

RESEARCH ARTICLE

Chemokine and chemokine receptors: a comparative study between metastatic and nonmetastatic lymph nodes in breast cancer patients

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ABSTRACT. Background: Lymph nodes (LNs) are among the first sites of tumor metastasis. The expression of chemokines and chemokine receptors in LNs are involved in cancer prognosis and are considered to be good predictors of tumor progression. The main aim of this study was to assess the expression of important, tumor-promoting chemokines and chemokine receptors in LNs of breast cancer patients. **Methods:** LNs were isolated from eighteen women diagnosed with breast cancer. Data were compared between positive and negative LNs. Expression of chemokines and chemokine receptors were determined by quantitative real-time PCR (qRT-PCR) and flow cytometry. **Results:** Results of qRT-PCR showed that all chemokines, in particular MCP-1, IL-8, SDF-1 and CXCL13, and chemokine receptors CXCR3, CXCR4 and CCR5 showed greater mRNA expression in LN⁺ compared to LN⁻ samples. However, these differences were not statistically significant. IL-8 and CXCR5 gene transcripts had significantly higher expression in LN⁺ patients with stage III compared to those with stage II tumors (P value = 0.04). Results of flow cytometry analysis showed a higher, significant presence of CD69⁺, CCR5⁺ and CD3⁺CCR5⁺ cells in LN of LN⁺ compared to LN⁻ breast cancer patients (P value < 0.05). Expression of MCP-1 was higher in LN⁺ patients, which was near significance (P value = 0.07). **Conclusion:** Our findings provide additional information on the expression of essential chemokines and chemokine receptors in LN and on their relationships to important prognostic factors in breast cancer. These findings have important implications for immunotherapeutic interventions in the treatment of breast cancer.

Key words: breast cancer, lymph node, chemokine, chemokine receptor, metastasis

Lymph nodes (LNs) are among the first sites of cancer metastasis. Although LN involvement is one of the most important prognostic indicators, the exact mechanisms have not been clearly identified. Chemokine and chemokine receptor expression has been reported as the main mediator that orchestrates the metastasis of tumor cells to LNs. Several studies have proposed an important role for CCR7 in the metastasis of different tumors including breast, gastric, colorectal, and head and neck cancers to the sentinel LNs (SLNs) where CCL21, the CCR7 ligand, is highly expressed [1-3]. Muller *et al.* have demonstrated that CCR7 may help the tumor cells find their way toward the LNs; whereas CXCR4 may facilitate their metastasis from LNs to distant sites [4]. Thus, disruption of the CCR7/CCL21 axis by the use of antagonistic agents may show therapeutic effects by preventing LN metastases in the early stages of tumor development [1]. The den-

sity of SDF-1⁺ and CXCR4⁺ nodal cells were higher in involved than non-involved LNs in patients with squamous cell carcinoma of the oral cavity (OSCC) and of the lip (LSCC) [5]. The significant expressions of CXCR4, CCR7 and CCR10 were shown in melanoma compared to primary melanocytes. The ligands of these receptors, SDF-1, CCL21 and CCL27/CTACK have been demonstrated to be expressed at high levels in their metastatic sites such as LNs [6]. Another chemokine receptor mediating the metastasis of tumors to the LNs is CXCR3 [7, 8]. A number of studies have reported the expression of RANTES and MCP-1 in tumor-infiltrated LNs as well as breast tumor cells and distant sites of metastases [9]. Based on these reports, we investigated further the expression of a variety of chemokines and chemokine receptors in positive and negative LNs that were isolated from patients with various stages of breast cancer.

SUBJECTS, MATERIALS AND METHODS

Patients

Lymph nodes were isolated from 18 breast cancer patients, of which 10 were LN⁺ and 8 were LN⁻. The clinicopathological characteristics of patients studied are presented in *table 1*. The median age of the patients was 51 and the mean age was 49 ± 11.5 years. All data were compared between positive and negative nodes and with clinicopathological characteristics of patients. Samples were taken and examined after patients gave fully informed consent.

Table 1

Clinicopathological characteristics of breast cancer patients used for isolation of LNs.

