RESEARCH ARTICLE

Is the Tumor Infiltrating Natural Killer Cell (NK-TILs) Count in Infiltrating Ductal Carcinoma of Breast Prognostically Significant?

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Abstract

Purpose: The aim of this study was to investigate the prognostic significance of the CD56+NK-TIL count in infiltrating ductal carcinoma (IDC) of breast. Material and Methods: Immunohistochemistry (IHC) was performed using antibodies specific for CD56 on formalin-fixed and paraffin-embedded tissue sections of 175 infiltrating ductal carcinomas (IDC) of breast. Distribution of intratumoral and stromal CD56+NK-TILs was assessed semi-quantitatively. Results: A low intratumoral CD56+count showed significant and inverse associations with tumor grade, stage, and lymph node status, whereas it had significant and direct association with response to treatment indicating good prognosis. These patients had better survival (χ^2=4.80, p<0.05) and 0.52 fold lower death rate (HR=0.52, 95% CI=0.28-0.93) as compared to patients with high CD56+ intratumoral count. The association of survival was insignificant with low CD56 stromal count as compared to high CD56 stromal count (χ^2=1.60, p>0.05). Conclusion: To conclude, although NK-TIL count appeared as a significant predictor of prognosis, it alone may not be sufficient for predicting the outcome considering the fact that there exists a crosstalk between NK-TILs and the other immune infiltrating TILs.

Keywords: CD-56 - NK TILs - infiltrating ductal carcinoma (IDC) breast

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Introduction

Breast cancer is the most common cause of cancer death in women worldwide (Key et al., 2001), and represents the leading or second most leading cancer in females in India (Ferlay et al., 2010; D'Souza et al., 2013a; 2013b). Currently, two major challenges of breast cancer research are; to understand the interrelation between breast cancer and anti-tumor immunity, and to identify candidates whose targeting would contribute to enhance anti-tumor efficiency (Mamessier et al., 2012).

The cancer tissue is invaded by a mixed population of immune cells, including T-cells, B-cells, natural killer (NK) cells and macrophages. For a long time NK cells were considered merely as relatively primitive killers, but now they are seen not only as bonafide actors in innate immunity but are also important cells that shape and influence the adaptive immune responses (Poli et al., 2009). NK cells are effector lymphocytes of innate immune system that act by limiting the growth and dissemination of the tumor and are known to have an immunoregulatory role.

An established marker for NK cells, CD56, is an integral membrane protein and an isoform of neural adhesion molecule (NCAM). It is expressed by a number of normal cells, including a variety of neuroectodermal derivatives and natural killer cells. It is abundantly present in the growing as well as in the adult brain. In the diagnostic armamentarium, CD56 is a specific histological immune marker for malignant nervous tumors like medulloblastoma and astrocytoma, malignant NK/T-cell lymphomas (NK/T-NHLs), and neuroendocrine carcinoma. Its over expression in malignant cells is linked with an aggressive tumor type, insufficient therapeutic response, and a reduced survival time in different malignancies including lymphoblastic and myeloid leukemias (ALLs/AML), malignant melanoma (Johnson, 1999; Abbott et al., 2004), and different cancers (Pujol et al., 1993; Zoltowska et al., 2001; Daniel et al., 2003 Choi et al., 2004; Cho et al., 2006; Evans et al., 2006;). It has been reported in different types of epithelial malignancies and sarcomas like Ewings /PNET (McKenzie et al., 1981; Lipinski et al., 1987; Gardner et al., 1998; Farinola et al., 2003).

Well characterized in blood, NK cells are also present in various tissues and different sites of NK development

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and maturation have been discovered. Correlation between NK cell infiltrates and prognosis has been reported in various tumors. However, tumor-infiltrating NK cells are yet poorly characterized (Fregni et al., 2012). Mamessier et al. (2011) have shown that breast tumor progression involves NK cell dysfunction and that breast tumors mold their environment to evade NK cell anti-tumor immunity.

The aim of the present study was to evaluate CD56+ tumor infiltrating natural killer cell count, their localization, distribution and density in breast cancer patients and their influence on outcome and survival. The findings were correlated with tumor size, grade, lymph node status, Estrogen receptor (ER), Progesterone receptor (PR), Human epidermal growth factor receptor (Her-2/neu) status and response to treatment.

