

CLINICAL STUDY

The decrease in NKG2D+ Natural Killer cells in peripheral blood of patients with metastatic colorectal cancer

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Abstract: *Background:* Natural killer (NK) cells play important roles in the immune defense against tumors such as colorectal cancer. In humans, NKG2D is an activating immune receptor constitutively expressed in most cytotoxic lymphocytes including NK and CD8+ T cells. In this study, the expression of NKG2D molecule was investigated in peripheral blood NK cells from colorectal cancer patients and compared with healthy subjects. *Methods:* We studied 21 non-metastatic (low-grade), 17 non-metastatic (high-grade), 16 metastatic colorectal cancer patients, and 24 healthy controls. Peripheral blood samples were obtained to isolate peripheral blood mononuclear cells (PBMCs) and the percentage of peripheral blood NKG2D+CD3-CD56+ NK cells was analyzed by flow cytometry. The expression of NKG2D at mRNA level was also measured by real-time PCR in both, patients and control subjects. *Results:* The results showed a significant reduction in the percentage of NKG2D+NK cells as well as NKG2D mRNA expression in peripheral blood of metastatic colon cancer patients. *Conclusion:* This result suggests that decreased expression of activating NKG2D receptor in metastatic colorectal cancer might compromise NK cell function and allow tumor to evade immunity (Tab. 3, Fig. 4, Ref. 33). Text in PDF www.elis.sk. **Key words:** colorectal cancer, metastasis, NK cells, NKG2D.

Introduction

Colorectal cancer, also called colon cancer, is the third most commonly diagnosed cancer in the world, but its prevalence is higher in developed countries (1, 2). Based on Tumor/node/metastasis (TNM) system, this cancer is divided into four stages (I, II, III, and IV), which are considered non-metastatic (I–III) or metastatic (IV) CRC stages (3). Similar to other cancers, tumor grading of colorectal cancer includes low and high grade.

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Acknowledgements: Our thanks go to the vice chancellor for research, Isfahan University of Medical Sciences for supporting this research (project registration number: 189022). Also, we would like to thank all patients and healthy volunteers who participated in this study.

Natural Killer (NK) cells are large granular lymphocytes (LGLs), which play an important role in primary protection from viruses and tumors. For this purpose, NK cells apply both activating and inhibitory receptors, which regulate their activity through a balance between inhibitory and stimulatory signal cascades. NKG2D (Natural Killer Group 2D; CD314), also mentioned as NKG2D receptor, belongs to C-type lectin-like superfamily and is expressed on the surface of NK cells as the primary activating receptor. It is also expressed on CD8+T cells, $\gamma\delta$ T cells and some CD4+T cells as a co-stimulator receptor (4–6).

The NKG2D ligands (NKG2DLs) consist of a diverse array of proteins that are structurally related to MHC class I. Several studies indicate that NKG2D engagement by NKG2DLs induces proliferation, survival, and cytotoxic activity in NK cells (7, 8). Consequently, NKG2D/NKG2DLs interaction may represent an important activation to launch the immune strong response against malignant cells (9). NKG2D ligands are also extensively expressed on solid tumors and some forms of leukemia through induction of cancer-related pathways and oncogenes, but not on normal cells (10, 11). Hence, owing to the loss of these ligands in normal cells and their expression in some types of cell stress such as in viral infections and cancers, NKG2D is considered as one of molecules involved in immune surveillance (12–14).

Interestingly, it has been shown that NKG2DLs shedding from tumor cell surface happens in diverse malignant cancers, including advanced hepatocellular carcinoma, colon, prostate, renal, and breast cancer, as well as in hematopoietic tumors (15–19). Release of these ligands in serum may end in down-modulation of NKG2D through facilitating NKG2D internalization and lyso-

Tab. 1. Demographic characteristics of patients and healthy subjects.

Feature	Normal	Non-metastatic (low-grade)	Non-metastatic (high-grade)	Metastatic
Total number	24	21	17	16
Sex				
female	10	9	6	4
male	14	12	11	12
Age (years)				
minimum	29	40	43	44
maximum	65	71	71	72

Tab. 2. Hematologic parameters of patients and healthy subjects.

