RESEARCH ARTICLE

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Reduced Expression of Natural Killer Cell-Related Activating Receptors by Peripheral Blood Mononuclear Cells from Patients with Breast Cancer and Their Improvement by Zoledronic Acid

Leila Rahimian[1], Behjat Kalantary-Khandany[2], Maryam Nemati[3,4], Samane Hoseini-Shahrestanak[1], Najmeh Aminizadeh[5], Abdollah Jafarzadeh[1,6]*

Abstract

Background/aim: Natural killer (NK) cell receptors affect the NK cell-mediated elimination of malignant cells. In this experimental study the effect of Zoledronic acid (ZOL) was investigated on the expression of NK activating-(NKP46 and NKG2D) and inhibitory (KIR2DL1) receptors by Phytohaemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMCs) from breast cancer (BC) patients. **Materials and Methods:** Peripheral blood mononuclear cell-extracted RNA from thirty breast cancer women and twenty-five healthy subjects was analyzed for gene expression of NKP46, NKG2D and KIR2DL1 using real time-PCR. Then, the PBMCs from BC patients were cultured in the presence of PHA with 5 μg/ml, 10 or 20 μg/ml of ZOL for 32 hours and expression of the aforementioned receptors was determined. **Results:** Expression of NKP46, NKG2D and NKP46/KIR2DL1 ratio in BC women were lower than healthy group ($P<0.01$, $P<0.04$ and $P<0.05$, respectively). NKP46 expression was up-regulated by PHA-stimulated PBMCs treated with 10 μg/ml and 20 μg/ml of ZOL compared with PHA-stimulated cultures ($P<0.01$ and $P<0.05$, respectively). NKG2D expression remarkably increased by PHA-stimulated cultures treated with 5 μg/ml, 10 μg/ml and 20 μg/ml of ZOL compared with PHA-stimulated cultures ($P<0.05$ and $P<0.02$ and $P<0.04$, respectively). **Conclusion:** Expression of NK cell-related activating receptors decreased in BC patients. ZOL can improve the expression of NK activating receptors.

Keywords: Breast cancer- Zoledronic acid- natural killer cells- NKP46- NKG2D- KIR2DL1

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Introduction

Breast cancer (BC) comprises approximately 24.2% of all women's cancers and more than 620000 deaths are related to this malignancy, worldwide (Abdel-Latif and Youness, 2020). Some determinants such as human epithelial growth factor receptor 2 (Her2), estrogen receptor (ER) and progesterone receptor (PR) are important markers for characterization of BC diversity, prognosis and determination of the treatment program (Inoue and Fry, 2016). The Her2-positive BC exhibits greater tumor growth and aggression than other subtypes (Abdel-Latif and Youness, 2020). Moreover, the stages of BC are determined according to the primary tumor size, involvement of lymph node and distant metastasis (Palacios-Arreola et al., 2014).

On the basis of the immune surveillance phenomenon, one of the essential tasks of the immune system is to identify and kill cancer cells (Balouchi-Anaraki et al., 2018; Malmberg et al., 2017). Some abnormalities in the immune-related parameters were observed in patients with BC (Jafarzadeh et al., 2015a; Jafarzadeh et al., 2016; Jafarzadeh et al., 2015b; Khalife et al., 2018). Immunotherapy using various forms of immunomodulators can boost the host immune system against tumor-induced immunosuppression (Masoumi et al., 2022; Pemula Gowtham, 2021). In the immune system, the natural killer (NK) cell-mediated immune surveillance acts as the first line of defense against malignant cells (Malmberg et al., 2017; Meza Guzman et al., 2020). NK cells consist 5–10% of peripheral blood lymphocytes and are characterized by the absence of T cell receptor (TCR) and the positivity for the cell surface expression of CD16 and CD56 markers (Rezvani and Rouce, 2015). A wide board of both inhibitory and activating receptors regulates the functions of NK cells. The balance between

*[1]Department of Immunology, Medical School, Kerman University of Medical Sciences, Kerman, Iran. [2]Department of Hematology-Oncology and BMT, Kerman University of Medical Sciences, Kerman, Iran. [3]Immunology of Infectious Diseases Research Center, Research Institute of Basic Medical Sciences, Rafsanjan University of Medical Sciences, Rafsanjan, Iran. [4]Department of Hematology and Laboratory Sciences, Para-Medical School, Kerman University of Medical Sciences, Kerman, Iran. [5]Department of Histology, School of Medicine, Islamic Azad University Branch of Kerman, Kerman, Iran. [6]Department of Immunology, Medical School, Rafsanjan University of Medical Sciences, Rafsanjan, Iran. *For Correspondence: Jafarzadeh14@yahoo.com*

signals coming from activating and inhibitory receptors regulates the capability of NK cells to kill target cells (Meza Guzman et al., 2020).

The natural killer group 2D (NKG2D) and natural cytotoxicity receptor NKP46 are among the most efficient activation receptors on NK cells which bind to their ligands on tumor or virally-infected cells (Kumar, 2018). The MHC class I-related chain (MICA and MICB) and the UL16 binding proteins (ULBP1-6) are recognized by the NKG2D receptor (Duan et al., 2019; Kumar, 2018). Normal tissues do not express these ligands, but they are induced in some diseases, including cancers (Kumar 2018). Another NK cell-related activating receptor NKP46 lead to the cell activation through binding to vimentin and heparin (Konjevic et al., 2016).

