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ORIGINAL PAPER

Circulating CD16+CD56+ nature killer cells indicate the prognosis of colorectal cancer after initial chemotherapy

Feng Cui[1,2] · Di Qu[2] · Ruya Sun[2] · Kejun Nan[1]

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Abstract

As the prognosis of colorectal cancer (CRC) does not always coincide with the pathology and/or surgical findings, a reliable noninvasive prediction tool for the prognosis of CRC is needed. Patients admitted for initial treatment of CRC between January 1, 2015 and December 31, 2015 were retrieved and reviewed. Records of circulating CD16+CD56+ natural killer (NK) cells were analyzed before and after the initial chemotherapy of FOLFOX plan. Patients were followed up until June 30, 2019. One hundred and twenty-four cases after the FOLFOX chemotherapy were enrolled into this study. There were no significant differences in gender, age, or number of metastasis cases between the survival group and the nonsurvival group ($p > 0.05$), but significant differences in pre-chemotherapy, post-chemotherapy, and the differences between pre- and post-chemotherapy circulating CD16+CD56+NK cells between the survival group and the nonsurvival group ($p < 0.01$, $p < 0.01$, and $p < 0.05$, respectively) were observed. For the prediction of survival and nonsurvival CRC cases, the Areas Under the Curve were 0.626 and 0.759 in the Receiver-Operating Characteristic curves for the pre- and post-chemotherapy circulating CD16+CD56+NK cells, respectively. Using an optimal cutoff value of 11.8% in post-chemotherapy circulating CD16+CD56+NK cells to differentiate survival and nonsurvival cases, the odds ratio was 0.12 (0.05, 0.27), $p < 0.001$. The percentages of both pre-chemotherapy and post-chemotherapy circulating CD16+CD56+NK cells were negatively correlated with the prognosis of CRC. The percentage of post-chemotherapy circulating CD16+CD56+NK cells was able to effectively predict the prognosis of CRC cases.

Keywords Colorectal cancer · Nature killer cells · Prognosis

Introduction

With an incidence of nearly one million and fatality of nearly 700,000 per year, colorectal cancer (CRC) has been ranked the fourth most deadly cancer in the world [1, 2]. Along with the up to 25% unresectable cases at the time of diagnosis, and 50% recurrence rate in early-stage cases following surgery [3], an accurate prediction of the prognosis of CRC is warranted.

Traditionally, the staging and prognosis of CRC mainly rely on pathology after surgical procedures [4]. However, as the prognosis of CRC does not always coincide with the pathology and/or surgical findings, challenges have been proposed against the traditional TNM system of staging [5]. An immunoscore for the classification and prognosis of CRC has been reported [6]. Despite this, complementary and noninvasive biomarkers in the diagnosis of CRC have been reported [7]—a reliable prediction tool for the prognosis of CRC after initial chemotherapy is still lacking.

The immune system has been reported to be involved in the development and progression of CRC [8]. Immune infiltration of different immune cells in CRC and other solid cancers in the digestive system has been shown to be related with metastasis and prognosis [9], whereas the circulating

✉ Kejun Nan
1806524503@qq.com

Feng Cui
cuifeng0915@126.com

Di Qu
a58339431a@163.com

Ruya Sun
sunruya@sina.com

[1] Department of Oncology, The First Affiliated Hospital of Xi'an Jiao Tong University, No. 277, Yanta West Road, Xi'an 710061, Shanxi, China

[2] Department of Oncology, The Second Affiliated Hospital of Harbin Medical University, Harbin, China

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immune cells may reflect the local immune response in the tumor microenvironment [10], thereby providing important information regarding disease severity in CRC [11]. Natural killer (NK) cell is an important subset of the immune cells. The activity of NK cells is triggered by a dynamic equilibrium between activating and inhibitory signals received and processed by cell surface receptors. Therefore, NK cells are considered interesting targets for translational and clinical studies [12].

