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ORIGINAL ARTICLE

Assessment of changes in expression and presentation of NKG2D under influence of MICA serum factor in different stages of breast cancer

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Abstract Breast cancer is the most common cancer in women worldwide. In this study, we correlated the serum level of major histocompatibility complex class I-related chain A (sMICA) with expression and presentation of NKG2D receptors on NK cells among patients with breast cancer. Peripheral blood (PB) samples were collected from 49 healthy and 49 breast cancer patients before surgery and chemotherapy. The expression and presentation of NKG2D were assessed using qRT-PCR and flow cytometry, respectively. Furthermore, sMICA levels were determined using ELISA. In flow cytometry, whole blood samples were stained with anti-CD56/NKG2D/CD3 and the obtained results were analyzed using WinMDI software. In addition, SPSS software was used for statistical analysis of data. Significantly higher levels sMICA were detected in the sera of the majority of cancer patients in contrast to healthy volunteers ($P<0.001$). The expression and presentation of NKG2D receptor were significantly lower than those in healthy persons, and with an inverse correlation to sMICA and positively correlated with tumor stage. Our study showed that sMICA may have an important role in diminishing the expression and presentation of NKG2D receptor in breast cancer patients and proposes the notion that sMICA can be a target candidate for treatment of breast cancer.

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Keywords Breast cancer · NKG2D · sMICA · Flow cytometry · qRT-PCR

Abbreviations

MIC	Major histocompatibility complex class I chain-related protein
qRT-PCR	Quantitative real-time PCR
CT	Threshold cycle
FITC	Fluorescein isothiocyanate
PE	Phycoerythrine
KIR	Killer immunoglobulin-like receptors
ELISA	Enzyme-linked immunosorbent assay

Introduction

Despite advances in medical science, breast cancer remains the most common malignancy in women globally, and the occurrence of breast cancer is increasing in developing countries [1]. An estimated 1.38 million women, accounting for nearly a quarter (23%) of all cancers diagnosed in women, were diagnosed with breast cancer in 2008 [2–4]. According to a report of the Iran Ministry of Health and Medical Education, breast cancer is the most commonly diagnosed cancer in Iranian women [5], and the incidence of the disease is rising with patients being relatively younger (approximately 10 years) than their Western counterparts [6, 7]. Iran has the highest rates of growth of breast cancer in the Middle East, and annually 8 per 100,000 women suffer from this disease [8]. According to the latest report of the Iran Ministry of Health and Medical Education, the newly diagnosed of all breast cancer cases in 2009, 525 (23.1%) were in the province of Khuzestan[5]. The role immune system in the control of

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progression of a malignant disease is well established, and immune suppression induced by cancer is the primary cause of cancer progression [9]. Natural killer (NK) cells are the first line of antitumor immune defense, which represent the main effector subpopulation of the innate immune system [8]. Unlike tumor-specific T cells, NK cells are defined by their capacity to kill certain tumor target cells without prior sensitization or MHC restriction [9, 10]. These cells characteristically express CD56 and lack CD3 [11]. NK cells have opposing functions mediated by activating and inhibitory receptors, the balance of which ultimately determines the susceptibility of target cells to lysis [12, 13]. Among the activating receptors, natural killer 2 member D (NKG2D) plays an important role in NK-mediated cytotoxicity against transformed cells and in immune surveillance of tumors [14, 15]. Furthermore, NKG2D costimulates NK cell activity by binding to the cell surface glycoprotein of MHC class I chain-related gene (MICA), leading to induction of immune response against tumor cells [16, 17]. This ligand is broadly expressed by epithelial tumors but not in healthy tissue [17, 18].

Recent evidence suggests that several immune escape mechanisms exist by which tumor cells evade NKG2D-mediated immunosurveillance. Downregulation of NKG2D expression, which directly affects tumor cell lysis, is likely a novel tumor immune escape mechanism. One of the reasons probably is that the accumulation of MICA in serum (sMICA) by proteolytic shedding may lead to the down-modulation of NKG2D through the facilitation of NKG2D internalization and lysosomal degradation. Because NKG2D-mediated immune responses critically depend on NKG2DL surface levels, the release of soluble MICA (sMICA) can reduce immunogenicity of tumor cells thus causing impairment of lysis of tumor cells and disease progression [19–22].

In this study, the tumor escape mechanism in breast cancer, which is one of the typical epithelial tumors, was investigated by correlating the serum MICA level and the expression and presentation of NKG2D on NK cells in blood samples from breast cancer patients and compared these with healthy donors. A correlation analysis was also performed to explore the relationship between sMICA levels and NKG2D receptor expression and presentation.