The killer immunoglobulin-like receptors (KIRs) are a very polymorphic family of NK cell receptors that bind to class I molecules of MHC and trigger inhibitory or activating signals (Duan et al., 2019; Kumar, 2018). KIRs are designated according to the number of Ig-like domains (2D or 3D) that they have within their extracellular parts and the existence of a long or short (L or S) cytoplasmic region that induce activating or inhibitory signals, respectively (Duan et al., 2019). KIR2DL1 is a member of the KIRs family with inhibitory activity for NK cells that recognizes some alleles of HLA-C (Duan et al., 2019).

Zoledronic acid (ZOL) as the most powerful nitrogen-containing bisphosphonates suppresses osteoclast genesis through prevention of the RANKL expression on osteoblasts, used in patients with osteoporosis or low bone mass (Huang et al., 2019). ZOL also exhibits antitumor effects via suppression of the proliferation, induction of apoptosis, prevention of angiogenesis, reduction of tumor cells adhesion to the bone, reduction of tumor cells invasion and migration, and enhancement of the antitumor immune response (Zekri et al., 2014).

Zoledronic acid is used for treating multiple forms of osteoporosis, hypercalcemia of malignancy, multiple myeloma, bone metastases from solid tumors, and Paget's disease of bone. The main action of zoledronic acid is inhibiting the resorption of bone. Moreover, ZOL induces the expression of phosphor antigens in which stimulate γδ T-cells. Therefore, the expression of phosphor antigens is up-regulated in ZOL-treated tumor cells and they become more vulnerable to killing by γδ T-cells (Mattarollo et al. , 2007). ZOL also induces γδ T- cells to IFN-γ-producing effector lymphocytes, which may elicit more efficient antitumor responses (Dieli et al., 2003). ZOL not only increases the sensitivity of tumor cells to killing by γδ T-cells but also enhances the number of γδ T cells when was co-administered with IL-2 (Marten et al., 2007; Naoe et al., 2010; Zekri et al., 2014). Moreover, ZOL inhibits the migration, expansion and functions of pro-tumor Treg cells (Liu et al., 2019; Liu et al., 2016; Sarhan et al., 2017). There are also reports indicating that ZOL has the capacity to prevent the differentiation of the pro-tumor myeloid-derived suppressor cells (MDSC) and tumor-associated macrophages (TAM) (Melani et al., 2007; Tang et al., 2020).

The expression of the NK cell-related activating receptors (NKp46 and NKG2D) and an inhibitory receptor (KIR2DL1) has not been adequately evaluated in BC, yet. In our previous study, it was observed that PHA improves the NK cell activity and reinforces the expression of their activating receptors (Sheikhi et al., 2014). The stimulatory effects of the PHA on the NK cell activity of PBMCs were also indicated (Sheikhi et al., 2011). It was also indicated that ZOL potentiates the expression of pro-inflammatory cytokines by LPS-activated macrophages (Muratsu et al., 2013). According to our knowledge, the effects of ZOL on the expression of NK cell-related functional receptors have not also been evaluated, yet. The aim was to investigate the expression of two activating receptors (NKP46 and NKG2D), and an inhibitory receptor (KIR2DL1) in newly diagnosed patients with BC. The effects of ZOL on the expression of these receptors by PBMCs isolated from BC patients were also investigated. Furthermore, the association of the expression of NK cell receptors with BC stages and ER, PR, Her-2 status was evaluated to clear any associations.

Materials and Methods

Subjects

For this experimental study, thirty women with BC (mean age: 48.95 ± 11.43 years) were chosen among patients who were referred to the Oncology Unit of the Shahid Bahonar Hospital affiliated to Kerman University of Medical Sciences. The patients were newly diagnosed (untreated) and enrolled before chemotherapy, radiotherapy or immunotherapy. The presence of BC and its staging was confirmed by expert oncologists based on the principles of the Sixth Edition of the American Joint Committee on Cancer (AJCC) (Greene et al., 2002). Moreover, 25 healthy women (mean age: 47.75 ± 10.03 years), without any acute or chronic illnesses, were randomly recruited among blood donors of Kerman Blood Transfusion Center. Ethics Committee of Kerman University of Medical Sciences confirmed the investigation with the ID number of IR.KMU.REC.1394.413 and the informed written consent was also obtained from participants before blood sampling.

Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

5-10 ml of heparinized peripheral blood was collected from healthy donors and BC patients. The PBMCs were isolated using centrifugation over Lymphosep (Biosera, UK). Briefly, the blood samples were diluted with the same volume of phosphate-buffered saline (PBS) to obtain a 1:2 dilution. The diluted blood was carefully placed on the same volume of Lymphosep solution and was then centrifuged at 3,000 rpm for 30 minutes at 20°C. Upon centrifugation, the PBMC layer was carefully transferred to a new tube and washed using Roswell Park Memorial Institute Medium (RPMI) 1640 media. The PBMCs cells were re-suspended in 2 mL of RPMI 1640. A fraction of PBMCs was used for total RNA extraction and analysis of gene expression before in vitro culturing. Another fraction of PBMCs from patients with BC was used for in vitro culturing in the presence of PHA without or with ZOL.

