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Lower expression of CXCR4 in lymph node metastases than in primary breast cancers: Potential regulation by ligand-dependent degradation and HIF-1α ☆

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Abstract

Stromal-derived factor-1 (SDF-1) is a unique ligand of the CXC chemokine receptor 4 (CXCR4), which is critically involved in the metastasis of breast cancer. High levels of SDF-1 in the common destination organs of metastasis, such as the lymph nodes, lungs, liver, and bones, attract CXCR4-positive tumor cells. The interaction between SDF-1 and CXCR4 leads to the activation of specific signaling pathways, allowing for homing and metastatic progression. However, regulation of CXCR4 expression at the metastatic organ site is not well-documented. We detected the expression of CXCR4 and hypoxia inducible factor (HIF)-1α in breast tumor tissues by immunohistochemical staining and analyzed SDF-1 in primary tumors and lymph nodes using real-time RT-PCR. Compared to the corresponding metastasized tumors in the lymph nodes, primary invasive carcinomas showed more intense staining for CXCR4, particularly on the cellular membrane. Both primary tumors and lymph node metastases exhibited higher levels of CXCR4 expression compared to non-neoplastic breast tissues. Therefore, we hypothesized that the tumor environment in the lymph nodes may cause the reduction of CXCR4 levels in the metastatic tumor cells because of: (1) high SDF-1 levels and (2) lower levels of HIF-1α. Our in vitro data demonstrated that high levels of SDF-1 can induce the internalization and degradation of CXCR4 through the lysosome pathway. In addition, lower levels of HIF-1α in the lymph node metastases, probably induced by the less hypoxic environment, further lowered CXCR4 levels. These results indicate that ligand-dependent degradation and lower HIF-1α levels may be potential causes of lowered levels of CXCR4 in the lymph nodes compared to the primary tumors. Our study suggests that CXCR4 levels in tumor cells are regulated by its microenvironment. These findings may enhance our ability to understand the biological behavior of breast cancers.

Keywords: CXCR4; HIF-1α; Breast cancer; SDF-1; Lymph node metastasis

Chemokines are a superfamily of small cytokines that induce, through their interaction with G-protein-coupled receptors, cytoskeletal rearrangements and directional migration of several cell types [1–3]. These secreted proteins direct the precise homing of various subsets of hematopoietic cells to specific anatomical sites [4–7]. In particular, stromal cell-derived factor-1 (SDF-1 or CXCL12) is a chemokine that interacts specifically with CXCR4, a major co-receptor for the entry of the T cell-tropic HIV [8–11], and a critical mediator of breast cancer metastasis [12]. Previous studies have shown that the in vitro invasion and in vivo metastasis of breast cancer cells can be blocked in animal

☆ *Abbreviations:* CXCR4, CXC chemokine receptor-4; HIF-1α, hypoxia-inducible factor 1α; SDF-1, stromal-derived factor-1; DAB, diaminobenzidine; RT-PCR, reverse transcription-polymerse chain reaction.

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models by using anti-CXCR4 antibodies, CXCR4 antagonists, or silencing the gene expression of *CXCR4* with small interfering RNA [12–14]. These data support the necessity of the CXCR4/SDF-1 interaction in breast cancer metastasis, and further suggest that CXCR4 is a critical target in the prevention of metastasis. CXCR4 expression was also found to correlate with the metastasis of invasive ductal carcinomas [12,15]. In addition, Schmid et al., found elevated CXCR4 levels in 13 out of 14 ductal carcinomas in situ (DCIS) and in all seven ductal carcinomas [16]. CXCR4 was initially thought to be a membrane protein; however, immunohistochemical results of CXCR4 in breast cancer tissues showed that its subcellular localization could vary, e.g., on the membrane, in the cytoplasm, or even in the nucleus [15,16]. Based on these findings, CXCR4 could serve as a novel biomarker for breast cancer metastasis.

Although several investigations demonstrated the involvement of CXCR4 in both the metastasis and growth of various tumors, the relationship between CXCR4 subcellular localizations and expression levels during metastatic progression has not yet been determined. To investigate this issue, we analyzed CXCR4 expression in primary breast tumors and lymph node metastases in archived patient tissue samples. We observed that the frequency of CXCR4 membranous localization and staining intensity were higher in primary tumors than in the lymph nodes of the corresponding metastatic tumors. To study the cause of this discrepancy, we analyzed the levels of two critical factors, SDF-1 and HIF-1α, in primary tumors and the lymph nodes using real-time RT-PCR and immunostaining methods. The results indicated that high SDF-1 and low HIF-1α levels correlated with reduced CXCR4 expression of metastases in the lymph nodes compared to primary tumors.