Materials and methods

Subjects

Blood samples (5 ml) were obtained from 49 women with infiltrating ductal carcinoma of the breast and confirmed by histological investigations in the surgery department of the hospitals in Ahvaz, Iran. The sample size was determined based on statistical calculation and the prevalence of disease in our region. All breast cancer patients were newly diagnosed cases and none of patients had received chemotherapy, radiotherapy, or immunotherapy before sampling. Data on histological grade, clinical stage, and the presence of organ metastasis were provided from the hospital reports of the 49 patients. Table 1 shows the distribution of patients according to clinical criteria. The majority of the patient population had low-grade, early stage (I, II), and negative metastasis, position left, and aged ≤50 group.

Blood samples from 49 healthy volunteer women without a history of malignancies or autoimmune disorders were also collected as control. The mean age of the patients and healthy control group were 45 years (ranged 24 to 73 years) and 33 years (ranged 21 to 60 years), respectively.

RNA isolation and reverse-transcription polymerase chain reaction

Two milliliters of peripheral blood samples with EDTA, as an anticoagulant, was obtained from patients and control subjects, and total RNA was isolated from blood cells by using RBC lysis and RNX Plus reagent treatment (Cinnagen, Iran) according to the manufacturer's instructions. To the resulting sediment, 100 μl cold PBS and then l cc RNX plus reagent was added and the solution obtained was gently stirred to be completely transparent. Three hundred microliters of chloroform was added to the solution and centrifuged at 14,000 rpm, 4 °C, for 20 min. After adding isopropanol and ethanol and centrifugation, supernatants were removed and were given time to dry completely, RNA quality was assessed by 1 % agarose gel electrophoresis, and the quantity of the extracted RNA samples was estimated at 260 and 280 nm by spectrometry (Eppendorf Biophotometer, Germany). Then RNA was treated with DNase I before cDNA synthesis to avoid DNA contamination. First-strand cDNA synthesis was performed from 5 μg of total RNA, using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania).

Table 1 Distribution characteristics of patients with breast cancer according to different clinical criteria

Number (%)	Characteristics
31 (63)	Age ≤50
18 (27)	Age ≥50
33 (67)	Position left
16 (33)	Position right
6 (12.2)	Stage 1
31 (63.2)	Stage 2
25.5)) 12	Stage 3
36 (73.9)	Grade low
11 (22.4)	Grade high
2	Metastasis
47	Negative metastasis
49	Total

Quantitative real-time RT-PCR

The abundance of NKG2D gene transcripts was determined in triplicates by quantitative real-time PCR (qRT-PCR), using Applied Biosystems Step one™ and Step one Plus™ Real Time PCR systems. Expression of GAPDH housekeeping gene was used as a reference for the level of target gene expression. Each PCR reaction was performed in a final volume of 20 μl and contained 1 μg of the cDNA product, 1 μl of each primer 50 nm, and 2× reaction mixtures consisting of Fast Start DNA polymerase, reaction buffer, dNTPs, and SYBR green I (Applied Biosystems). Table 2 shows the forward and reverse primers of genes. Thermal cycling for all the genes was initiated with a denaturation step at 95 °C for 10 min, followed by 40 cycles (95 °C for 3 s and 60 °C for 30 min).

Amplification efficiency and standard curve analysis

For each target gene, efficiency of the real-time PCR reaction was calculated from the slope of the standard curve. Standard curves were plotted by Ct values of serial dilutions of cDNA and PCR product containing the genes of interest amplicon against the logarithm concentration of input template DNA.

Analysis

In order to assess changes, NKG2D transcripts in the peripheral blood were compared with the corresponding values from healthy control samples, CT average of triplicate samples were read with ABI step one system, and relative expression was plotted and analyzed using Rest 2008 v2.0.7.

Flow cytometry

Two milliliters of heparinized peripheral blood samples was obtained from patients and control subjects. Three-color flow cytometry analyses were performed using Becton Dickinson FACS Calibur and then analyzed with the WinMDI (2.8) software. One milliliter of peripheral blood samples with heparin, as an anticoagulant, was obtained from patients and control subjects, and the flow cytometry analyses were freshly carried out.

Table 2 Forward and reverse primers of genes for real-time PCR amplification

Sequence	Primer
ACTGTGGCCCATGTCCTAAA	NKG2D F
GGTTGGGTGAGAGAATGGAG	NKG2D R
CACCATCTTCCAGGAGCGAG	GAPDH F
GCAGGAATTGCTGAT	GAPDH R

Table 3 Relative expression of NKG2D in peripheral blood cells of breast cancer patients generated by Rest RG 2008 software

P(H1) result	Expression	% Reaction efficiency	Type	Gene
	1.000	93	REF	GAPDH
<0.001DOWN	0.016	89	TRG	NKG2D

Monoclonal antibodies

PE/Phycoerythrin conjugated anti-CD56 (eBioscience catalog no. 15003842), PECy5/Cyanine5 conjugated anti-CD3 (eBioscience catalog lot no: 12056742), and fluorescein isothiocyanate conjugated anti-NKG2D (eBioscience/FITC catalog no. 11587873) antibodies and isotype controls were used.