In vitro culturing of PBMCs in the presence of PHA and/or ZOL

The PBMC viability was assessed using the trypan blue exclusion method and the viability >95% was accepted for experiments. After three times washing, the PBMCs from patients with BC were re-suspended in RPMI-1640 supplemented with 10.0% heat inactivated-fetal bovine serum (Gibco Life Technologies Ltd, UK), and streptomycin and penicillin antibiotics with 100 µg/ml and 100 U/ml, respectively. The PBMCs were cultured at 1×10^6 cells/ml in 24 flat-bottomed well microtiter plates (Nunc, Denmark), in the existence of 10 µg/ml PHA without ZOL or with 5 µg/ml, 10 µg/ml or 20 µg/ml of ZOL. The doses of PHA and ZOL were selected according to previous studies (Bringmann et al., 2007; Campbell et al., 2019; Liu et al., 2019; Sheikhi et al., 2014). The PBMCs were incubated in a 5% CO_2 incubator for 32 hours at 37°C. After incubation, the PBMCs were harvested and the RNA extraction was done.

Quantitative real-time PCR

Trizol reagent (Bionner, Korea) was used to extract total RNA from the PBMCs. A spectrophotometer was also used to determine the quantity and purity of the extracted RNA according to its absorbance at 280 nm and 260 nm. The RNA quality was determined using electrophoresis on the agarose gel.

The conversion of the extracted RNA to complementary DNA (cDNA) was done using a cDNA synthesis kit (Applied Biosystems, USA). The protocol of the cDNA synthesis was included: 70°C for 10 minutes (without reverse transcriptase), 20°C for 1 minute (in the presence of reverse transcriptase), 42°C for 60 minutes and eventually the process was terminated by an additional step at 95°C for 10 minutes to halt the reverse transcriptase activity.

A real-time PCR system (Applied Biosystems, USA) was employed to assess the gene expression of NKP46, NKG2D and KIR2DL1 using a SYBR green master mix (Applied Biosystems, USA), mixed with 200 ng of synthesized cDNA with 2 µL proper primers (10 pmol stock). The used primers were synthesized by Bionner Company (Korea) (Table 1).

The thermal cycler-related PCR program was designed as 95 °C for 2 minutes; 40 sequential cycles at 95 °C for 15 seconds, and 60 °C for 30 seconds; and finally 72°C for 30 seconds. The β-actin gene was also used as an internal control and the amounts of the NKP46, NKG2D and KIR2DL1 expression were calculated by the $2^{-\Delta\Delta Ct}$ formula. The PCR products were also electrophoresed and visualized on a 1% agarose gel.

Statistical analysis

The data were exhibited as mean ± SEM. The comparisons of the variables were done using statistical software (SPSS version 18, Chicago, IL, USA) by ANOVA, Student's t test, Kruskal-Wallis and Mann-Whitney U tests as appropriate. The differences were regarded meaningful, when the P values were <0.05.

Results

The expression of NKP46 by PBMCs from BC patients and healthy groups

The fold change of the gene expression of NKP46 by PBMCs from women with BC and healthy subjects were demonstrated in Table 2. The NKP46 expression in women with BC was significantly lower than in the healthy control group (P<0.01). The NKP46 expression in patients who were in stages I, II and III was significantly lower as compared with healthy women (P<0.01, P<0.01 and P<0.03, respectively) (Table 2 and Figure 1A).

The expression of NKP46, NKG2D and KIR2DL1 according to ER, PR and Her-2 status was summarized in Table 3. The expression of NKp46 in ER+ and PR+ patients was significantly lower than ER- and PR- patients, respectively (P<0.05).

The effects of ZOL on the expression of NKP46 by PBMCs from BC patients

The NKP46 mRNA was significantly overexpressed in PHA-stimulated PBMCs treated with 10 µg/ml and 20 µg/ml of ZOL in comparison with PHA-stimulated cultures (without ZOL treatment) (P<0.01 and P<0.05, respectively). The expression of NKP46 mRNA in PHA-stimulated PBMCs treated with 5 µg/ml of ZOL was higher than PHA-stimulated cultures (without ZOL treatment), but the difference was not significant (Table 4 and Figure 1B). No significant difference was observed between BC patients-collected PBMCs treated with 20 µg/ml of ZOL and healthy subjects-collected PBMCs concerning the NKP46 expression, when data were normalized against the PBMCs collected from healthy individuals.

Table 1. The Used Primers for the Gene Expression of *NKP46, NKG2D* and *KIR2DL1* by PBMC from Healthy Subjects and Breast Cancer Patients

Genes	Primer sequences	PCR product size (bp)
NKP46	Forward primer: 5′-ACGGGACTCCAGAAAGACCAT-3′	66
	Reverse primer: 5′-CAGGCCCATCCGAAGGA-3′	
NKG2D	Forward primer: 5′-GGCTCCATTCTCTCACCCA-3′	79
	Reverse primer: 5′-TAAAGCTCGAGGCATAGAGTGC-3′	
KIR2DL1	Forward primer: 5′-TTCTCCATCAGTCGCATGAC-3′	96
	Reverse primer: 5′-GTCACTGGGAGCTGACAC-3′	
β-Actin	Forward primer: 5′-GCATGGGTCAGAAGGATTC-3′	104
	Reverse primer: 5′-GTCCCAGTTGGTGACGAT-3′	