In the present study, we analyzed circulating CD16+CD56+NK cells in CRC patients before and after initial chemotherapy, trying to figure out the value of CD16+CD56+NK cells in the prediction of the prognosis of CRC.

Methods

This retrospective cohort study was carried out at the Department of Oncology of the second Affiliated Hospital of Harbin Medical University, which is a tertiary hospital in Northeast China. Institutional Ethics Committee approval was obtained from the second Affiliated Hospital of Harbin Medical University before data collection and analysis, and informed consent was obtained from patients on admission.

Clinical records of patients who were admitted for initial chemotherapy of CRC between January 1, 2015 and December 31, 2015 were retrieved and reviewed. Patients were followed up until June 30, 2019. Included patients should have pre-chemotherapy (the most recent one before chemotherapy) and post-chemotherapy (the most recent one after the first cycle of chemotherapy) NK cell data available, as well as histologically confirmed primary CRC. Staging was based on the Tumor Node Metastasis (TNM) terminology [13]. The FOLFOX plan was employed for chemotherapy in all enrolled patients, with the following details: Oxaliplatin 85 mg/m^2 IV infusion, given as a 120 min IV infusion, concurrent with leucovorin 400 mg/m^2 (or levoleucovorin 200 mg/m^2) IV infusion, followed by 5-FU 400 mg/m^2 IV bolus, followed by 6-h 5-FU 2400 mg/m^2 infusion every 2 weeks for 6 months [13]. Patients with unclear diagnosis, or complicated with other caner, or were admitted after previous chemotherapy treatments for CRC, or with other chronic diseases (such as cardiovascular diseases, endocrine diseases), or with viral or bacterial infections were excluded.

NK cells were analyzed through flow cytometer. Briefly, peripheral venous blood were collected from all participants in a heparin-coated tube and kept at 2–8 °C. One hundred µl of freshly collected blood was transferred into a flow-specific tube. Twenty µl of BD Multitest 6-color TBNK reagent (Ref # 644611, BD, USA, including CD3 FITC, CD16 PE+CD56 PE, CD45 PerCP-Cy5.5, CD4 PE-Cy7, CD19 APC, and CD8 APC-Cy7.) was added for flow cytometry study according to the manufacture manual, within the panel of which CD16+CD56+ specifically quantified NK cells within the lymphocyte population. The mixture was incubated at room temperature for 15 min in the dark, and treated with 2.5 ml of red blood cell lysis buffer (Sigma-Aldrich) for 10 min at room temperature in the dark. The mixture was washed twice with PBS buffer, resuspended, and fixed in 0.5 ml PBS containing 0.9% formaldehyde (Sigma-Aldrich), and analyzed using FACSCanto II flow cytometer system (BD Biosciences) by FACSDiva™ software version 8.0 (BD Biosciences). At least 20,000 cells were analyzed in P1 gate from each sample.

Statistical analysis

Discrete data were expressed as number of cases (percentages) and analyzed using χ^2-test or Fisher's exact test, along with odds ratio (OR) and 95% confidence interval (95% CI), whichever was applicable. Continuous data were shown as mean ± standard deviation (SD), and were analyzed using t test. Area under the Receiver-Operating Characteristic (ROC) curve was used to show the value of prediction. Pearson correlation was used for correlation analysis. Kaplan–Meier plot was used for survival analysis. SPSS24.0 (IBM Corp, Armonk, NY) was used for statistical analysis. A two-tailed $p < 0.05$ is considered significantly different.

Results

During the study period, seven hundred and eight CRC patients were admitted to our hospital, including eighty-two of stage I, one hundred and ninety-eight of stage II, two hundred and forty-nine of stage III, and one hundred and seventy-nine of stage IV patients. After excluding 12 cases of incomplete data, one hundred and twenty-four cases after the chemotherapy of FOLFOX plan were enrolled into this study (Fig. 1). There were no significant differences in gender, age, or number of metastasis cases between the survival group and the nonsurvival group ($p > 0.05$, Table 1).

