EVALUATION OF THE ACTIVITY OF PERIPHERAL BLOOD NATURAL KILLER CELLS IN HEALTHY SUBJECTS AND BREAST CANCER PATIENTS

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Abstract

Objectives: To study Natural Killer (NK) cell activity (NK-IFNγ) and some indicators related to immunological characteristics of peripheral blood NK cells in healthy people and breast cancer patients. ***Methods:*** We evaluated NK cell activity through its secretory characteristics (NK-IFNγ) using ATGen's commercial NK VUE Test® (ATGen kit) on 35 healthy subjects (medical staff) and 132 cancer patients treated at Vietnam National Cancer Hospital from August 2020 to February 2023. At the same time, we collected peripheral blood samples to conduct immunological characterization of peripheral blood NK cells based on the flow cytometer system (number, expression of the activation and inhibitory receptors such as NKG2A, NKG2D), and some other biomarkers (CEA, CA 15.3). ***Results:*** The secretory activity of peripheral blood NK cells (NKA-IFNγ) in breast cancer patients (1,013.46 ± 1,115.87 pg/mL) was statistically significantly lower than that in the healthy group (2,571.38 ± 827.52 pg/mL) ($\overline{X}$ ± SD) ($p < 0.001$). The proportion of breast cancer patients with NKA-IFNγ activity of a very low level (≤ 200 pg/mL) was 40.9% and that of the healthy subjects was 0%. There was no relationship between NKA-IFNγ and peripheral blood NK cell count, and the expression levels of the activating receptor NKG2D and the inhibitory receptor NKG2A. Patients with CEA and

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CA 15.3 serum levels above the normal limits accounted for relatively low percentages (10.6% and 6.1%). ***Conclusion:*** Breast cancer patients with very low NK cell activity accounted for a significant proportion (40.9%). NKA-IFNγ activity could be used as a potential tool for monitoring the immune system health status of breast cancer patients before and after receiving treatment interventions.

Keywords: NKA-IFNγ activity; NKG2A; NKG2D; Breast cancer.

INTRODUCTION

Breast cancer is the most common cancer and the second leading cause of death among women worldwide [1]. There are many treatment approaches, such as surgery, chemotherapy, radiation therapy, targeted therapy, and immunotherapy that have significantly reduced mortality rates and recurrence rates of breast cancer. In terms of immunotherapies, checkpoint inhibitors (such as anti-PD-1/PD-L1, anti-CTL-4) and adoptive cell transfusion have been receiving considerable interest in the treatment of breast cancer.

The immune system in humans plays an important role in eliminating pathogens such as cancer-causing viruses or malignant cells. There are two important groups of immune cells responsible for removing malignant cells in the body: Cytotoxic lymphocytes (TCD8+) and Natural Killer cells (NK). The function of malignant-cell elimination by NK and TCD8+ cells may be suppressed during the interaction between the immune system and cancer cell emergence. From the hypothesis, understanding the altered cancer-resistance markers of immune cells in cancer patients and healthy people is an important issue and needs to be studied.

NK cells play an important role in the immune response against cancer by identifying and killing cancer cells with reduced or no MHC-class I expression [2, 3]. The decision to kill a cell in the body depends on the balance between the two groups of receptors in NK cells: The activating receptor group (such as NKG2D) and the inhibitory receptor group (NKG2A). Activated NK induces direct cytotoxicity through the release of perforin and granzyme, and also controls the immune response by secreting cytokines such as Interferon-gamma (IFNγ) and TNF (tumor necrosis factor).

In lung or prostate cancer patients, some studies have shown that peripheral NK cells demonstrated significantly reduced cytotoxic function and IFNγ secretion function phenotypes as compared to the healthy subjects [4, 5]. These studies used a new reliable method of analyzing NK cell activity via its IFNγ secretion (NK VUE kit) [4, 5]. From these, it is suggested that the assessment of the secretory activity of peripheral blood NK cells by the NK VUE kit (NKA-IFNγ) could be used as an aid in the diagnosis as well as in the treatment of cancer. However, not much information on the activity and immunophenotype of NK cells in healthy Vietnamese women and breast cancer patients has been published.

In this study, we investigated NKA-IFNγ activity and surface marker characteristics of peripheral blood NK cells and some other serum biomarkers in female Vietnamese healthy participants and breast cancer patients.