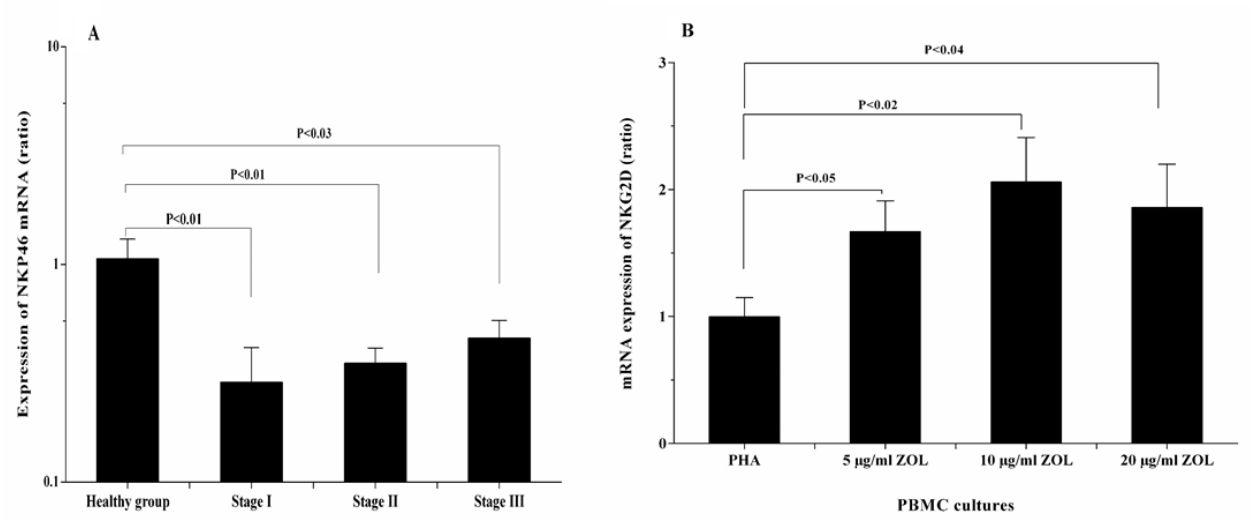

Figure 1. The mRNA Expression of NKP46 by PBMC. 1A: The expression of NKP46 in healthy subjects and patients with breast cancer according to tumor stages. The NKP46 expression by PBMC from patients with stage I, stage II and stage III were significantly lower as compared with healthy women. 1B: The effects of ZOL on the expression of NKG2D. The expression of NKG2D was significantly increased in PHA-stimulated PBMCs treated with 5, 10 and 20 µg/ml of ZOL in comparison with PHA-stimulated cultures.

Table 2. The Fold Change of the Gene Expression of *NKP46, NKG2D* and *KIR2DL1* in Patients with Breast Cancer According to Tumor Stages

No.	Expression of NKp46 mRNA	Expression of NKG2D mRNA	Expression of KIR2DL1 mRNA	Expression of NKP46/KIR2DL1 ratio	Expression of NKG2D/KIR2DL1 ratio	P values
3	0.28 ± 0.12	0.69 ± 0.29	0.38 ± 0.21	1.91 ± 0.69	6.63 ± 2.38	*0.01
10	0.35 ± 0.06	0.44 ± 0.12	0.65 ± 0.35	2.95 ± 1.19	5.09 ± 3.95	**0.42
17	0.45 ± 0.09	0.48 ± 0.13	1.17 ± 0.42	4.36 ± 1.08	2.79 ± 0.71	***0.81
30	0.40 ± 0.05	0.48 ± 0.08	0.91 ± 0.27	3.50 ± 0.69	4.19 ± 1.42	†0.05, ††0.25
25	1.06 ± 0.24	1.06 ± 0.23	1.00 ± 0.27	20.09 ± 7.57	12.76 ± 6.05	

*, **, ***, † and †† represent the differences of the expression of NKP46, NKG2D, KIR2DL1, NKP46/KIR2DL1 ratio and NKG2D/KIR2DL1 ratio among breast cancer patients with different stages, respectively.

The expression of NKG2D by PBMCs from BC patients and healthy groups

The NKG2D expression in patients with BC was significantly decreased in comparison with healthy individuals (P<0.01) (Table 2). The NKG2D expression in patients with stages II and III was significantly lower than

