

# Decreased NKG2D Expression on Natural Killer Cells in Gastric Cancer Patients

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## SUMMARY

NKG2D is an activating receptor of natural killer (NK) cells. NKG2D expression have not been studied on NK cells of gastric cancer patients so far. Therefore, we aimed to investigate NKG2D of NK and T cells in peripheral blood of patients with gastric cancer.

Twelve chemotherapy and radiotherapy naive patients with gastric cancer and 25 age-matched healthy volunteers were enrolled in this study. NKG2D expressions on NK and T cells were evaluated by three color flow cytometry. The hematological and flow cytometric parameters were compared between cancer patients and healthy subjects. Hemoglobin and hematocrite values were decreased in patients compared with those of controls. Other hematological parameters, T cells (CD56-CD3+ cells) and NK cells (CD56+CD3- cells) were not different between groups. The percentage of NKG2D positivity in NK cells was significantly lower in gastric cancer patients than healthy subjects (84.4% ± 9.5% vs 90.2% ± 8.2%; p= 0.02, respectively), whereas NKG2D positive T cells were similar.

NKG2D expression is decreased on NK cells in gastric cancer patients. Further studies which explore the biological significance of lower NKG2D expression and its putative anti-tumor role in cancer patients should be established.

**Key Words:** NKG2D, NK cells, Immunity, Gastric cancer, KIR

## ÖZET

### Mide Kanseri Hastalarında Doğal Öldürücü Hücrelerde Azalmış NKG2D Ekspresyonu

NKG2D Doğal Öldürücü (NK) hücrelerin bir aktivatör reseptörüdür. Daha önce mide kanser hastalarında NK hücrelerde NKG2D ekspresyonu çalışılmamıştır. Bu nedenle mide kanser hastalarının periferik kanında NK hücreler ve T lenfositlerde NKG2D varlığını araştırmayı amaçladık.

Daha önce kemoterapi ya da radyoterapi görmemiş 12 mide kanser hastası ile 25 yaş ortalaması denk sağlıklı gönüllü çalışmaya alınmıştır. NK hücreler ve T lenfositlerdeki NKG2D ekspresyonu üç renkli akım sitometri ile çalışılmıştır. Hematolojik ve akım sitometrik veriler kanser hastaları ve sağlıklı bireyler arasında karşılaştırılmıştır. Hemoglobin ve hematokrit değerleri hastalarda kontrolden daha düşüktü. Diğer hematolojik parametreler, T lenfositler (CD56-CD3+ hücreler) ve NK hücreler (CD56+CD3- hücreler) gruplarda farklı değildi. T lenfositlerde NKG2D pozitif hücreler farklı değilken mide kanser hastalarında NK hücre NKG2D pozitiflik yüzdesi sağlıklı bireylerden daha düşüktü (Sırasıyla %84.4 ± %9.5 vs %90.2 ± %8.2; p= 0.02).

Mide kanser hastalarında NK hücrelerde NKG2D ekspresyonu azalmıştır. Bu düşük NKG2D ekspresyonunun biyolojik önemini ve onun olası anti-tümör rolünü ortaya koyacak daha ileri çalışmalara gereksinim vardır.

**Anahtar Kelimeler:** NKG2D, NK hücreleri, İmmünite, Gastric kanser, KIR

## INTRODUCTION

Natural Killer (NK) cells are important factors of the immunity against cancer.<sup>1</sup> The activity of NK cells is closely regulated by balance of inhibitory and activating receptors.<sup>1</sup> NKG2D is a C-type lectin-like activating receptor and it is expressed in NK cells, CD8+  $\alpha\beta$  T cells and  $\gamma\delta$  T cells.<sup>1,2</sup> NKG2D is a receptor for MICA and MICB (MHC class I chain-related A and B) and RaeT1 (Retinoic acid early transcript 1).<sup>1</sup> MICA and MICB proteins play no role in antigen presentation, but show a highly restricted expression in humans. They are mostly found in the gastrointestinal epithelium<sup>3</sup>; but they are upregulated in many cell types under conditions of stress (e.g. heat shock), infection and during tumorigenesis.<sup>1</sup>

MICA is expressed in malignant cells of various tumors.<sup>3,9</sup> Accumulation of shed form of MICA (SMICA) in serum may lead to down-modulation of NKG2D through facilitation of NKG2D internalization and lysosomal degradation.<sup>1</sup> Moreover, NKG2D expression is decreased on  $\gamma\delta$  T cells in advanced cancer patients.<sup>2</sup> It has been shown that NKG2D expression on both circulating and tumor-infiltrating CD8+ T cells is down-regulated in gastric cancer patients.<sup>10</sup> However, status of NKG2D expression on NK cells is not known in the peripheral blood of those patients. Thus, in this study, NKG2D expression on circulating NK cells has been investigated in gastric cancer patients.

