Activation of STAT3 is involved in malignancy mediated by CXCL12-CXCR4 signaling in human breast cancer

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Abstract. The chemokine receptor CXCR4 and signal transducer and activator of transcription 3 (STAT3) play an important role in breast cancer malignancy and metastasis. However, it remains unknown whether STAT3 can be activated by CXCR4 in human breast cancer. The expression levels of CXCR4, STAT3 and p-STAT3 in 208 breast cancer tissues and 26 tumor-adjacent tissues were examined by immunohistochemistry. Flow cytometry, western blot analysis and immunoprecipitation were used to study activation of STAT3 by CXCL12-CXCR4 signaling in human breast cancer cell lines. The expression levels of CXCR4, STAT3 and p-STAT3 were higher in the breast cancer samples than these levels in the tumor-adjacent samples. The combined expression of CXCR4 and p-STAT3 was correlated with TNM stage, tumor size, lymph node metastasis and histological grade of breast cancer. In the breast cancer cells, CXCL12 treatment increased the expression of p-STAT3. The CXCR4 antagonist AMD3100 and the Janus kinase 2 (JAK2) antagonist AG490 inhibited the CXCL12-induced increase in the phosphorylation of STAT3. Furthermore, CXCL12 promoted direct binding of JAK2 to CXCR4. Our findings suggest that activation of the JAK2/STAT3 pathway via CXCL12-CXCR4 signaling plays an important role in breast cancer malignancy and metastasis. Targeting the CXCL12-CXCR4/JAK2/STAT3 signaling pathway may be a potential therapeutic strategy for the treatment of breast cancer.

Introduction

Breast cancer is one of the most common malignant cancers in women, and is one of the leading causes of cancer-related deaths worldwide. Distant metastasis is the main cause of breast cancer-related mortality (1). However, the mechanisms leading to breast cancer metastasis remain poorly understood. Breast cancer commonly metastasizes to certain organs such as the lymph node, bone marrow, and lung, which exhibit enhanced secretion of cytokines, but rarely to other organs with low expression of cytokines, such as the kidney and skin. This characteristic feature highlights the important role played by chemokines and their receptors in breast cancer metastasis (2,3).

CXCR4 and its ligand CXCL12 (also called SDF-1 α) play a critical role in breast cancer carcinogenesis and metastasis, and targeting the CXCL12-CXCR4 signaling pathway is a potential therapeutic strategy for the treatment of breast cancer (3,4). Activation of CXCL12-CXCR4 signaling has been reported to promote survival, proliferation, adhesion, chemotaxis and migration of breast cancer cells (5). The binding of CXCL12 to CXCR4 results in activation of several signaling pathways in breast cancer including the MAPK/ERK1/2 (6,7) and PI3K/AKT (8,9) pathways. It has been reported that CXCL12-CXCR4 signaling activates the Janus kinase 2 (JAK2) signal transducer and activator of transcription 3 (STAT3) pathway (10,11), and STAT3 inactivation inhibits murine breast cancer metastasis (12). However, it remains unclear whether activation of the JAK2/STAT3 pathway is involved in the CXCL12-CXCR4 signaling axis in human breast cancer.

STAT3, a transcription factor that belongs to the STAT family, has been found to be overexpressed and constitutively activated in many malignant tumors (13-15). STAT3 has been regarded as an oncogene, and constitutive activation of STAT3 can lead to abnormal cell proliferation and malignant transformation (16,17). JAK2 can recruit and activate STAT3, which translocates to the nucleus and regulates transcription of a variety of genes that are associated with proliferation, differentiation, apoptosis and metastasis of many types of cancer cells (18,19). However, it remains unclear whether the JAK2/STAT3 pathway is coupled to CXCL12-CXCR4 signaling. It has been reported that in response to CXCL12, CXCR4 activates the JAK2/STAT3 pathway (10,11,20). However, Moriguchi *et al* reported that CXCL12-CXCR4 signaling is independent of the JAK2/STAT3 pathway in primary lymphocytes (21). Furthermore, STAT3 activation

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is associated with the CXCR4 signaling pathway in many types of cancers such as cervical carcinoma (22), small cell lung cancer (23) and bladder cancer (24). However, Lee *et al* reported that CXCL12 failed to activate STAT3 in gastric cancer cells (25). Constitutively activated STAT3 was found in primary tumors from high risk breast cancer patients (26), and is associated with breast cancer growth and metastasis in xenograft models (27,28). However, it remains unclear whether CXCL12-CXCR4 signaling is coupled to the JAK2/STAT3 pathway in human breast cancer.

In the present study, we aimed to investigate the association between CXCR4 and STAT3, particularly p-STAT3, in breast cancer tissues from Chinese patients, and to identify the JAK2/STAT3 pathway in CXCL12-CXCR4 signaling in human breast cell lines. We performed immunohistochemistry to examine the expression of CXCR4, STAT3 and p-STAT3 in 208 patients with breast cancer, and analyzed their correlation with clinicopathological characteristics of these patients. We found that the expression levels of CXCR4 and STAT3, particularly p-STAT3, were increased with increased TNM stage and lymph node metastasis. The combined expression of both CXCR4 and p-STAT3 was correlated with breast cancer malignancy. Furthermore, we found that inhibition of CXCR4 or JAK2 prevented CXCL12-induced phosphorylation of STAT3 in breast cancer cell lines. Our results suggest that activation of the JAK2/STAT3 pathway by CXCL12-CXCR4 signaling may play an important role in breast cancer malignancy, and targeting the CXCL12-CXCR4/JAK2/STAT3 signaling pathway may be a potential therapeutic strategy for the treatment of breast cancer.