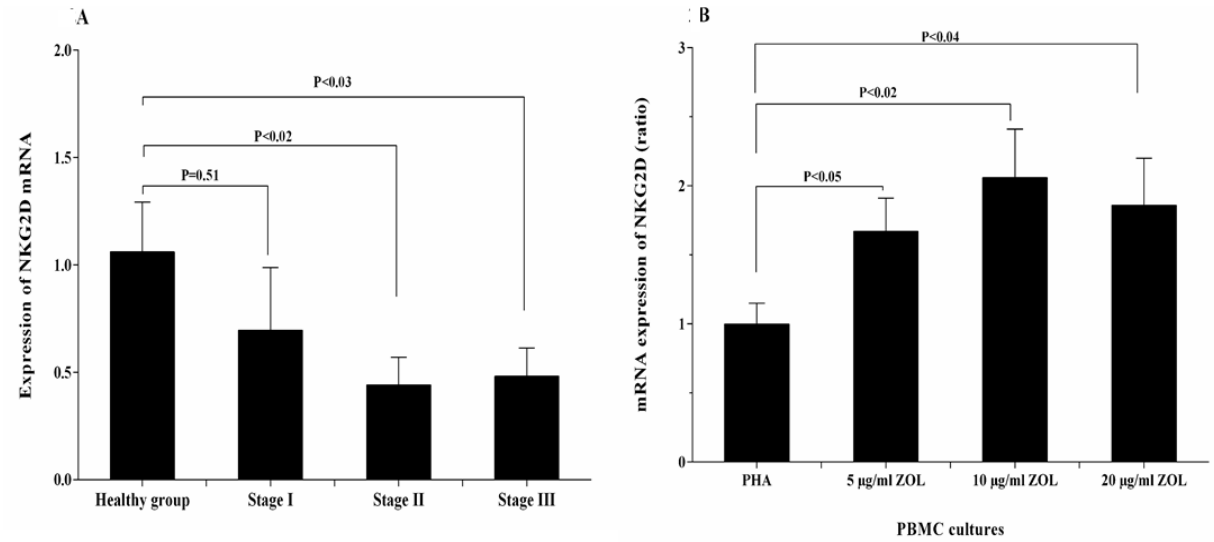

Figure 2. The mRNA Expression of NKG2D by PBMC. 2A: The mRNA expression of NKG2D by PBMC from healthy subjects and patients with breast cancer according to tumor stages. The NKG2D expression by PBMC in breast cancer patients with stage II and stage III were significantly lower than healthy women. 2B: The effects of ZOL on the expression of NKG2D by PBMCs from patients with breast cancer. The expression of NKG2D was significantly increased in PHA-stimulated PBMCs treated with 5, 10 and 20 µg/ml of ZOL in comparison with PHA-stimulated cultures.

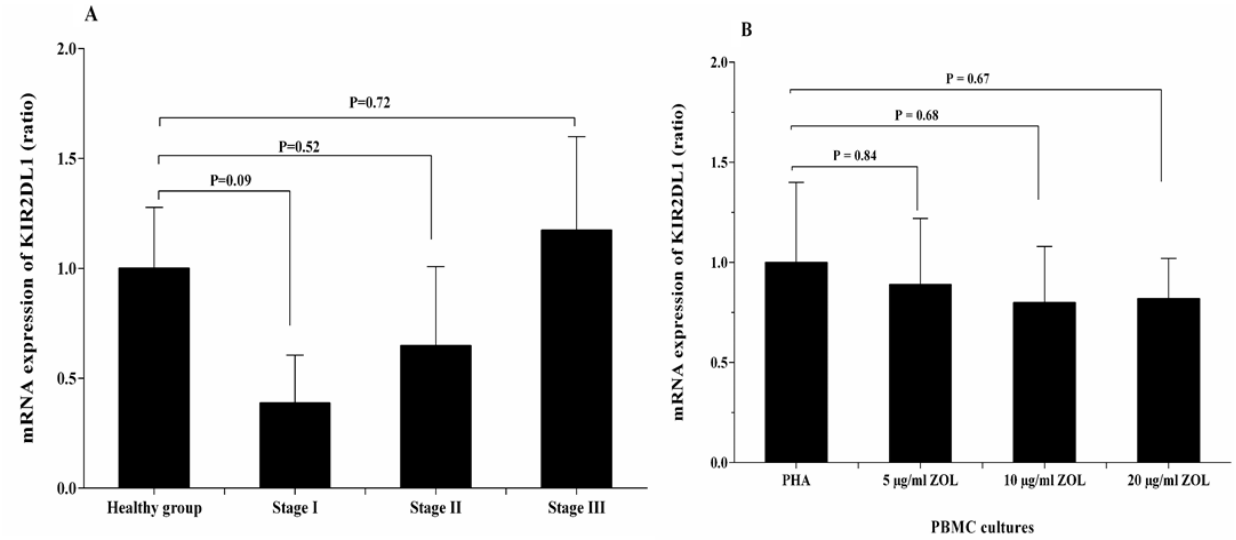

Figure 3. The mRNA Expression of KIR2DL1 by PBMC. 3A: Comparison of the mRNA expression of KIR2DL1 by PBMC from healthy subjects and patients with breast cancer according to tumor stages. There was no significant difference between breast cancer patients having stage I, stage II or stage III and healthy subjects regarding the KIR2DL1 expression. 3B: The effects of ZOL on the expression of KIR2DL1 by PBMCs from patients with breast cancer. The expression of KIR2DL1 did not significantly differed between PHA-stimulated PBMCs treated with 5, 10 and 20 µg/ml of ZOL and PHA-stimulated cultures.

healthy women (P<0.02 and P<0.03, respectively). The difference of the NKG2D expression between BC patients with stage I and healthy individuals was not significant, although this parameter was decreased in patients (Table 2 and Figure 2A).

The expression of NKG2D in triple-positive (TP) patients was significantly increased as compared with triple-negative (TN) patients (P<0.02). Furthermore, the expression of NKG2D in PR+ patients was higher than PR- patients, but the difference was not significant (P=0.08) (Table 3).

The effects of ZOL on the expression of NKG2D by PBMCs from BC patients

The expression of NKG2D mRNA was significantly increased in PHA-stimulated PBMCs treated with 5 µg/ml, 10 µg/ml and 20 µg/ml of ZOL in comparison with PHA-stimulated cultures (without ZOL treatment) (P<0.05 and P<0.02 and P<0.04, respectively) (Table 4 and Figure 2B). No significant difference was observed between BC patients-collected PBMCs treated with 20 µg/ml of ZOL and healthy subjects-collected PBMCs concerning the NKG2D expression, when data were normalized against the PBMCs collected from healthy individuals.

