ORIGINAL RESEARCH

Circulating CD16+ CD56+ Natural Killer Cells in Colorectal Cancer and Their Correlation with Survival

Gülcin SAHINGOZ ERDAL[1,2], Nilgun ISIKSACAN[2,3]*, Tamay Seda TASCI[2,3], Murat CİKOT[4], Pınar KASAPOGLU ATAR[2], Nursel KOCAMAZ[5]

1. Department of Oncology, Bakirkoy Dr. Sadi Konuk Training and Research Hospital, Saglik Bilimleri University
2. Department of Immunology, Hamidiye Health Science Institute , Saglik Bilimleri University
3. Department of Biochemistry and Immunology, Bakirkoy Dr. Sadi Konuk Training and Research Hospital, Saglik Bilimleri University;
4. Department of General Surgery, Bakirkoy Dr. Sadi Konuk Training and Research Hospital, Saglik Bilimleri University
5. Department of Internal Medicine, Bakirkoy Dr. Sadi Konuk Training and Research Hospital, Saglik Bilimleri University, Istanbul, TURKIYE

***Corresponding Author: Nilgun ISIKSACAN**; E-mail: nisiksacan@gmail.com

Abstract

Background

Natural killer cells, which play a role in the innate immune system, are cells in the early defence system against infections and tumours. The aim of this study was to investigate the role of NK cells in stage 3 colorectal cancer (CRC) and their relationships to serum carcinoembryonic antigen (CEA) levels, obesity, and survival.

Method

CD16+CD56+ NK cells were analyzed by flow cytometry in newly diagnosed stage 3 CRC patients and healthy controls. Associations with survival, obesity, and serum CEA were evaluated.

Results

The rate of NK cells was found to be lower in the CRC patients than in the healthy control subjects. The reduced NK cell rate was associated with reduced survival. A negative correlation was determined between NK cells and age, serum CEA levels, and obesity at the time of diagnosis.

Conclusion

As the study results suggest that the NK cell rate could be effective in predicting prognosis in stage 3 CRC patients, they could also be of guidance for further studies with larger patient populations.

Key words: carcino-embryonic antigen, Natural killer cells, immune system, colorectal cancer

Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related deaths worldwide, and the primary treatment approaches are surgery and chemotherapy[1,2]. The reasons that the results of existing treatments are not sufficiently satisfactory in metastatic patients, who are common, include that the immune system does not have sufficient anti-tumour efficacy[3,4]. T-lymphocytes, B-lymphocytes, and Natural Killer (NK) cells, which function in the immune system, are important components of the host anti-tumour immune response. These cells play very important roles in the onset, development, and progression of cancers[5,6]. The NK cells play a role in the innate immune system, and work in collaboration with the adaptive immune system in the eradication of malignant cells and large granular lymphocytes in the early defence against infections and tumours[7,8].

The relationship between NK cell activity and cancer has been a subject of research for many years, and low NK cell activity has been shown to be associated with a higher risk of cancer. In previous studies, NK cell activity has been seen to be reduced in CRC patients compared to healthy control subjects[9-11]. Research has been conducted on the relationship between NK cell activity in cancer patients and disease recurrence, metastasis, or low survival. Together with the increase in immunotherapy as a part of cancer treatment in recent years, the anti-tumour immune mechanisms in CRC continue to be a subject of research[12,13].

The effect of obesity and chronic inflammation emerging associated with obesity on the immune system and disease survival has also become a subject of research. The results of previous studies have shown that obesity and the chronic inflammation it causes are modifiable prognostic factors in CRC[14].

Carcinoembryonic antigen (CEA) is an important tumour marker in CRC follow-up and can be used as a predictor of disease survival. In previous studies, high preoperative CEA levels have been accepted as an independent predictor of overall survival and disease-free survival[15,16].

The evaluation of circulating CD16+CD56+ NK cells in patients with newly diagnosed stage 3 colorectal cancer (CRC), assessing their relationship to survival, and investigating associations with obesity and serum CEA levels were the objectives of this study.