Materials and methods

Patients and tissue samples. The Ethics Committee of China Medical University approved this study. Human breast cancer samples of 208 female patients with primary breast cancer were collected at the Department of Surgical Oncology and The Department of General Surgery of the First Affiliated Hospital of China Medical University between 2003 and 2010. Some of the samples were used in our previous studies (29,30). The median age of the breast cancer patients was 51 years (range, 29 to 85 years). Of the 208 patients, the stage and the histological grading of the cancer were evaluated according to the TNM staging system and the Elston-Ellis modification of Scarff-Bloom-Richardson grading system, respectively. Clinicopathological data including patient age, menopausal status, tumor size, tumor type, lymph node metastasis, and the status of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (HER2) were retrospectively retrieved from the medical records. Of the 208 patients, 182 patients had invasive ductal carcinoma, and 10 had invasive lobular carcinoma. Other patients presenting with tumors of less incidence were categorized into one group, including 3 patients with cribriform carcinoma, 3 patients with micropapillary carcinoma, 2 patients with mucinous carcinoma, 2 patients with medullary carcinoma, 2 patients with papillary carcinoma, 1 patient with tubular carcinoma and 3 patients with ductal carcinoma *in situ*. This study included 26 tumor-adjacent samples as controls. The breast tissues outside the cancer loci were selected as tumor-adjacent samples. The diagnosis of breast cancer was confirmed by pathological staining. The tumor-adjacent samples exhibited no tumor texture histologically. All patients did not undergo radiation therapy, chemotherapy and hormonal therapy.

Cell culture. Human breast cancer cell lines (MDA-MB-231, BT-549, and MCF-7) were obtained from the American Type Culture Collection (ATCC), and maintained according to ATCC's recommendation. To investigate the effect of CXCR4 on the phosphorylation of STAT3, human breast cancer cells were treated with 100 ng/ml CXCL12 (350-NF/CF; R&D Systems Inc.) for 0, 2, 5, 15 and 30 min after serum-starvation for 4 h. To investigate whether CXCR4 or JAK2 mediates activation of STAT3 by CXCL12, cells were pretreated with the CXCR4 antagonist AMD3100 (10 μM, A5602; Sigma-Aldrich St. Louis, MO, USA) or the JAK2 inhibitor AG490 (25 μM, 658401; Calbiochem) for 2 h before treatment with CXCL12.

Immunohistochemistry. Tissue sections (4-μm thick) were obtained from formalin-fixed and paraffin-embedded tissue blocks from the control and breast cancer samples. Sections were washed in xylene to remove the paraffin, and rehydrated with serial dilutions of alcohol, followed by a wash in PBS solution. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide in methanol at 37˚C for 20 min. Sections were then incubated with primary antibodies against CXCR4 (1:100 dilution, ab2074; Abcam), STAT3 (1:50 dilution, #4904; Cell Signaling Technology) and p-STAT3 (Tyr705) (1:200 dilution, #9145; Cell Signaling Technology) overnight at 4˚C. After the primary antibody was washed off, sections were incubated with goat anti-rabbit biotin-conjugated secondary antibodies (1:1000 dilution; Dako, Glostrup, Denmark) for 30 min at 37˚C. The tissue sections were then incubated with streptavidin horseradish peroxidase for 30 min at 37˚C. DAB (3,3-diaminobenzidine) substrate was applied to the sections, and then sections were counterstained with hematoxylin. Sections in which primary antibodies were omitted were used as negative control.

Immunostaining was examined under a light microscope by two pathologists blinded to the experimental conditions. Agreement on the scores between the two pathologists was nearly 100%. In cases in which the pathologists disagreed to the score, the immunohistochemical scoring was repeated by both pathologists until the same score was achieved. The immunoreactivity was evaluated using a scoring system according to the percentage of stained cells and the intensity of the immunoreactivity. The intensity of immunoreactivity was scored as follows: 0 for no staining, 1 for weak staining, 2 for moderate staining, and 3 for strong staining. The percentage of stained cells was scored as follows: 0 for <10%, 1 for 10-29%, 2 for 30-49%, 3 for 50-74%, and 4 for ≥75%. The final immunoreactive score was determined by multiplying the intensity score with the score for the percentage of positively stained cells. The minimum score was 0 and the maximum score was 12. Low and high expression of CXCR4, STAT3 and p-STAT3 was defined by a final score of <6 and ≥6, respectively.

Flow cytometry. Flow cytometric analysis was performed on a FACSCalibur (Becton-Dickinson, Franklin Lakes, NJ, USA).