Materials and methods

Cell culture and internalization of CXCR4. Human breast carcinoma cell line MDA-MB-231 was cultured in 5% CO_2 at 37 °C in RPMI-1600 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Sigma), 50 U/ml of penicillin, and 50 μg/ml of streptomycin (Invitrogen, Carlsbad, CA). Ten thousand cells were seeded per well in an eight-well slide chamber 24 h prior to serum-starvation (0.5% FBS). Following overnight serum starvation, cells were treated with various concentrations of SDF-1β (100 and 200 ng/ml; R & D Systems, Minneapolis, MN) at 37 °C for 30 min. Then, the cells were fixed and immunostained after removing SDF-1β by washing the cells with serum-free medium at pH 3.0 for 1 min. The detailed procedure for CXCR4 immunofluorescence with the biotin-labeled CXCR4 antagonist, TN14003, was described previously [13]. The immunofluorescence images were acquired using the Quips Smart Capture (Vysis) software and a Zeiss confocal laser fluorescence scanning microscope with a 63× oil objective.

Tissue samples. The human breast cancer tissue array was purchased from Imgenex (Cat #IMH-304/CB2), which contained 40 primary breast tumors, 10 metastatic carcinomas in the lymph nodes, and 10 normal breast tissues adjacent to the tumor site. In addition, we obtained 56 archived patient tissue samples from the Avon Tissue Bank for Translational Genomics Research at Grady Memorial Hospital in Atlanta, GA. These included 10 pairs of related primary and lymph node metastatic tumors, 24 primary tumor samples of different stages, and 12 normal breast samples. The source and characteristics of tissues are summarized in Table 1.

Immunohistochemical staining. For immunohistochemical staining of CXCR4, formalin-fixed and paraffin-embedded tissue sections were heated at 58 °C for 45 min. These specimens were washed with xylene three times for 5 min each, followed by washes with 100%, 95%, and 75% ethanol, and then rinsed with PBS. The detailed staining procedure with the biotinylated-CXCR4 antagonist, TN14003, was described previously [13]. All protocols for human tissue studies were reviewed and approved by the Institutional Review Board (IRB) at Emory University. The intensity of staining (brown color) was scored semi-quantitatively as follows: +, weak; ++, medium; +++, strong; and ++++, very strong. Samples with a staining score of ++ and greater were considered CXCR4-positive. Additionally, another immunostaining score was calculated by the multiplication of the percentage of positive tumor cells (0–100) by the staining intensity (grade 1–4), producing a total range of 0–400 [17]. Membrane or cytoplasm staining was also recorded. Samples in which a combination of membrane and cytoplasm staining was observed, with a cellular membrane staining ring surrounding the cytoplasm staining, were classified as a membrane-strong staining represented by M > C. Conversely, if cytoplasm staining was dominant with little membrane staining, it was represented by C > M. For HIF-1α, the detailed procedure of immunostaining was described previously using the monoclonal anti-HIF-1α antibody purchased from Novus Biologicals (Littleton, CO) [18]. The grading system for HIF-1α was as follows: 4+ (>30% of nuclei staining); 3+ (20%–30% of nuclei staining); 2+ (10%–20% of nuclei staining); and 1+ (<10% of nuclei staining).

RNA isolation and real-time reverse transcription PCR (RT-PCR) analysis for SDF-1 from formalin-fixed and paraffin-embedded tissues. RNA extraction from formalin-fixed and paraffin-embedded tissues was based on a previous description [19]. Five 10-μm formalin-fixed paraffin-embedded tissue sections were heated at 58 °C for 30 min. The specimens were deparaffinized by incubating in 1.0 ml of xylene three times for 5 min each, followed by washes with 100%, 95%, and 75% ethanol, and then rinsed with PBS. Then, the tissues were air-dried for 10 min. According to Godfrey et al., digestion of the fixed tissues is a key step in isolating optimum RNA. The tissues were digested overnight at 37 °C in 100 μl of digestion solution plus 20 μl of 10% SDS and 40 μl of 80 mg/ml of proteinase K. Another 40 μl aliquot of 80 mg/ml proteinase K was added, followed by vortexing and incubation again overnight at 37 °C. The digested tissues were then processed to prepare total RNA using Trizol Reagent (Invitrogen) following the manufacturer's instructions. Despite the improved RNA extraction protocol, RNA obtained from fixed tissues