## MATERIALS AND METHODS

### Patients and Study Design

Twelve patients with gastric cancer (Patient group) and 25 age-matched healthy volunteers (Control group) were enrolled in this study. None of the patients had received radiotherapy or chemotherapy before the study. Exclusion criteria were as follows: previous malignancy, concomitant chronic infectious disease including human immunodeficiency virus, tuberculosis, immunosuppressive medications, and metastases of bone or bone marrow.

Demographic and clinical data were recorded. Peripheral blood was collected before the time of treatment for flow cytometry and complete blood count. Blood samples were studied freshly. Hematologic and flow cytometry data were compared between the two groups.

## Flow Cytometry

Three-color flow cytometry analyses were performed using Becton Dickinson FACSCalibur, and then analyzed with the Cell Quest and WinMDI software. Peripheral blood samples were obtained from patients and control subjects and the flow cytometry analyses were freshly carried out.

**Monoclonal Antibodies:** Peridinin chlorophyll protein conjugated anti-CD3 (BD Bioscience Catalog No:345766), fluorescein isothiocyanate conjugated anti-CD56 (Santa Cruz Catalog No:sc-7326), and phycoerythrin conjugated anti-NKG2D (eBioscience Catalog No:12-5878) antibodies and isotype controls were used.

**Cell Preparation and Surface Staining:** Human peripheral blood mononuclear cells were isolated by Histopaque (Sigma Catalog No:1077) gradient centrifugation. 100  $\mu$ L aliquots were transferred to polypropylene test tubes (12 x 75 mm; BD Bioscience Catalog No: 352052). Cells were washed twice in phosphate binding solution (PBS), and then incubated with 20  $\mu$ L conjugated monoclonal antibodies or isotype controls. After washing twice, cells were fixed with PBS then analyzed by flow cytometry with BD FACS Calibur.

**Analysis:** Anti-CD56/anti-NKG2D/anti-CD3 triple staining was used for flow cytometric analysis. In lymphocytes, gated anti-CD56/anti-CD3 histogram, CD3+CD56- cells (as T cells) and CD3-CD56+ cells (as NK cells) were calculated. Then, NKG2D+ cells were counted in T cells and NK cells via FL2 (NKG2D-PE) histogram.

## Statistics

Statistical analyses were performed by SPSS software. Non-parametric tests (Fisher's exact test and Mann Withney-U test) were used to compare demographic, hematologic and flow cytometric parameter between the groups.

## RESULTS

### Patient Characteristics and Clinical Data

Patient group comprised 12 subjects (10 males and 2 females) with gastric cancer and the control group consisted of 25 healthy volunteers (18 males and 7 females). Six patients had metastatic cancer.

**Table 1.** Characteristics of the study population

		Patients	Controls
Number		12	25
Sex	Female	2	7
	Male	10	18
Age	Median	73	65
	Minimum	29	44
	Maximum	81	82
Histopathological classification	Intestinal type	5	
	Diffuse type	7	
	Total	12	
Stage	Stage IA	0	
	Stage IB	3	
	Stage II	2	
	Stage IIIA	1	
	Stage IIIB	0	
	Stage IV	6	
Primer Treatment Modality	Follow up	2	
	Chemotherapy	3	
	Adjuvant chemoradiotherapy	1	
	Supportive care	4	
	Treatment denial	2	
ECOG Performance Status	0		3
	1		5
	2		2
	3		2

Three patients were given chemotherapy and one patient received adjuvant chemoradiotherapy after the study. Characteristics of all the individuals are summarized in Table 1.

#### ***Hematological Parameters and Counts of T and NK Cells***

Hemoglobin and hematocrite values were decreased in patient group when compared with the control group. White blood cell count, absolute number of neutrophils, lymphocytes, and monocytes were not different between the groups (Table 2).

T cell (CD56-CD3+ population) percentage in total lymphocytes and NK cell (CD56+CD3- population) percentage in total lymphocytes were similar between the two groups (Table 2).

#### ***NKG2D Expression on Circulating T and NK Cells***

The percentage of NKG2D expression in NK cells of gastric cancer patients ( $84.4\% \pm 9.5\%$ ) was significantly lower than that of healthy subjects' ( $90.2\% \pm 8.2\%$ ) ( $p:0.02$ ) (Figure 1 and 2A). NKG2D+ cell percentages in T cells were not different between patient and control groups ( $45.9\% \pm 20.5\%$  vs  $44.4\% \pm 14.4\%$ ;  $p= 0.06$  respectively) (Figure 1 and 2B).