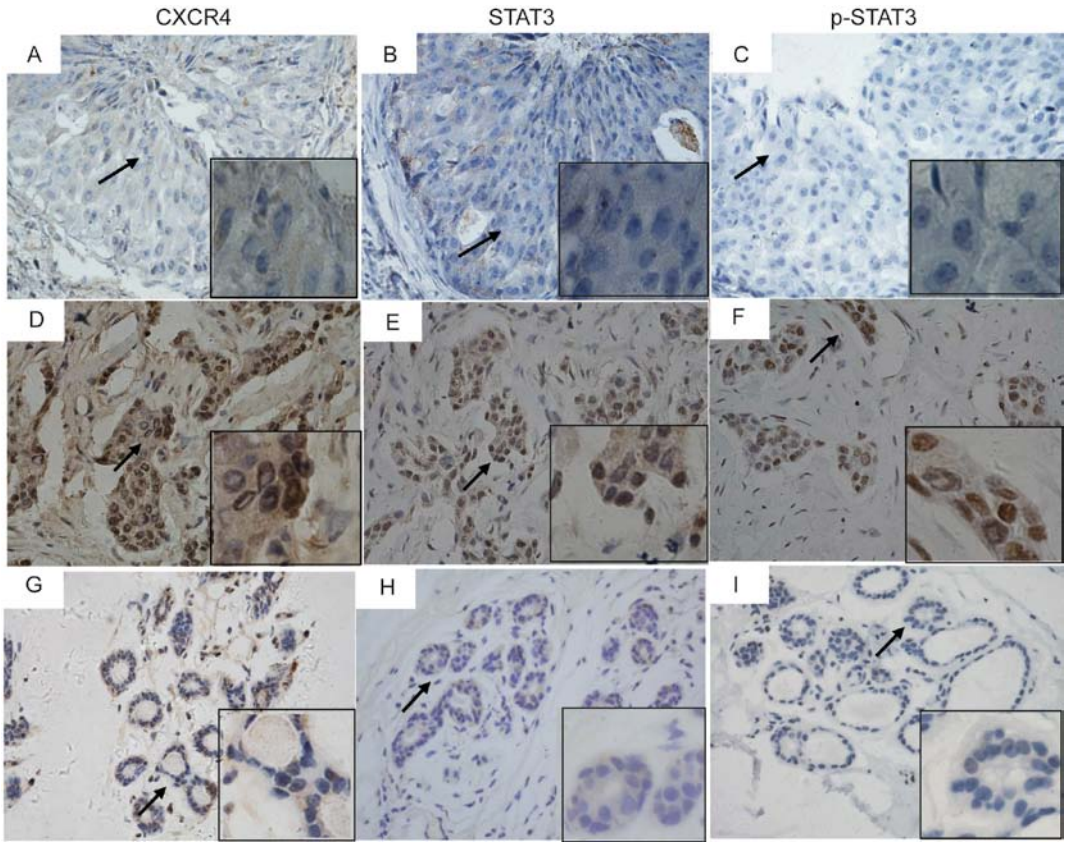

Figure 1. Immunohistochemical staining of CXCR4 (A, D and G), STAT3 (B, E and H), and phospho-STAT3 (Tyr705) (C, F and I) in breast cancer tissues (A-F) and tumor-adjacent tissues (G-I). Representative micrographs of low expression (A-C), and high expression (D-F) of CXCR4, STAT3 and p-STAT3 in consecutive sections of breast cancer tissues, and the expression of CXCR4, STAT3, and p-STAT3 in tumor-adjacent tissues (G-I). Magnification; x400. Areas indicated by an arrow are magnified in inserts (x1,000).

Cells (1x10^6 cells/ml) were fixed with 4% paraformaldehyde, and then washed and resuspended in PBS containing 0.5% bovine serum albumin. Cells were permeabilized with 0.1% Triton-X 100, and incubated with the primary antibodies against CXCR4 (1:100 dilution) for 30 min at room temperature. Isotype rabbit IgG was used as a control. After washing, cells were labeled with Alexa Fluor 488-conjugated secondary antibodies (Molecular Probes, A21206; Invitrogen) for 1 h at 4˚C. Cells were subsequently analyzed by flow cytometry.

Western blot analysis. Human breast cancer cells were homogenized on ice in RIPA lysis buffer (Beyotime, Nantong, China) containing a cocktail of protease and phosphatase inhibitors (Sigma-Aldrich). Proteins were resolved by SDS-PAGE, and transferred onto polyvinylidene fluoride membranes by electroblotting. The membranes were incubated with primary antibodies against CXCR4 (1:2,000 dilution), STAT3 (1:4,000 dilution) and p-STAT3 (1:2,000 dilution) at 4˚C with gentle shaking overnight. GAPDH was used as a loading control. The membranes were then incubated with horseradish peroxidase-linked goat anti-rabbit secondary antibodies (dilution 1:5,000) at room temperature for 2 h. Bands were visualized using a chemiluminescence detection system.

Immunoprecipitation. MCF-7 cells were solubilized on ice with lysis buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1% Triton X-100) supplemented with a cocktail of phosphatase and proteinase inhibitors (Sigma-Aldrich). Lysates were centrifuged at 10,000 x g for 15 min at 4˚C. The supernatants were incubated with antibodies against CXCR4 (1:100 dilution) or non-specific rabbit IgG (control), and protein A-agarose at 4˚C overnight. The immunoprecipitates were collected by centrifugation, and the agarose pellet was suspended in 2X sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer. The expression of JAK2 was determined by western blotting, using antibodies against JAK2 (1:1,000 dilution, #3230; Cell Signaling Technology).

Statistical analysis. Statistical analyses were performed using SPSS 11.5. The numerical data are presented as mean and standard deviation. Student t-test or one-way analysis of variance (ANOVA) was used to compare the difference in the means among two or more groups, respectively. Categorical data were compared with Pearson Chi squared tests. The Spearman's correlation analysis was applied to assess the association of the expression of CXCR4 with the expression of STAT3 and p-STAT3. Probability (P)-values <0.05 were considered to indicate statistically significant results.

Results

Expression of CXCR4, STAT3 and p-STAT3 in the breast cancer tissues. The clinicopathological characteristics of 208 patients with breast cancer are shown in Table I. Of the 208 breast cancer patients, the TNM stage, tumor size, lymph node metastasis, and histological grading were recorded in 194, 193,

Table I. Correlation of the expression of CXCR4, STAT3 and p-STAT3 with the clinicopathological parameters of the breast cancer cases.