Materials And Methods

Patients' information was collected in accordance with the Declaration of Helsinki and ethical rules. This prospective, observational study included patients diagnosed with CRC

https://dx.doi.org/10.4314/mmj.v37i3.8

in 2019 (Group 1), and an age and gender-matched healthy control group (Group 2). The patients in Group 1 were selected from those diagnosed with stage 3 operable CRC according to the American Joint Committee on Cancer (AJCC) TNM Staging System (tumor, node, metastasis) for Colon Cancer (8th ed., 2017) criteria, with involvement of at least 1 lymph node and no distant organ metastasis. Patients were excluded from the study if they were aged <18 years, were pregnant, were unwilling to participate in the study, had diabetes, acute or chronic renal failure, acute or chronic liver disease, chronic inflammatory disease, congenital or acquired immune deficiency, autoimmune disease, acute infection, or were using immunosuppressive drugs.

A record was made for each patient of demographic data, height and weight. Body mass index (BMI) was calculated as body weight divided by height squared (kg/m2) and a value of >30 was recorded as obese. Group 1 included patients diagnosed with stage 3 CRC, who had not previously received anticancer treatment, had no metastasis, and were planned to undergo surgery. The healthy volunteers included in Group 2 as the control group were selected from those attending the general internal medicine polyclinic for a routine check-up, who underwent colonoscopy for general screening purposes or a disorder in bowel habits with normal results and had no comorbid diseases.

Written informed consent was obtained from all the study participants. A venous blood sample of 2ml was taken by an experienced nurse in the morning hours for flow cytometry. The CEA levels at the time of diagnosis were recorded from patient files for the patient group. CEA level was measured by immunoassay (Roche Diagnostics, Mannheim,. Germany, Cobas 8000) according to manufacturer instructions. The patient group was followed up at regular intervals for 3 years, and survival and non-survival were recorded from telephone calls, hospital records, or the deaths registration system. For those who developed mortality, the cause of death was recorded.

Flow cytometry

NK cell analysis was performed with flow cytometry. A peripheral venous blood sample was withdrawn from each participant into an EDTA-coated tube, then 100 µl of the freshly collected blood was transferred into a flow specific tube. For each of the following titrated antibodies, CD3 fluorescein isothiocyanate (FITC) (A07746), CD16 phycoerythrin-cyanine (PC5) (A07767), CD56 phycoerythrin (PE) (A07788), and CD45 allophycocyanin (APC) (IM2473) [Beckman Coulter, USA], 5µl was added according to the manufacturer's protocol. The mixture was incubated at room temperature for 15 min in the dark, then treated with 500 µl of red blood cell lysis buffer (Beckman Coulter, Optilyse C-A11895) for 10 min at room temperature in the dark. The mixture was washed twice with flow sheath via centrifugation, re-suspended with 0.5 ml flow sheath (8546859) and analyzed using the Navios flow cytometer system (Beckman Coulter) with Kaluza software (Beckman Coulter).

Table 1. Participants' early clinical and demographic characteristics

		Patient group n (%)	Control group n (%)
Gender	Female	32 (46.8)	31 (45)
	Male	38 (54.2)	38 (55)
Age (years)	Median (Min-Max)	57 (37-88)	56.5 (38-87)
Obesity	Absent	60	59
	Present	10 (14.2)	10 (14.4)
Mortality	Absent	43 (61.5)	
	Present	27 (38.5)	

Table 2. Comparison of circulating CD16 and CD56 NK cell subsets between CRC patients (group 1) and healthy controls (Group 2)

% (Lymphocyte gate)		Group 1 (n=70)	Group 2 (n=67)	P
$CD16^+$	Mean±SD	10.91±7.60	24.92±14.56	[a]0.001**
$CD56^+$	Mean±SD	12.72±12.54	25.24±14.02	[a]0.001**
$CD16^+CD56^+$	Mean±SD	7.13±6.08	20.69±10.54	[a]0.001**
$CD16^+CD56^-$	Mean±SD	3.51±3.18	8.29±6.56	[a]0.001**

[a]Mann Whitney U-Test **p<0.01

https://dx.doi.org/10.4314/mmj.v37i3.8

Table 3. Association between NK cell subsets and 3-year survival in CRC patients in Group 1

(%)		3-year survival status		p
		Surviving (n=43)	Non-surviving n=27)	
$CD16^+$	Mean±SD	13.61±7.92	6.00±3.82	[b]0.001**
$CD56^+$	Mean±SD	15.92±13.49	7.18±9.37	[b]0.001**
$CD16^+CD56^+$	Mean±SD	8.82±6.10	3.36±2.67	[b]0.001**
$CD16^+CD56^-$	Mean±SD	4.27±3.45	2.27±2.37	[b]0.008**