Material and Methods

The study included a total of 175 histologically proven cases of infiltrating ductal carcinomas (IDC) of breast, received from the Department of General Surgery, King George's Medical University, Lucknow, UP, India, after informed written consent and institutional ethical clearance. Detailed histopathological examination was done in the Department of Pathology, King George's Medical University UP, Lucknow. Demographic details, clinical history, complete general/local examination and epidemiological risk factors including family history, clinical staging as per International Union Against Cancer (UICC) and American Joint Committee on Cancer (AJCC) criteria (Wittekind et al., 2001; Greene et al., 2002), (Stages T1-T4), tumor grading (I-IV) as per Nottingham Grading System (Tavassoli et al., 2003), lymph node status, ER, PR, and HER-2/neu were recorded. Tumor histological grades and clinical stages were grouped together as low histological grade (I & II), high histological grade (III & IV), early clinical stages (T1 & T2) and advanced clinical stage (T3 & T4) for ease of calculation in relatively low number of cases. All patients had a pre-operative tissue diagnosis of IDC either by fine needle aspiration cytology and/or core biopsy of the breast. All patients underwent surgery with axillary lymph node dissection, and none of these patients had received pre-operative antitumor therapy. No patient had evidence of active infection or inflammatory disease.

Immunohistochemistry was performed using antibody against CD56 (R&D System) and Novolink Min Polymer Detection system (Novacastra, Leica Biosystem Newcastle Ltd, UK). Formalin-fixed, paraffin-embedded tissues sections (3-4μm thick) were taken on 3-aminopropyltriethoxysilane (APTES) coated glass slides. Sections were deparaffinized in xylene followed by hydration in graded ethanol. Antigen retrieval was performed by heating sections at 100˚C for 20min in 0.01M citrate buffer (pH 6.0) using an EZ antigen retriever system (Biogenex, USA). Endogenous peroxidase was blocked by incubating sections with 0.3% hydrogen peroxide for 5 min and the nonspecific binding sites were blocked with a protein block for 5 min. Sections were covered with primary antibody and the slides were incubated in moist chamber overnight at 4˚C. Slides were then washed with Tris Buffer Saline (TBS, pH 7.4), followed by a 30 minutes incubation with post primary block at room temperature. Sections were washed twice in TBS followed by incubation with Novolink polymer for 30 min at room temperature. After three washes in TBS sections were treated with DAB chromogen (3, 3' diaminobenzidine tetrahydrochloride) for 5-10min in the dark. Sections were counterstained with haematoxylin, dehydrated with ethanol and xylene, and mounted permanently with DPX. Negative control slides omitting the primary antibody were included in all batches. Section from tonsillar tissue served as positive control for CD56.

Sections of each immunostained tumor were viewed at low magnification, and the highest density of positive cells in five best areas of intratumoral and stromal areas were selected. These areas were then assessed at higher magnification (×20 objectives) with a grid overlaid over

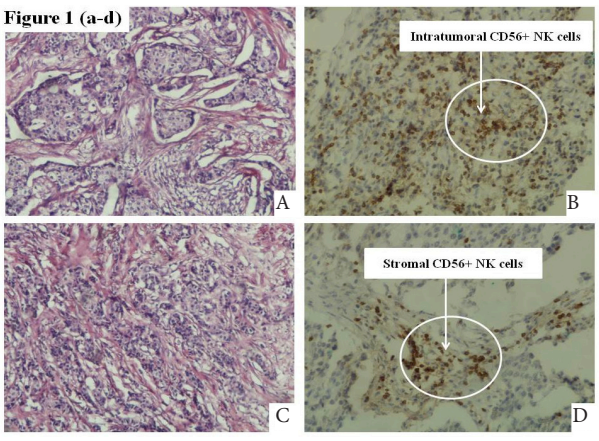

Figure 1. A) Histological Section from Infiltrating Ductal Carcinoma (IDC) Breast Showing Intratumoral TILs (Hematoxylin & Eosin x20); B) Histological Section from IDC Showing Intratumoral CD56+NK cells (Immunostain x20); C) Histological Section from IDC Showing Both Intratumoral and Stromal TILs (Hematoxylin & Eosin x20); D) Histological Section from IDC Showing Stromal CD56+NK Cells (Immunostain x20)