Table 1
Source and characteristics of tissue specimens

	Total	Source		T stage		Sex		Age	
		Tissue array	Archived tissues	T1 & T2	T3 & T4	Male	Female	<50	⩾50
Primary tumor	74	40	34	35	39	3	71	42	32
Lymph node metastases	20	10	10	11	9	0	20	11	9
Normal breast tissues	22	10	12	—	—	0	22	10	12
Total	116	60	56	—	—	3	113	63	53

might contain different sized fragments because of the formalin-fixing and paraffin-embedding procedure. To increase sensitivity, we designed a smaller amplicon size (212 bp). The human SDF-1-specific primers for the 212 bp amplicon were 5′- CTGGGTTTGTGATTGCCTCT and 5′-CACCAGGACCTTCTGTGGAT (GenBank Accession No. E09668). The procedures for real-time PCR were described previously [13]. In each run, a dilution series of a calibration sample (one of the primary tumor samples) and β-actin mRNA were run along with the unknown samples from the tissues. Relative levels of SDF-1 mRNA were calculated from the quantity of unknown sample and the quantity of the calibrated PCR sample.

Statistical analysis. All statistical significances in this study were determined by the Student's t test and χ test. P value $\leqslant 0.05$ is considered to be significant.

Results

Over-expression of CXCR4 in breast cancer tissues

We analyzed 74 primary breast carcinomas and 20 lymph node metastatic samples for CXCR4. Eighty-five out of 94 (90%) breast carcinoma samples were positive for CXCR4 ($\geqslant 2+$). Six representative CXCR4 immunostaining examples from primary tumors and lymph node metastases are shown in Fig. 1A and B. Three out of 22 (14%) "normal" breast tissue samples, obtained from regions adjacent to the tumor, demonstrated positive staining ($= 2+$). In contrast, CXCR4 levels were low in all normal breast tissues obtained from patients without any malignancies. Thus, the tumor microenvironment may have induced the moderately elevated CXCR4 levels ($\sim 2+$) in the normal tissues next to the tumors. However, the immunostaining intensity was lower in the normal tissues adjacent to the tumors compared to the tumors themselves.

Reduction of CXCR4 levels in metastatic tumors in lymph nodes

We adapted "an expression score" by multiplying the percentage of positive tumor cells by their intensity of staining [17]. For example, if the staining intensity grade of a sample is 3+, and the percentage of positive cells is 90%, then the total score will be 270. The average CXCR4 score in primary tumors with immunostaining was 264, and was significantly higher than the 160 average score of the metastatic lymph nodes group ($P < 0.001$) (Fig. 1B). We observed that 61% of the primary tumors expressed elevated levels of CXCR4 (3+ or 4+) while only 30% of the tumors in the lymph nodes exhibited a 3+ or 4+ staining grade ($P < 0.001$) (Table 2).