#### **DISCUSSION**

NK cells have an important role in surveillance and elimination of tumor cells. These cells are able to distinguish between normal and diseased cells; and maintain self-tolerance. In this process, inhibitory and activating receptors of NK cells play role.<sup>11</sup>

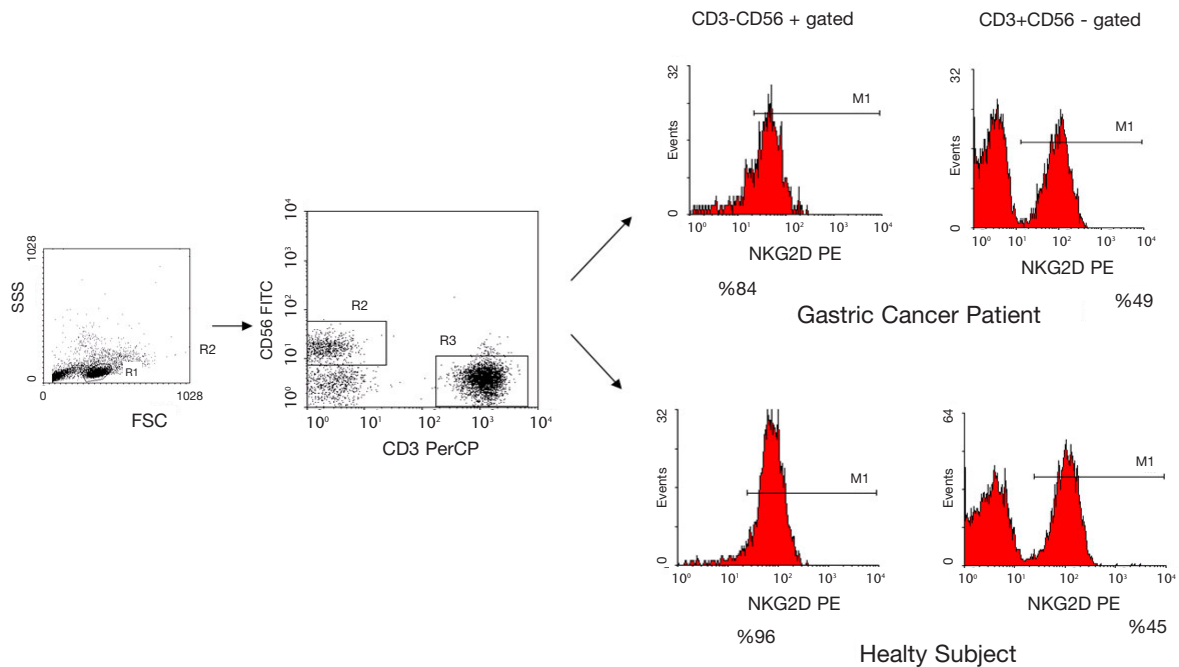
**Table 2.** Hematologic and flow cytometric parameters\*

		Control	Patients	p
White Blood Cells	(/μL)	7812 ± 1493	7508 ± 1626	0.62
Neutrophils	(/μL)	4914 ± 1275	4972 ± 1345	0.94
Lymphocyte	(/μL)	2255 ± 519	1914 ± 548	0.09
Monocytes	(/μL)	472 ± 505	475 ± 222	0.28
Hb	gr/dl	13.9 ± 1.1	12.2 ± 2.1	0.01
Hct	%	41.56 ± 3.46	37.25 ± 5.44	0.01
Platelets	(x1000/μL)	266 ± 112	320 ± 142	0.12
T cells in lymphocytes	%	69.1 ± 9.6	64.7 ± 13.5	0.41
NK cells in lymphocytes	%	7.9 ± 6.4	13.7 ± 10.6	0.08

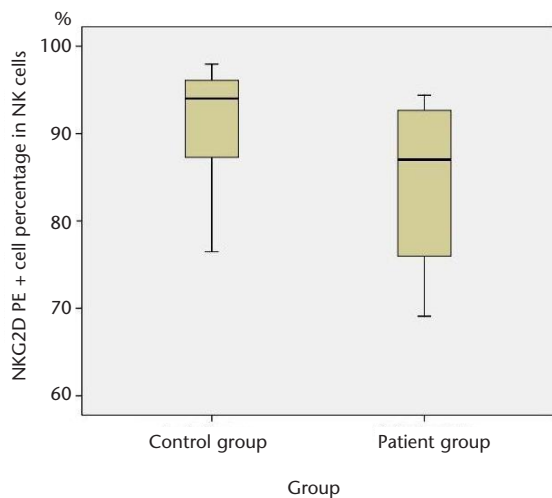
\* Mean ± standard deviation

Most of the inhibitory receptors expressed on NK cells are specific for MHC class I molecules. MHC class I molecules are expressed by nearly all normal cells. Accordingly, self-recognition of MHC class I by inhibitory receptors avoids the attack of NK cell to that cell. This molecule expression is down-regulated or absent in tumor cells, leading to the lysis of tumor cells by NK cells.