Analysis

Anti-CD56/anti-NKG2D/anti-CD3 triple staining was used for flow cytometry 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 NK cells via FL1 (NKG2D-FITC) histogram.

ELISA

Serum levels of sMICA were determined by our previously described sandwich ELISA [16, 23]. MICA ELISA kit (Abcam, UK) MICA Human ELIZA Kit-ab59569 was used to detect sMICA in serum samples, according to the manufacturer's protocol. Briefly, after covering the 96-well flat-bottom plates with capture anti-MICA mAb, standard serial dilutions and serum samples were added to each well. Detection anti-MICA mAb was added to the wells. HRP-conjugated anti-

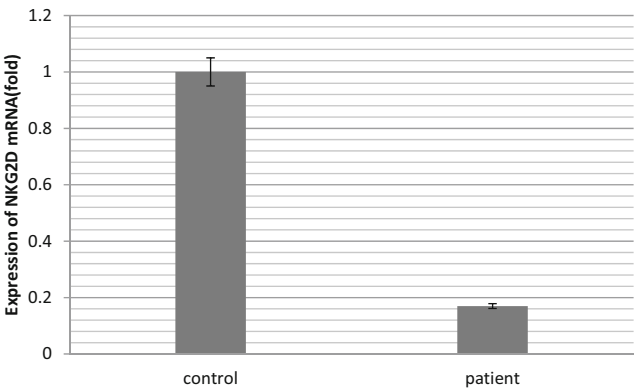

Fig. 1 Fold change of mRNA gene expression of NKG2D receptor in blood cells taken from cancer patients compared with healthy controls ($P<0.01$, paired t test)

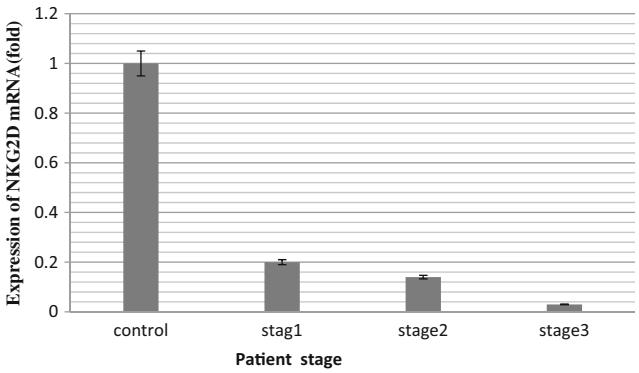

Fig. 2 Fold change of mRNA gene expression of NKG2D receptor relative to reference gene in patients with different stages of breast cancer compared with healthy controls ($P<0.01$, paired t test)

human Ab was added and color was developed using tetramethylbenzidine system. Absorbance values (at A450) by duplicate were plotted against dilutions and expressed as picograms per milliliter (normalized to log10).

Statistics

Statistical analysis of all data was done using SPSS software version 16 (Chicago, IL, USA). The sMICA levels and percentages of NKG2D-expressing NK were expressed as mean±SD. Statistical comparisons among different groups were performed using a parametric test (t test) for NKG2D expression and presentation and non-parametric test (Mann-Whitney) for sMICA levels. Non-parametric Spearman analysis was performed to correlate sMICA levels with NKG2D expression and presentation. Additionally, parametric variance analysis (ANOVA) was performed to correlate sMICA levels and NKG2D expression and presentation with the stage of the disease. For analysis of the data, we used post hoc method (Tukey HSD). For all the statistical analysis, $P<0.05$ was considered statistically significant.

Results

NKG2D gene expression

Efficiency of the GAPDH and NKG2D was calculated as 93 and 89%, respectively. Comparison of the findings indicates that NKG2D in the sample group is different from the control group. The relative expression of NKG2D is downregulated in the patient group in comparison to control by a mean factor 0.016 (SE range=0.002–0.082, $P<0.001$, Table 3 and Fig. 1). Also, parametric variance (ANOVA) analysis of NKG2D expression with stage disease showed a decrease in the expression of NKG2D with progression of disease. Indeed, this decrease positively correlated with the stage of tumor (Figs. 2 and 3 and Table 4).