The expression of KIR2DL1 by PBMCs from BC patients and healthy groups

No significant difference was observed between BC patients and healthy subjects with respect to the expression of KIR2DL1 (Table 2). Moreover, there was no significant difference between BC patients with stages I, II or III and healthy subjects regarding the KIR2DL1 expression. Although, the patients with advanced tumor stages had elevated expression of the inhibitory receptor KIR2DL1,

Table 3. Gene Expression of NKP46, NKG2D and KIR2DL1 in Patients with Breast Cancer According to Their Clinicopathological Parameters Status

Markers	Marker status	No.	Expression of NKp46 mRNA	Expression of NKG2D mRNA	Expression of KIR2DL1 mRNA	Expression of NKP46/ KIR2DL1 ratio	Expression of NKG2D/KIR2DL1 ratio	P values
Estrogen receptor	Negative	9	0.50 ± 0.08	0.25 ± 0.09	1.2 ± 0.60	3.07 ± 1.13	6.04 ± 4.41	*0.05, **0.10, ***0.60, †0.70, ††0.39
	Positive	21	0.36 ± 0.07	0.56 ± 0.1	0.73 ± 0.28	3.66 ± 0.86	3.36 ± 0.71	
Progesterone receptor	Negative	10	0.51 ± 0.06	0.22 ± 0.07	1.04 ± 0.49	3.63 ± 1.29	5.32 ± 3.60	*0.05, **0.08, ***0.89, †0.88, ††0.54
	Positive	20	0.33 ± 0.07	0.62 ± 0.11	0.82 ± 0.32	3.41 ± 0.81	3.50 ± 0.77	
HER-2	Negative	12	0.37 ± 0.11	0.51 ± 0.15	0.66 ± 0.32	4.12 ± 1.02	3.81 ± 1.48	*0.78, **0.38, ***0.22, † 0.54, ††0.53
	1+	8	0.31 ± 0.07	0.49 ± 0.11	0.68 ± 0.26	2.19 ± 1.37	2.19 ± 1.05	
	2+	3	0.43 ± 0.5	0.27 ± 0.12	0.89 ± 0.77	2.35 ± 0.83	0.77 ± 0.53	
	3+	7	0.50 ± 0.09	0.52 ± 0.20	1.4 ± 0.82	3.74 ± 1.75	3.05 ± 1.43	
Triple status	TP	9	0.19 ± 0.05	0.49 ± 0.07	1.38 ± 0.68	1.26 ± 0.33	2.27 ± 0.95	*0.63, **0.02, ***0.96, †0.79, ††0.49
	TN	3	0.13 ± 0.08	0.08 ± 0.03	1.45 ± 1.29	1.15 ± 0.21	1.23 ± 1.05	

*, **, ***, † and †† represent the differences of the expression of NKP46, NKG2D, KIR2DL1, NKP46/KIR2DL1 ratio and NKG2D/KIR2DL1 ratio according the specified marker status of the breast cancer marker, respectively; TN, triple negative (ER, PR and HER2 negative); TP, triple positive (ER, PR and HER2 positive).

