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Original Article

High Level of CXCR4 in Triple-Negative Breast Cancer Specimens Associated with a Poor Clinical Outcome

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Despite high sensitivity to chemotherapy, the prognosis for triple-negative breast cancer (TNBC) remains poor because of its high rate of metastasis and low sensitivity to endocrine therapy. CXCR4 expression has been reported in many subtypes of human breast cancers, but it remains unknown whether CXCR4 is expressed in TNBC and whether CXCR4 expression in TNBC could be a prognostic indicator. TNBCs tissues were formalin fixed, paraffin embedded and hematoxylin-eosin (H&E) stained. Immunohistochemical staining was utilized to determine the CXCR4 expression in those specimens. Statistical analyses were performed using SPSS16.0 software to reveal the correlation of CXCR4 expression in TNBC specimens and cancer recurrence and cancer-related death. Our results showed that there was a strong association between CXCR4 overexpression and both menopause and the histological cancer grade of TNBC patients (p values were separately 0.004 and 0.001). The 5-y disease-free survival (DFS) and the 5-y overall survival (OS) were 57.69% and 58.33% for the low-CXCR4 group versus 42.11% and 44.74% for the high-CXCR4 group, respectively ($p = 0.031$ and 0.048). CXCR4 overexpression plays an important role in triple-negative breast cancers, and may be a predictor of poor prognosis.

Key words: CXCR4, immunohistochemical staining, triple-negative breast cancer

Triple-negative breast cancer (TNBC) is a description often used by clinicians to describe tumors lacking expression of hormone receptors (HRs) and human epidermal growth factor receptor 2 (HER-2). Despite their higher sensitivity to chemotherapy than the other subtypes, prognosis for TNBC remains poor, because of its high rate of metastasis and low sensitivity to endocrine therapy [1, 2]. One study conducted by Liedtke *et al.* clearly demonstrated that the poor overall survival (OS) of TNBC was derived from those patients with chemoresistant disease, unfortunately representing more than 50% of TNBC [3]. This observation underscores the need for novel, biological markers and targets for patients that are not sensitive to existing chemotherapies. Impairment of the BRCA1 pathway, mutation of p53, and overexpression of epidermal growth factor receptor (EGFR), caveolin-1 and so on have all been implicated in TNBC [4–10]. Recently, significantly high N-cadherin and TOP2A expression were shown in TNBC with lymphatic infiltration [11].

The fact that breast cancer is not uniform but

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consists of several different subtypes with different molecular profiles, biological behavior and risk profiles; this variability poses a challenge for its clinical management. Very few of the many individual prognostic markers evaluated are sufficiently powerful on their own to merit clinical use. Therefore, there will continuously to be a need to identify new markers that are prognostic and useful for therapy. Chemokine (C-X-C motif) receptor 4 (CXCR4), a seven-transmembrane G protein-coupled chemokine receptor, has been shown to play a pivotal role in the pathogenesis of metastatic breast cancer [12–18]. CXCR4 on cancer cells could direct their migration to organs, *i.e.* liver, bone, and lung, that express high concentrations of chemokine ligand 2 (CXCL-2).

This study was to determine the association of CXCR4 expression with the prognosis of TNBC, and to clarify whether CXCR4 overexpression in tumor specimens can predict the outcomes for TNBC patients.

Materials and Methods

Patients and tissue specimens. Utilization of all 148 TNBC specimens was approved by our hospital Internal Review Board (IRB). From January 1995 to December 2011, there were 719 cases of breast cancer with complete clinico-pathologic data including specimens. All 148 cases of TNBC within that group were used in this study. Clinical data of the 148 TNBC patients were recorded prospectively, including the age at diagnosis, menstruation status, stage of disease, treatment protocol, surveillance protocol compliance, and study endpoints. Primary endpoints were cancer recurrence and cancer-related death. All tissue samples were formalin fixed and paraffin embedded. Hematoxylin-eosin (H&E) slides, pathology reports, and other medical records were reviewed to confirm the diagnoses as well as to establish the clinicopathologic parameters of the tumors, such as age, menopause, tumor size, histological grade (evaluated by Nottingham combined histology grade system [19, 20]), axillary lymph node metastasis, and patient survival. Treatment and surveillance protocols were standardized to ensure study homogeneity. Surveillance protocol consisted of a thorough physical examination every 3 months for 3 years, every 6 months in year 4 and 5, and annually thereafter.