Serum level of MICA protein in patient groups

A significantly higher sMICA level was measured in patients with breast cancer when compared with healthy donors. The level of sMICA in the healthy group was 60.14±16.22 pg/ml, and the mean and SEM values for the cancer group patients was 200.15±45.28 pg/ml

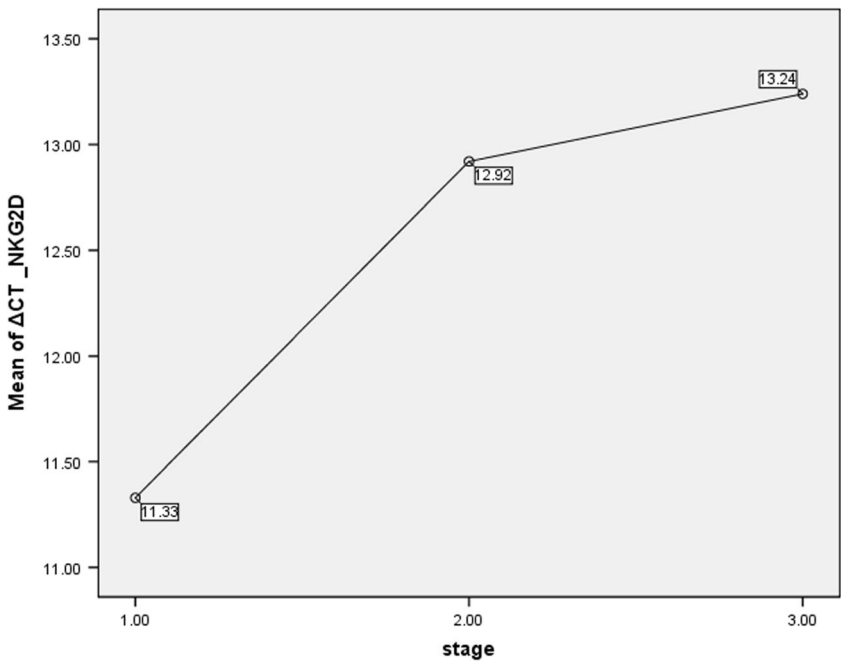

Fig. 3 Mean of ΔCT-NKG2D expression levels relative to reference gene with stage by ANOVA correlation analysis. Negative relationship between the levels of NKG2D expression and disease progression was found

Table 4 Parametric variance analysis (ANOVA) was showed to correlate NKG2D expression with the stage of the disease using post hoc method (Tukey HSD *$P<0.05$)

Tukey HSD Dependent variable	(I) stage 1	(J) stage 1	Mean difference (I–J)	SE	*P*
CT_NKG2D	1	2	−0.49733	0.51127	0.598
		3	−1.68603*	0.56424	0.012
	2	3	−1.18869*	0.37961	0.008

($P<0.001$). Furthermore, there was a close correlation between sMICA level and the stage of the disease ($P<0.001$) (Fig. 4 and Table 5).

NKG2D presentation on NK cells

In order to determine NKG2D presentation on NK cells on lymphocytes, we gated the CD56+CD3− population (as NK cells). Then, NKG2D+ cells were counted in NK cells via FL1 (NKG2D-FITC) histogram (Fig. 5). The percentage of NKG2D presentation in NK cells of breast cancer patients was significantly lower (29.64±1.92) than that of healthy subjects (62.41±2.52) (Fig. 7). Additionally, NKG2D presentation on NK cells was significantly negatively correlated with the severity of the disease ($P<0.001$) (Fig. 6 and Table 6).