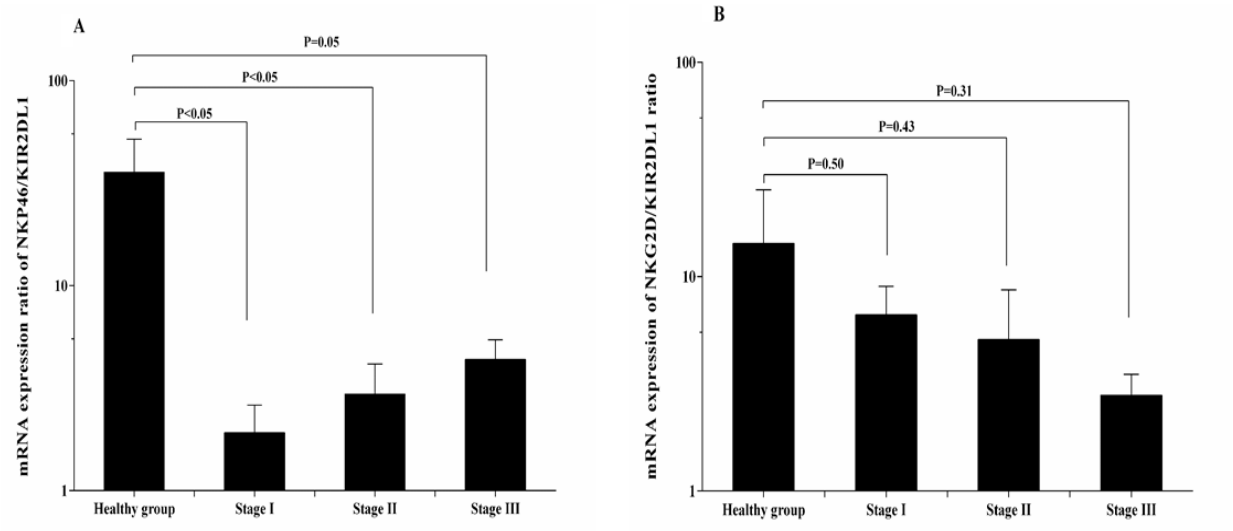

Figure 4. The mRNA Expression Ratios of NKP46/KIR2DL1 and NKG2D/KIR2DL1 by PBMC. 4A: Comparison of the mRNA expression ratio of NKP46/KIR2DL1 by PBMC from healthy subjects and patients with breast cancer according to tumor stages. The expression ratio of NKP46/KIR2DL1 by PBMC from patients with stage I, stage II and stage III were significantly lower as compared with healthy women. 4B: Comparison of the mRNA expression ratio of NKG2D/KIR2DL1 by PBMC from healthy subjects and patients with breast cancer according to tumor stages. There was no significant difference between breast cancer patients with stage I, stage II or stage III and healthy subjects regarding the NKG2D/KIR2DL1 ratio.

Table 4. The Expression of NKG2D, NKP46 and KIR2DL1 in zoledronic acid-treated PHA-stimulated PBMCs from Patients with Breast Cancer

Markers	PBMCs treated with				P vlue
	PHA	PHA+ 5 µg/ml ZOL	PHA+ 10 µg/ml ZOL	PHA+ 20 µg/ml ZOL	
NKP46	1.04 ± 0.28	1.71 ± 0.71	2.04 ± 0.46	1.93 ± 0.57	*=0.23; **=0.01; ***=0.05
NKG2D	1.00 ± 0.15	1.67 ± 0.24	2.06 ± 0.35	1.86 ± 0.34	*=0.05; **=0.02; ***=0.04
KIR2DL1	1.00 ± 0.40	0.89 ± 0.33	0.80 ± 0.28	0.82 ± 0.20	*=0.84; **=0.68; ***=0.67

*, ** and *** represents the differences of the expression of a specified marker between PHA-stimulated PBMCs and cultures treated with 5 µg, 10 µg and 20 µg of zoledronic acid (ZOL), respectively.

the differences were not significant (Table 2 and Figure 3A). The expression of KIR2DL1 was not affected by the clinic pathological status of cancer (Table 3). The expression of KIR2DL1 mRNA did not significantly differ between PHA-stimulated PBMCs treated with 5 µg/ml, 10 µg/ml and 20 µg/ml of ZOL and PHA-stimulated cultures (without ZOL treatment) (Table 4 and Figure 3B).

The expression of NKG2D/KIR2DL1 and NKP46/KIR2DL1 ratios in PBMCs from BC patients and healthy groups

The expression of NKP46/KIR2DL1 and NKG2D/KIR2DL1 mRNA ratios from women with BC and healthy subjects was also demonstrated in Table 2. The expression ratios of NKP46/KIR2DL1 and NKG2D/KIR2DL1 in patients with BC were lower than in healthy control group, but it reached to a significant level only for NKP46/KIR2DL1 (P<0.05). The expression NKP46/KIR2DL1 in patients with stages I, II and III were lower as compared with healthy women (P<0.05, P<0.05 and P= 0.06, respectively) (Table 2 and Figure 4A). There was no significant difference between BC women with stages I, II or III and healthy individuals concerning the expression of NKG2D/KIR2DL1, although this ratio was lower in all stages (Table 2 and Figure 4B). The expression of NKG2D/KIR2DL1, and NKP46/KIR2DL1 did not influence by the ER, PR and Her2 status of cancer (Table 3). The expression of NKP46/KIR2DL1 and NKG2D/KIR2DL1 ratios in ZOL-treated cultures was higher than only PHA-stimulated cultures, but differences were not significant. The expression of KIR2DL1 was remarkably greater in the BC patients-collected PBMCs treated with 20 µg/ml of ZOL compared with that in healthy subjects-collected, when data were normalized against the PBMCs collected from healthy individuals (P<0.05).

Discussion

The results of the present investigation indicated that the gene expression of the NK cell-related activating receptors NKP46 and NKG2D was significantly decreased in newly-diagnosed patients with BC in comparison with healthy group. Diminished expression of NKG2D was demonstrated in patients with BC which is in accordance with our results (Nieto-Velazquez et al., 2016; Roshani et al., 2016). Diminished expression of NKP46 and NKG2D was also indicated in other malignancies such as nasopharyngeal carcinoma, non-small cell lung cancer and gastric cancer (Han et al., 2018; Xu et al., 2018; Yu et al., 2014). It has been also indicated that the expression of NKP46 and NKG2D in tumor-relapsed BC patients

was lower than in non-relapsed patients. Furthermore, the patients with higher survival time exhibited more expression of activating receptors (Ascierto et al., 2013). The importance of the NKG2D receptor in immune surveillance has been demonstrated in NKG2D-deficient mice (Guerra et al., 2008). Downregulation of NKp46 and NKG2D expression by NK cells attributed to l-kynurenine, which is produced by tryptophan degradation by a tumor-derived indolamine-2, 3-dioxygenase (Della Chiesa et al., 2006; Konjevic et al., 2016).