The predictive value of circulating CD16+CD56+NK cells in the prognosis of CRC

There were significant differences in pre-chemotherapy, post-chemotherapy, and the difference between pre- and post-chemotherapy circulating CD16+CD56+NK cells between the survival group and the nonsurvival group ($p < 0.01$, $p < 0.01$, and $p < 0.05$, respectively, Table 1).

The receiver-operating characteristic (ROC) curve was employed to show the role of circulating CD16+CD56+NK cells in the prognosis of CRC. When comparing pre-chemotherapy circulating CD16+CD56+NK cells in survival

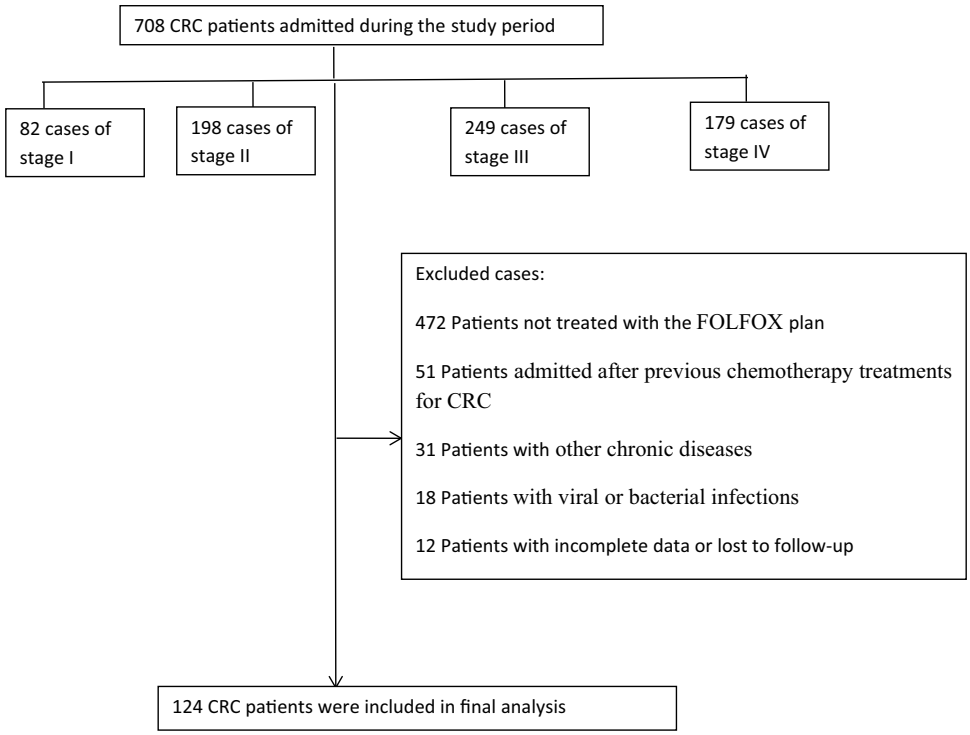

Fig. 1 Flow chart of included and excluded cases in the present study

Table 1 Clinical characteristics of enrolled patients

Parameters	Survival ($n=76$)	Nonsurvival ($n=48$)	p value
Male	48	30	0.94*
Age (years)[a]	54.7 ± 15.2	51.1 ± 13.2	0.173**
Metastasis cases	24	20	0.25*
Ptreatment CD16+CD56+NK %	17.5 ± 7.5	13.7 ± 7.3	< 0.01**
Posttreatment CD16+CD56+NK %	17.0 ± 7.2	10.8 ± 4.7	< 0.01**
Difference CD16+CD56+NK%	0.6 ± 7.3	2.9 ± 4.1	0.045**

[a]Mean ± SD

*χ^2 test

**t test

and nonsurvival CRC cases (Fig. 2), the Area Under the Curve (AUC) was 0.626. When comparing post-chemotherapy circulating CD16+CD56+NK cells in survival and nonsurvival CRC cases (Fig. 2), the AUC was 0.759.