	Healthy Control	Non - metastatic (low-grade)	Non-metastatic (high-grade)	Metastatic
WBC $\times 10^3/\mu\text{l}$	6.85 \pm 1.53	6.56 \pm 2.21	6.85 \pm 1.83	7.8 \pm 2.33
Lymphocyte $\times 10^3/\mu\text{l}$	2.6 \pm 0.88	2.06 \pm 0.67	3.23 \pm 0.61	4.48 \pm 0.77
RBC $\times 10^6/\mu\text{l}$	5.11 \pm 0.52	4.72 \pm 0.56	4.75 \pm 0.66	4.79 \pm 0.55
PLT $\times 10^3/\mu\text{l}$	211 \pm 59	195 \pm 53	182 \pm 67	225 \pm 99
Hb g/dl	14.43 \pm 1.99	12.66 \pm 1.62	12.52 \pm 2.13	2.64 \pm 1.79
Hct %	44.89 \pm 5.69	39.7 \pm 4.7	38.56 \pm 1.34	40.97 \pm 5.91

somal degradation, and this strategy has been assumed to be an interesting mechanism applied by cancer cells to evade the tumor immunosurveillance (20).

In the current research, we studied NKG2D expression in peripheral blood NK cells acquired from metastatic, non-metastatic colorectal cancer patients, and healthy individuals.

We postulated that the percentages of NKG2D+ NK cells were diminished concomitantly with progression of the cancer.

Methods

Samples from patients and controls

Heparinized peripheral blood was obtained from 54 patients with colorectal cancer attending the gastroenterologic clinic of Al-zahra, khanevadeh, and seid-al-shohada hospitals, affiliated to Esfahan University of Medical Sciences, as well as from 24 healthy volunteers matched as to age and sex (Tab. 1). Hematologic features of patients and healthy subjects are compared among the groups in Table 2, including white blood cell count (WBC), total lymphocytes, red blood cell count (RBC), platelet count (PLT), hemoglobin (Hb) and hematocrit (Hct). Written consent was obtained from all patients and healthy controls, and the study was approved by the Ethical Committee of Esfahan University of Medical Sciences. Disease diagnosis was based on symptoms including constipation in recent 6 months, history of familial polyposis, anus bleeding and positive colonoscopy results in relation to non-metastatic patients, and history of colorec-

tal cancer with current tumor dissemination to other organs in metastatic patients. Patients with established colonoscopic and histopathological diagnosis of non-metastatic (low-grade), non-metastatic (high-grade) and metastatic colorectal cancer were enrolled in this study. Also, in our research, the scale applied to grading of non-metastatic colorectal cancer contained low grade (where the tissue of cancer was similar to normal colorectal cancer) and high grade (where the tissue of cancer appeared to be very abnormal).

Peripheral blood mononuclear cell (PBMC) isolation

The most common method for PBMC isolation, Ficoll-Hypaque PBMC separation, was applied. Briefly, fresh peripheral whole blood samples with heparin coagulant were diluted in one-to-one ratio by PBS/2%FBS solution. Then, diluted blood sample was gently laid on Ficoll-Hypaque layer (sigma, St Louis, MO, USA) and finally PBMC was separated from this solution via density centrifugation at 2800 rpm for 20 min. The viability of isolated cells was more than 95 % as assessed by Trypan blue exclusion test.

Flow cytometry

PBMC staining for FACS analysis was done with optimized amount of the following fluorochrome conjugated mAbs: CD3-PerCp, CD56-FITC, NKG2D-PE, PerCp Mouse IgG1 κ Isotype Control, FITC Mouse IgG2b κ Isotype Control, and PE Mouse IgG1 κ Isotype Control. All mAbs with the exception of NKG2D-PE and PE Mouse Isotype control (eBioscience; San Diego, CA; USA) were purchased from BD Bioscience (San Jose, CA, USA). The isolated cell suspension was adjusted at 10^5 – 10^6 cells/ml and incubated with anti CD3, anti CD56 and anti NKG2D for 30 min at 4 °C. Simultaneously, daily, we incubated a tube with isotype controls for these three color staining. Ultimately, cells were fixed with 0.5 % paraformaldehyde solution if evaluation was delayed. A three-color analysis on FACS caliber (BD) was used to demonstrate the NKG2D expression on CD56+CD3- as NK cells.

Tab. 3. Primer sequences used in Real-time PCR test F: Forward, R: reverse.