Characteristics (%)	
Tumour type	
Infiltrative ductal carcinoma (IDC)	8 (44.4)
Non-infiltrative ductal carcinoma (Medullary & invasive carcinomas)	8 (44.4)
Missing	2 (11.1)
Estrogen receptor	
Positive	10 (55.5)
Negative	5 (27.8)
Missing	3 (16.7)
Progesterone receptor	
Positive	10 (55.5)
Negative	5 (27.8)
Missing	3 (16.7)
HER2	
Positive	10 (55.5)
Negative	5 (27.8)
Missing	3 (16.7)
Lymph node involvement	
Positive	10 (55.6)
Negative	8 (44.4)
Tumor size (cm)	
T1 (≤ 2)	3 (16.7)
T2 (2-5)	13 (72.2)
T3 (> 5)	0 (0)
Missing	2 (11.1)
Stage	
0	0 (0)
I	4 (22.2)
II	9 (50)
III	3 (16.7)
IV	0 (0)
Missing	2 (11.1)
Histological grade	
Low grade (I and II)	12 (66.7)
High grade (III)	2 (11.1)
Missing	4 (22.2)

Isolation of LNs

LNs were isolated from breast cancer patients; tumor cell metastases were pathologically examined both grossly and through H&E staining. Then, each LN was dissected in a Petri dish that contained 2 mL sterile RPMI (Biosera, UK) until all cells were released. RPMI that contained lymphocytes and disseminated tumor cells were transferred slowly on Ficoll-hypaque (Biosera, UK) and centrifuged at 2,800 rpm for 20 minutes. The resultant ring was isolated, washed with phosphate-buffered saline (PBS), and the viability of isolated cells was verified with trypan blue (Sigma, USA).

RNA extraction, cDNA synthesis and quantitative real-time polymerase chain reaction (qRT-PCR)

A total of 1×10^6 lymphocytes were subjected to RNA extraction by the phenol chloroform method. cDNA was synthesized using a cDNA Synthesis Kit (Fermentas, Canada). Then, expression of RANTES, SDF-1, CXCL13, IP-10, IL-8, MCP-1, CXCR4, CXCR7, CXCR3, CXCR5, CCR5, CCR7 and CCR4 mRNAs were detected by a quantitative real-time polymerase chain reaction (qRT-PCR) using a Bio-Rad thermal cycler. Approximately 2 μ L of cDNA was amplified in each 25 μ L PCR reaction mix that contained 2X SYBR Green Master Mix (Fermentas, Canada), 10 pmol each of forward and reverse primers, and DEPC-treated water. All data were compared to those from the beta actin housekeeping gene. Forward and reverse primers were designed with Primer 3 software.

Flow cytometry

For flow cytometry analysis, 10×10^6 cells were incubated for five hours with brefeldin (BD Biosciences, USA) at 37°C in a CO₂ incubator. Cells were washed twice with PBS and the following mAb combinations were used: CCR5-FITC/RANTES-PE/CD3-Percp, MCP-1-PE/CD3-Percp, CD69-FITC/IP-10-PE/CD3-Percp, intracellular CXCR4-PE/CD3-Percp, extracellular CXCR4-PE/CD3-Percp (BD Biosciences). Briefly, cells were initially stained with 5 μ L of FITC-conjugated mouse anti-human CCR5 and CD69, PE-conjugated mouse anti-human CXCR4 and Percp-conjugated mouse anti-human CD3 antibodies for the assessment of surface expression of the above mentioned molecules. To exclude any non-specific bindings, cells were additionally stained with isotype-matched mouse monoclonal antibodies (BD Biosciences, USA). After 30 minutes incubation at room temperature, cells were washed twice with PBS. For intracellular staining, 400 μ L of the 1% cell fixative was added, and then incubated for five minutes at 4°C after which it was washed with 1 mL PBS at 300 g for five minutes at 4°C. We added 500 μ L of 0.2% saponin to the cells, which were allowed to incubate for five minutes at 4°C and then centrifuged. Next, 1 mL of saponin was added and cells were washed. Cells were either stained with 5 μ L of PE-conjugated mouse anti-human RANTES, MCP-1, IP-10 or CXCR4 antibodies. After 30 minutes of incubation on ice, cells were washed with 1 mL of saponin followed by 500 μ L of PBS at 300 g for five minutes at 4°C. Finally, 500 μ L PBS was added and approximately 20,000 events were collected and further analyzed with the use of WinMDI 2.5 software.

