

# Investigation of NK Cell Population in Peripheral Blood and Tumor Lesions of Patients with Breast Cancer

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## ABSTRACT

**Background:** Alteration in peripheral blood lymphocytes (PBLs) is usually investigated to provide an evidence of the host immune responses to tumor antigens. The tumor infiltrating NK cells interact most closely with the tumor cells and more accurately reflect tumor host interactions. **Objective:** To analyze peripheral blood and tumor associated Natural Killer (NK) cells in patients with breast cancer by immunophenotyping. **Methods:** Twenty women suffering from breast cancer were examined; 12 of them were confirmed histologically to be invasive ductal carcinoma. PBL and tissue samples from patients and matched control group were processed for analysis by flow cytometry. **Results:** Results of PBL analysis indicated a significant ( $P < 0.05$ ) increase in both the total number and activated NK cells in invasive ductal carcinoma patients compared to normal controls. No significant differences were noticed in the percent of NK cells and their activation marker expression in intra tumor lesion of the invasive ductal carcinoma and other tumors compared to benign lesions, however a decrease in the total NK number and activated NK cells was observed with progression of the tumor. **Conclusion:** Data of this investigation conclude that the total and activated NK cell number increase in peripheral blood of patients with breast cancer. The relationship between peripheral blood and intratumor NK cells needs more clarification, however, a decrease in intratumor NK cell number and their activation status occurs with tumor progression.

**Keywords:** Tumor Immunity, Breast Cancer, Tumor Associated Natural Killer Cells

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## INTRODUCTION

Invasive ductal carcinoma is the most common type of breast cancer in Iran. The immune system is capable of responding to cancer as evidenced by systemic, regional, and intratumoral leukocyte activation. For individual patients there is no predictable relationship between leukocyte composition, or function, and the prognosis of the disease (1).

Infiltrated leukocytes to neoplastic tissues are a target for therapeutic intervention (2). These cells are endowed with tumor destructive features, exerted directly on neoplastic cells or via soluble mediators which affect components of the tumor tissue (3). Although T cells are the most tumor infiltrating immune cells (4), the activity status of different cytotoxic leukocytes (mononuclear, phagocytic and NK cells) encompasses tumor cells resistant to chemotherapeutic agents (2).

Impaired immune responses occur frequently in cancer patients, but the mechanism of the induced immune defects remains poorly understood (5). In clinical practice, reports have been made on immunosuppression even after surgical excision of primary tumor or at relapse. However, the relationship between undefined or overt metastasis and the host immune system has been sufficiently examined over a prolonged period (6).

It has been noted that the tumor host relationship and cell mediated immunity in the PBL does not often correlate with prognosis (7).

## MATERIALS AND METHODS

### Sampling

Twenty patients who underwent surgical procedure in Institute of Cancer of Imam-Khomeini Hospital, Tehran were studied. The age of patients ranged from 25-65 (mean 45.4) years. The surgical procedure undergone was radial mastectomy. Twelve of the patients were diagnosed histologically to be invasive ductal carcinoma (IDC) (60%), two of the patients were invasive lobular carcinoma (ILC) (10%), one patient was intra ductal carcinoma (IDCC), one patient was atypic medullary carcinoma (AMC) and one patient was fibrocystic. Three patients had benign tumors that were considered as a separate group. From each patient one centimeter tumor tissue (biopsies) was obtained. PBL of 10 matched control women (group1) and 8 cancer patients (4 IDC as group 2 and 4 other types of tumor as group3) were collected in EDTA-coated tubes prior to any chemoradiotherapy. The mononuclear cell fraction of blood samples were isolated by ficoll and processed for immunostaining (see below). The patients and control groups participated in this study after informed consent.

### Preparation of Tumor Cell Suspension

Biopsies of human solid tumor were cut into small pieces with forceps and scalpel. The pieces were rinsed twice with phosphate buffer saline, then 5ml of cocktail enzymes (0.05 mg/ml collagenase and 0.002 mg/ml DNase) were added and the mixture was placed in 37°C incubator for 2-4 hrs. The suspensions were passed through 150-micron stainless steel mesh. Cell were washed twice and labeled with monoclonal antibodies.

### **Immunophenotyping**

The following fluorescent monoclonal antibodies (mAb) (DAKO, Denmark) were used to identify leukocytes and NK cells in both PBL and tumor cell suspensions: anti-CD45<sup>+</sup> for detection of leukocytes; anti-CD45<sup>+</sup>/CD56<sup>+</sup> for detection of NK cells and anti- CD56<sup>+</sup>/CD25<sup>+</sup> for detection of activated NK cells.

