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Original Article

Clinical Significance of C-X-C Motif Chemokine Receptor 4 and Integrin αvβ6 Expression in Breast Cancer

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ABSTRACT

Purpose: C-X-C motif chemokine receptor 4 (CXCR4) and integrin αvβ6 play important roles in the malignant progression of multiple cancers. However, it remains unclear whether the expression of one or both proteins in breast cancer (BC) is of clinical significance. In this study, we investigated the expression of CXCR4 and integrin αvβ6 in BC tissues and their correlation with clinicopathological characteristics, including survival.
Methods: CXCR4 and αvβ6 expression in 111 BC tissues was examined by immunocytochemistry. Correlations between the expression of the 2 proteins and patient clinicopathological characteristic were investigated using the Kaplan–Meier method and the Cox proportional hazards model.
Results: CXCR4 and αvβ6 were overexpressed in BC tissue compared with normal breast tissue. Overexpression of both molecules was related to lymph node status ($p = 0.013$ and $p = 0.022$, respectively). αvβ6 overexpression was also associated with tumor size ($p = 0.044$). A positive correlation was detected between the expression of CXCR4 and αvβ6 ($r = 0.649$, $p = 0.001$), and co-overexpression of both molecules was associated with tumor size ($p = 0.018$) and lymph node metastasis ($p = 0.015$). Kaplan–Meier analysis revealed that overexpression of CXCR4, αvβ6, or both molecules was associated with short overall survival (OS; $p < 0.001$, $p < 0.001$, and $p = 0.009$, respectively) and disease-free survival (DFS; $p < 0.001$, $p = 0.005$, and $p = 0.019$, respectively). Multivariate analysis indicated that lymph node metastasis was an independent prognostic factor for unfavorable OS and DFS ($p = 0.002$ and $p = 0.005$, respectively), whereas co-overexpression of CXCR4 and αvβ6 was an independent prognostic factor only for OS ($p = 0.043$).
Conclusion: CXCR4 and αvβ6 may play synergistic roles in the progression of BC, and co-targeting of CXCR4 and αvβ6 could be a potential strategy for the prevention and treatment of BC.

Keywords: Breast neoplasms; Integrin alphaV; Prognosis; Receptors, CXCR4

INTRODUCTION

Breast cancer (BC) is the most common cancer afflicting women and the leading cause of cancer-related deaths in this population worldwide [1]. Although advances in the diagnosis

Conflict of Interest
The authors declare that they have no competing interests.

Author Contributions
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and treatment of BC have reduced its mortality rates, the overall prognosis of patients with distant metastasis is still unsatisfactory. Therefore, it is essential to identify new therapeutic and prognostic biomarkers of BC, which would also provide a better understanding of this malignancy at the molecular level.

Chemokine signaling is mediated by a family of G protein-coupled receptors, among which C-X-C motif chemokine receptor 4 (CXCR4) is one of the most commonly expressed on tumor cells. Müller et al.[2] have first documented the significant role of CXCR4 in BC metastasis, and since then, a growing body of evidence has shown that engagement of CXCR4 by its specific ligand C-X-C motif chemokine 12 (CXCL12) activates multiple intracellular pathways that contribute to metastasis and influence the clinical outcomes of various tumors [3,4].

The integrin family of cell surface adhesion molecules composed of 18 α and 8 β subunits that form at least 24 heterodimeric receptors. The β6 subunit forms only one integrin, αvβ6, which is exclusively expressed on epithelial cells. Notably, αvβ6 is expressed at high levels in several tissues during development, but in adults, it is mainly expressed during physiological tissue remodeling events, such as wound healing, and pathological events, such as carcinogenesis [5,6]. αvβ6-mediated intracellular signaling has been shown to modulate diverse processes with important roles in tumor progression, including cell proliferation, invasion, metastasis, and survival [7,8].

Recent evidence suggests that CXCR4–CXCL12 signaling may promote metastasis of colon cancer to the liver by upregulating αvβ6 expression [9], which prompted us to investigate the relationship between CXCR4 and αvβ6 in BC. Here, we evaluated the expression of both molecules, alone and in combination, in BC tissues and determined their prognostic significance, with the goal of providing new insights into our understanding of the molecular mechanisms underlying BC development and progression.