Table 1. The Clinicopathological Characteristics of Breast Cancer Patients (N=175)

Variables		No	(%)
Age (yrs)*		49.13±12.21 (25-86)	
Menstrual status**	Premenopausal	68	(38.9%)
	Postmenopausal	105	(60.0%)
Family history	No	165	(94.3%)
	Yes	10	(5.7%)
Grade	I-II	82	(46.9%)
	III-IV	93	(53.1%)
Stage	T1-T2	84	(48.0%)
	T3-T4	91	(52.0%)
Node	Negative	90	(51.4%)
	Positive	85	(48.6%)
Follow up	Well	105	(60.0%)
	Not well	45	(25.7%)
	Lost to follow up	25	(14.3%)

*Mean±SD (range), **2 patients were excluded because they had undergone hysterectomy

the section, with grid area of 0.56 mm^2. The number of positive intratumoral and stromal lymphocytes was quantified within the area of the grid by method described by Denkert et al. (2010) with slight modification. Intratumoral localization was defined as lymphocytes within tumor cell nests or in direct contact with tumor cells. Similarly the cells were counted in stromal areas.

Scoring of immune stained positive NK-TILs was done independently by two pathologists. CD56+NK-TILs were counted in five randomly selected areas at 20X magnification and the counts were averaged. NK-TIL count was recorded as:+(1-25 cells),++(26-50 cells), +++(≥51 cells) in the tumor and the stroma separately. Positive NK-TIL upto 25 cells were considered as low NK-TIL count and more than 25 cells (i.e.++,+++) were considered as high NK-TIL count. The histomorphology and immunostaining patterns of intratumoral and stromal CD 56+NK-TIL are shown in figure 1(a-d).

Details of clinical progress and survival of patients were obtained from the hospital records. The follow up period was 4 years. The survival status of only 150 (85.7%) patients was available. Of these 105 (70%) patients were relapse free survivors, 40 had tumor recurrence (26.67%) and 5 (3.33%) died. The overall survival (OS) time was calculated for each patient to the nearest month, taken from the time of diagnosis to the time of death or last recorded follow-up.

In statistical analysis continuous data were summarized as Mean±SD while discrete (categorical) data was expressed in percentage. The continuous variables were compared by Student's t test and the discrete variables by Fisher's exact test. Prognostic significance of each independent variable was evaluated by logistic regression analysis. Each model was adjusted with age, menstrual status and family history. Kaplan-Meier method was used to calculate overall survival proportion and the difference of survival between the two groups was performed by log-rank test. A two sided (α=2) $p<0.05$ was considered statistically significant. Analyses were performed using GraphPad Prism (version 5.0) and MINITAB (version 13.0) softwares.

Table 2. Association of Intratumoral and Stromal CD56 Count with Patients Demographic and Clinicopathological Characteristics

Variables	Intratumoral CD56 count			Stromal CD56 count		
	Low (n=90)	High (n=85)	p value	Low (n=104)	High (n=71)	p value
Age (yrs):						
25-35 yrs	14	12	0.361	19	7	0.242
36-50 yrs	41	31		39	33	
>50 yrs	35	42		46	31	
Menstrual status:						
Premenopausal	38	30	0.414	44	24	0.265
Postmenopausal	52	53		59	46	
Family history:						
No	86	79	0.457	98	67	0.97
Yes	4	6		6	4	
Grade*:						
I-II	56	26	0.001	54	28	0.104
III-IV	34	59		50	43	
Stage**:						
1-T2	52	32	0.008	55	29	0.118
T3-T4	38	53		49	42	
Node:						
Negative	56	34	0.003	57	33	0.279
Positive	34	51		47	38	
Combination of ER, PR and HER-2/neu*** (0=Negative,1=Positive):						
001	10	11	0.74	11	10	0.108
011	0	1		1	0	
101	8	4		8	4	
111	14	16		17	13	
000	13	14		10	17	
010	7	3		8	2	
100	2	3		2	3	
110	36	33		47	22	
Treatment response:						
Well	62	43	0.004	63	42	0.208
Unwell	15	30		22	23	
Lost to follow up	13	12	-	19	6	-