Using an optimal cutoff value of 11.8% in post-chemotherapy circulating CD16+CD56+NK cells to differentiate survival and nonsurvival cases, the OR was 0.12 (0.05, 0.27), $p < 0.001$, sensitivity was 73.7%, specificity was 75.0%, positive predictive value (PPV) was 82.4%, and negative predictive value (NPV) was 64.3%. Using the same cutoff value of 11.8% in post-chemotherapy circulating CD16+CD56+NK cells, a Kaplan–Meier plot was drawn showing a significant difference in survival curve below and above the cutoff value ($p < 0.01$ by Log Rank test, Fig. 3).

Correlations between circulating CD16+CD56+NK cells and prognosis of CRC

There were significant positive correlations between the pre-chemotherapy circulating CD16+CD56+NK cells and the survival rate of CRC cases ($p < 0.01$), between post-chemotherapy circulating CD16+CD56+NK cells and the survival rates of CRC cases ($p < 0.01$), as well as between the differences in the circulating CD16+CD56+NK cells and the survival rate of CRC cases ($p = 0.045$, Table 2).

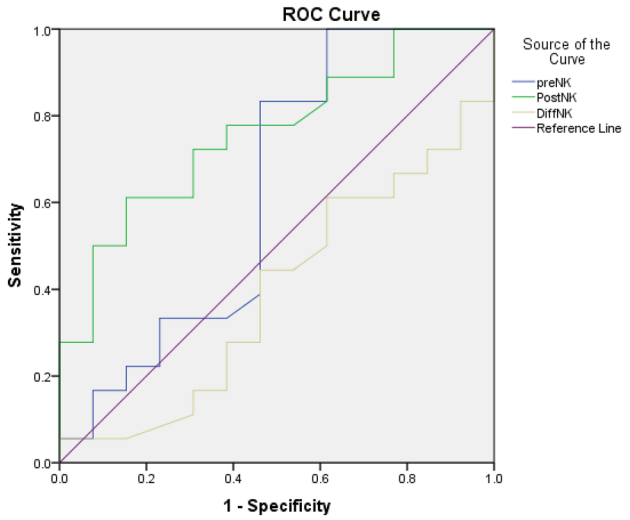

Fig. 2 Ptreatment, posttreatment, and the difference between pre- and posttreatment circulating CD16+CD56+NK cells in the prediction of prognosis of CRC cases analyzed by ROC curve

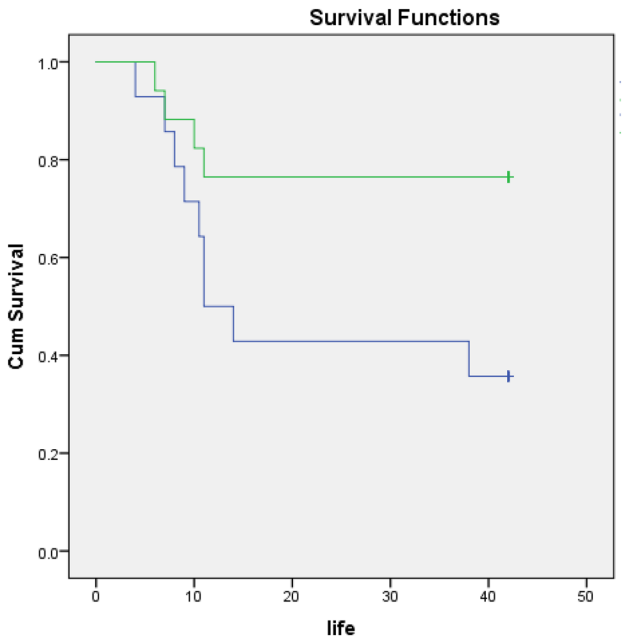

Fig. 3 Kaplan–Meier survival plot using posttreatment circulating CD16+CD56+NK cells threshold. Unit in *x*-axis is month. Green line represents survival with posttreatment circulating CD16+CD56+NK cells > 11.8%. Blue line represents survival with posttreatment circulating CD16+CD56+NK cells equal or less than 11.8%