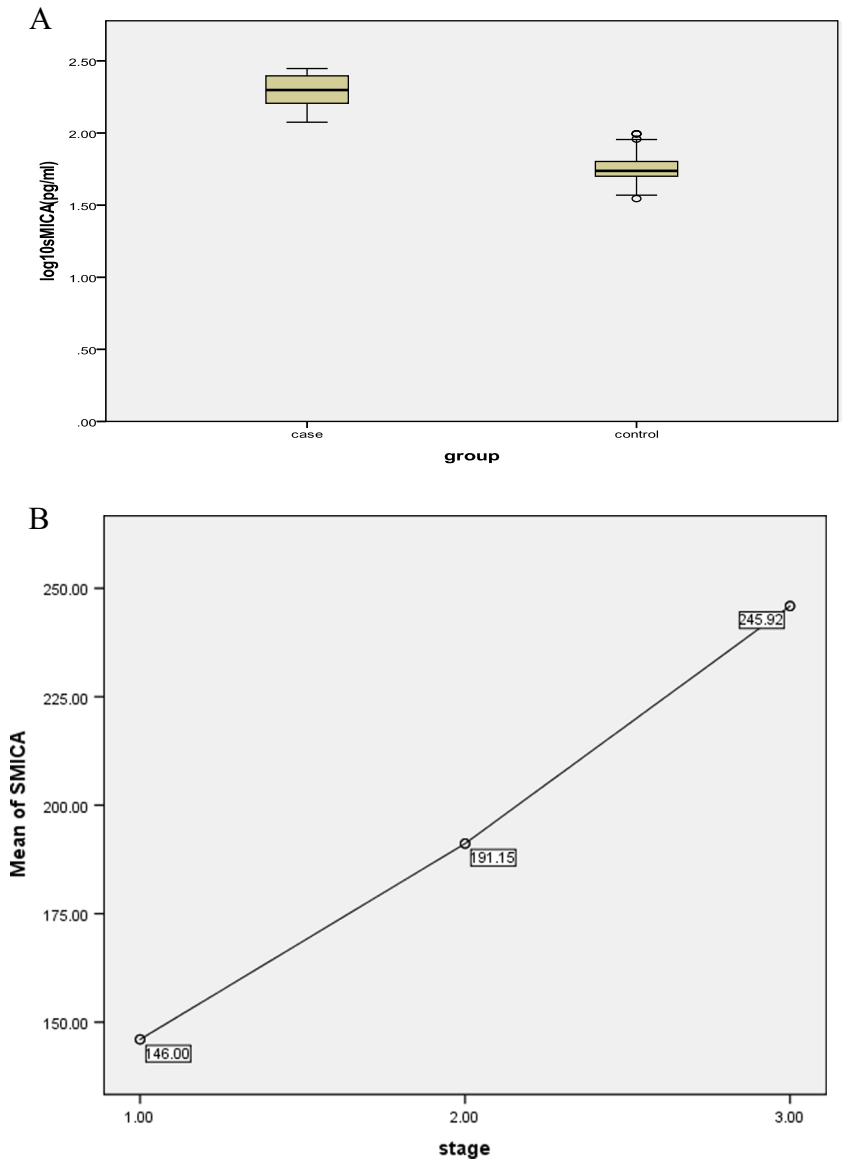

Fig. 4 **a** Mean serum level of major histocompatibility complex class I-related chain (A) sMICA in breast cancer patients and healthy controls (pg/ml normalized to log10, in each group). sMICA was determined by using ELISA assay. **$P<0.01$, Mann-Whitney test. **b** ANOVA correlation analysis of sMICA levels by stage indicates a positive relationship in the levels of sMICA with disease progression ($P<0.001$)

Table 5 Parametric variance analysis (ANOVA) was performed to correlate sMICA levels with the stage of the disease using post hoc method (Tukey HSD *$P<0.05$)

Tukey HSD Dependent variable	(I) stage 1	(J) stage 1	Mean difference (I–J)	SE	*P*
SMICA	1	2	−45.15000*	14.91046	0.011
		3	−99.91538*	16.45529	0.000
	2	3	−54.76538*	11.07077	0.000

Effect of sMICA on expression and presentation of NKG2D on NK cells

sMICA levels were negatively correlated with the number of NKG2D-presenting NK cells and NKG2D gene expression, respectively ($R^2=0.95$ and $R^2=0.99$, $P<0.001$) (Fig. 7).

Discussion

Cancer is a whole body disease, and cancer cells develop the capacity to evade the immune system by various mechanisms [24]. One of these well-studied mechanisms is production of inhibitory ligands such as MICA which induces reduction in expression and presentation of NKG2D receptors on NK cells. NKG2D receptor is one of the most important for NK cell activation. On the other hand, MICA ligand is considered vital for removing tumor cells possibly because of its high presence on the surface of the tumors when compared with other ligands that bind to the receptor [25].

Breast cancer is the most common cancer among females, and in terms of incidence, it ranks first, and data show that it is on the rise [26]. On the other hand, NK cells are the primary defense cells against cancer and NKG2D receptors are considered as the main activating receptors; inhibition or activation of this receptor by inhibition ligands, mainly MICA, may be responsible for the induction of immune system tumor escape [27]. This hypothesis is made on the basis that this ligand is only produced by cancer cells with epithelial origin such as lung, stomach, kidney, ovary, mouth, and breast [28]. Therefore, identification and quantification of this ligand may