Ethics approval. The specimens in our study are human breast cancer samples removed by surgery as part of the cancer treatment. Prior to the operation, patients granted consent for the use of the excised cancer tissue in medical or scientific research.

Immunohistochemical staining. Cancer specimens were cut and transferred to adhesive-coated slides. Before proceeding with the staining protocol, the slides were deparaffinized by heating at 55℃ for 30 min, followed by 3 washes of 5 min each with xylene, and rehydrated by a series of 5-min washes in 100%, 90%, 70% ethanol and phosphate-buffered saline (PBS). Antigen retrieval was performed by heating for 20 min at a constant temperature of 98℃ in 250 mL of 10-mM sodium citrate (pH 6.0), and endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 20 min. The 4 antibodies, introduced separately, were rabbit polyclonal antibody to CXCR4 (ab2074, Abcam, Cambridge, UK), mouse monoclonal antibody to the estrogen receptor (ab16460, Abcam, Cambridge, UK), and rabbit polyclonal antibodies to the progesterone receptor and HER2, (ab68195, ab2428; Abcam, Cambridge, UK); they were diluted 1 : 50 with goat serum separately. After incubation with the primary antibodies at room temperature for 1 h, the sections were washed with PBS 3 times for 5 min each, and incubated with a goat anti-rabbit/mouse IgG HRP polymer (ab30887 or ab2891; Abcam). After 3 more washes, DAB HRP substrate (ab64238; Abcam), was added for 1 min and counterstained with Mayer's hematoxylin. The samples were then dehydrated and sealed with cover slips. Negative controls were performed by omitting the primary antibodies. Immunostaining for estrogen receptor, progesterone receptor and HER2 was interpreted according to the Allred scoring system. This system consists of a proportion score (PS: 0, none; 1, <1/100; 2, 1/100 to 1/10; 3, 1/10 to 1/3; 4, 1/3 to 2/3; and 5, >2/3) and an intensity score (IS: 0, negative; 1, weak nuclear staining, faintly perceptible at high-power magnification; 2, intermediate nuclear staining; and 3, nuclei displaying strong staining with the appearance of an ink dot at low-power magnification). The PS and IS are added to obtain the total score (TS; range, 0, 2–8) [21], according to the ASCO/CAP guidance [22]. The immunostaining for CXCR4 was semiquantified by grading the staining proportion and staining density: the staining proportion was divided into grade 0 (less than 5%), grade 1 (6–25%

positive cells), grade 2 (26–50% positive cells), grade 3 (51–75% positive cells) and grade 4 (more than 75% positive cells) scored as 0, 1, 2, 3 and 4, respectively; and the staining density was divided into negative, pale yellow, yellow and brownish yellow, scored as 0, 1, 2 and 3, respectively. Then the 2 scores were multiplied and the samples divided into 4 groups based on the results: 0 negative (−); 1–4 weakly positive (+), 5–8 positive (++) and 9–12 strongly positive (+++). The negative (−) and weakly positive (+) results were classified as low expression and the positive (++) and strongly positive (+++) results were classified as high expression.

Statistical analysis. Statistical analyses were performed using SPSS16.0 software (IBM SPSS, Inc., Armonk, NY, USA) according to the software manual (http://www.unt.edu/rss/class/Jon/SPSS_SC/Manuals/v19/IBM%20SPSS%20Advanced%20Statistics%2019.pdf) to Author. PLS put (accessed June, 2012–June, 2014). Levels of CXCR4 expression, tumor size, and nodal status were correlated using the Spearman rank correlation. Survival analysis was performed using the Kaplan-Meier method, while the log-rank test was used to compare the curves and the Cox proportional hazard regression model was used for multivariate analysis. Risk ratios and 95% confidence intervals (CI) were calculated from the model. P values < 0.05 were considered statistically significant.