		CXCR4 expression n (%)			STAT3 expression n (%)			p-STAT3 expression n (%)		
Parameters	N[a]/208	Low	High	P-value[b]	Low	High	P-value[b]	Low	High	P-value[b]
Age (years)	208/208			0.563			0.749			0.189
<50	86	27 (31.4)	59 (68.6)		25 (29.1)	61 (70.9)		53 (61.6)	33 (38.4)	
≥50	122	43 (35.2)	79 (64.8)		33 (27.0)	89 (73.0)		64 (52.5)	58 (47.5)	
Menopause status	196/208			0.590			0.160			0.080
Pre-menopausal	83	31 (37.3)	52 (62.7)		29 (34.9)	54 (65.1)		53 (63.9)	30 (36.1)	
Post-menopausal	113	38 (33.6)	75 (66.4)		29 (25.7)	84 (74.3)		58 (51.3)	55 (48.7)	
Tumor type	208/208			0.850			0.080			0.970
Ductal	182	60 (33.0)	122 (67.0)		46 (25.3)	136 (74.7)		102 (56.0)	80 (44.0)	
Lobular	10	4 (40.0)	6 (60.0)		5 (50.0)	5 (50.0)		6 (60.0)	4 (40.0)	
Others	16	6 (37.5)	10 (62.5)		7 (43.8)	9 (56.2)		9 (56.2)	7 (43.8)	
TNM stage	194/208			**0.002**			**0.045**			**0.000**
I	29	16 (64.7)	13 (35.3)		11 (37.9)	18 (62.1)		21 (72.4)	8 (27.6)	
II	100	38 (38.0)	62 (62.0)		32 (32.0)	68 (68.0)		65 (65.0)	35 (35.0)	
III-IV	65	13 (20.0)	52 (80.0)		11 (16.9)	54 (83.1)		22 (33.8)	43 (66.2)	
Tumor size (cm)	193/208			0.193			0.999			0.080
≤2.0	53	23 (43.4)	30 (56.6)		15 (28.3)	38 (71.7)		31 (58.5)	22 (41.5)	
>2.0 to ≤5.0	98	32 (33.5)	66 (64.5)		28 (28.6)	70 (71.4)		62 (63.3)	36 (36.7)	
>5.0	42	11 (26.2)	31 (73.8)		12 (28.6)	30 (71.4)		18 (42.9)	24 (57.1)	
LNM	186/208			**0.025**			**0.040**			**0.023**
No	78	34 (43.6)	44 (56.4)		28 (35.9)	50 (64.1)		50 (64.1)	28 (35.9)	
Yes	108	30 (27.8)	78 (72.2)		24 (22.2)	84 (77.8)		51 (51.0)	57 (57.0)	
Histological grade	177/208			0.277			0.072			**0.000**
I	25	10 (40.0)	15 (60.0)		11 (44.0)	14 (56.0)		22 (88.0)	3 (12.0)	
II	131	46 (35.1)	85 (64.9)		35 (26.7)	96 (73.3)		71 (54.2)	60 (45.8)	
III	21	4 (19.0)	17 (81.0)		3 (14.3)	18 (85.7)		6 (28.6)	15 (71.4)	
ER	179/208			0.640			0.964			0.312
Negative	68	25 (36.8)	43 (63.2)		20 (29.4)	48 (70.6)		42 (61.8)	26 (38.2)	
Positive	111	37 (33.3)	74 (66.7)		33 (29.7)	78 (70.3)		60 (54.1)	51 (45.9)	
PR	179/208			0.415			0.978			0.752
Negative	72	22 (30.6)	50 (69.4)		21 (29.2)	51 (70.8)		40 (55.6)	32 (44.4)	
Positive	107	39 (36.4)	68 (63.6)		31 (29.0)	76 (71.0)		62 (57.9)	45 (42.1)	
HER2	164/208			0.307			0.852			0.584
Negative	53	21 (39.6)	32 (60.4)		17 (32.1)	36 (67.9)		32 (60.4)	21 (39.6)	
Positive	111	35 (31.5)	76 (68.5)		34 (30.6)	77 (69.4)		62 (55.9)	49 (44.1)	

[a]Number of cases for which corresponding data are available. [b]P-value obtained from Pearson Chi-square test. LNM, lymph node metastasis; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; STAT3, signal transducer and activator of transcription 3.

186 and 177 patients, respectively. ER, PR, and HER2 were examined in 179, 179 and 164 patients, respectively.

We studied the expression of CXCR4, STAT3, and p-STAT3 in 208 tumor samples and in 26 tumor-adjacent samples from patients with breast cancer, using immunohistochemistry (Fig. 1). The expression levels of CXCR4, STAT3, and p-STAT3 were higher in the breast cancer samples than these levels in the tumor-adjacent samples (Fig. 2). CXCR4 immunoreactivity showed low expression and high expression in 70 (33.6%) and 138 (66.4%) of the 208 breast cancer samples, respectively, and in 19 (70.1%) and 7 (26.9%) of the 26 tumor-adjacent samples, respectively (Fig. 2, P<0.001). The expression level of CXCR4 increased with increased TNM stage (P=0.025) and lymph node metastasis (P=0.039), but not with tumor size (P=0.193) and histological grade (P=0.277) (Table I). STAT3 immunoreactivity showed low expression

Table II. Correlation of the combined expression of CXCR4/STAT3 or CXCR4/pSTAT3 with TNM stage, tumor size, metastasis and histological grade in the breast cancer cases.

Parameters	CXCR4/STAT3 expression, n (%)				CXCR4/p-STAT3 expression, n (%)			
	Low/Low	Low/High or High/Low	High/High	P-value[b]	Low/Low	Low/High or High/Low	High/High	P-value[b]
TNM stage								
I	7 (24.1)	14 (48.3)	8 (27.6)	**0.002**	12 (41.4)	13 (44.8)	4 (13.8)	**<0.001**
II	18 (18.0)	34 (34.0)	48 (48.0)		29 (29.0)	45 (45.0)	26 (26.0)	
III-IV	5 (7.7)	14 (21.5)	46 (70.8)		10 (15.4)	17 (26.2)	38 (58.5)	
Tumor size (cm)								
≤2.0	10 (18.9)	20 (37.7)	23 (43.4)	0.348	16 (30.2)	24 (45.3)	13 (24.5)	**0.043**
>2.0 to <4.0	13 (13.3)	34 (34.7)	51 (52.0)		25 (25.5)	43 (43.9)	30 (30.6)	
≥4.0	7 (16.7)	9 (21.4)	26 (61.9)		10 (23.8)	10 (23.8)	22 (52.4)	
LNM								
No	15 (19.2)	33 (42.3)	30 (38.5)	**0.002**	24 (30.8)	35 (44.9)	19 (24.4)	**0.013**
Yes	15 (13.9)	24 (22.2)	69 (63.9)		25 (23.1)	34 (31.5)	49 (45.4)	
Histological grade								
I	7 (28.0)	8 (32.0)	10 (40.0)	0.078	10 (40.0)	12 (48.0)	3 (12.0)	**0.027**
II	19 (14.5)	44 (33.6)	68 (51.9)		33 (25.2)	52 (35.7)	46 (35.1)	
III	1 (4.8)	4 (19.0)	16 (76.2)		3 (14.3)	6 (28.6)	12 (57.1)	

