{bright}$ NK cells on POD30 in CRC patients correlates

with better clinical outcomes and enhanced recovery of NKA in those with advanced-stage disease. These findings not only offer potential implications for the use of postoperative NKA and circulating NK cell subset analyses in patient risk stratification and the development of treatment strategies but also provide a foundation for CRC research. This includes molecular classification to guide precision therapy by interpreting NKA data and analysis of NK cell subsets. Further research is necessary to confirm the role and function of recovered NK cells following the surgical removal of CRC tumors and to investigate potential confounding factors that may affect NKA recovery.

CRediT authorship contribution statement

Jeng-Fu You: Writing – review & editing, Writing – original draft, Software, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Cheng-Chi Lee:** Resources, Methodology, Investigation, Conceptualization. **Yun-Shien Lee:** Resources, Methodology, Investigation, Formal analysis, Data curation. **Yih-Jong Chern:** Resources, Investigation, Data curation. **Chun-Kai Liao:** Software, Resources, Formal analysis, Data curation. **Hung-Chih Hsu:** Writing – review & editing, Writing – original draft, Supervision, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgements

We acknowledge the Chang Gung Memorial Hospital and University for their support in this research. This work was supported by grants from Chang Gung Memorial Hospital (CMRPG3J1531 to Jeng-Fu You).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2024.102198.

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