MATERIALS AND METHODS

1. Subjects

The study recruited 132 breast cancer patients who came for examination and treatment at the Vietnam National Cancer Hospital (Tan Trieu campus) and 35 healthy medical staff, from August 2020 to February 2023.

* *Inclusion criteria:* Female patients were diagnosed with breast cancer via histopathological examination for the first time; the histopathological types were classified by using an immunohistochemistry assay (IHC); The healthy female medical staff have received periodical health check-ups at the hospital, and have been classified into the health categories I and II; The patients and the healthy volunteers agreed to participate in the study.

* *Exclusion criteria:* Any patients who have been pregnant people, patients with medical diseases: liver failure, kidney failure, autoimmune diseases; Any patients who received or were receiving chemotherapy, radiation therapy, or immunosuppressive drugs; Of note, the healthy controls were not recruited with the intention to match age with the patient group, as some studies showed that there was no correlation between age and NKA-IFNγ [6].

2. Methods

* *Study design:* A cross-sectional descriptive study.

* *Histopathological diagnosis:*

Morphology of breast lesions was accurately diagnosed based on routine

methods being applied at the Center of Pathology and Molecular Biology, Vietnam National Cancer Hospital (Tan Trieu campus).

- Total blood count: 2mL of the human peripheral blood count percentage and the absolute number of lymphocytes from the peripheral blood samples were counted on the DxH 900 automatic hematology analyzer system. The tests were performed according to the routine techniques at the Department of Hematology - Microbiology, Vietnam National Cancer Hospital (Tan Trieu campus).

- Analysis of NK cells on a flow cytometer system: 2mL peripheral blood was stained with fluorescent antibodies detecting CD45, CD3, CD56, NKG2A, NKG2D with antibody dilution ratio and incubation conditions as recommended by the manufacturer (Biolegends). Then, the blood sample was incubated with RBC lysis buffer of Biolegends to break down red blood cells before being analyzed by the ACEA Novocyte flow cytometer at the Department of Immunology, Vietnam Military Medical University.

- Evaluation of natural killer (NK) cell activity in peripheral blood according to ATGEN's NKA VUE commercial kit: Peripheral blood NK cell activity (NKA-IFNγ) was determined by ELISA technique using ATGEN's NK VUE Test® commercial kit according to the method described by Lee S. et al. and was performed at the Department of Immunology, Vietnam Military Medical University [4].

Briefly, 1mL of the human peripheral blood was incubated in the NK Vue tube *in vitro* (containing NK cell-specific activating recombinant cytokines) for 24 hours; the concentration of Interferon (IFN)-gamma in the harvested supernatant after the end of incubation was quantified by ELISA technique (measurement unit: pg/mL). The level of NKA-IFNγ activity was considered normal when the NKA-IFNγ was above/equal to 500 pg/mL; low, when NKA-IFNγ was from 200 - 500 pg/mL; and, very low when NKA-IFNγ was below/equal to 200 pg/mL [4].

- Evaluation of some biochemical cancer biomarkers: Quantification of breast cancer markers, including CEA, CA15.3 from 1mL of peripheral blood.

On Cobas 6000 automatic immunoassay system by chemiluminescence immunoassay method was performed according to the routine techniques at the Department of Biochemistry - Immunology, Vietnam National Cancer Hospital (Tan Trieu campus).

** Data analysis:*

The collected data were analyzed on Microsoft Excell software. We used the Student's T-test to compare the data of the two groups, $p < 0.05$ was considered to be statistically significant. We use the correlation test on Excell software (r); interpret r between ± 0.5 and ± 1 as strong correlation, r between ± 0.3 and ± 0.49 as moderate correlation, r greater than 0 to ± 0.29 as weak or no correlation (r = 0).

RESULTS AND DISCUSSION

Regarding some NK cell immunophenotypic characteristics assessed by flow cytometry, we found that:

About % NKG2A: The population of NK cells expressing NKG2A (inhibitory receptor) accounted for about 30.56 ± 14.05 and 23.9 ± 11.67 (Mean ± SD) in the healthy group and the breast cancer group, respectively, although p-value was less than 0.05, the difference was relatively low (*Table 1*). The data were quite similar to the published data by Phan et al. (2016) in the Korean group [7].