[b]P-value obtained from Pearson Chi-square test. LNM, lymph node metastasis.

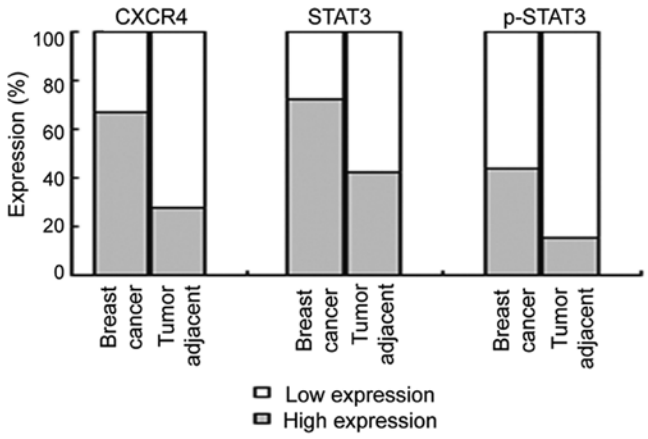

Figure 2. The low and high expression of CXCR4, STAT3 and p-STAT3 in breast cancer samples and tumor-adjacent samples. The expression levels of CXCR4, STAT3 and p-STAT3 in breast cancer samples were significantly higher than levels in the tumor-adjacent samples (Pearson Chi-square; P<0.001 for CXCR4, P=0.002 for STAT3, P=0.003 for p-STAT3).

and high expression in 58 (27.9%) and 150 (72.1%) of the 208 breast cancer samples, respectively and in 15 (57.7%) and 11 (42.3%) of 26 tumor-adjacent samples, respectively (Fig. 2, P=0.002). The expression level of STAT3 increased with increased TNM stage (P=0.045) and lymph node metastasis (P=0.040), but not with the tumor size (P=0.999) and histological grade (P=0.072) (Table I). p-STAT3 immunoreactivity showed low expression and high expression in 117 (56.2%) and 91 (43.8%) of the 208 breast cancer samples, respectively and in 22 (84.6%) and 4 (15.4%) of the 26 tumor-adjacent samples, respectively (Fig. 2, P=0.003). The expression of p-STAT3 was increased with increased TNM stage (P<0.001), lymph node metastasis (P=0.023) and histological grade (P<0.001), but not with the tumor size (P=0.080) (Table I). In addition, the expression levels of CXCR4, STAT3, and p-STAT3 did not significantly differ in regards to patient age, menstruation status, tumor type, ER status, PR status and HER2 status.

Association of the combined expression of CXCR4/STAT3 and CXCR4/p-STAT3 with clinicopathological characteristics of the breast cancer. We further investigated the correlation of the expression of CXCR4 with the expression of STAT3 and p-STAT3. A positive correlation was observed between the expression of CXCR4 and STAT3 (r=0.260, P<0.001) and between the expression of CXCR4 and p-STAT3 (r=0.300, P<0.001). These results suggest that the signaling pathway that involves CXCR4 and STAT3 may play a role in breast cancer carcinogenesis.

We then investigated the association of the combined expression of CXCR4 and STAT3 (CXCR4/STAT3) or CXCR4 and p-STAT3 (CXCR4/p-STAT3) with the clinicopathological characteristics of the breast cancer cases (Table II). Compared with the combined low expression of CXCR4 and STAT3 (Low/Low) and the high expression of either CXCR4 or STAT3 (High/Low or Low/High), the combined high expression of CXCR4/STAT3 (High/High) was highly correlated with TNM stage (P=0.002) and lymph node metastasis (P=0.002), but not with the tumor size (P=0.348) and histological grade