METHODS

Patients and clinical samples

This study enrolled 111 women who were pathologically diagnosed with invasive ductal carcinoma at the First Affiliated Hospital of China Medical University between 2008 and 2009. Inclusion criteria were: 1) curative surgery and pathologically confirmed infiltrative ductal carcinoma; 2) no evidence of distant metastasis in preoperative examination and no preoperative anticancer treatment; 3) availability of complete medical information and follow-up data; and 4) availability of paraffin-embedded resected tissues. The median age at diagnosis was 52 years (range, 29–82). According to the clinical tumor-node-metastasis classification set by the 6th edition of the American Joint Committee on Cancer staging system, 44 patients had stage I disease, 50 had stage II, and 17 had stage III. This retrospective study was approved by the Ethics Committee of the First Affiliated Hospital of China Medical University (Institutional Review Board approval No. AF-SOP-07-1.1-01); the requirement for informed patient consent was waived.

Immunohistochemistry

Formalin-fixed, paraffin-embedded BC tissues and adjacent normal tissues were cut into 4-μm-thick sections, and immunohistochemical staining was performed with the

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streptavidin-peroxidase method. In brief, sections were heated at 65°C for 2 hours, dewaxed in xylene, rehydrated in a graded alcohol series, and washed with phosphate-buffered saline (PBS). The sections were then subjected to high-pressure antigen retrieval for 2 minutes in citrate buffer (pH 6.0), and endogenous peroxidase was blocked with hydrogen peroxide. The sections were incubated with primary antibodies against CXCR4 (ab1670, 1:200 dilution; Abcam, Cambridge, UK) or integrin αvβ6 (bs-5791R, 1: 400; Bioss Inc., Beijing, China) at 4°C overnight. As negative controls, sections were incubated with PBS instead of the primary antibodies. The sections were then incubated with secondary antibodies (Maixin-Bio, Fuzhou, China) at 37°C for 30 minutes, and antibody binding was visualized by addition of 3,3-diaminobenzidine.

Evaluation of immunohistochemical staining

All sections were randomly and blindly evaluated by 2 pathologists. Staining of the cell membranes and cytoplasm was considered positive expression. Staining was evaluated using a semi-quantitative scoring system [10] that takes into account the intensity and extent of staining. Intensity was scored as: 0, no staining; 1, weak; 2, moderate; and 3, high staining. The extent of staining (percentage of stained tumor cells) was scored as: 1, 1%–25%; 2, 26%–50%; 3, 51%–75%; and 4, 76%–100%. The final immunohistochemistry score (IHS) was the product of the intensity score and the extent of staining score and ranged from 0 to 12. For correlation analysis, patients were assigned to either a high (IHS ≥ 4) or low (IHS < 4) αvβ6 or CXCR4 expression group.

Statistical analysis

Statistical analyses were performed using SPSS 20.0 software (IBM, Armonk, USA). Graphical representations were performed using GraphPad Prism 7.0 software (GraphPad Software, San Diego, USA). The relationships between CXCR4, αvβ6, and clinicopathological factors were analyzed by the χ^2 test. Spearman's rank correlation analysis and the Mann–Whitney test were applied to evaluate the relationship between CXCR4 and αvβ6 expression. The Kaplan–Meier method was used to calculate overall survival (OS) and disease-free survival (DFS), and differences were evaluated using the log-rank test. Univariate and multivariate analyses were performed using the Cox proportional hazards regression model. All tests were 2-sided, and a p-value of < 0.05 was considered statistically significant.

RESULTS

CXCR4 and αvβ6 expression in BC tissues

The expression of CXCR4 and αvβ6 in 111 BC samples and 40 adjacent normal tissues was analyzed by immunohistochemical staining. Both CXCR4 and αvβ6 were mainly located in the cell membranes and cytoplasm of BC cells (**Figure 1**). CXCR4 was overexpressed in 58 (52.3%) of the BC tissues examined, and αvβ6 was overexpressed in 43 (38.7%) BC tissues. Notably, 40 (36.0%) of BC tissues overexpressed both CXCR4 and αvβ6. All 40 samples of adjacent normal tissues were negative for both proteins (**Table 1**).