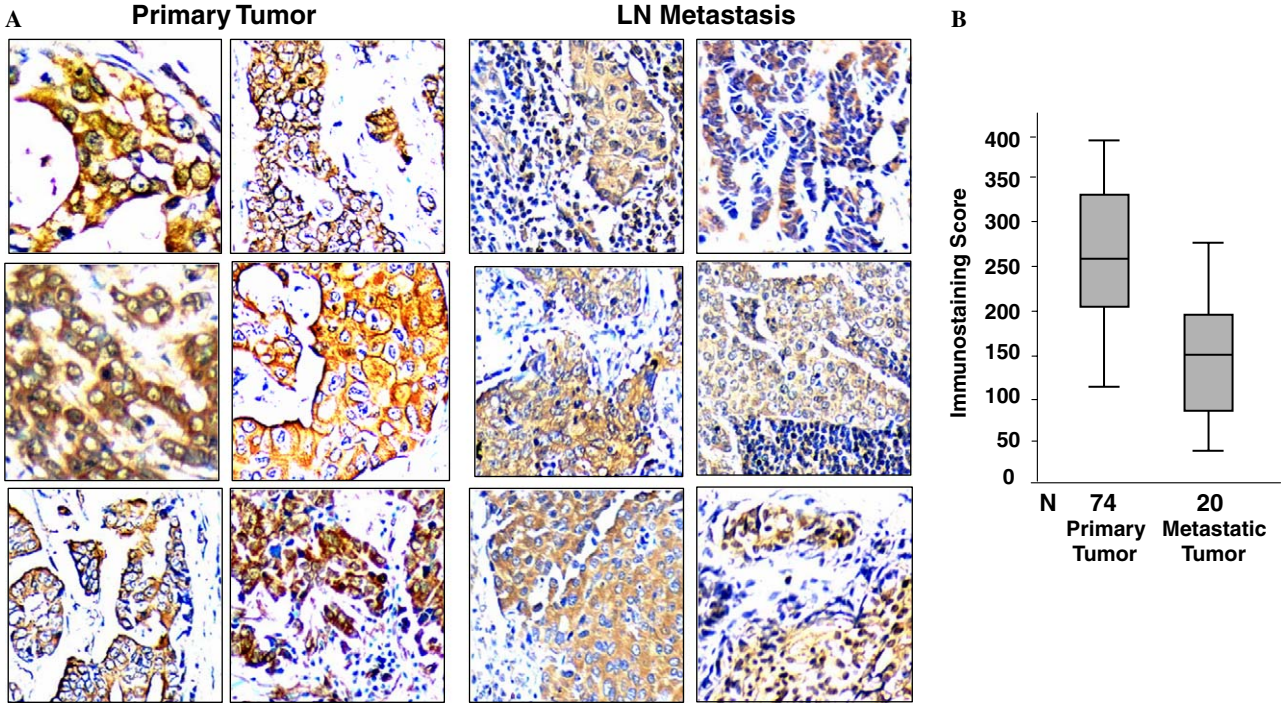

Fig. 1. CXCR4 expression levels are higher in primary tumors than lymph node metastases. (A) Representatives of the immunostained primary breast cancer and lymph node metastases with CXCR4. CXCR4 protein is depicted by the brown color (DAB), whereas nuclei counterstaining is represented by the blue color. (B) A box and whisker plot diagram showing the expression levels (immunostaining scores) of CXCR4 from both primary and metastatic breast tumors in lymph nodes by immunohistochemical staining. The scores were calculated by multiplying the percentage of positive tumor cells (1–100) by the staining intensity (1–4), producing a score range of 0–400. Horizontal lines in the boxes represent the median value of CXCR4 immunostaining scores from each group. The immunostaining scores of lymphoid metastases are significantly lower than those of the primary tumors ($P < 0.001$). The top and bottom edges of the boxes indicate the score values from the 75th and the 25th percentile, respectively. Whiskers represent the highest and lowest values. The range is shown as a vertical line.

Table 2
Summary of immunostaining of primary and metastatic breast cancer tissues with CXCR4

	CXCR4 staining		
	1 & 2+	3 & 4+	*P* value
Primary tumor	29 (39%)	45 (61%)	<0.0001
Lymph node metastases	14 (70%)	6 (30%)	

Immunostaining patterns of CXCR4 in primary tumors and lymph node metastases

The subcellular localization and expression pattern of CXCR4 that accompanies breast cancer progression has not yet been established. Two immunostaining patterns for CXCR4 were found in this study: those containing predominantly membrane staining found mainly in primary tumors and primarily cytoplasmic staining found mostly in lymph node metastases (Fig. 1A). In primary tumors, 58% of the samples exhibited predominantly membranous staining while 80% metastatic tumors in lymph nodes showed predominantly cytoplasmic staining (Table 3). Furthermore, the majority of the lymph node metastases showed an overall lower intensity of CXCR4 expression (Fig. 1A and Table 2).

SDF-1 induces the internalization of CXCR4 and degradation through the lysosome pathway