[a]Mann Whitney U Test **p<0.01

Table 4. Relationship between NK cell subsets and serum CEA levels in patients with CRC in the Group 1 Patients

(n=70)		CEA
$CD16^+$	R	-0.258
	P	0.035*
$CD56^+$	R	-0.235
	P	0.055
$CD16^+CD56^+$	R	-0.252
	P	0.039*

r=Spearman Correlation Coefficient *p<0.05

Table 5. Relationship between NK cell subsets and serum CEA levels in patients with CRC in Group 1 Patients

(n=70)		Obesity		P
		Present (n=10)	Absent (n=60)	
$CD16^+$	Mean±SD	9.21±5.95	21.09±8.70	[a]0.001**
$CD56^+$	Mean±SD	11.80±12.65	18.23±10.77	[a]0.023*
$CD16^+CD56^+$	Mean±SD	6.09±5.23	13.35±7.34	[a]0.001**
$CD16^+CD56^-$	Mean±SD	3.11±2.78	5.94±4.36	[a] 0.025*

[a]Mann Whitney U Test *p<0.05 **p<0.01

At least 20,000 cells were analyzed in the lymphocyte gate. NK cells were defined as CD45+ CD3- CD16+CD56+.

Statistical Analysis

Data obtained in the study were analyzed statistically using NCSS 2007 software (Number Cruncher Statistical System, Kaysville, UT, USA). The conformity of numerical data to normal distribution was assessed with the Shapiro-Wilk test and graph examinations. Descriptive statistics were stated as mean ± standard deviation or median, minimum and maximum values for continuous data and as number (n) and percentage (%) for categorical data. The Mann Whitney U-test was applied in the comparisons of two groups of numerical variables not showing normal distribution, and in the comparison of more than two groups, the Kruskal-Wallis test and Dunn Bonferroni test were used. In the evaluations of relationships between quantitative variables, Spearman correlation analysis was used. A value of $p<0.05$ was accepted as statistically significant. Power analysis was performed using the G*Power (v3.1.7) program to determine the number of samples.

At the beginning of the study, 10 people in each group were taken and the pilot study was calculated as 0.928, and it was calculated that there should be at least 20 people in each group and 40 people in total in order to achieve 80% power at the $\alpha=0.05$ level.

Results

Evaluation was made of a total of 139 participants, as 70 patients diagnosed with CRC and 69 healthy control group subjects. The age and gender distribution were seen to be balanced between the two groups. The whole sample comprised 76 males and 63 females with a median age of 57 years (range, 37-88 years). In each group, 10 subjects were classified as obese. Cancer-related mortality developed during the study follow-up period in 27 patients in Group 1 (Table 1).

The measurements of CD16+, CD56+, CD16+ CD56+, CD16+ CD56- were determined to be statistically significantly lower in the CRC group patients than in the healthy control group ($p=0.001$; $p<0.01$).

https://dx.doi.org/10.4314/mmj.v37i3.8

In both groups no statistically, significant difference was determined between the genders in respect of the CD16+, CD56+, CD16+CD56+ and CD16+CD56- measurements (p>0.05).

A statistically significant negative correlation at a strong level was determined between patient age in the CRC group and the CD16+ CD56+ measurements (as age increased CD16+ CD56+ values decreased) (r=-0.321; p=0.007; p<0.01) (Table 2).

The CD16+, CD56+, CD16+ CD56+, CD16+ CD56- values of the non-surviving cases at the end of 3 years were determined to be statistically significantly lower than those of the surviving cases (p=0.001; p=0.001; p=0.001; p=0.008, respectively) (Table3).

A statistically significant negative correlation at a weak level was determined between the serum CEA values of the patients and the CD16+ CD56+ measurements (as CEA value increased, the CD16+ CD56+ values decreased) (r=-0.252; p=0.039; p<0.05) (Table 4).

The CD16+, CD56+, CD16+ CD56+, CD16+ CD56- values of the obese patients were determined to be statistically significantly lower than those of non-obese cases (p=0.001; p=0.023; p=0.001; p=0.025, respectively) (Table 5). In the control group, NK cell values were found to be significantly lower in the presence of obesity (p <0.05).