*Grades were grouped together as low histological grade (I & II), high histological grade (III & IV); ** Stages were grouped together as early clinical stages (T1 & T2) and advance clinical stage (T3 & T4); *** Estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER-2/neu)

Results

The clinicopathological characteristics of 175 breast cancer patients are summarized in Table 1. Age of these patients ranged from 25-86 years with an average of 49.13±12.21 years. Menstrual history was available only for 173 patients as 2 women had undergone hysterectomy. Majority (60.0%) of the subjects were postmenopausal. All patients enrolled in the study had infiltrating ductal carcinomas with 53.1% showing a higher grade (III, IV), 52.0% with higher stage (T3, T4) and 51.4% with negative

Table 3. The 4 Year Overall Survival According to Intratumoral and Stromal CD56 Counts

Comparisons of survival curve		Intratumoral CD56 count	Stromal CD56 count
Log rank test:	χ^2	4.8	1.6
	DF	1	1
	p value	0.029	0.206
Median survival (months)			
	Counts-Low	43	43
	Counts-High	31	31
	Ratio	1.39	1.39
	95% CI	0.85 to 1.93	0.83to1.94
Hazard ratio	Ratio	0.52	0.69
	95% CI	0.28 to 0.93	0.37to1.24

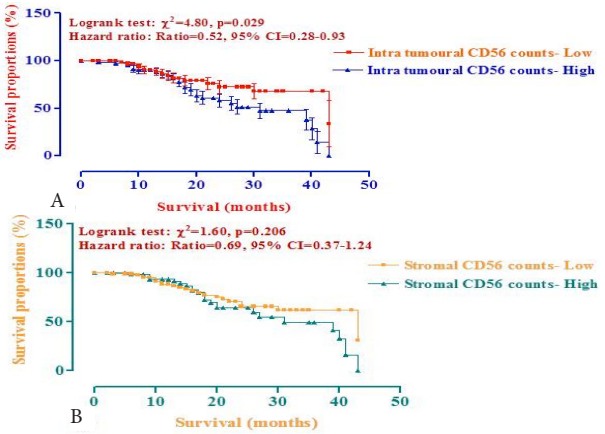

Figure 2. Overall Treatment Response Survival According to CD56 Intratumoral A) and Stromal B) Counts

lymph node status.

Table 2 shows low and high intratumoral and stromal CD56+ NK-TIL counts in relation to clinicopathological characteristics. The low intratumoral CD56+counts showed significant and inverse association with tumor grade, stage, and lymph node status ($p<0.05$), whereas it had significant association with response to treatment indicating good prognosis ($p<0.05$). Age, menstrual status, family history of breast cancer and combination of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER-2/neu) were not significantly associated with either intratumoral or stromal CD56+counts.

The response to treatment and survival analysis using log rank test for a follow up period of 4 year; well (disease free survival) versus unwell (recurrence+death) is shown in Table 3, figure 2. The patients with low CD56 intratumoral count had better survival than high CD56 intratumoral count ($\chi^2=4.80$, $p<0.05$), with 0.52 fold lower death rate as compared to patients with high CD56 intratumoral count (HR=0.52, 95% CI=0.28-0.93).

The association of survival was insignificant with low CD56 stromal count as compared to high CD56 stromal count ($\chi^2=1.60$, $p>0.05$). The patients with low CD56 stromal count, had 0.69 fold lower death rate as compared to patients with high CD56 stromal counts (HR=0.69, 95% CI=0.37-1.24).

Discussion

A Natural killer (NK) cells play an important role in the innate immune response against cancer, particularly in the elimination of tumor metastases and small tumors. Compared with blood NK cells, only a limited amount of information is available on tissue infiltrating NK-TILs in breast cancer (Jochems and Schlom, 2011), which usually present more severe phenotypic and functional alterations compared with circulating or non-cancerous tissue-infiltrating NK counterparts (Fregni et al., 2012). Although the number of NK cells coming into contact with neoplastic cells constitute a small percentage of the infiltrating lymphocytes, they can play an important role in the prognosis of tumors (Stojanovic and Cerwenka, 2011).