CXCR4 membrane staining was found to be decreased in breast cancer cells residing in the lymph nodes. SDF-1 levels have been reported to be high in normal lymph nodes [12]. We hypothesized that the high levels of SDF-1 in the lymph nodes may play a role in the down-regulation of membrane and total CXCR4 expression levels. We analyzed SDF-1 mRNA levels of 10 primary tumors as well as their matching metastases in lymph node tissues. The results confirmed that SDF-1 levels are indeed elevated in the lymph nodes compared to the primary sites ($P < 0.001$) (Fig. 2). In the absence of SDF-1, CXCR4 was detected predominantly on the membrane of MDA-MB-231 breast cancer cells grown in vitro (Fig. 3A). However, when MDA-MB-231 cells were incubated with 100 ng/ml or 200 ng/ml SDF-1β for 30 min and then fixed in ice-cold acetone, CXCR4 was found in the cytoplasm and even in the nuclei in confocal micrographs (Fig. 3A), suggesting ligand-mediated internalization of the surface receptor. We further explored the impact of SDF-1 on CXCR4 levels by exposing serum-starved (0.5% FBS) MDA-MB-231 cells to 100 or 200 ng/ml SDF-1β for 48 h and measuring CXCR4 protein levels by Western blotting. CXCR4 protein levels were significantly decreased with the addition of 100 ng/ml or 200 ng/ml SDF-1β in vitro (Fig. 3B). To investigate a mechanism by which elevated levels of SDF-1 could down-regulate CXCR4 levels, we analyzed the ability of chloroquine to inhibit proteolysis

Table 3
Summary of CXCR4 subcellular localization

	M > C	C > M	*P* value
Primary tumor	43 (58%)	31 (42%)	<0.0025
Lymph node metastases	4 (20%)	16 (80%)	

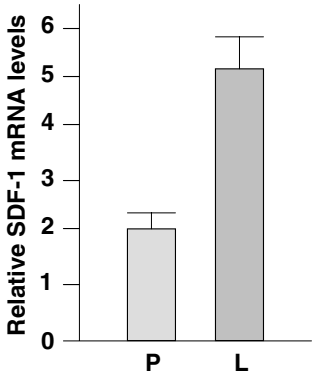

Fig. 2. Comparison of SDF-1 mRNA expression levels between 10 breast primary tumors (P) and their paired lymph nodes with metastases (L) by real-time RT-PCR analysis ($P < 0.001$).

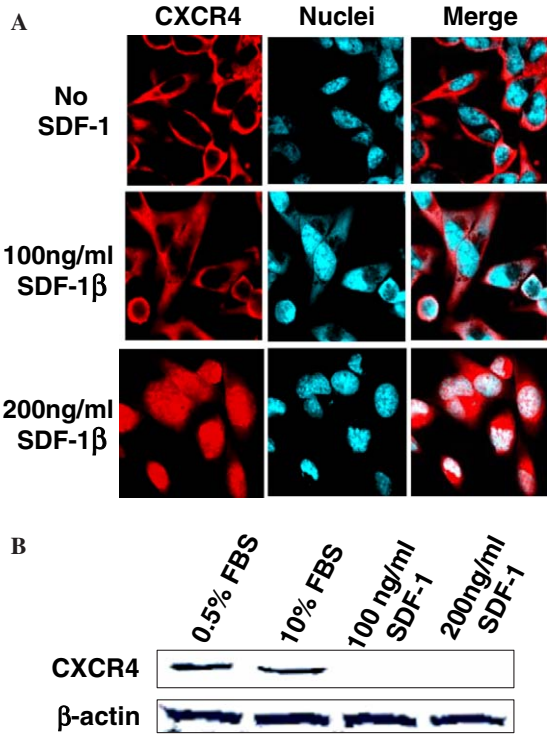

Fig. 3. SDF-1 induced internalization of CXCR4 and mediated degradation of CXCR4. (A) Cells were immunostained using biotin-labeled CXCR4 antagonist after pre-incubation with 100 or 200 ng/ml SDF-1β for 30 min compared to no SDF-1 treatment. Fluorescent images were scanned by a Zeiss confocal microscope. Original magnification was 63×. Red, rhodamine staining, represents CXCR4; blue, nuclei counterstaining. (B) Western blot analysis for MDA-MB-231 cells incubated with 100 or 200 ng/ml SDF-1β for 48 h demonstrated CXCR4 protein degradation by SDF-1 48 h after 100 and 200 ng/ml treatment. Cells incubated with 10% FBS did not exhibit any degradation. β-Actin was used as a loading control.