We established the reference immunophenotypic pattern using standard procedures. In this study 100µl of each blood/tissue samples was immunostained with 10µl mAbs directly conjugated with Fluorescein Isothiocyanate (FITC) or R-Phycoerythrin (RPE) in Q-Prep apparatus, then, three immunopreps were added automatically, 0.7 ml immunoprep A (Formic Acid 1.2ml/L), 0.32ml immunoprep B (Sodium Carbonate 6.0g/L, Sodium Chloride 14.5 g/L, Sodium Sulfate 31.3 g/L) and 0.14 ml immunoprep C (Paraformaldehyde 10.0 g/L, Phosphate Buffer). Each sample was then kept at 2-8 °C in the dark before analyzing in 24 hrs (8).

### **Flowcytometric Analysis**

Cell samples were measured on a coulter flowcytometer with serial filter configuration. The analysis was focused on the myeloid areas of the forward and side scatters. Double stained cells were analyzed using coulter software.

### **Statistics**

In this study one-way analysis of variance (ANOVA) and Kruskal-Wallis nonparametric test were employed using SPSS software.

## **RESULTS**

### **PBL Natural Killer Cells in Patients and Controls**

In order to estimate the percent of leukocyte (CD45<sup>+</sup>cells) and NK cells (CD45<sup>+</sup>CD56<sup>+</sup> cells) in the PBL of patients suffering from breast cancer, the protocol shown in table 1 was employed. Results of PBL indicated a significant (P<0.05) increase in the total NK cell percent in invasive ductal carcinoma compared to normal group. Whereas a significant (P<0.05) decrease was observed in other tumors (other than invasive ductal carcinoma) compared to normal group (Table1).

In the case of activated NK cells (CD56<sup>+</sup> CD25<sup>+</sup> cells ) results indicated that the mean percent of CD56<sup>+</sup> CD25<sup>+</sup> cells in the PBL of both invasive ductal carcinoma and other breast tumors were significantly (P<0.05) increased compared to the normal group (Table1).

### **Percentage of Tumor Associated NK Cells**

The results of intratumor NK cells are summarized in table2. No significant differences were noticed in the percent of NK cells in intratumor lesion of the invasive ductal carcinoma and other tumors compared to benign ones.

In order to detect the activated tumor associated NK cells, anti-CD25 mAb was used. Results indicated no significant difference between patients with malignant and those with benign tumors.

**Table 1. The percentage of the total and activated NK cells in the peripheral blood of the breast cancer patients and normal group<sup>a</sup>**

Subject	No. of cases	% CD45 <sup>+</sup>	% CD45 <sup>+</sup> CD56 <sup>+</sup>	% CD45 <sup>+</sup> CD56 <sup>+</sup> /CD45 <sup>+</sup>	% CD56 <sup>+</sup> CD25 <sup>+</sup>	% CD56 <sup>+</sup> CD25 <sup>+</sup> /CD56 <sup>+</sup>
Group 1 Normal	10	85.4	2.4	2.8	0.1	4.1
Group 2 IDC Patient	4	79.4	2.97 <sup>b</sup>	3.7 <sup>b</sup>	1.55 <sup>b</sup>	52.1
Group 3 ILC+AMC+IDCC Patients	4	89.8	1.67 <sup>b</sup>	1.8 <sup>b</sup>	0.3 <sup>b</sup>	17.9 <sup>b</sup>

<sup>a</sup> analysis was done in Lymphocyte gate

<sup>b</sup> indicate a significant difference in comparison with normal group1 (P<0.05)

IDC: invasive ductal carcinoma, ILC: invasive lobular carcinoma

AMC: atypic medullary carcinoma, IDCC: intra ductal carcinoma

**Table 2. Immunostatus of tumor associated NK cells in breast cancer patients<sup>a</sup>**

subjects	No. of cases	% CD45 <sup>+</sup>	% CD45 <sup>+</sup> CD56 <sup>+</sup>	% CD45 <sup>+</sup> CD56 <sup>+</sup> /CD45 <sup>+</sup>	% CD56 <sup>+</sup> CD25 <sup>+</sup>	% CD56 <sup>+</sup> CD25 <sup>+</sup> /CD56 <sup>+</sup>
Group 1 IDC Patient	12	14.7	2.18	14.8	1.35	62
Group 2 ILC+AMC+IDCC Patients	5	16	2.4	15	1.54	64
Group 3 Benign breast tumor	3	17.5	2.3	13.1	1.5	65.2