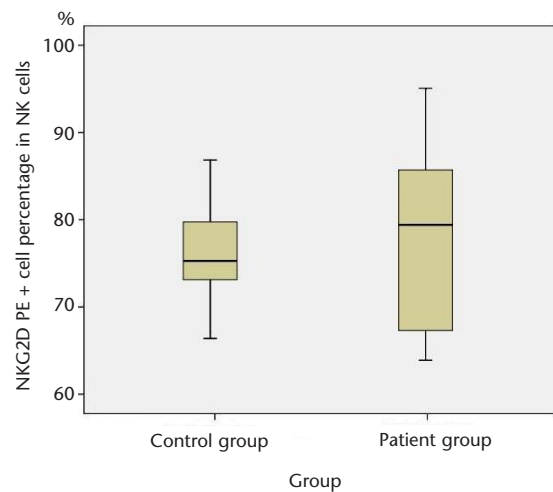
In humans, known inhibitory receptors are killer cell immunoglobulin-like receptors (KIR), CD94/NKG2A receptor, and receptors for non-MHC class I ligands (e.g. 2B4 receptor). The stimulatory receptors are high-affinity Fc receptor, NKG2D, NKp46, NKp44, and NKp30. These receptors recognize self-ligand expressed by diseased cells.<sup>11</sup>



**Figure 1.** NKG2D (+) cells in NK cells and T cells. Anti-CD56/anti-NKG2D/anti-CD3 staining was used for flow cytometric analysis. In lymphocytes gated anti-CD56/anti-CD3 histogram, CD3+CD56- cells (as T cells) and CD3-CD56+ cells (as NK cells) were calculated. Then, NKG2D+ cells were counted in T cells and NK cells via FL2 (NKG2D-PE) histogram.



**Figure 2A.** NKG2D (+) percentage (in NK cells) among patient and control groups.



**Figure 2B.** NKG2D (+) percentage (in T cells) among patient and control groups.

NKG2D is a C-type lectin-like activating receptor and expressed in NK cells, CD8+  $\alpha\beta$  T cells and  $\gamma\delta$  T cells.<sup>1,2,12</sup> In our study, we have found that NKG2D expression was decreased on circulating NK cells in the peripheral blood of gastric cancer patients. To our best notice, this has not been reported before. Previously, we have investigated  $\gamma\delta$  T cells in advanced stage cancer patients.<sup>2</sup> Although the percentages of total  $\gamma\delta$  T cells, V $\gamma$ 2-TCR expressing  $\gamma\delta$  T cells, and apoptotic  $\gamma\delta$  T cells were not different from healthy subjects; NKG2D expression in  $\gamma\delta$  T cells was significantly reduced in advanced stage cancer patients.

Osaki et al. have studied NKG2D expression in CD8+ T cells in gastric cancer patients.<sup>10</sup> They found NKG2D expression in CD8+ T cells lower than those in normal controls. Moreover, they showed that NKG2D expression on CD8+ T cells in the tissue of gastric cancer was lower than that of circulating CD8+ T cells. In that study, NK cells were not investigated. Arreygue-Garcia et al. compared NKG2D-expressing NK and T cells in patients with cervical cancer or precursor lesions with those from healthy donors.<sup>13</sup> They demonstrated decreased NKG2D expression in both NK cells and T cells. In our study, we did not find any difference between gastric cancer patients and healthy subjects with regard to NKG2D expression of T cells.

MICA, MICB, and RaeT1 are known as NKG2D ligands (NKG2DL).<sup>1,11</sup> Expression of NKG2DL in healthy cells is restricted. MICA is upregulated on cells during tumorigenesis and it is a stress cell marker.<sup>1</sup> NKG2D/MICA interaction is an important activation pathway to trigger the immunity against tumor cells. MICA is frequently observed on epithelial tumor cells including gastric cancer.<sup>3-9</sup> Recent studies showed that sMICA occurs in sera of patients with malignant epithelial tumors, including liver, colon, prostate, kidney, and breast cancer.<sup>5,11,13-17</sup> The appearance of sMICA in serum is correlated with down-regulated NKG2D expression.<sup>11</sup> The mechanism of this process is internalization of NKG2D and lysosomal degradation. However, Osaki et al. did not demonstrate any difference in sMICA levels between gastric cancer patients and normal control subjects.<sup>10</sup> Consequently, the cause of decreased NKG2D expression in gastric cancer may be due to the accumulation of other NKG2DL. Likewise, Song et al investigated soluble RaeT1 from a human gastric cancer line and demonstrated that sRaeT1 suppressed NK cell activity via down-regulating NKG2D expression.<sup>18</sup>

In conclusion, NKG2D expression is decreased on NK cells in gastric cancer patients. Further studies which explore the biological significance of lower NKG2D expression and its putative anti-tumor role in cancer patients should be established.

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