Primer sequence	Target
GGC TCC ATT CTC TCA CCC A	NKG2D-F-primer
TAA AGC TCG AGG CAT AGA GTG C	NKG2D-R-primer
TCCAAGGCTCAGGTACTTCAAAG	NKp44-F-primer
GGGCGGGTACTGGCATCT	NKp44-R-primer
GAA GGT GAA GGT CGG AGT	GAPDH-F-primer
CAT GGG TGG AAT CAT ATT GGA A	GAPDH-R-primer

Real-time PCR analysis

Cell pellet was separated from whole blood after using cell lysing buffer and centrifuging. Then, total RNA was extracted by Guanidinium thiocyanate-phenol-chloroform method (Sinagen, Iran). Consequently, oligo (dT) primed cDNA (Vivantis Tech-

nologies, Malaysia) was prepared and relative quantification value (RQ value) of gene expression was performed in real-time PCR using SYBR green. To internally standardize the levels of gene expression, the GAPDH housekeeping gene was used. All PCR amplification was performed using the ABI Real Time PCRSystem (Perkin-Elmer Applied Biosystems, California, USA) in a 25 µl final volume, using primers at 300 nM. PCR reactions were performed in triplicate as follows: 55 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s, and 60 °C for 1 min.

The fluorescent signal was measured and plotted during each 60 °C annealing and extension step for all samples. Using the cycle threshold (the number of PCR cycles required for the fluorescent dye to be detectable) and the constructed standard curve for each cDNA, the relative amounts of GAPDH and NKG2D cDNA in each sample were determined. Primers are summarized in Table 3.

Statistics

Statistical analysis was performed using the SPSS21 statistical software. The results were evaluated by One-way ANOVA and Kruskal-Wallis tests. Probability values of less than 0.05 were regarded significant. The results presented in text, tables and figures represent mean standard deviation (SD).

Results

Subjects

Based on TNM system, patients were classified either as non-metastatic (low-grade) (n = 21), non-metastatic (high-grade) (n = 17), or metastatic (n = 16) groups (Tab. 2). All patients were en-

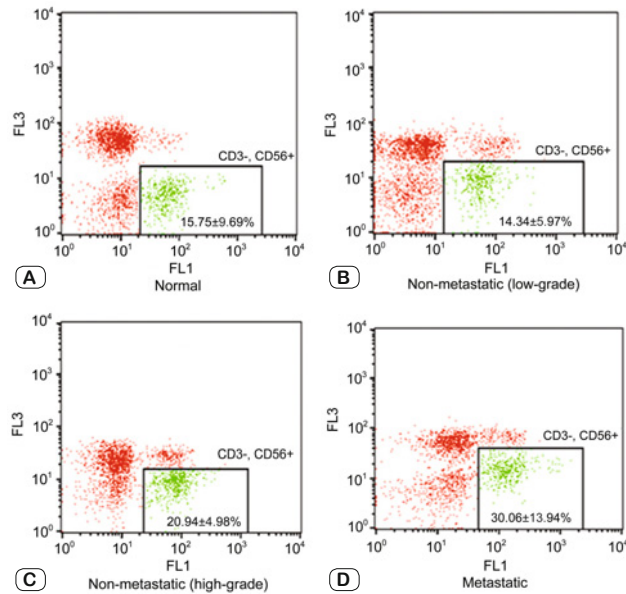


Fig. 1. Gating on NK cells based on CD3-CD56+ panel and mean±SD in relation to NK cell percentage in lymphocytes of PBMC in study groups: A) control B) non-metastatic (low grade) C) non-metastatic (high grade) and D) metastatic of colorectal cancer.