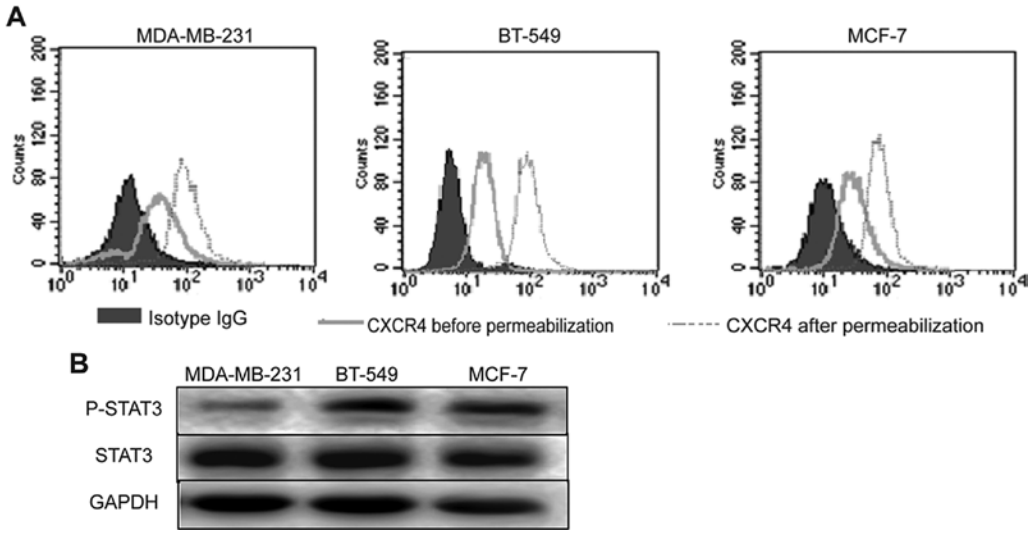

Figure 3. Endogenous expression of CXCR4, STAT3 and p-STAT3 in breast cancer cell lines. (A) Flow cytometric analysis of MDA-MB-231, BT-549 and MCF-7 cells. The cell surface expression of CXCR4 was analyzed using anti-CXCR4 antibodies. The total expression of CXCR4 was detected after cells were permeabilized. (B) Representative western blot analysis showing the expression of STAT3 and p-STAT3 in MDA-MB-231, BT-549 and MCF-7 cells. The experiments were repeated three times.

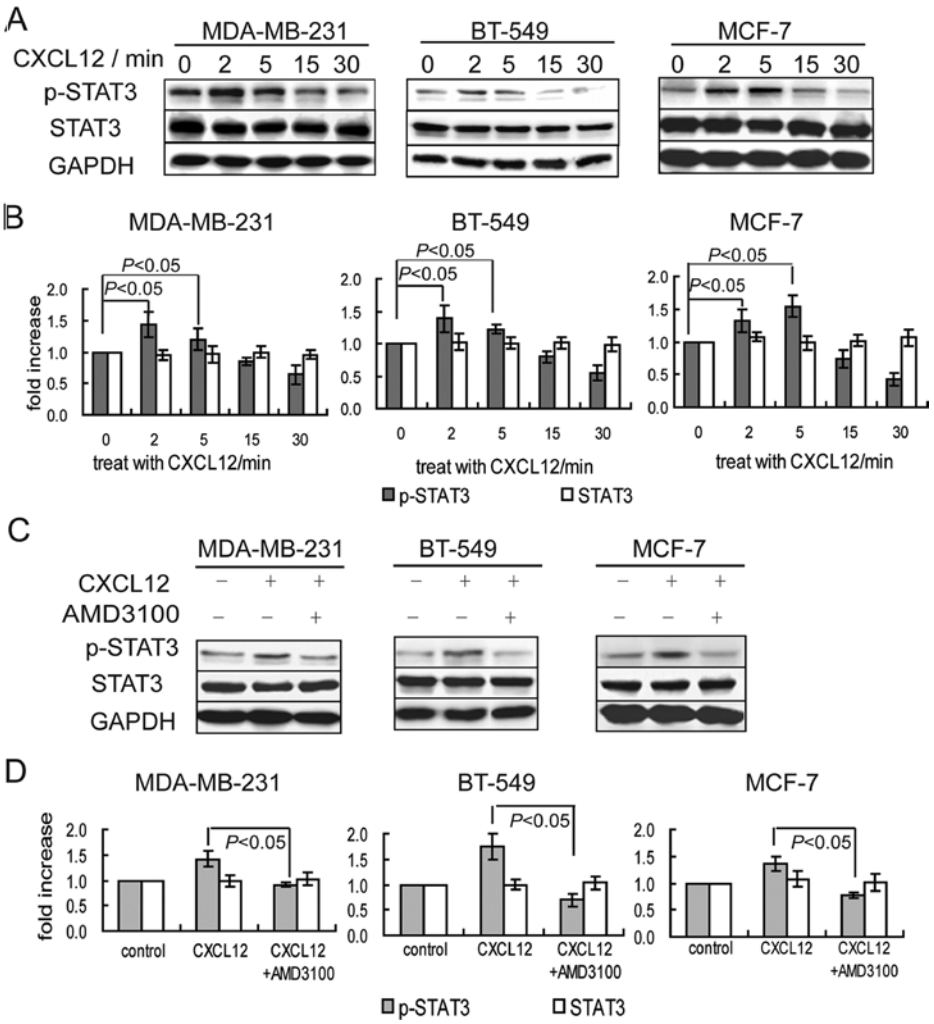

Figure 4. Activation of STAT3 by CXCL12 in breast cancer cells. (A) Representative western blot analysis showing the expression of STAT3 and p-STAT3 in MDA-MB-231, BT-549 and MCF-7 cells treated with CXCL12 (100 ng/ml) for 0, 2, 5, 15 and 30 min. GAPDH was used as a loading control. (B) Quantification of p-STAT3 and STAT3 expression normalized to GAPDH; n=3. (C) Representative western blot analysis showing the expression of STAT3 and p-STAT3 in MDA-MB-231, BT-549 and MCF-7 cells treated with CXCL12 (100 ng/ml) in the presence or absence of AMD3100. Cells were treated with AMD3100 (10 μM) for 2 h followed by stimulation with CXCL12 for 2 min (MDA-MB-231 and BT-549) or 5 min (MCF-7). GAPDH was used as a loading control. (D) Quantification of p-STAT3 and STAT3 expression normalized to GAPDH; n=3.