Statistical analysis

Expression of chemokine and chemokine receptor gene transcripts were evaluated with the nonparametric Mann-Whitney test using SPSS software v. 15 (Chicago, USA). The relative amounts of gene transcripts were determined from the $2^{-\Delta CT}$ formula. Relative expression was plotted and evaluated by means of Prism 5 software (Inc; San Diego CA, USA, 2003). In all statistical analyses, a P value < 0.05 was regarded as significant.

RESULTS

Chemokine and chemokine receptor gene transcripts in LN⁺ and LN⁻ samples

Expressions of chemokines and chemokine receptors were compared between LN⁺ and LN⁻ patients by qRT-PCR and flow cytometry. Results of qRT-PCR showed that all chemokines had more mRNA expression in LN⁺ compared to LN⁻ patients. MCP-1, SDF-1, CXCL13 and IL-8 had 4-, 3.8-, 3.7- and 3-fold higher expressions in LN⁺ patients; these differences were not statistically significant (P value > 0.05; figure 1).

The expression levels of chemokine receptor mRNA in LN⁺ and LN⁻ breast cancer patients are presented in figure 2. In LN⁺ patients, CXCR3 had a 2-fold higher mRNA expression, CCR5 had a 2.7-fold higher, and CXCR4 had a 1.5-fold higher mRNA expression compared to LN⁻ patients.

A significant correlation was observed between the expression of MCP-1 and RANTES in LN⁺ compared to LN⁻ samples (P value < 0.05).

Flow cytometry analyses

Expression of CXCR4 (intracellular and surface), IP-10, CD69, CCR5, RANTES and MCP-1 in LNs of LN⁺ and LN⁻ breast cancer patients was analyzed by flow cytometry. The schematic representation of flow cytometry analyses of one of the patients is shown in figure 3. As a result, the expressions of CD69 and CCR5 were significantly higher in LNs of LN⁺ compared to LN⁻ breast cancer patients. The mean \pm SEM for CD69 was 24 ± 2.3 for LN⁺ and 17 ± 0.7 for LN⁻ patients (P value = 0.03). Mean \pm SEM

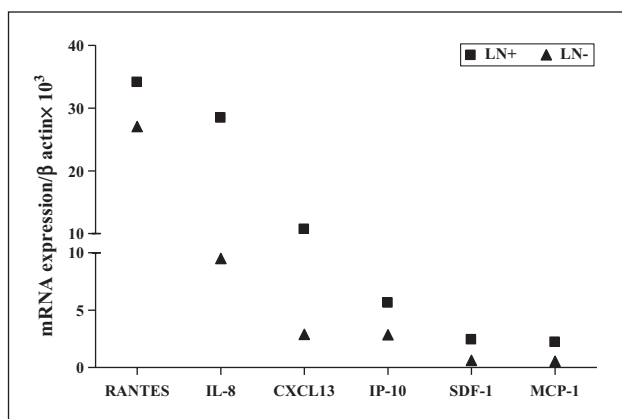


Figure 1

Expression of chemokine mRNA in LN⁺ and LN⁻ breast cancer patients. Data are shown as the median of $2^{-\Delta CT}$. P value > 0.05 for all molecules.

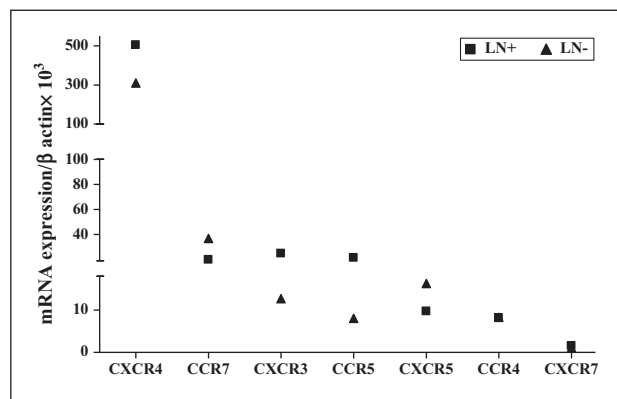


Figure 2

Expression of chemokine receptor mRNA in LNs of LN⁺ and LN⁻ breast cancer patients. Data are shown as the median of $2^{-\Delta CT}$. P value > 0.05 for all receptors.