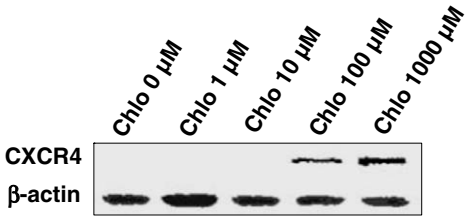

Fig. 4. Chloroquine, an inhibitor of lysosome function, blocked CXCR4 degradation mediated by its ligand, SDF-1. Following a 30 min incubation of various concentrations of chloroquine, MDA-MB-231 cells were incubated for 48 h with 200 ng/ml of SDF-1. The treated cells were collected for Western blot analysis for CXCR4. The results showed that chloroquine blocked the degradation of CXCR4 induced by SDF-1 in a dose-dependent manner.

function of lysosomes in cultured cells. Incubation of MDA-MB-231 cells with chloroquine for 30 min prior to SDF-1 treatment blocked degradation of CXCR4 mediated by its ligand in a dose-dependent manner (Fig. 4).

Correlation between HIF-1α and CXCR4 expression in primary tumors and lymphoid metastases

As CXCR4 has been reported to be up-regulated by HIF-1α, we speculated whether lower CXCR4 levels found in metastasized tumor cells may relate to a better oxygenated tumor environment with lower HIF-1α levels in the lymph nodes versus the primary solid tumors. Our results suggest that HIF-1α expression levels are higher in the primary tumors than their matching lymph node metastases. Seventy percent of primary tumors appeared HIF-1α-positive ($\geqslant 3+$) while only 10% of lymph node metastases were found to be HIF-1α-positive, as summarized in Table 4. Two representative micrographs of HIF-1α immunostaining from the primary tumors and their corresponding lymph node metastases are shown in Fig. 5. In particular, there was a significant correlation found between HIF-1α and CXCR4 expression in both the lymph node metastases and primary tumors ($R = 0.6455$, $P < 0.05$).

Table 4
Summary of HIF-1α immunostaining of primary and metastatic breast cancer tissues

	HIF-1 staining		
	1 & 2+	3 & 4+	*P* value
Primary tumor	22 (30%)	52 (70%)	<0.0001
Lymph node metastases	18 (90%)	2 (10%)	

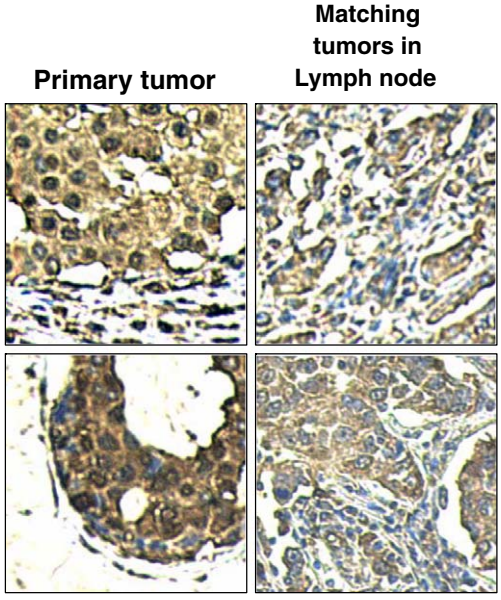

Fig. 5. The representative micrographs of HIF-1α immunostaining in the matching primary tumors and their counterpart in the lymph nodes. HIF-1α protein is shown in brown color (DAB). The results are summarized in Table 4.

Discussion

CXCR4 is a member of the G-protein coupled-receptor family that is characterized by seven transmembrane domains coupled to G-proteins. Like most surface receptors, CXCR4 is internalized to the cytoplasmic domain by the binding of its ligand [20–22]. Previously, we demonstrated that a CXCR4 antagonist (TN14003) effectively inhibited the interaction between CXCR4 and SDF-1 by binding to the ligand binding site on CXCR4 at nanomolar concentrations. We also showed that use of biotin-labeled TN14003 for immunohistochemical staining was superior to that of commercially available antibodies (R and D Systems) [13]. By utilizing this imaging probe, we were able to determine not only the staining intensity but also the subcellular localization of CXCR4 proteins. We found that both primary and metastatic tumors in the lymph nodes over-expressed CXCR4 while normal breast tissues only expressed low levels of CXCR4. Our results also indicated that lymph node metastases expressed lower levels of CXCR4 than primary tumors. Similar to previous observations [15,23], we identified that CXCR4 found in patient tissue samples was not limited to the cell membrane, but was also observed frequently in the cytoplasm, and occasionally in the nucleus. Pure membrane expression in vivo was rarely seen. In cultured cells, we identified that SDF-1 binding to CXCR4 induced the translocation of CXCR4 to the cytoplasm and even to the nucleus at higher concentrations of SDF-1. This phenomenon is comparable to the mechanism observed for the epidermal growth factor receptor (EGFR), another membrane receptor. Lin et al. have demonstrated that when the epidermal growth factor (EGF) binds to the EGFR, EGFR translocates to the nucleus, binds to the promoter region of the cyclin D1 gene, and increases its transcription [24]. Thus, the nuclear translocation of EGFR correlates well with cell proliferation. It will be of interest to examine whether CXCR4 plays a similar biological role when it translocates to the cytoplasm and the nucleus. Stronger membrane staining was found in primary tumors than in their corresponding lymph node metastases. Also, more homogeneous cytoplasmic staining was observed in lymph node