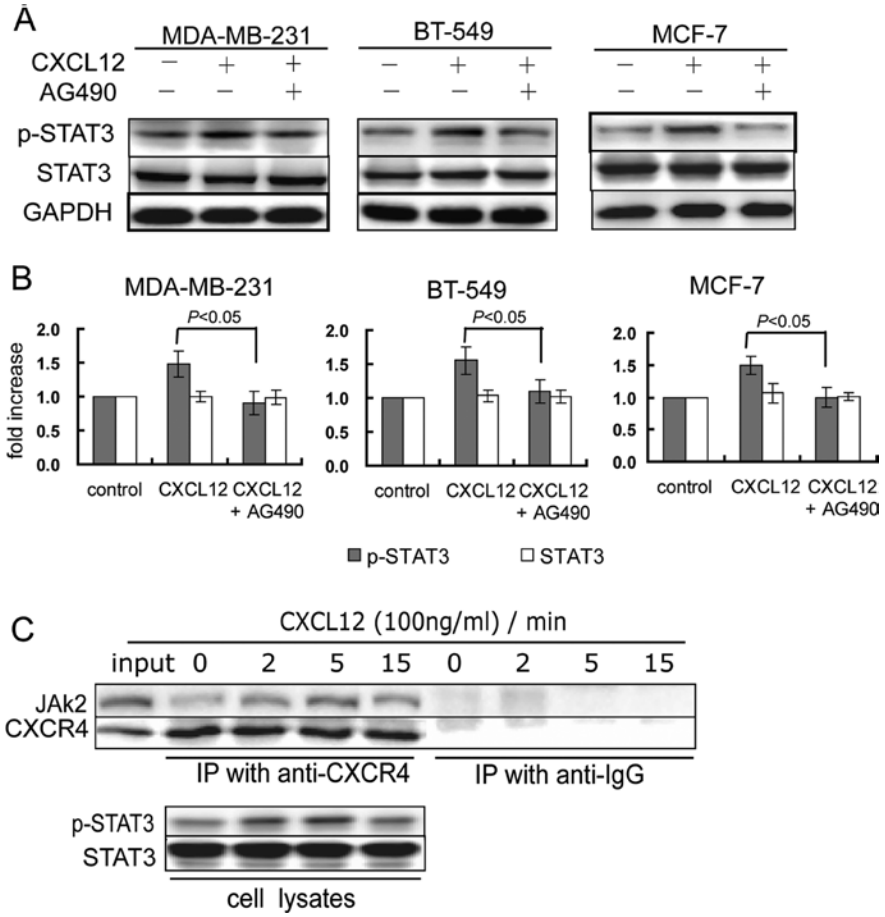

Figure 5. CXCL12-CXCR4 signaling activates the JAK2/STAT3 pathway. (A) Representative western blot analysis showing the expression of STAT3 and p-STAT3 in MDA-MB-231, BT-549 and MCF-7 cells treated with CXCL12 (100 ng/ml) in the presence or absence of AG490. Cells were treated with AG490 (25 μM) for 2 h followed by stimulation with CXCL12 for 2 min (MDA-MB-231 and BT-549) or 5 min (MCF-7). GAPDH was used as a loading control. (B) Quantification of p-STAT3 and STAT3 expression normalized to GAPDH; n=3. (C) Immunoprecipitation detecting the direct binding of Janus kinase 2 (JAK2) to CXCR4. MCF-7 cells were treated with CXCL12 (100 ng/ml) for 0, 2, 5 or 15 min. Cell lysates were immunoprecipitated with CXCR4 antibodies or istotype IgG, and the expression of JAK2 was determined by the western blot analysis. The expression of p-STAT3 and STAT3 in cell lysates was detected by western blot analysis. The experiments were repeated three times.

(P=0.078). Compared with the combined low expression of CXCR4 and p-STAT3 (Low/Low) and the high expression of either CXCR4 or p-STAT3 (High/Low or Low/High), the combined high expression of CXCR4/p-STAT3 (High/High) was highly correlated with TNM stage (P<0.001), tumor size (P=0.043), lymph node metastasis (P=0.013) and histological grade (P=0.027).

CXCR4-mediated activation of JAK2/STAT3 in human breast cancer cell lines. The positive correlation of CXCR4 expression with the expression level of STAT3 and p-STAT3 in breast cancer suggests that p-STAT3 may be regulated by CXCR4. To investigate the signaling pathway that is involved in activation of STAT3 by CXCR4, three human breast cancer cell lines (MDA-MB-231, BT-549 and MCF-7) were analyzed for their expression of CXCR4, STAT3 and p-STAT3, using flow cytometry and western blot analysis. Flow cytometric analysis showed that CXCR4 was endogenously expressed in the cell membrane and cytoplasm of the MDA-MB-231, BT-549 and MCF-7 cells (Fig. 3A). Western blot analysis showed constitutive expression of STAT3 and p-STAT3 in all the breast cancer cell lines (Fig. 3B).

To test whether CXCR4 promotes the phosphorylation of STAT3, human breast cancer cells were treated with the CXCR4 agonist CXCL12 for 0, 2, 5, 15 and 30 min. The expression of p-STAT3 in these cells was significantly increased at 2 to 5 min after CXCL12 treatment, and gradually declined at 15 to 30 min after CXCL12 treatment (Fig. 4A and B). Pretreatment with the CXCR4 antagonist AMD3100 inhibited the CXCL12-induced increase in the phosphorylation of STAT3 (Fig. 4C and D). These results suggest that the activation of CXCR4 promoted the expression of p-STAT3 in breast cancer cells.

STAT3 has been shown to be activated by JAK2 (11,31). We then investigated whether CXCR4 mediates activation of STAT3 via JAK2. Pretreatment with the JAK2 inhibitor AG490 inhibited CXCL12-induced phosphorylation of STAT3 (Fig. 5A and B), suggesting that JAK2 mediated CXCR4-induced activation of STAT3. Furthermore, we investigated whether CXCR4 directly interacted with JAK2 in MCF-7 cells, using immunoprecipitation. In the absence of CXCL12, JAK2 was immunoprecipitated with CXCR4. After CXCL12 treatment for 2 to 5 min, the binding of JAK2 to CXCR4 was increased, accompanied by an increase in the expression of

p-STAT3 (Fig. 5C), suggesting that CXCR4 directly activated JAK2. These results suggest that CXCR4 mediates the JAK2/STAT3 activation in breast cancer cells.