for CCR5 was 15 ± 4.3 in LN⁺ patients and 5 ± 1.1 in LN⁻ patients (P value = 0.04). Expression of MCP-1 was also higher in LN⁺ patients, of which the P value was approximately significant. The mean \pm SEM for MCP-1 was 2 ± 0.95 in LN⁺ versus 0.07 ± 0.07 in LN⁻ patients (P value = 0.07). No difference was found in the expression of other molecules studied between positive and negative nodes (figure 4).

Co-expression of described molecules with CD3 was also studied. As presented in figure 5, expression of CD3⁺CCR5⁺ was significantly higher in LNs from LN⁺ compared to LN⁻ patients (P value = 0.04). Mean \pm SEM for CD3⁺CCR5⁺ was 10 ± 3 in LN⁺ and 3 ± 0.6 in LN⁻ patients (P value = 0.04). Also, expressions of CD3⁺CD69⁺ lymphocytes were higher in this group of patients; the P value for these differences was almost significant (P value = 0.07). No difference was noted in the expressions of CD3⁺CXCR4⁺, CD3⁺IP-10⁺, CD3⁺RANTES⁺, and CD3⁺CD69⁺IP10⁺ between LN⁺ and LN⁻ patients.

Association between the expression of chemokine/chemokine receptors in LNs and clinicopathological characteristics of patients

Age

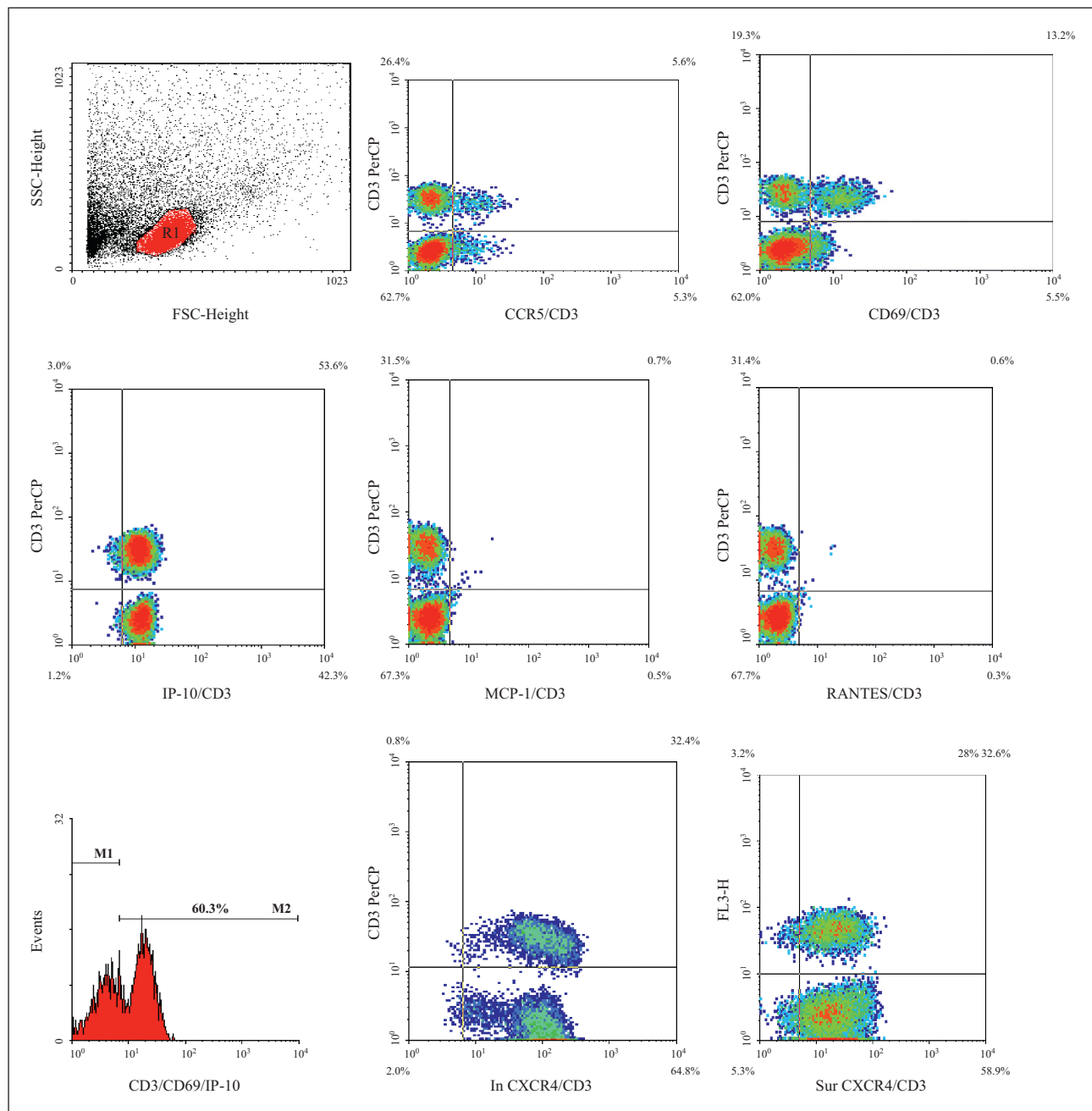
There was a significant positive relationship between the expressions of CCR4, CXCR4 (P value = 0.03), CCR5, CXCR7 (P value = 0.05), CCR7, CXCL13 (P value = 0.02) and LN⁺ patients between the ages of 50-80 years old. No such correlations were observed in LN⁻ patients (P value > 0.05).