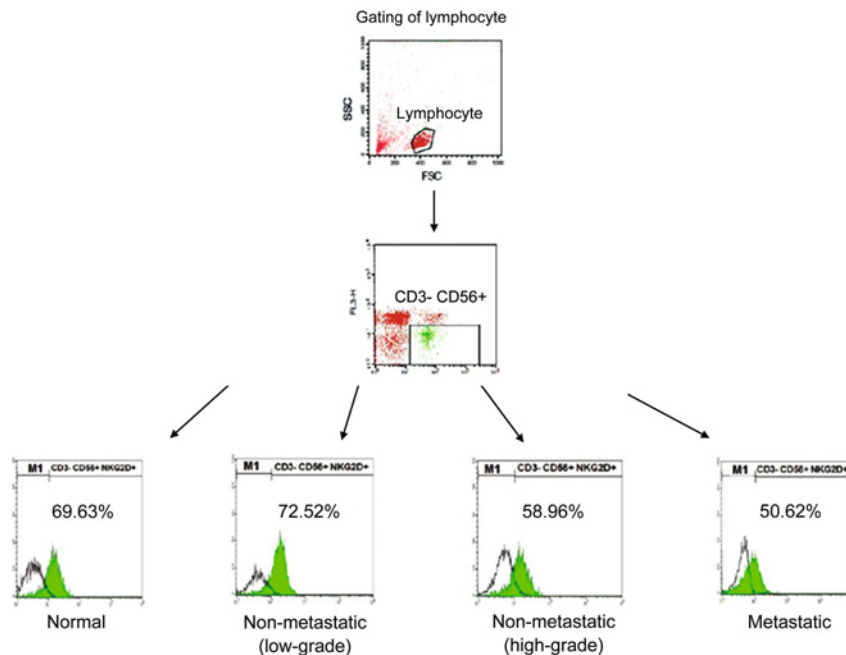


Fig. 2. Three color flowcytometric analysis of NKG2D+CD3-CD56+ NK cells in representative patient and control samples. Lymphocytes were gated in FSc (Forward light scatterer)-SSc (Side light scatterer) dot plot and then CD3-CD56+ gated in CD3-PerCp intensity vs. CD56-FITC intensity. Histograms show the frequency of NKG2D+NK cells and MFI of NKG2D on these cells in the upper of each histogram. Colorless histograms represent isotype control.

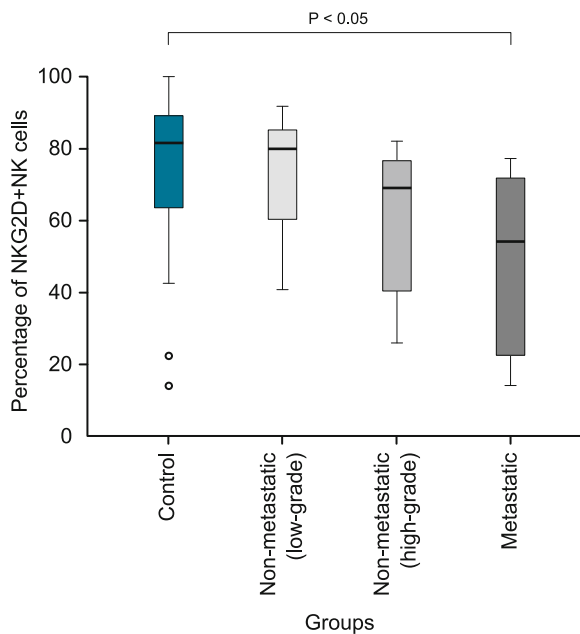


Fig. 3. The percentage of NKG2D+ NK cells were reduced by progression of colon cancer. $p < 0.05$ was considered significant.

rolled in our study before surgery or chemotherapy to minimize the negative effect of these factors on their immune status.

Hematologic parameters

Among these features, the lymphocyte count and Hct in peripheral blood of patients with metastatic cancer showed significant increase ($(4.48 \pm 0.77) \times 10^3/\mu\text{l}$; $p < 0.001$ and $40.97 \pm 5.91\%$; $p < 0.001$, respectively) when compared with non-metastatic patients and healthy controls.

Gating of NK cells based on CD3-CD56+ panel

The percentage of NK cells in peripheral blood lymphocytes of colorectal cancer patients showed a rising trend and it was significantly higher in metastatic colorectal cancer patients ($30.06 \pm 13.94\%$) than in control subjects ($15.75 \pm 9.69\%$; $p < 0.0001$) (Fig. 1).

Reduced numbers of NKG2D+ NK cells

As shown in Figure 1, the percentage of NKG2D+ NK cells in all patients and healthy donors was determined using flow cytometry. A decreased percentage of NKG2D+ NK cells was detected in patients with non-metastatic (high-grade) and metastatic colon cancer compared to controls; however, only the decrease in the percentage of NKG2D+ NK cells in metastatic group was statistically significant ($p = 0.02$) (Fig. 2).

Down-regulation of NKG2D mRNA expression

Although NKG2D mRNA levels were decreased during colorectal cancer progression, it was diminished significantly only in the metastatic group (0.82 ± 0.013 RQ value) compared to the normal group ($p < 0.001$) (Fig. 3).

Discussion

NK cells play critical role in immune surveillance and elimination of tumor cells. These cells can distinguish stressed cells from normal cells and maintain self-tolerance through function of their activating and inhibitory receptors. In normal conditions, the inhibitory receptors bind to their ligands (self- MHC class I molecules) on surface of healthy host cells and lead to NK cell inhibition; as opposed to the latter, in stressed cells, the decreased expression of ligands for inhibitory receptors (missing-self) or increased expression of ligands for stimulatory receptors (induced-self) cause NK cell activation and tumor cell lysis (21–23).