Our study showed significant and inverse association of low intratumoral CD56+NK-TIL count with tumor grade, stage, and lymph node status, whereas it had significant association with response to treatment indicating good prognosis. This observation appears interesting, because inspite of an inverse correlation between NK-TILs and tumor stage and grade, the prognosis was good and was statistically significant. The possible explanation for this finding could be an interplay or interaction of NK-TILs with some other factors, for example like cytokines associated with other immune cells (Kim et al., 2007). We have also observed increased cytokine levels (IFNγ, IL-4 and TNFα) in patients with IDC (unpublished observation). The clinical relevance of these observations needs to be studied further.

NK cells are usually not found in large numbers in advanced human neoplasms, indicating that they do not normally home efficiently to malignant tissues (Levy et al., 2011). NK cell infiltration has been found to be associated with variable clinical outcome and survival in different tumors (Levy et al., 2011). In majority of the studies, high numbers of immune cells, such as natural killer (NK) cells or CD8+T cells, infiltrating the tumors have been shown to correlate with an improved prognosis for cancer patients (Coca et al., 1997; Deschoolmeester et al., 2010). Elevated CD56+NK cell count has been reported to be associated with a lower risk of progression in prostate cancer (Gannon et al., 2009), and with poor patient outcome in renal cell carcinoma (Daniel et al., 2003). In the present study, analysis of response to treatment and survival, using log rank test after a 4 year follow up period, showed that the patients with low CD56 intratumoral count had better survival than high CD56 intratumoral count ($\chi2=4.80$, $p<0.05$), with 0.52 fold lower death rate as compared to patients with high CD56 intratumoral count (HR=0.52, 95% CI=0.28-0.93). The unexpected association between low CD56 intratumoral and better patient survival observed in our study could be attributed to the nature of tumor infiltrating NK cells,possibly their subset distribution, phenotype, potential interaction with other immune cells and the overall influence of the tumor microenvironment on NK cell function (Kim et al., 2007). Mamessier et al. (2011) observed that the NK cells infiltrating the breast tumors differed with respect to their CD56Bright and CD56Dim phenotype as compared with healthy mammary tissue.

Changes in breast tumor stroma may have a crucial role in disease progression and outcome (Finak et al., 2008). We observed insignificant association of survival with low CD56 stromal count as compared to high CD56 stromal count ($\chi^2=1.60$, $p>0.05$). The patients with low CD56 stromal count had 0.69 fold lower death rate as compared to patients with high CD56 stromal counts (HR=0.69, 95% CI=0.37-1.24). Several stroma-derived factors, including TGF-β1, involved in tumor-induced reduction of normal NK cell function have been identified (Mamessier et al., 2011). Multiple, distinct biological responses have been reported to be differentially present within the stroma of individuals in outcome-linked categories. Tumor stroma samples from the good-outcome cluster overexpressed a distinct set of immune-related genes, including T cell and NK cell markers indicative of a Th1–type immune response (Finak et al., 2008).

In the present study we observed inverse relation of low intratumoral CD56+NK-TILs with prognosis in IDC i.e. patients had better prognosis and survival with low intratumoral counts. Considering the fact that non T CD56+ and non leukocyte CD45+cells might also give nonspecific staining for CD56+the NK-TIL count should have been high rather than being so low. This observation needs to be studied in all histological subtypes of breast cancer and also at molecular level.

The limitation of the present study was that we used only immunohistochemistry for detection of NK-TILs and the cell count was done only in cases of IDC. Better option would have been double staining with CD3 and CD56 or CD57 specific antibodies or direct staining with anti-NKp46 antibody and/or flow cytometry using CD56 (Non-T) and NKG2D for evaluation of NK cell activity.

However, flow cytometry requires fresh tissue and also direct visualisation of intratumoral and stromal TILs is not possible with flow cytometry. IHC, as done in the present study, had the distinct advantage of morphologically observing the NK cells in tumor and stromal areas.

To conclude, although the NK-TIL count apparently appears to be a significant predictor of prognosis, it alone may not be sufficient for predicting the outcome considering the fact that there exists a crosstalk between NK-TILs and the other immune infiltrating TILs.

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