The expression of NKP46 and NKP46/KIR2DL1 in BC patients with stages I, II and III and the expression of NKG2D in BC with stages II and III were lower than those in healthy women. Thus, diminished expression of NKP46 and NKG2D may contribute to tumor establishment and development. During tumor progression, tumor cells escape from the recognition and killing by NK cells through the acquisition of several evading mechanisms (Cheng et al., 2013). Some of the evasion ways are functional impairment of MIC/NKG2D signaling through the production of TGF-β by tumor cells (Waldhauer and Steinle, 2008). Our findings suggest that during tumor establishment and development, tumor cells may utilize mechanisms to escape from NK cell-mediated recognition through the downregulation of the activating receptors.

In this study, no remarkable difference was found between women with BC and healthy subjects concerning the KIR2DL1 expression. According to our knowledge, this is the first report concerning the expression of KIR2DL1 in patients with BC. In one study from India, it has been indicated that in patients with oral squamous cell carcinoma the expression of inhibitory receptor KIR2DL1 was increased when compared with healthy group (Dutta et al., 2014). Elevated expression of KIR2DL1 and its ligand (HLA-C) was also correlated with susceptibility to melanoma and its progression (Konjevic et al., 2016).

We indicated that the expression of NKP46/KIR2DL1 ratio was decreased in BC patients compared with the control group. The activating- and inhibitory receptors of NK provide signals that determine the activation or inhibition of NK cells. The down-regulation of activating receptors in combination with up-regulation (or unchanged) expression of inhibitory receptors leads to the impairment of NK cell activity contributing to the tumor development.

The expression of NKP46 in ER+ and PR+ patients were significantly lower than ER- and PR- patients, respectively. The expression of NKG2D in PR+ patients was also lower than PR- patients, but the difference was not significant (P=0.08). The reasons for these associations remain to be clear in future investigations. In our previous study, a negative association was also observed between the serum levels of chemokine CXCL10 with the expression of ER and PR (Jafarzadeh et al., 2016). In accordance with our results, an inverse correlation was also indicated between the expression of a number of cytokines such as IL-1β, IL-2, IL-6, IL-8, IL-10, IL-13, IFN-γ, MCP-1, MIP-1β, TNF-α were with the ER and PR expression in BC patients (Chavey et al., 2007).

The reducing effects of estradiol on the activation of nuclear factor (NF)-κB has been reported (Liu et al., 2014) which may be an account for the negative association between immune/inflammatory parameters with ER status. We have also observed that the expression of NKG2D in TP patients was significantly increased as compared with TN patients. However, no correlation was found between the expression of NK receptors and Her2 status. Accordingly, it seems that the expression of the aforementioned NK receptors did not influence by Her2 status of BC. More studies with a greater sample size need to clear the exact association and accountability mechanisms.

We found that the NKP46 expression by PHA-stimulated PBMCs treated with 10 and 20 μg/ml of ZOL was significantly raised in comparison with non-treated PBMCs. Moreover, the expression of NKG2D in PBMCs treated with 5 μg/ml, 10 μg/ml, and 20 μg/ml of ZOL were higher than o non-treated PBMCs. Thus ZOL can improve the NK activity against tumor cells. In agreement with our findings, it was revealed that ZOL enhances direct NK cytotoxicity and ADCC against squamous cell carcinoma of the head and neck, and lymphoma cell lines (Maniar et al., 2010).

ZOL may directly and/or indirectly influence the NK cells. The indirect effects of ZOL on the NK cells may be mediated through activation of γδ T cells, DCs and monocytes. Maniar et al. showed that the elevated NKG2D expression on NK cells occurs after their co-culturing with ZOL-treated γδ T-cells (Maniar et al., 2010). Diminished NK cell activity and NKG2D expression were also attributed to the inhibitory effects of TGF-β, IL-13 and IL-10 secreted in the tumor microenvironment, by tumor cells, TAM, MDSC and Treg cells (Balouchi-Anaraki, 2018; Castriconi et al., 2003; Konjevic et al., 2016). As mentioned, ZOL exerts inhibitory impacts on the pro-tumor TAM, MDSC and Treg cells (Liu et al., 2019; Liu et al., 2016; Melani et al., 2007; Sarhan et al., 2017; Tang et al., 2020).

Furthermore, ZOL induces the IFN-γ production by monocytes leading to the enhancement of TRAIL expression on NK cells. ZOL-induced NK cells exhibit the greater capacity to eliminate TRAIL-sensitive tumors (Sarhan et al., 2013). ZOL-treated DCs also stimulate NK cells to secrete greater quantities of IFN-γ, to express higher NKG2D and to exhibit potent anti-tumor cytotoxicity (Nussbaumer et al., 2011; Su et al., 2010).

In conclusion, the expression of activating receptors was decreased in women with BC which may imbalance the activating and inhibitory signaling, which impairs the NK cell activity and causes tumor development. The expression of activating receptors may be influenced by some BC parameters such as stages, ER and PR. ZOL can improve the expression of NK cell-related activating receptors. Therapeutic strategies need to be developed in attempts to increase the expression of the NK cell-related activating receptors and diminish inhibitory receptors.

Author Contribution Statement

Conceptualization and study design: AJ; Data Collection: LR, BKK, MN, SHS; Data analysis and interpretation of results: AJ, LR; Draft manuscript

preparation: AJ, LR, NA; Writing-review and editing: AJ; All authors reviewed the results and approved the final version of the manuscript.

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Ethical issue

This study has been approved by Ethical Committee of Kerman University of Medical Sciences with a registered ethical code of IR.KMU.REC.1394.413.

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Conflict of interest

The authors declare no conflict of interest.

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