Correlations between CXCR4 and αvβ6 expression in BC tissue and clinicopathological characteristics

As shown in **Table 2**, CXCR4 overexpression was significantly associated with lymph node metastasis ($p = 0.013$), whereas αvβ6 overexpression correlated with tumor size ($p = 0.044$) and lymph node metastasis ($p = 0.022$). Co-overexpression of both CXCR4 and αvβ6 was

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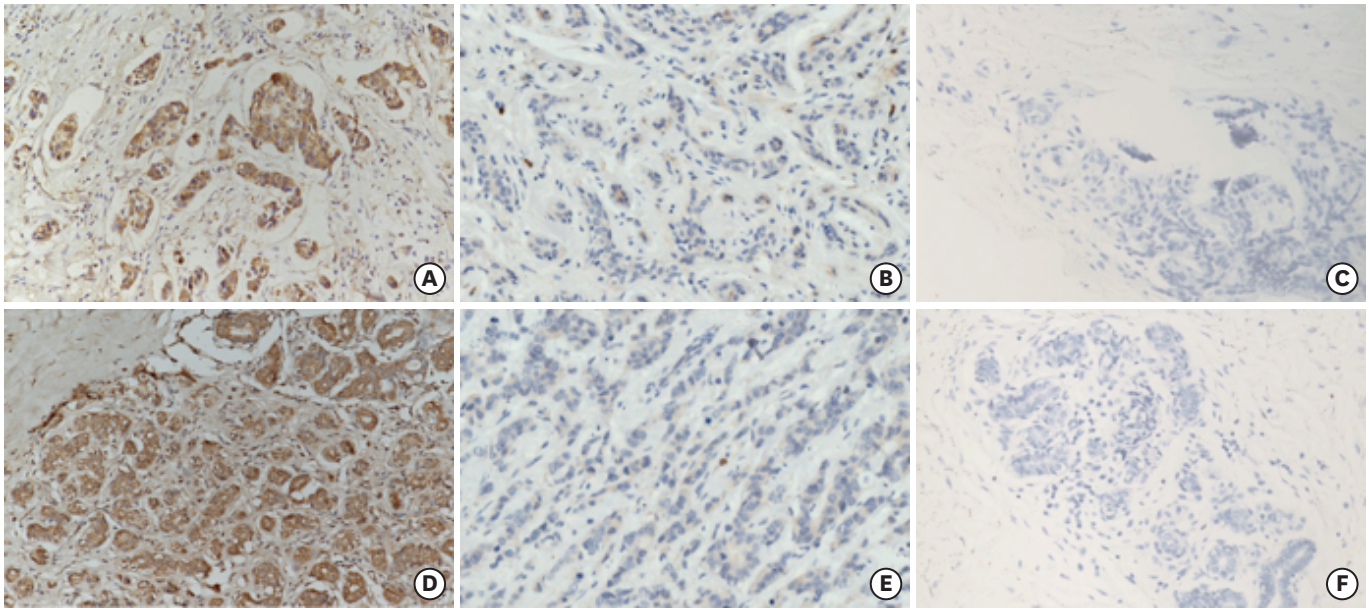

Figure 1. Immunohistochemical staining of CXCR4 and αvβ6 in BC and normal breast tissue (×200). (A) CXCR4 positive staining in BC. (B) CXCR4 negative staining in BC. (C) CXCR4 negative staining in normal breast tissue. (D) Integrin αvβ6 positive staining in BC. (E) Integrin αvβ6 negative staining in BC. (F) Integrin αvβ6 negative staining in normal breast tissue.
BC = breast cancer; CXCR4 = C-X-C motif chemokine receptor 4.

Table 1. Clinicopathological characteristics of patient samples and expression of CXCR4 and integrin αvβ6 in breast cancer

Characteristics	No. of cases
Age (yr)	
< 60	91 (82.0)
≥ 60	20 (18.0)
Tumor size (cm)	
≤ 2	44 (39.6)
> 2	12 (10.8)
Lymph node metastasis	
Yes	47 (42.3)
No	64 (57.7)
Histological grade	
1	31 (27.9)
2 + 3	80 (72.1)
ER expression	
Negative	38 (34.2)
Positive	73 (65.8)
PR expression	
Negative	44 (39.6)
Positive	67 (60.4)
HER-2 amplification	
Yes	25 (22.5)
No	86 (77.5)
CXCR4 expression	
Low	53 (47.7)
High	58 (52.3)
Integrin αvβ6 expression	
Low	68 (61.3)
High	43 (38.7)
Co-overexpression of CXCR4 and integrin αvβ6	
Yes	40 (36.0)
No	71 (64.0)

Values are presented as number (%).
ER = estrogen receptor; PR = progesterone receptor; HER-2 = human epidermal growth factor receptor 2; CXCR4 = C-X-C motif chemokine receptor 4.