In our research, a significantly rising trend was shown in metastatic colon cancer patients (Fig. 1). Different studies display various changes in percentage of peripheral blood NK cells in diverse cancers. In one study on colon cancer, all patients without metastasis displayed a significantly increased percentage of NK cells in the peripheral blood when compared to control patients. In contrast, patients with already established metastasis did not display changes in the number of NK-cells when compared to control patients (24). Garcia-Iglesias clarified that although peripheral blood NK cell percentage did not significantly vary among HGSIL (High grade squamous intraepithelial lesions), LGSIL (Low-grade squamous intraepithelial lesions) and healthy women, patients with invasive squamous cervical cancer had significantly lower NK cell percentage in comparison with HGSIL patients (25). The other study on breast cancer indicated a significant increase in the total NK cell percent in invasive ductal carcinoma (IDC) compared to that in normal group (26).

NKG2D molecules are one of important receptors on NK cells and cytotoxic T cells (CD8+ and $\gamma\delta\text{T}$) and their binding to the ligands can activate alone cytotoxicity of these cells (27). It has been previously shown that the expression of NKG2D on NK cells from colon cancer patients was decreased in comparison with

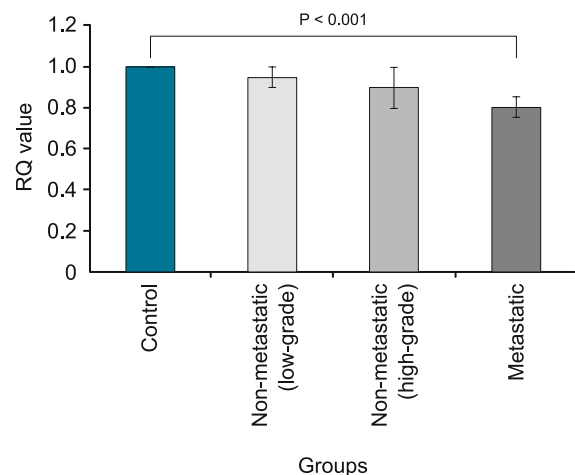


Fig. 4. NKG2D expression in the peripheral blood of patients and control groups. A sample from the normal subjects was applied as a calibrator after normalizing versus GAPDH. Bars represent the mean \pm SD relative expression as RQ value. $p < 0.05$ was considered significant.

normal donors (16). Moreover, a significant lower expression of NKG2D on NK cells from cervical cancer carcinoma (metastatic) patients in comparison with healthy women was reported previously (25). In a similar manner, we observed that the percentages of NKG2D+ NK cells as well as their mRNA expression were decreased during tumor progression, indicating a significant decrease in metastatic colon cancer patients (Fig. 4).

Down-regulation of NKG2D expression on NK cells in metastatic colorectal cancers could be considered a tumor evading mechanism, which has been reported for several tumors previously. It has been shown that reduced expression of NKG2D on NK cells was correlated with high serum levels of major histocompatibility complex class I-related chain A (MICA), the most important membrane-bounded NKG2DL in high-grade cancers (28). MICA is prevalent only in low-grade cancers and may be released into the tumor stroma and circulation in high-grade cancers as soluble MICA (sMICA) (29). In fact, soluble MICA is regarded as a soluble decoy receptor and it is not likely produced in the early stages of cancer. As a result, primary cancers with low number of mutations are good targets for NK and CD8+ T cells activity, while secondary mutations that occur in the later stages of cancer mediate sMICA production or silencing the ligand expression and provide a possibility of immunologic evasion for cancer cells. Thus, MICA-NKG2D works like a double-edged sword in various stages of cancer (29, 30).

TGF- α 1 could also influence the surface NKG2D expression. It has been reported that TGF- β 1 present in plasma of lung and colorectal cancer patients affects NK cell activity via NKG2D down-modulation (31). By silencing TGF- β 1 and - β 2 genes in malignant glioma cells, Friese et al showed that these tumor cells did not down-regulate the NKG2D expression on NK cells (a NK cell line). Furthermore, a strong surface MICA expression on tumor cells was also observed (32). These results support the firm role of TGF- β in the MICA/NKG2D pathway. It is well known that this cytokine is largely produced by many cancer cells and it is also common with high levels in colorectal cancer patients (33).

In conclusion, our study reveals reduced numbers of NKG2D+ NK cells in patients with metastatic colorectal cancer by flow cytometric assay. Moreover, since NKG2D-NKG2DLs system requires precise regulation. Therefore inappropriate expression of NKG2D ligands might compromise NK cell activation, and it can be considered a double-edged sword role for this system in various stages of colorectal cancer. Targeting specific factors involved in MICA shedding from cell surfaces or directly blocking sMICA in circulation could be a clinically important strategy to boost the anti-tumor response against colorectal cancer.

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Received June 14, 2014.
Accepted January 20, 2015.