Results

Identification of triple-negative breast cancer specimens by IHC. From 719 cases of breast cancer, 148 triple-negative breast cancer (TNBC) specimens were identified by immunohistochemical staining with rabbit polyclonal antibodies to estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER-2). Fig. 1 shows representative immunohistochemical stainings of the estrogen receptor, progesterone receptor, and HER-2. Detailed clinicopathological characteristics of our 148 patients are shown in Table 1.

CXCR4 expressions in TNBC. CXCR4 expression was evaluated by immunohistochemical staining in 148 cases of TNBC. A representative CXCR4 immunohistochemical staining is shown in Fig. 2 CXCR4 overexpression was observed in varying degrees in the TNBC specimens; strong associations with CXCR4 overexpression were found for menopausal status and the histological grade of cancer of the TNBC patients (p values, 0.004 and 0.001, respectively) while age ($p = 0.198$), tumor size ($p = 0.134$) and axillary lymph node metastasis ($p = 0.484$) were not associated with the CXCR4 overexpression in TNBC specimens (Table 1).

Correlations between CXCR4 overexpression and poor prognosis in TNBC. To reveal the correlations between the CXCR4 overexpression and prognosis in TNBC, the Kaplan-Meier method was utilized to calculate the overall survival and disease-free survival of the 2 groups of breast cancer patients. As shown by the Cox Proportional hazard model, CXCR4 overexpression was a significant risk for cancer recurrence and cancer-related death (Table 2 and 3). Patients whose cancer specimens showed elevated CXCR4 had a worse survival rate (Fig. 3) than those showing low CXCR4 levels. The 5-y disease-free survival (DFS) rates for the low- and high-CXCR4 groups were 57.69% and 42.11%, respectively ($p = 0.031$). The median DFS was 55 months for the low-CXCR4 group and 48 months for the high-CXCR4 group. The 5-y overall survival (OS) rates for the low- and high-CXCR4 groups were 58.33% and 44.74%, respectively ($p = 0.048$); the median OS was 75 months for the low-CXCR4 group and 57 months for the high-CXCR4 group.

To further confirm that high CXCR4 overexpression in cancer specimens is a novel independent prognostic indicator of a poor cancer outcome in patients with TNBC, we performed a Cox proportional hazard model to determine the relative risks of cancer recurrence (Table 2) and cancer death (Table 3) between CXCR4 expressions and known clinicopathologic factors. Patients whose tumors had high CXCR4 expression had a 2.02-fold and 1.97-fold increases in relative risk of cancer recurrence and cancer-related death, respectively, compared with those whose tumors had low CXCR4 expression (95% CI = 1.04 to 3.92; $p = 0.037$; 95% CI = 0.98 to 3.91; $p = 0.046$). In comparison, patients whose cancer had a high histological grade had 2.73-fold and 3.24-fold increase in relative risk of cancer recurrence and cancer-related death compared with those having a lower histological grade (95% CI = 1.26 to 6.18; $p = 0.003$; 95% CI = 1.14 to 7.24; $p = 0.017$).