Discussion and Conclusion

Since a 1997 study by Coca et al., it has been known that widespread infiltration of NK cells within the tumour is related to a positive tumour outcome in patient with colorectal carcinoma. Studies conducted at that time stated that the presence of an increased number of NK cells within the tumour could be an important variable for disease prognosis, especially in patients with TNM stage 3 disease[17]. Many studies of the relationship between CRC and NK cells have been conducted to date. Cancer and the NK cell rate, the relationship between the function of NK cells and cancer, the relationship between NK cells in the tissue or in circulation and outcome and survival, and subtypes of NK cells have become subjects of research. In the current study, CD16+CD56+ positive cells of the CD45+CD3- portal in peripheral blood were accepted as NK cells, and the relationship with disease survival was evaluated.

According to Xiong et al. (2018), there were notable differences between tumor and nearby normal tissues for several Tumor Infiltrating Immune Cell (TIIC) clusters, including NK cells. This suggests that NK sub-clusters may play a part in colorectal tumorigenesis[18]. Similarly, Spacek et al. discovered that patients with stage II–III CRC had fewer CD16+CD56+ NK cells[19]. Together with several cytokines and chemokines, the anti-tumour innate immune response represents an important mechanism in tumour formation.

The combination of reduced NK cells and cancer has been shown in several studies. The results obtained from a study conducted in 2000 suggested that low cytotoxic activity of NK cells was associated with increased cancer risk and played a role in the innate immunological host defence mechanisms against cancer[20]. Consistent with these data in literature, in the current study circulating NK cells were found to be at a significantly lower level in CRC patients compared to the healthy control subjects.

In a 2018 study by Odaka et al., of stage II CRC patients, the number of NK cells (CD56 positive cells) in the lymph nodes was reported to be related to the prognosis of the patients[21]. Several studies have investigated the relationship between NK cell count and the survival of CRC patients. In an analysis of 157 CRC patients by Coca et al., patients with extensive CD57+NK cell intratumoural infiltration were found to have longer survival[17]. Tang et al. also showed the prognostic value of NK cells in CRC patients[22]. In the current study, the NK cell rates of patients surviving at the end of 3 years were seen to be significantly higher than those of non-survivors in patients diagnosed with stage 3 CRC. The NK cell rate before treatment could be predictive of prognosis in stage 3 CRC patients.

Previous studies have reported a relationship between serum CEA level used as a tumour marker and survival in CRC patients[23]. In the current study, a negative correlation was determined between the serum CEA level and the peripheral blood NK level. These findings support the view that a low NK rate in circulation could potentially predict a negative prognosis. Moreover, it can be considered that a combination of NK cell percentage with these variables could increase the prognostic value.

It is now known that the immune system weakens with age, and the number and activity of immune cells changes with development of the disease in cancer patients[19,24]. In the study by Tang et al., a negative correlation was observed between NK cell percentage and patient age. It has been thought that this prognostic marker could work in collaboration with other immune markers in CRC and could decrease together with ageing[22]. Consistent with the data in literature, a strong negative correlation was determined between CRC patient age and the NK cell rate in the current study. Although this change in NK cells supports the increase in the incidence of CRC with increasing age, it suggests that decreased NK cells have an effect on the prognosis of this disease in advanced age, as well as many factors affecting the prognosis of CRC.

Obesity is another well-established risk factor for CRC. It is known to increase mortality and has been associated with dysfunction of the immune system[25–27]. In our study, the NK percentages were lower in obese patients both in the CRC and control groups. This further supports the relation between obesity, immune dysfunction and CRC risk.

Limitations of this study can be said to be the relatively low number of patients, that NK cell analysis was only performed once in the patients, NK cell change during disease follow-up was not evaluated, and that other immune cell subtypes were not evaluated. Although this study focused on stage III CRC patients, it would be valuable for future research to investigate whether similar associations between NK cell levels and prognosis can be observed in metastatic colorectal cancer. Such studies could further broaden the clinical relevance of NK cell findings.

In conclusion, the role of NK cells in CRC is complex, but in both clinical and pre-clinical studies, it seems clear that NK cells can affect both the risk of developing cancer and prognosis after treatment of CRC. The results of this study demonstrated that the NK cell rates were lower in patients with CRC than in patients without cancer. Low levels of NK cells are associated with decreased survival. There was found to be a negative correlation between NK cells and patient age at diagnosis, serum CEA levels and the presence of obesity. As these results suggest that NK cell rates could be predictive of prognosis in stage 3 CRC patients, they can be of guidance for further larger-scale studies in this area.

https://dx.doi.org/10.4314/mmj.v37i3.8

Conflicts of interest

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

Funding

No funding. The authors report no involvement in the research by the sponsor that could have influenced the outcome of this work.

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