Vascular invasion

There were a significant relationship between the expressions of CCR5 (P value = 0.04), CXCL13 (P value = 0.02) and SDF-1 (P value = 0.04) in LN⁺ patients and vascular invasion, however no such correlations were observed in LN⁻ patients (P value > 0.05).

Stage

Of all chemokines and chemokine receptors, IL-8 and CXCR5 were expressed significantly more in LN⁺ patients diagnosed with stage 3 disease compared to those with stage 2 disease (P value = 0.04). No such correlations were observed in LN⁻ patients (P value > 0.05).

**Figure 3**

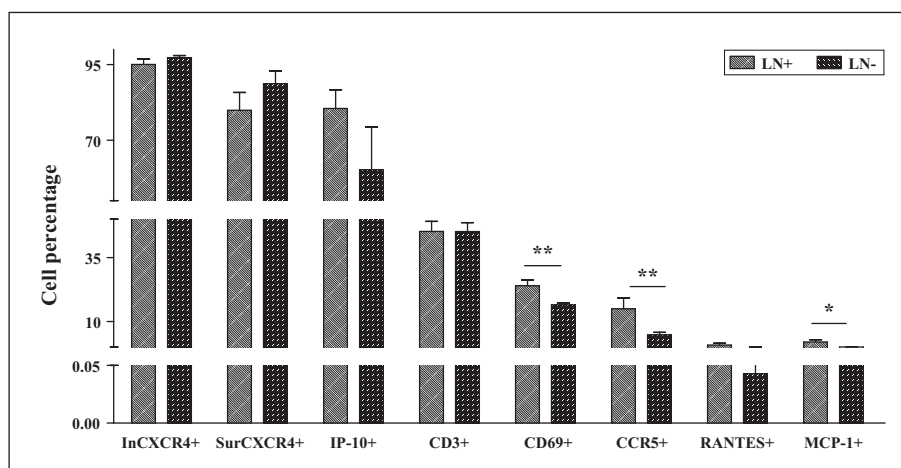
A schematic representation of flow cytometry analysis in LNs of one of the patients studied.