About % NKG2D: The population of NK cells expressing NKG2D (activated receptor) accounted for about 94.2 ± 2.64 and 94.55 ± 1.64 ($\overline{X}$ ± SD) in the healthy group and breast cancer group, respectively (no difference, $p > 0.05$) (*Table 1*). These data were also quite similar to the published data of Phan et al. (2016) in the Korean group [7]. Although NKG2D was expressed on almost all peripheral blood NK cells, NK cells in the tumor group had a higher median fluorescence intensity (MFI) level of NKG2D (NKG2D MFI) than that in the healthy group ($p < 0.05$) (*Table 1*). It is necessary to re-examine and further clarify the molecular biological mechanism why NK cells in cancer patients have higher levels of NKG2D activating receptor expression than those in healthy groups.

Table 1. Some immunological characteristics of NK cells and the levels of other cancer markers in breast cancer patients and healthy donors.

Criteria	Breast cancer group (n = 132) ($\overline{X}$ ± SD)	Healthy group (n = 35) ($\overline{X}$ ± SD)	p*
Age	53.67 ± 11.55	38.03 ± 9.18	< 0.001
# Lympho (million/mL)	2.31 ± 0.76	2.34 ± 0.68	> 0.05
% TCD3	61.57 ± 9.91	69.33 ± 7.11	< 0.001
# TCD3 (million/mL)	1.39 ± 0.48	1.63 ± 0.55	< 0.05
% NK	16.09 ± 9.11	15.36 ± 6.91	> 0.05
# NK (million/mL)	0.4 ± 0.33	0.34 ± 0.14	> 0.05
% NKG2A	23.9 ± 11.67	30.56 ± 14.05	< 0.05
% NKG2D	94.55 ± 1.64	94.2 ± 2.64	> 0.05
NKG2D MFI	5321.00 ± 948.07	4946.74 ± 785.70	< 0.05
NKA-IFNγ (pg/mL)	1013.46 ± 1115.87	2571.38 ± 827.52	< 0.001
CEA (ng/mL)	3.08 ± 3.97	1.42 ± 0.82	< 0.001
CA 15.3 (U/mL)	16.24 ± 10.30	14.11 ± 5.31	> 0.05
Stage, n (%)			
0, I	33 (25.0)		
II	74 (56.07)		
III, IV	25 (18.93)		

*#: Quantity; %: Percentage; MFI: Median fluorescence intensity; *: T-test method.*

Regarding peripheral blood NK cell secretion activity (NKA-IFNγ): Studying a group of healthy women with a sample size (n = 35), the average age was 38 (range 25 - 57), we found that the NKA-IFNγ level was 2571.38 ± 827.52 pg/mL ($\overline{X}$ ± SD). Of these, 34/35 (97.14%) had NKA-IFNγ level of greater than 500 pg/mL (the manufacturer's normal threshold value) (*Table 1*).

According to the manufacturer's announcement, NKA-IFNγ level of between 200 - 500 pg/mL is considered to be low as a warning value, and NKA-IFNγ below/equals 200 pg/mL is very low [4]. In this study, we determined a threshold of 200 pg/mL as the cut-off threshold dividing subjects with normal and very low NKA-IFNγ activity, similar to some previous authors [8].

Studying cancer subjects (n = 132), we found that the NKA-IFNγ level was 1013.46 ± 1115.87 pg/mL (Mean ± SD), statistically significantly lower than that of the healthy group ($p < 0.001$) (*Table 1*). Up to 54/132 cancer cases, accounting for 40.9 % of cancer subjects, had NKA-IFNγ activity ≤ 200 pg/mL (very low NKA-IFNγ), while we did not detect any cases in healthy subjects that had NKA-IFNγ ≤ 200 pg/mL; the difference was statistically significant ($p < 0.001$) (*Table 2*).

Table 2. Characteristics of NK cell activity (NKA-IFNγ) in the breast cancer patients and the healthy group.

NK cell activity (NKA-IFNγ)	**Breast cancer group (n = 132)**		**Healthy group (n = 35)**		**Total (n = 167)**	**p***
	n	**%**	**n**	**%**	**n (%)**	
NKA-IFNγ ≤ 200 pg/mL (very low NKA-IFNγ)	54	40.9	0	0	54 (32.3)	< 0.001
NKA-IFNγ > 200 pg/mL	78	59.1	35	100	113 (67.7)	
Total	132	100	35	100	167 (100)	

Interestingly, most of the cancer subjects (about 80%) were only at a relatively early stage of the disease (0, I, II), but more than 40% of the group had very low NKA-IFNγ**.** This partly suggested that the altered peripheral blood NK cell activity may be present in the early stages of breast cancer (*Tables 1, 2*).