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Table 2. Relationships among the expression of CXCR4, integrin αvβ6 and clinicopathological factors in breast cancer

Characteristics	No. of cases	CXCR4 expression		*p*-value	Integrin αvβ6 expression		*p*-value	Co-overexpression of CXCR4 and integrin αvβ6		*p*-value
		Low	High		Low	High		Yes	Other	
Age (yr)				0.079			0.254			0.151
< 60	91	47	44		58	33		30	61	
≥ 60	20	6	14		10	10		10	10	
Tumor size (cm)				0.121			0.044			0.018
≤ 2	44	25	19		32	12		10	34	
> 2	67	28	39		36	31		30	37	
Lymph node metastasis				0.013			0.022			0.015
Yes	47	16	31		23	24		23	24	
No	64	37	27		45	19		17	47	
Histological grade				0.176			0.082			0.066
1	31	18	13		23	8		7	24	
2 + 3	80	35	45		45	35		33	47	
ER expression				0.954			0.264			0.480
Low	38	18	20		26	12		12	26	
High	73	35	38		42	31		28	45	
PR expression				0.700			0.677			0.729
Low	44	22	22		28	16		15	29	
High	67	31	36		40	27		25	42	
HER-2 amplification				0.073			0.540			0.346
Yes	25	8	17		14	11		11	14	
No	86	45	41		54	32		29	57	

CXCR4 = C-X-C motif chemokine receptor 4; ER = estrogen receptor; PR = progesterone receptor; HER-2 = human epidermal growth factor receptor 2.

significantly associated with larger tumor size ($p = 0.018$) and lymph node metastasis ($p = 0.015$). However, there were no correlations between overexpression of either protein and age, histological grade, estrogen receptor expression, progesterone receptor expression, or human epidermal growth factor receptor 2 (HER-2) status (**Table 2**).

Correlation between CXCR4 and αvβ6 expression in BC

CXCR4 and αvβ6 expression in BC were significantly positively correlated; thus, of the 58 BC tissues overexpressing CXCR4, 40 (69.0%) also overexpressed αvβ6 (Spearman rank correlation $r = 0.649$, p=0.001) (**Table 3**). After assigning the 111 patients into low (< 4 IHS) or high (≥ 4 IHS) expression of CXCR4 groups, αvβ6 expression, based on the IHS score, was found to be significantly higher in the CXCR4-high group than in the CXCR4-low group ($p < 0.001$, **Figure 2**).

Prognostic significance of CXCR4 and αvβ6 expression in BC

The 111 patients were followed up for a median duration of 114 months (range, 5–127 months). During the follow-up period, 33.3% (37/111) of patients experienced distant metastasis. Associations between CXCR4 and αvβ6 expression and patient outcome were evaluated using the Kaplan–Meier method and log-rank test. As shown in **Figure 3**, the OS and DFS were shorter for patients with high CXCR4 expression compared with low

Table 3. Correlation between CXCR4 and integrin αvβ6 in patients with breast cancer

Integrin αvβ6 expression	CXCR4 expression		Total
	Low	High	
Low	50 (94.3)	18 (31.0)	68
High	3 (5.7)	40 (69.0)	43
Total	53	58	111

Values are presented as number (%).
CXCR4 = C-X-C motif chemokine receptor 4.