Discussion

The NK cell population can generally be determined by CD56. The subpopulation of $CD56^{bright}$ NK cells is associated with immunoregulation and production of proinflammatory cytokines and, on the other hand, $CD56^{dim}$ NK cells exert cytotoxic activity [14]. The CD16 (FcγRIII) on NK cells is involved in antibody-dependent cell-mediated cytotoxicity [15], and as such, the CD16-positive population excludes the involvement of certain NK cells correlated with T or B cells [16].

Table 2 The correlations between circulating CD16+CD56+NK cells and prognosis of CRC

NK cells at different stages (%)	*p* value*
Ptreatment NK	< 0.01
Posttreatment NK	< 0.01
Difference NK	0.045

*Pearson correlation

Immune infiltration in CRC lesions is associated with clinical outcomes. A recent study has shown the diversity of functionally distinct cell types during the immune response in CRC cases [17]. The number of NK cells (CD56-positive cells) in lymph nodes has been reported to be associated with the prognosis of patients with stage II CRC [18]. Another study showed a decrease of peripheral CD16+CD56+NK cells in stage II and III CRC cases, although with limited number of participants and heterogeneous staging information [19]. In our present study, we analyzed the pre-chemotherapy and post-chemotherapy circulating CD16+CD56+NK cells. The chemotherapy plan was taken into account, as well as the number of metastasis cases in the survival group and the nonsurvival group. On top of these, with a larger number of enrolled cases, our study is statistically more convincing.

Some studies used CD3−CD56+ as markers for NK cells. One study found that "percentage of CD16+NKT-like cells was independently associated with shorter disease-free survival in CRC patients" [20]. However, some of the enrolled patients have already received radiological treatment before NK cells were collected, which might introduce heterogeneity into the rest of patients without treatment. Moreover, only a limited number of patients were enrolled, and the stratification by different stages as well as $CD56^{bright}$ and $CD56^{dim}$ NK cell population further minimized the statistical power. Another study showed a negative correlation between peripheral NK cells and the TNM staging of CRC. Significant differences in NK cells between the healthy and early stages (I&II) and the healthy and late stages (IV) were found [21]. This inconsistency in trend (no difference found between healthy and stage III) might be attributed to the small number of enrolled patients. In our study, we enrolled more patients, and only patients without previous treatment were eligible for analysis, which might disclose the natural body response after the initial chemotherapy. Thus, our data are more homogeneous and convincing.

In our study, we also excluded cases with viral or bacterial infections. NK cells have activating receptors and inhibitory receptors on the surface of cell membrane [22]. Both activating [23–25] and inhibitory receptors [26, 27] are necessary to clear viral infections. On the other hand, the stimulation of direct Toll-like receptors (TLRs) TLRs on NK cells has also been reported to be involved in NK cell activation [28]. Therefore, exclusion of viral or bacterial infection cases will minimize the influence of those cases in the analysis of CRC cases.

Other prediction strategies of the prognosis of CRC have been reported based on immunohistochemistry staining of lesions for gene mutation or polymorphism [29, 30], or activation [31]. Circulating biomarkers for the prognosis of CRC include microRNA or methylation [32, 33]. Serum metabolomics analysis has also been investigated [34].

Conclusion

In conclusion, we found that the percentage of both pre-chemotherapy and post-chemotherapy circulating CD16+CD56+NK cells was negatively correlated with the prognosis of CRC. Using a cutoff value of 11.8%, the percentage of post-chemotherapy circulating CD16+CD56+NK cells was able to effectively predict the prognosis of CRC cases.

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Data availability Original data could be obtained by contacting the corresponding author.

Compliance with ethical standards

Conflict of interest All the authors declare there are no conflicts of interest involved in the submission of this manuscript, and the manuscript has been approved by all authors for publication.

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