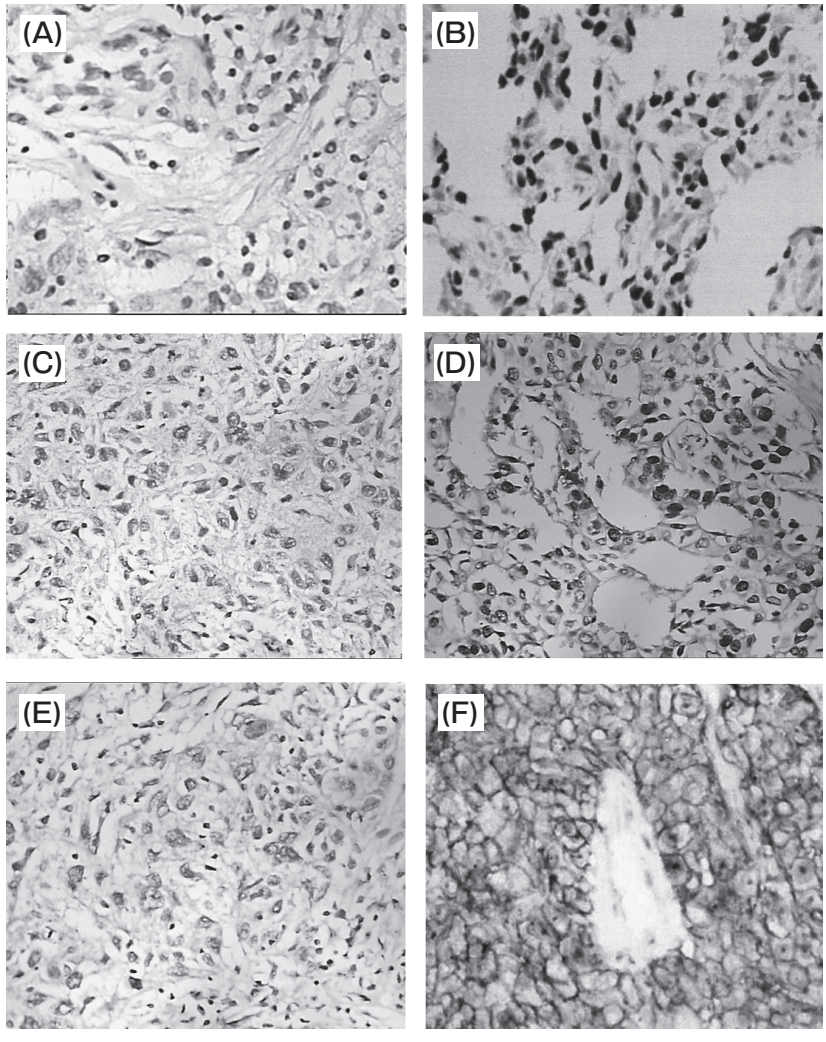

Fig. 1 Representative immunohistochemical staining for estrogen receptors, progesterone receptors, and HER-2 expression in breast cancer specimens. (A), (C), (E): negative expression of the estrogen receptor, progesterone receptor, and HER-2 by immunohistochemical staining. (B), (D), (F): positive expression of the estrogen receptor, progesterone receptor, or HER-2 by immunohistochemical staining.

Discussion

Breast Cancer (BC) is increasingly recognized as a heterogeneous disease exhibiting substantial variations with regard to biological behavior and requiring distinct therapeutic interventions. Steroid hormone receptors (HRs) such as estrogen receptor (ER) and progesterone receptor (PgR) in concert with the oncogene ErbB-2/human epidermal growth factor receptor 2 (HER-2) are critical determinants of these BC subtypes, and the presence of HRs is thought to indicate a good prognosis [23]. HER-2 expression is also perceived as a favorable predictive factor because trastuzumab has become such a potent therapeutic approach in HER-2-positive BC [24–26]. TNBC is characterized by a lack of expression of ER, PgR and HER-2. Thus, to date, chemotherapy remains the only possible therapeutic option in the adjuvant or metastatic setting of TNBC. A recent analysis indicates that TNBC carries a distinct molecular profile when compared with HR-positive BC. Thus, clarifying the molecular events responsible for triple-negative aggressive behavior might assist us to better identify high-risk individuals and develop target-specific thera-

Table 1 Relationship of CXCR4 expression and clinicopathological characteristics in TNBC