On the other hand, when comparing the value of NKA-IFNγ with some other biomarkers, such as CEA and CA 15.3, we found that:

CEA usually fluctuates below 5 ng/mL in healthy subjects; We observed that about 14/132 cancer patients, accounting for 10.61 %, had CEA greater than 5 ng/mL, and 3.8% of the cancer patients had CEA > 7.5 ng/mL [9]. (The level of 50% higher than the upper limit of the normal CEA reference range) (*Table 3*).

Meanwhile, as discussed above, 40.9% of the tumor group had very-low NKA-IFNγ levels (≤ 200 pg/mL). This suggests a better sensitivity of NKA-IFNγ than that of CEA and CA 15.3 (*Table 3*).

Table 3. Characteristics of NK cell activity (NKA-IFNγ), CEA, CA 15.3 in the breast cancer patients and the healthy subjects.

Criteria	Breast cancer group (n = 132)		Healthy group (n = 35)	
	n	%	n	%
NKA-IFNγ ≤ 200 pg/mL	54	40.9	0	0
CEA > 5 ng/mL	14	10.61	0	0
CEA > 7.5 ng/mL	5	3.78	0	0
CA 15.3 > 30 U/mL	8	6.06	0	0
CA 15.3 > 40 U/mL	5	3.78	0	0

In this study, we also evaluated the correlation between the result of peripheral blood NK cell activity assay and other immunological & biochemical indicators.

We found that peripheral blood NK activity measured by the NK VUE kit was independent of the number of peripheral blood NK cells (r = 0.063) (*Table 4*). This again shows when using the NK VUE kit, the levels of IFN-gamma secretion by Promoca-induced peripheral blood NK cells were independent of input NK-cell count, similar to that described by Lee et al. (2014) [4]. At the same time, NKA-IFNγ was also not associated with activating or inhibitory receptor expression levels of peripheral blood NK cells (*Table 4*).

Table 4. Evaluation of the correlation between NKA-IFNγ and some other immunological and biochemical indicators.

Criteria	NKA-IFNγ	Classification of correlation levels
Age	-0.349	moderate
# NK	0.063	weak
% NKG2A	0.204	weak
% NKG2D	0.061	weak
NKG2D MFI	0.064	weak
CEA (ng/mL)	-0.142	weak
CA 15.3 (U/ml)	-0.028	weak

#: quantity; %: percentage

Through the study of immunophenotypic characteristics and secreting activity of NK cells in healthy subjects and breast cancer patients, we found that NKA-IFNγ activity has been changed relatively clearly in the breast cancer group compared with that in the healthy group with approximately 40.9% of breast cancer subjects having very low NKA-IFNγ (≤ 200 pg/mL) (*Table 1, 2*). The very-low NK cell activity may affect the immunological surveillance of cancerous-cell emergence and growth. Therefore, this study suggests the need to focus more on the immunotherapies to harness NK cell function or activity in this group of breast cancer patients with a very-low NKA-IFNγ activity and at the same time, to evaluate the

clinical improvement of the disease. This test could be a potential tool to monitor the clinical response to the treatment in breast cancer patients as some centers around the world are approaching [10]. Our study has certain limitations on the relatively low sample size and the difference in the mean age between the two groups of healthy and breast cancer subjects.

CONCLUSION

The secretory activity of peripheral blood NK cells (NKA-IFNγ) in the breast cancer group was statistically significantly lower than that in the healthy group ($p < 0.001$).

The rate of NKA-IFNγ activity was very low (≤ 200 pg/mL) in the breast cancer patients accounting for 40.9%, while no NKA-IFNγ cases were detected in the healthy group.

There was no relationship between NKA-IFNγ activity and peripheral blood NK cell count or the expression levels of the activating receptor NKG2D and the inhibitory receptor NKG2A.

NKA-IFNγ activity could be a potential aid in monitoring the immune status of breast cancer patients before and after treatment interventions.

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