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expression ($p < 0.001$ for OS and DFS) and for patients with high αvβ6 expression compared with low expression ($p < 0.001$ for OS, $p = 0.005$ for DFS). Moreover, we divided the patients into 3 groups based on CXCR4 and αvβ6 expression: group 1, with CXCR4 (high) and αvβ6 (high) (n = 40); group 2, CXCR4 (high) and αvβ6 (low)/CXCR4 (low) and αvβ6 (high) (n = 21);

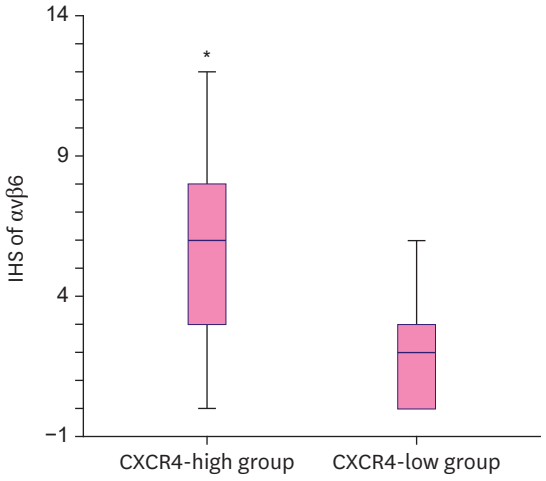

Figure 2. Box-and-whisker graph demonstrated that IHS of integrin αvβ6 in CXCR4-high group was significantly higher than CXCR4-low group.
IHS = immunohistochemistry score; CXCR4 = C-X-C motif chemokine receptor 4.
*$p < 0.001$.