Clinicopathological features	CXCR4		*p*-value
	Low	High	
Age (yr)			
≤50	26 (36.11)	46 (63.89)	0.198
>50	20 (26.32)	56 (73.68)	
Menopause			
Premenopausal	33 (41.25)	47 (62.5)	0.004
Postmenopausal	13 (19.12)	55 (80.88)	
Tumor size (cm)			
T1	8 (32.00)	17 (68.00)	0.134
T2	18 (40.91)	26 (59.09)	
T3	15 (31.91)	32 (68.09)	
T4	5 (15.63)	27 (84.37)	
Axillary lymph node metastasis			
Negative	19 (34.55)	36 (65.45)	0.484
Positive	27 (29.03)	16 (70.97)	
Histological grade			
I/II	30 (44.78)	37 (55.22)	0.001
III	16 (19.75)	65 (80.25)	

Table 2 CXCR4 and Cancer recurrence (Cox Proportional hazard model)

Factors	Relative risk	95% C.I.	Significance
Age	0.73	0.31–1.57	0.28
High CXCR4	2.02	1.04–3.92	0.037
Tumor size	0.84	0.56–1.39	0.68
Histological grade	2.73	1.26–6.18	0.003
Lymph node metastasis	2.97	1.47–4.55	0.058

Table 3 CXCR4 and Cancer-related death (Cox Proportional hazard model)

Factors	Relative risk	95% C.I.	Significance
Age	0.84	0.37–1.63	0.39
High CXCR4	1.97	0.98–3.91	0.046
Tumor size	1.03	0.68–1.62	0.82
Histological grade	3.24	1.14–7.24	0.017
Lymph node metastasis	2.84	1.23–6.11	0.071

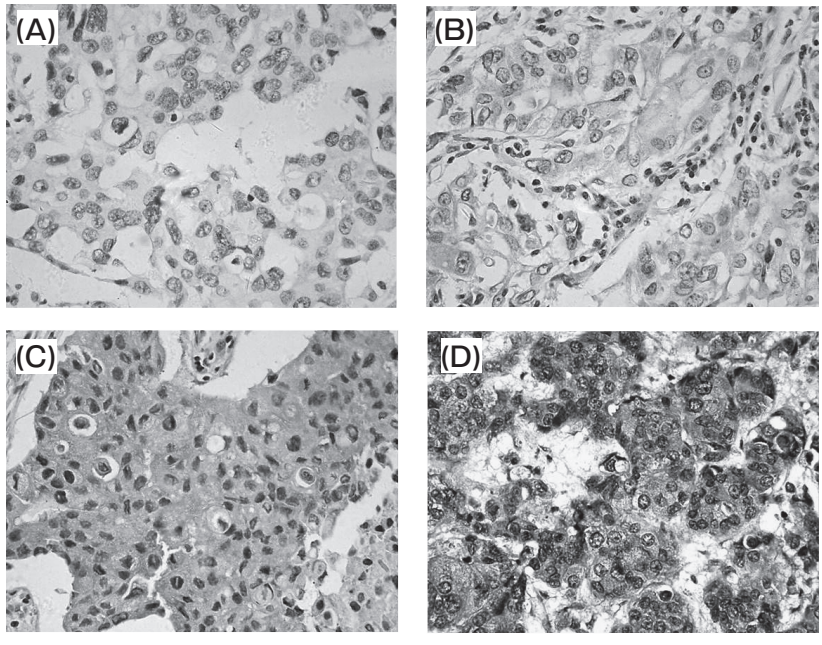

Fig. 2 Immunohistochemical staining of CXCR4 expression in the TNBC specimens in this study. (**A**), negative expression of CXCR4 in TNBC (−); (**B**), weakly positive expression of CXCR4 in TNBC (+); (**C**), positive expression of CXCR4 in TNBC (++); (**D**), strongly positive expression of CXCR4 in TNBC (+++).

pies.