Discussion

CXCR4 and STAT3 have been known to play an important role in growth, progression, angiogenesis and metastasis of many tumor types (3,18,32). In breast cancer, high CXCR4 expression has been found to be associated with poor prognosis (2,33), and the CXCL12-CXCR4 signaling axis mediates breast cancer metastasis (34,35). It is known that constitutively activated STAT3 is associated with breast cancer growth and metastasis (27,28). In addition, it has been reported that CXCR4 contributes to murine breast cancer growth and metastasis via modulation of STAT3 (36). However, the association of CXCR4 and STAT3 has not been studied in breast cancer patients. In the present study, we investigated the expression of CXCR4, STAT3 and p-STAT3 in 208 breast cancer patients, and analyzed their correlations with clinicopathological features of these patients. We found that the expression of CXCR4, STAT3 and p-STAT3 was higher in the breast cancer tissues than that in the tumor-adjacent tissues. The combined expression levels of CXCR4/p-STAT3 were positively associated with TNM stage, tumor size, lymph node metastasis and histological grade. Furthermore, we found that CXCL12-CXCR4 induced phosphorylation of STAT3 via JAK2 in the breast cancer cell lines. Our results suggest that the CXCL12-CXCR4/JAK2/STAT3 signaling pathway may play an important role in breast cancer malignancy and metastasis.

CXCR4 expression has been found to be upregulated in primary breast cancers, and is associated with breast cancer metastasis (37-39). Consistent with previous studies, we found that CXCR4 was highly expressed in breast cancer tissues, and the CXCR4 expression was correlated with TNM stage and lymph node metastasis. These results suggest that increased receptor numbers, at least in part, contribute to breast cancer metastasis. It has been reported that upregulation of CXCR4 receptors is induced by several oncogenic events such as hypoxia (40), HER2 overexpression (41), EGFR variant-mediated invasion (42) and TGFβ1 signaling (43), suggesting that CXCR4 mediates many oncogenic signals that lead to breast cancer metastasis. CXCL12, the CXCR4 ligand, attracts tumor cells expressing CXCR4 to organs such as the lymph node and bone marrow that produce CXCL12, thereby mediating metastasis. Our finding that CXCR4 expression is correlated with the lymph node metastasis in breast cancers highlights the important role played by CXCL12-CXCR4 signaling in breast cancer metastasis.

Several downstream signaling pathways including the JAK2/STAT3 pathway have been reported to be activated by CXCL12-CXCR4 signaling in breast cancer cells and xenograft animal models, and contribute to tumor angiogenesis, tumorigenesis and metastasis (10-12). In the present study, we found that the expression level of STAT3 was correlated with that of CXCR4, and the combined high expression of CXCR4 and STAT3, especially CXCR4 and p-STAT3, was correlated with TNM stage and lymph node metastasis of breast cancer, suggesting that the signaling pathway that involves STAT3 is critical for CXCR4-mediated breast cancer metastasis. This finding is in accord with a previous study by Ling *et al* showing that STAT3 was involved in CXCR4-mediated breast cancer growth and metastasis in a xenograft animal model (36). Furthermore, we also found that the combined high expression level of CXCR4 and p-STAT3 was correlated with tumor size and histological grade. This finding is consistent with evidence that constitutively activated STAT3 participates in breast carcinogenesis in cell lines and xenograft animal models (27,28,44). Our study suggests that activation of STAT3 by CXCR4 is important in human breast cancer malignancy.

In the present study, we investigated the potential signaling pathway that involves activation of STAT3 by CXCR4 in human breast cancer cell lines after treatment with CXCL12. We found that inhibition of either CXCR4 or JAK2 prevented CXCL12-induced phosphorylation of STAT3, suggesting that CXCL12-CXCR4 activated STAT3 via JAK2 in breast cancer cells. Our study agrees with previous studies that the JAK2/STAT3 pathway is activated by binding of CXCL12 to CXCR4 in human T cells (10), HEK cells expressing CXCR4 (11), and human acute lymphoblastic leukemia cells (20). However, it has been reported that CXCL12-CXCR4 cannot activate JAK2 in primary lymphocytes (21). The discrepancy may be due to different coupling mechanisms underlying activation of JAK2/STAT3 in different cells. In breast cancer cells, it is known that the JAK2/STAT3 pathway is activated by interleukin-6 (45,46) and osteopontin (47). In the present study, we found that the JAK2 antagonist inhibited CXCL12-induced phosphorylation of STAT3 in three breast cancer cell lines, suggesting that the JAK2/STAT3 pathway was coupled to CXCL12-CXCR4 signaling in human breast cancer cells. Furthermore, we found that JAK2 was immunoprecipitated with CXCR4, further suggesting that CXCR4-mediated activation of JAK2 acts via direct interaction between JAK2 and CXCR4. The N-terminal part of the third intracellular loop in CXCR4, which is critical for CXCR4-mediated JAK2 activation, may be the binding site for JAK2 (11).

In summary, we found that the expression of CXCR4, STAT3 and p-STAT3 was higher in breast cancer tissues than in tumor-adjacent tissues. The expression levels of STAT3 and p-STAT3 were correlated with the expression levels of CXCR4 and clinicopathological characteristics of the breast cancer patients. The combined expression level of CXCR4 and p-STAT3 was correlated with TNM stage, tumor size, lymph node metastasis and histological grade, suggesting that activation of STAT3 by CXCR4 may play an important role in breast cancer malignancy. In addition, in breast cancer cell lines, we found that CXCL2-CXCR4 signaling activated the JAK2/STAT3 pathway. The CXCR4 or JAK2 inhibitor effectively prevented CXCL12-induced activation of STAT3. Targeting the CXCL12-CXCR4/JAK2/STAT3 signaling pathway may represent a potential therapeutic strategy for the treatment of breast cancer.

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