DISCUSSION

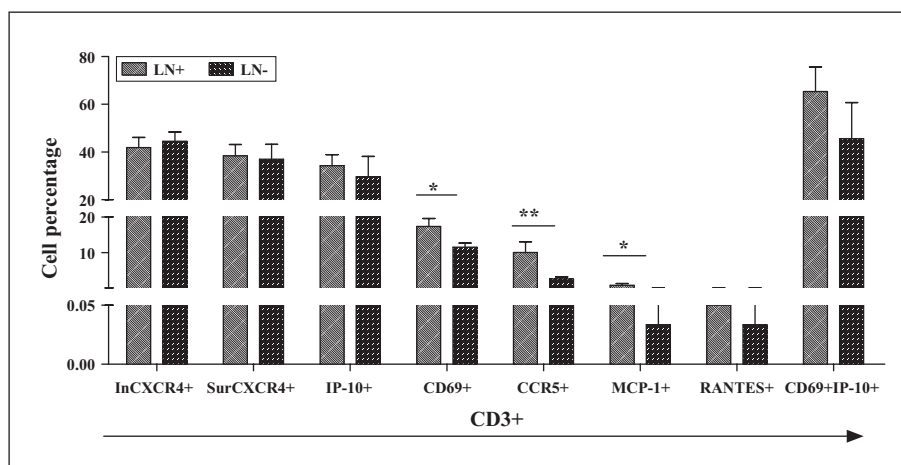
Several reports have implicated a significant correlation between chemokine/chemokine receptor expression in LNs and cancer prognosis. Expression of chemokines in draining LNs leads to the metastasis of tumor cells and recruitment of activated T lymphocytes bearing cognate chemokine receptors. However, recruiting T cells have immunoinhibitory roles and a T regulatory-like phenotype [10]. It has been reported that the immune response in SLNs is downregulated by tumor cells that produce immunomodulators, leading to immunosuppression of the SLN. The fewer numbers and maturity of dendritic cells (DCs) and T cells in addition to the production of immunoregulatory cytokines such as IL-10 and TGF- β in SLNs have been suggested to be the leading cause for this immunosuppression [11]. Increased expres-

sions of CCL21, SDF-1, IP-10, RANTES, MCP-1 and CCL27 have been reported in draining LNs from different types of cancers [1, 5, 6, 9]. Based on these studies, the chemokine/chemokine receptor axis is important for metastasis of tumor cells to the LNs; more information is needed to design the appropriate therapeutic approaches for inhibition of metastases.

In the present study, the expression profiles of RANTES/CCR5, SDF-1/CXCR4/CXCR7, CXCL13/CXCR5, IP-10/CXCR3, IL-8, MCP-1, CCR7 and CCR4 in positive and negative lymph nodes of breast cancer patients were determined. Results of qRT-PCR showed higher expressions of IL-8, CXCL13, MCP-1 and CXCR4 mRNAs in LN⁺ patients. A statistically significant correlation was found between the expressions of CCR5, CXCL13, SDF-1 and vascular invasion in LN⁺ patients. IL-8 and CXCR5 were expressed significantly more in

**Figure 4**

Expression of CXCR4 (intracellular and surface), IP-10, CD69, CCR5, RANTES and MCP-1 in LNs of LN⁺ and LN⁻ breast cancer patients. Data are shown as mean \pm SEM of cell percentages. ** Statistically significant; * Near significance.

**Figure 5**

Co-expression of described molecules with CD3 in LNs of LN⁺ and LN⁻ breast cancer patients. Data are shown as mean \pm SEM of cell percentage. ** Statistically significant; * Near significance.

LN⁺ patients with stage 3 tumors compared to those with stage 2 tumors. No such correlations were observed in LN⁻ patients. Thus expressions of SDF-1, IL-8, CXCL13, CXCR5 and CCR5 in the LNs of these breast cancer patients might be more important than other chemokine/receptors studied for LN involvement, resulting in a poorer prognosis for patients with breast cancer in our area.