The chemokine receptor CXCR4 is a 7-transmembrane G protein-coupled receptor that has been reported to be involved in the pathogenesis of metastatic breast cancer [12-18]. CXCR4 has been found to be highly expressed in breast cancer cells. Motility and migration of breast cancer cells expressing CXCR4 can be induced when they are exposed to the

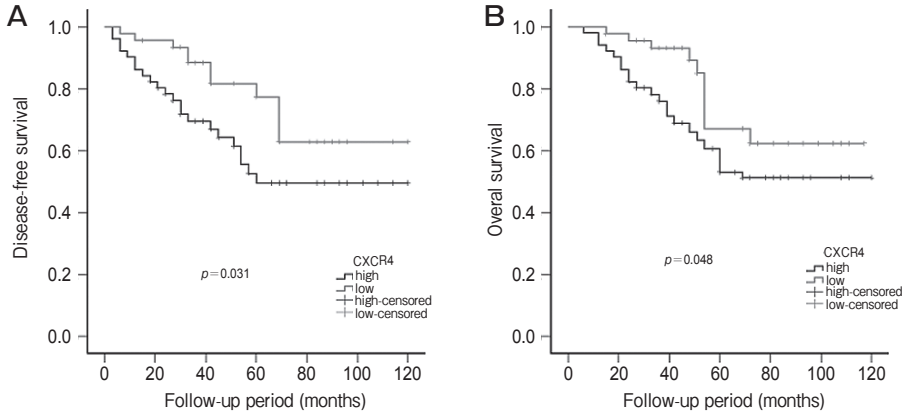

Fig. 3 Influence of CXCR4 overexpression on disease-free or overall survival in triple-negative breast cancers. This Kaplan-Meier survival curve compares the disease-free and overall survival, based on the degree of CXCR4 overexpression in patients with triple-negative cancers (**A** and **B**). Note that TNBC patients whose tumors showed high CXCR4 expression levels had a worse disease-free or overall survival compared with those whose tumors with low CXCR4 expression ($p = 0.031$ and 0.048).

CXCR4 ligands, namely, stromal derived factor 1 (SDF-1) [12]. The clinical utility of CXCR4 as an indicator of tumor metastasis has also been defined [12–18, 27]. Chu *et al.* found high CXCR4 expression in TNBC by western-blotting analysis of fresh frozen cancer tissue [27]. To confirm whether CXCR4 is an important factor in the clinical setting of TNBC, we performed immunohistochemical staining for CXCR4 on paraffin-embedded, formalin-fixed TNBC tissues, and then evaluated its role in triple-negative breast cancer prognosis. We found high levels of CXCR4 expression in the primary triple-negative breast cancers. Therefore, CXCR4 may be an important parameter for TNBCs. CXCR4 was also a strong indicator of TNBC outcome: high CXCR4 expression was associated with a significantly worse disease-free survival and overall survival than tumors with low CXCR4 expression (Fig. 3 and Table 2–3), as indicated by the univariate analysis model (Kaplan-Meier and log-rank test) and the multivariate Cox proportional hazard model (Tables 4 and 5). It has been reported that CXCR4 regulates tumor cell growth, migration and metastases in lungs, brains and bones [12–16] although the details about metastases other than lymph-node metastases are not complete. This migration might also be the main cause of the poor outcome of TNBC. Therefore, in the future it will be helpful to research the correlation of CXCR4 overexpression with metastases in lungs, bones or other organs. In this study, we noted that both CXCR4 overexpression and histological grade were significant predictors of cancer recurrence and cancer-related death on multivariate analysis ($p = 0.037$ and 0.046, Tables 2 and 3), as has also been observed by others [27], but there was no correlation of CXCR4 overexpression with lymph node metastases ($p = 0.058$ and 0.071) (Table 1). Whether this is due to a statistical limitation or indicates a real biological phenomenon is not known.

In conclusion, our findings indicate that CXCR4 overexpression plays an important role in triple-negative breast cancers, and may be an indicator of poor prognosis.

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