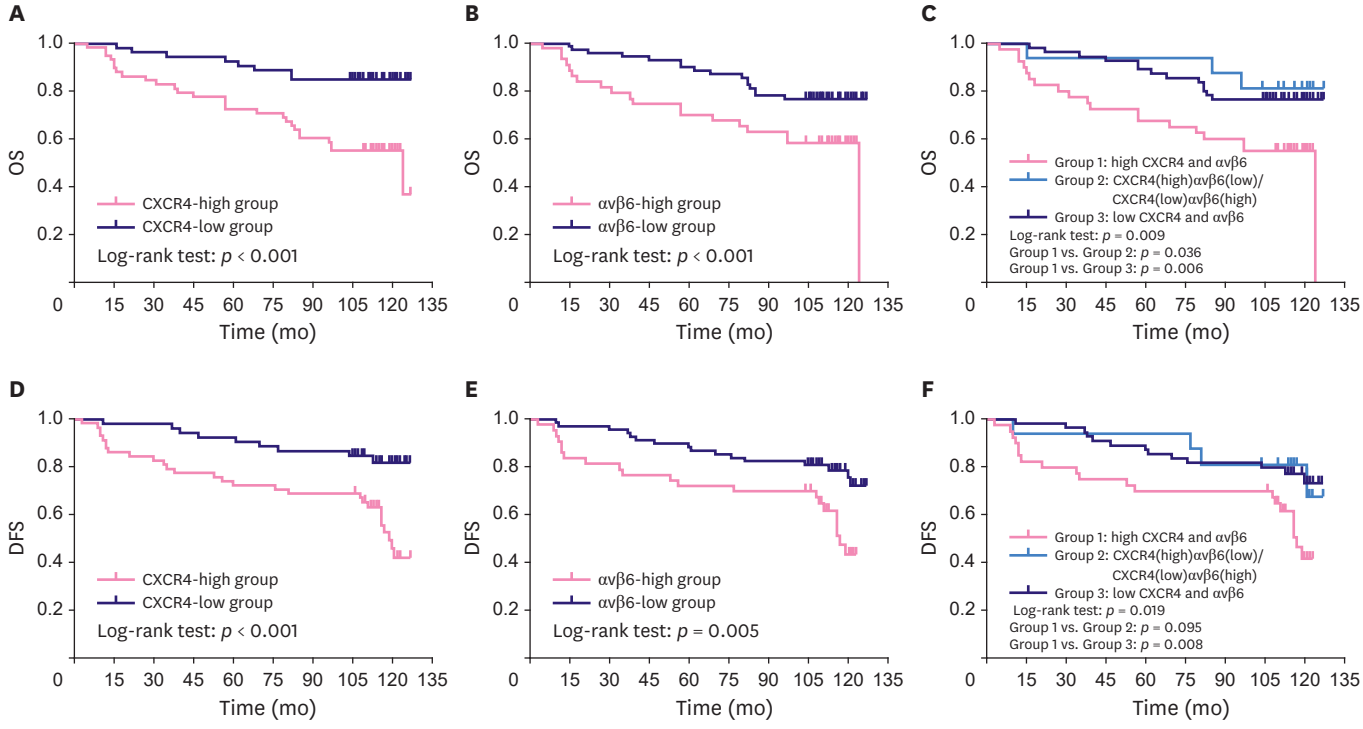

Figure 3. Kaplan–Meier curves for OS and DFS in patients with breast cancer (111 cases) according to CXCR4 and integrin αvβ6 expression. *p*-values determined using log-rank test. (A, D) Kaplan–Meier curves for OS and DFS according to CXCR4-expression status (n = 111). (B, E) Kaplan–Meier curves for OS and DFS according to integrin αvβ6 expression status (n = 111). (C, F) Kaplan–Meier curves for OS and DFS according to combined expression of CXCR4 and integrin αvβ6 status (n = 111).
OS = overall survival; DFS = disease-free survival; CXCR4 = C-X-C motif chemokine receptor 4.

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and group 3, with CXCR4 (low) and αvβ6 (low) (n = 50). Importantly, the 40 patients co-overexpressing CXCR4 and αvβ6 had a significantly poorer prognosis than the other groups (p = 0.009 for OS, p = 0.019 for DFS) (**Figure 3C and F**).

Next, we assessed the prognostic value of CXCR4 and αvβ6 expression and other clinicopathological characteristics using a Cox proportional hazards model. Univariate analysis revealed that lymph node metastasis (p < 0.001 for OS and DFS), histological grade (p = 0.007 for OS, p = 0.009 for DFS), CXCR4 overexpression (p = 0.001 for OS, p = 0.002 for DFS), αvβ6 overexpression (p = 0.012 for OS, p = 0.007 for DFS), and CXCR4 and αvβ6 co-overexpression (p = 0.003 for OS, p = 0.006 for DFS) predicted a poorer prognosis. Tumor size correlated only with shorter OS (p = 0.035) (**Table 4**). In multivariate analysis, only lymph node metastasis (p = 0.002 for OS, p = 0.005 for DFS) and CXCR4 and αvβ6 co-overexpression (p = 0.043 for OS) remained independent prognostic factors for BC patients (**Table 5**).

DISCUSSION

The incidence of BC has been slowly increasing and it poses a major threat to women's health worldwide. A better understanding of the molecular mechanisms underlying this malignancy would contribute to earlier diagnosis and better treatment options. In the present study, we found that both CXCR4 and αvβ6 were overexpressed in BC compared with adjacent normal breast tissues, and that overexpression of either molecule correlated significantly with clinicopathological factors and patient survival. Moreover, we detected a positive correlation between the expression of CXCR4 and the expression of αvβ6 in BC tissue.

Table 4. Univariate analysis of OS and DFS in breast cancer

Variable	Univariable analysis		
	HR	95% CI	p-value
OS			
Age (< 60 vs. ≥ 60)	1.242	0.542–2.844	0.608
Tumor size (> 2 cm vs. ≤ 2 cm)	2.259	1.058–4.823	0.035
Lymph node metastasis (yes vs. no)	5.126	2.392–10.987	< 0.001
Historical grade (2 & 3 vs. 1)	5.109	1.536–16.696	0.007
Surgery (MRD vs. other approaches*)	0.616	0.255–1.490	0.282
Chemotherapy (yes vs. no)	3.357	0.459–24.569	0.233
Endocrine therapy (yes vs. no)	0.874	0.440–1.735	0.700
Radiation therapy (yes vs. no)	1.074	0.536–2.044	0.849
CXCR4 expression (high vs. low)	3.891	1.757–8.613	0.001
αvβ6 expression (high vs. low)	2.365	1.208–4.630	0.012
Co-overexpression of CXCR4 and αvβ6 (yes vs. no)	2.729	1.394–5.342	0.003
DFS			
Age (< 60 vs. ≥ 60)	1.141	0.500–2.602	0.754
Tumor size (> 2 cm vs. ≤ 2cm)	1.792	0.885–3.627	0.105
Lymph node metastasis (yes vs. no)	4.242	2.087–8.624	< 0.001
Historical grade (2 & 3 vs. 1)	3.984	1.410–11.258	0.009
Surgery (MRD vs. other approaches)	0.710	0.296–1.715	0.444
Chemotherapy (yes vs. no)	3.837	0.525–28.025	0.185
Endocrine therapy (yes vs. no)	0.985	0.487–1.885	0.900
Radiation therapy (yes vs. no)	1.150	0.600–2.206	0.673
CXCR4 expression (high vs. low)	3.348	1.578–7.104	0.002
αvβ6 expression (high vs. low)	2.461	1.283–4.723	0.007
Co-overexpression of CXCR4 and αvβ6 (yes vs. no)	2.495	1.305–4.771	0.006