metastases, which may be caused by increased translocation of CXCR4 mediated by high levels of SDF-1 in the lymph nodes. Internalization of CXCR4 may activate important signaling pathways, including the Akt pathway, which would impact the ability of the tumors to survive. In addition, ligand-mediated degradation of CXCR4 started 3 h after treatment with SDF-1 [27] while CXCR4/SDF-1-mediated Akt phosphorylation began approximately 5 min after SDF-1 treatment. This demonstrates that ligand-mediated CXCR4/SDF-1 degradation does not impact activation of other signaling pathways. Combined, these results suggest that a reduction in membrane staining of CXCR4 in lymph node metastases may be associated with the progression of breast cancer metastasis.

Currently, the failure of endocrine treatments in some breast cancer patients with ER-positive primary tumors is believed to result from a loss of estrogen receptors in malignant cells [25,26]. Trastuzumab is a well-known antibody therapy agent against breast cancers with human epidermal growth factor receptor-2 (HER-2) over-expression. However, it has been shown that less than half of the patients with elevated HER-2 levels respond to Trastuzumab treatment. Though tumor resistance to Trastuzumab is not well clarified, one possible explanation may be the reduced HER-2 levels in the metastasized tumor cells compared to the primary tumor cells [27]. In a similar situation, our data demonstrated that overall expression of the CXCR4 protein was reduced in lymph node metastases with predominantly CXCR4 cytoplasmic staining. We have explained two potential mechanisms behind weaker CXCR4 expression in breast cancer cells that have metastasized to the lymph nodes compared to those found at the primary site. An inhibitor of lysosome function, chloroquine, blocked the degradation of CXCR4 mediated by SDF-1. Thus, these data support our hypothesis that high levels of SDF-1 in the lymph nodes lead to CXCR4 internalization and further degradation of the CXCR4 protein through the lysosome pathway. Two previous reports have also shown the down-regulation of CXCR4 by SDF-1 [21,28]. In addition, the HIF-1α levels in the primary tumors are higher than those of the metastasized tumor cells in the lymph nodes, which may suggest a more hypoxic environment in the primary tumors than the metastasized tumors in the lymph nodes. This may be one of the causes of lowered expression levels of CXCR4 in lymph node metastases. Hypoxia has been shown to increase CXCR4 expression through positive regulation by HIF-1α and negative regulation by von Hippel-Lindau tumor suppressor protein (VHL) [28]. It has been demonstrated that CXCR4 plays a critical role in the metastasis of cancer cells and, therefore, it is an important target for therapies that inhibit metastatic progression. Our findings of heterogeneous expression of CXCR4 in primary tumors and lymph node metastases contribute greatly to the building evidence for designing therapies which target CXCR4.

In conclusion, we observed that total CXCR4 levels decreased as breast cancer cells metastasized to the lymph nodes and the localization of the CXCR4 protein changed from being a combination of strong membranous and cytoplasmic to homogeneously cytoplasmic as the cancer cells migrated to the lymph nodes. We found that these phenotypes are attributed to elevated levels of SDF-1 in lymph nodes and reduced expression levels of HIF-1α in metastases. SDF-1 both down-regulated membranous CXCR4 expression and degraded the internalized CXCR4 receptors through the lysosome pathway. Lower HIF-1α levels, probably induced by the less hypoxic microenvironment in the lymph nodes, further reduced CXCR4 levels in the lymph node metastases. Our study suggests that analysis of the subcellular location and intensity of CXCR4 staining in primary tumors and lymph node metastases may enhance our ability to predict the biologic behavior of breast cancer and to devise a treatment plan accordingly.

Acknowledgments

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