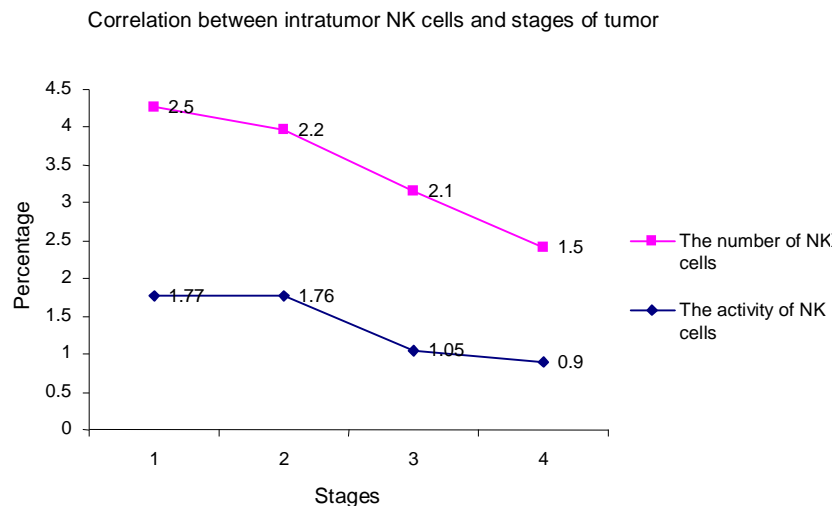
<sup>a</sup> the analysis was done as ungated and results were not significant

IDC: invasive ductal carcinoma, ILC: invasive lobular carcinoma

AMC: atypic medullary carcinoma, IDCC: intra ductal carcinoma

**Correlation between Intratumor NK Cells and Clinical Tumor Staging**

In order to assess the correlation between the intratumor total and activated NK cells with different stages of tumor, patients with stages 1, 2, 3 and 4 were evaluated. Results in figure 1 indicate a significant (P<0.05) decrease in both total number and the activated NK cells at the tumor site in stages 3 and 4 compared to stage 1.



**Figure1.** Correlation between intratumor NK cells and stages of tumor

## DISCUSSION

The great majority of tumors infiltrating lymphocytes (TILs) are CD3<sup>+</sup> T cells. NK cells and B lymphocytes are usually very few in neoplastic tissues (4, 9). At least in some immunogenic tumors, TILs contain a higher proportion of tumor specific effector lymphocytes in comparison to peripheral blood lymphocytes (PBLs). Tumor antigen recognition by TILs is manifested by tumor killing or cytokine production (10, 11).

Interleukin-12 (IL-12) has potent antitumor activities via NK and cytotoxic T cells. However, the underlying molecular mechanisms are poorly understood. Shi X and coworkers reported the genome-wide analysis of gene expression in a primary murine mammary carcinoma model that resembles human breast cancer, following the therapeutic application of recombinant IL-12, which restricted tumor growth and metastasis (19). IL-12 was able to curtail neovascularization in the tumor as well as enhance the number of tumor-infiltrating lymphocytes (20).

In this investigation we found that the peripheral blood NK cell number of patients with IDC and activated NK cells from patients with IDC or other tumor types were significantly increased compared to the normal group (Table1). In concordance with this study Murta et al. reported that patients with advanced breast cancer showed increases in B and NK cells in PBLs (20).

Despite of these differences in peripheral blood, no differences were observed in total intratumor NK and activated NK cells when patients with malignant tumors were compared with those with benign ones. Some investigators have believed that PBL natural killer cells do not reflect the tumor host relationship and cell mediated immunity in the PBL does not often correlate with prognosis (7, 22). Functional analysis of TILs and PBLs have been also indicated that cellular immunity is down regulated at the site of tumor whereas cytotoxic immune response as a part of cellular immunity predominates in peripheral blood. (21).

We found a significant negative correlation between the both the total number and activated NK cells and the stage of tumor (Fig.1). This finding is in agreement with previous reports on murine models which reported that NK cells infiltrate the lesions of tumor at a very early stage (13). Other studies have also indicated a low percent of NK cells in the intratumor infiltration (13, 14) and NK cells presence isolated from the infiltrate (4). Kuroda et al. found that the medulary carcinoma of the breast contained very few NK cells (15). In contrary to our findings, however, Vgenopoulou et al. showed that high-grade carcinomas exhibited higher numbers of endotumoral NK cells and B-cell aggregates (16).

Recently, we reported that TAMs and PBL mononuclear cells were not different in breast cancer patients and the control group (23), but here our results indicated that the total number and the activation marker expression of PBL Natural Killer cells significantly increased, at least, in IDC type of breast cancer while the total number and activation marker of these cells decreased with tumor progression. Additional studies concerning the tumor infiltrating T, B and NK cells are required to clarify further relationship between these cells in the breast cancer and also their cytolytic function against tumor cells.

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