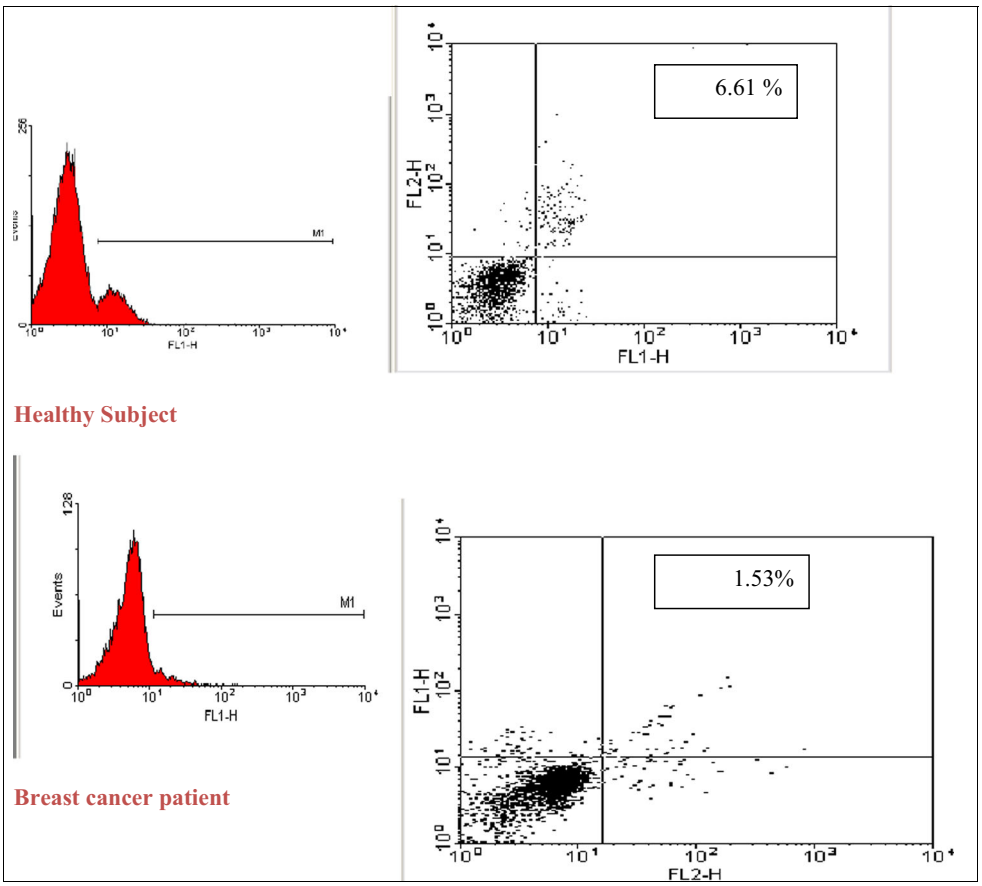

Fig. 5 Flow cytometric illustration of the percentage of NKG2D+CD56+CD3− receptors on NK cells among healthy control and breast cancer patient groups

be a useful marker for diagnosis and for assessment of progression of breast cancer.

Previous studies that reported on the relationship between serum level of SMCA and presentation of NKG2D receptors have been studies in various epithelial origins; however, this association has not been specifically studied in breast cancer patients. Martin and co-workers reported a significant increase in the serum MICA level, by ELISA method, in women suffering from cervical cancer [29]. However, no significant changes in the level of NKG2D receptor presentation, measured by flow cytometry method, were found on both NK and T peripheral blood cells. On the other hand, reduction in presentation of NKG2D receptors on NK peripheral blood cells was found among patients suffering from gastric cancer [30].

Breast cancer has been chosen because, in addition to its widespread presence, studies have shown that MICA primarily has a high expression by originated epithelial tumor cells. Because the expression of MICA is not noticeable in normal body conditions, it can be considered as a tumor biomarker [31]. During the determination of the serum levels of sMICA using the ELISA technique, a significant difference was found between the serum levels of this biomarker and the control group, thus indicating its increase in the patients. Furthermore, to determine the present rate of NKG2D receptor on NK cells in PB, flow cytometry techniques were used to confirm the RT-PCR results. The rate of NKG2D receptor appearance was shown to decrease significantly in breast cancer patients when compared with the controls. Moreover, the