Pioneers of the concept that chemokine receptors promote tumor metastasis are Muller and his colleagues. Their investigation of the expression of chemokine receptors in human breast cancer has shown high expression of the CXCR4 chemokine receptor and its ligand SDF-1 in metastasis-prone tissues such as LNs [4]. This has been confirmed by other researchers who have shown the importance of both CXCR4 and CCR7 in metastases to LNs in breast cancer patients [12-14]. Cabioglu *et al.* have found additional markers such as CXCR4 and HER2-neu to be good predictors of LN involvement in breast cancer. They proposed that positive LNs had higher expressions of cytoplasmic CCR7, CXCR4 and HER2-neu compared to negative LNs, however nuclear CXCR4 expression has been found in LN⁻ tumors. Co-expression of cytoplasmic CXCR4 with HER2-neu has been observed as the only

factor associated with involvement of four or more LNs in LN⁺ tumors [12]. It has been demonstrated that expression of MCP-1 leads to LN metastasis and tumor recurrence in both papillary thyroid carcinoma and squamous cell carcinoma of the head and neck [15, 16]. RANTES and MCP-1 have been reported as highly expressed chemokines in tumor-infiltrated LNs, causing metastases of CCR5- and CCR2-expressing breast tumor cells to draining LNs [9]. A significant association was suggested between expressions of RANTES and MCP-1 and breast malignancy. MCP-1 significantly supports the release of RANTES, and co-expression of these two chemokines leads to advanced pathological stages of breast cancer [17]. Consistent with these findings, we have detected higher expressions of RANTES and MCP-1 in LN⁺ samples, and observed a significant, positive correlation between MCP-1 and RANTES expressions in LN⁺, but not LN⁻ samples.

Results of flow cytometry analyses have shown higher expressions of IP-10⁺, CD69⁺, CD3⁺CD69⁺, CCR5⁺, CD3⁺CCR5⁺, RANTES⁺, MCP-1⁺ and CD3⁺MCP-1⁺ cells in LN⁺ compared to LN⁻ patients. These differences were statistically significant for the expressions of CD69⁺ CCR5⁺ and CD3⁺CCR5⁺ cells. CD69, as a signal transduction receptor, is an earlier activation marker involved

in proliferation and function of lymphocytes, NK cells and platelets [18]. It has been previously shown that breast cancer patients who survived longer following active, specific immunotherapy had lower numbers of CD69⁺ and CD4⁺CD69⁺ cells compared with those who died [19].

Interestingly, when metastatic tumor-draining LNs and non-metastatic tumor-draining LNs of patients with early cervical cancer were assessed, the number of CD4⁺Foxp3⁺ Tregs with the activated phenotype (HLA-DR⁺ and CD69⁺) were significantly higher in metastatic tumor-draining LNs [10]. Metastatic tumor-draining LNs are believed to contain higher numbers of CD8⁺Foxp3⁺ Tregs and plasmacytoid dendritic cells (pDC), which downregulate T cell responses. It has been suggested that metastatic tumor cells contribute to a tolerogenic response in tumor-draining LNs in cancer patients [10].

As the expression of CCR5 was significantly higher in our LN samples of LN⁺ patients, we have hypothesized that this chemokine receptor may be important for metastasis of breast tumor cells to the draining LNs; the use of an anti-CCR5 monoclonal antibody may be a useful therapeutic approach. Interestingly, Wysocki *et al.* have shown the important role of CCR5 on Treg functions in graft-versus-host disease (GVHD). They have shown that CCR5^{-/-} Tregs were less effective in preventing mortality from GVHD in animal models [20]. Since we have detected high, significant expressions of CD69⁺ and CD3⁺CCR5⁺ cells in LN⁺ samples, it is conceivable that the recruiting immune cells have an immunosuppressive phenotype that inhibits anti-tumor immune responses in metastatic LNs.

In conclusion, higher expressions of chemokines such as IL-8, CXCL13, MCP-1 and RANTES by immune cells may lead to the attraction of both tumor cells and more immune cells bearing corresponding receptors to draining LNs. Some of these chemokines may increase tumor cell survival as has been reported regarding CXCL13 [21]. Metastatic tumor cells provide a tolerogenic milieu in LNs that impair the function of activated immune cells and change their phenotype to an immunoregulatory-like phenotype. Therefore, chemokine/chemokine receptor axes would be considered immunotherapeutic targets in cancer metastases.

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