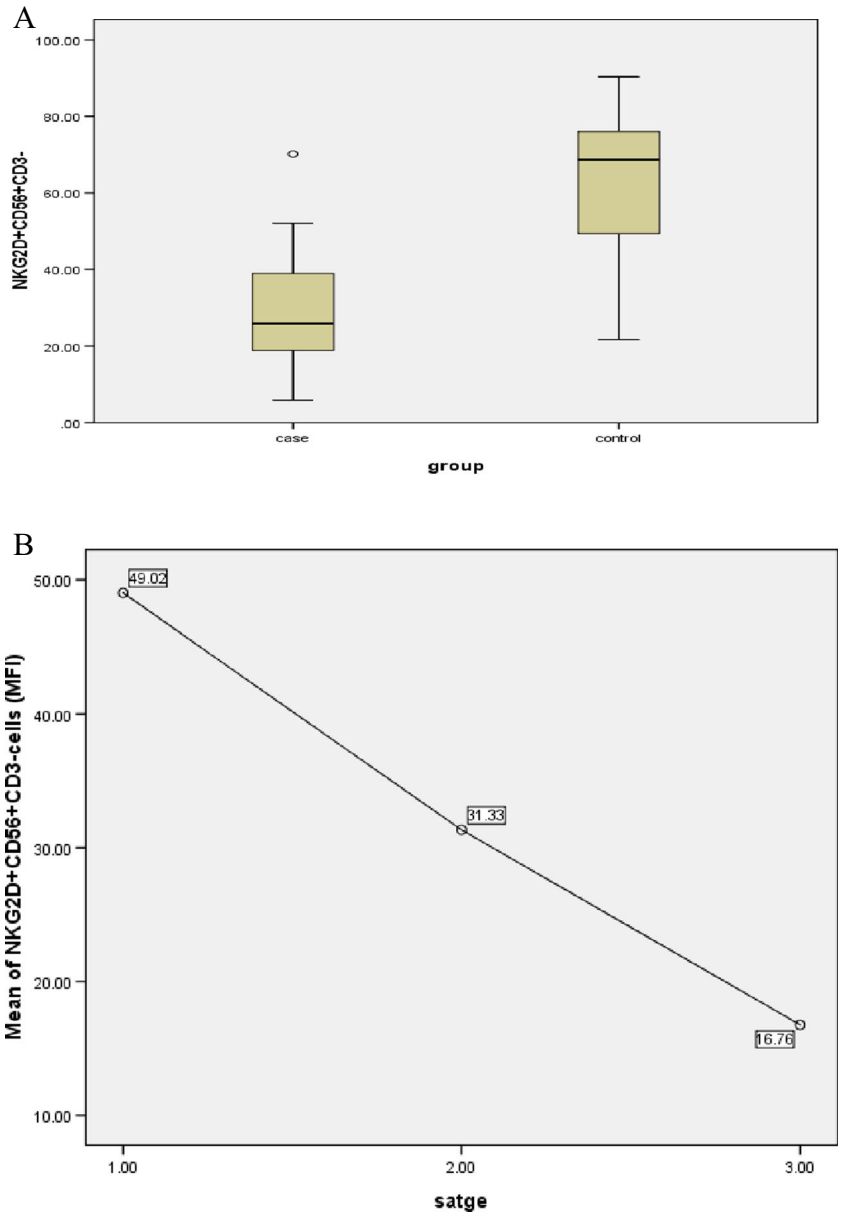

Fig. 6 **a** Mean percentage of NKG2D presentation on NKG2D+CD56+CD3− cells in cancer patients (29.64±1.92) compared to healthy controls (62.41±2.52, ***P*<0.001, Mann-Whitney test). **b** ANOVA correlation analysis of NKG2D presentation on NK cells by stage indicates a negative relationship between the levels of NKG2D presentation on NK cells and disease progression (***P*<0.001, Mann-Whitney test)

Table 6 Parametric variance analysis (ANOVA) was performed to correlate NKG2D presentation with the stage of the disease using post hoc method (Tukey HSD *$P<0.05$)

Tukey HSD Dependent variable	(I) stage 1	(J) stage 1	Mean difference (I–J)	SE	*P*
NKG2D+CD56+CD3−	1	2	17.68333*	4.27830	0.000
		3	32.22436*	4.72156	0.000
	2	3	14.54103*	3.17657	0.000

percentage of reduction in NKG2D receptor was found to be correlated with the progression of the disease. In the present study, we used RT-PCR technique which was found to be a reliable technique for quantitative measurement of this biomarker, and the current study is the first to compare the expression of this gene changes in the receptor of the patient with a normal control group.

The reduction of the number of the NKG2D receptor on the NK cells in advanced tumor stages is believed to be related to a reduction in the level of gene expression because a significant difference was observed between patients with different stages of breast cancer as well as with control normal subjects. For example, in a study on cervical cancer, which is the second most common cancer among women, conducted by Marten et al. [29] and in another study conducted by Holdenrieder on malignancy diagnosis, the role of sMICA in 512 patients with benign and malignant tumors was investigated and 52 patients were diagnosed with breast cancer [32]. The results of another study conducted by Karagoz on patients with gastric cancer come in accordance with those of the current study [30]. Previous studies have also confirmed that the presence of sMICA on the tumor surface reduced with disease progression owing to the loss of proteolytic process, thus leading to a lack of tumor recognition by the effective immune system. The present study proved that the enhanced serum levels of this sMICA and its connection to its receptor on the NK cell surface can be considered as a possible factor for the

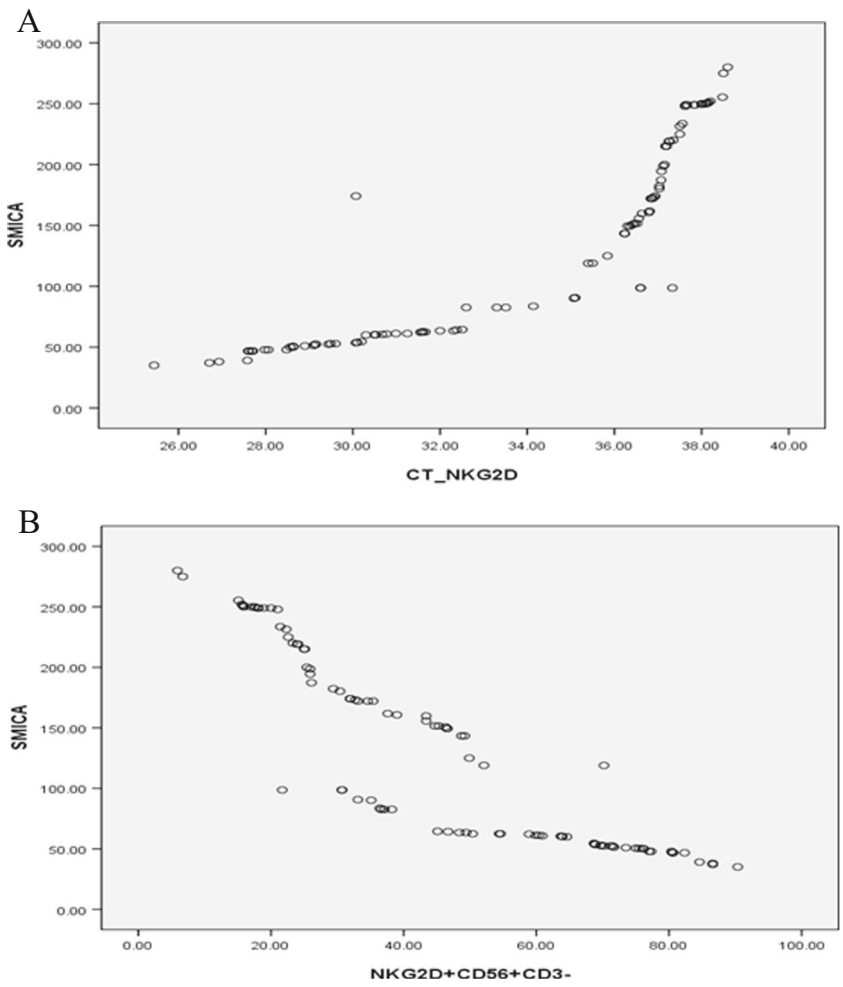

Fig. 7 Spearman's correlation analysis between sMICA levels and **a** NKG2D receptor expression ($R^2=0.99$, $P<0.001$) and **b** presentation on NK cells ($R^2=0.95$, $P<0.001$)

neutralization of the NKG2D-sMICA complex. In addition, with the progression of breast cancer, the reduction of activation signals to the NK cells was increased, resulting in the tumor escaping from the immune system [33, 34].

The results were analyzed to assess the relationship between the levels of these immunological biomarkers to the progression of tumor for validating their use in the diagnosis and prognosis of breast cancer stages. From the patient information and the analysis of the obtained results, a direct association was found between the serum sMICA levels and the stage of the disease and the expression, whereas an inverse relationship was observed for NKG2D receptor appearance. Previous studies on patients with prostate, oral squamous cell carcinoma, and colorectal cancers have also confirmed this hypothesis. These studies have also recommended the use of this biomarker as a diagnostic tumor marker for these types of cancers [21, 35, 36]. All these findings and the results from the current study confirm our hypothesis.

In the present study, we attempted to find if there is a correlation between serum sMICA level with the expression and presentation of NKG2D receptors among patients with breast cancer. The results showed that there was a significant inverse correlation between serum sMICA level and the level of expression and presentation of NKG2D receptors on the NK peripheral blood cells. This finding proposes the notion that one of the mechanisms of escape from the immune system of this type of cancer may be mediated by reduction in expression and presentation of this receptor on NK cells. Furthermore, these findings propose the notion that this protein marker can be used as an index for diagnosis and for prediction of the stage of progression of breast cancer.

Conclusions

The results obtained in this study showed the changes in expression levels and NKG2D appearance on NK cells and the correlation between MICA levels and breast cancer stage. Therefore, MICA can be used for diagnosis and treatment of the disease. This technique must be studied further with a wider scope to increase its reliability.

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