OS = overall survival; DFS = disease-free survival; CI = confidence interval; HR = hazard ratio; MRD = modified radical mastectomy; CXCR4 = C-X-C motif chemokine receptor 4.
*Other approaches were breast-conserving and total mastectomy plus sentinel lymph node biopsy.

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Table 5. Multivariate analysis of OS and DFS in breast cancer

Variable	Multivariable analysis		
	HR	95% CI	*p*-value
OS			
Age (< 60 vs. ≥ 60)	-	-	-
Tumor size (> 2 cm vs. ≤ 2 cm)	-	-	-
Lymph node metastasis (yes vs. no)	3.491	1.555–7.837	0.002
Historical grade (2 & 3 vs. 1)	-	-	-
Co-overexpression of CXCR4 and αvβ6 (yes vs. no)	2.030	1.023–4.029	0.043
DFS			
Age (< 60 vs. ≥ 60)	-	-	-
Tumor size (> 2 cm vs. ≤ 2 cm)	-	-	-
Lymph node metastasis (yes vs. no)	3.007	1.405–6.437	0.005
Historical grade (2 & 3 vs. 1)	-	-	-
Co-overexpression of CXCR4 and αvβ6 (yes vs. no)	-	-	-

OS = overall survival; DFS = disease-free survival; CI = confidence interval; HR = hazard ratio; CXCR4 = C-X-C motif chemokine receptor 4.

Binding of the chemokine CXCL12 to CXCR4 plays a well-established role in promoting tumor cell proliferation, invasion, and angiogenesis [11,12]. Accordingly, the CXCR4–CXCL12 signaling axis has been shown to contribute to the metastasis and clinical outcome of many different types of cancers [13,14]. Here, we found that CXCR4 overexpression in BC tissues was associated with lymph node metastasis, and shorter OS and DFS. These results are in agreement with previous studies in BC demonstrating that CXCR4 overexpression plays a prominent role in metastasis and correlates with unfavorable prognosis [15,16].

αvβ6 is a unique member of the integrin family in that its expression is restricted to epithelial cells and is usually expressed only during embryogenesis and under select conditions in adults; namely, during wound healing and in epithelial tumors [5]. αvβ6 has been shown to modulate cell invasion, inhibit apoptosis, regulate the expression of matrix metalloproteases (MMPs), and induce the production of transforming growth factor (TGF) β1 [17,18]. Overexpression of αvβ6 has also been associated with aggressive tumor behavior and poor survival in many types of cancer [7,19,20]. Upregulation of αvβ6 in myoepithelial cells in ductal carcinoma *in situ* not only induces its tumor-promoting functions via TGF-β and MMP9 signaling [21], but also induces its loss of response to a stiffening microenvironment, which lead to increase in tissue rigidity [22]. In turn, these changes result in a switch in myoepithelial cells from tumor suppressors to tumor promoters [23]. In support of these results, we found that αvβ6 overexpression in BC was significantly associated with tumor size, lymph node metastasis, and poorer survival. Another study has found that αvβ6 is upregulated in HER-2 overexpressing tumors and is an independent prognostic marker in HER-2 positive BC [24]. However, we did not detect a relationship between αvβ6 overexpression and HER-2 status or expression of estrogen or progesterone receptors. These discrepancies may be due to inter-study differences in the racial composition of the patient population, selection criteria, sample size, tissue preservation methods, staining antibodies, and data extraction methods.

Multiple factors contribute to the aggressive behavior of tumors. CXCR4-overexpressing tumor cells exhibit directional migration along a CXCL12 gradient in organs rich in this chemokine, such as lymph node, bone, lung, and liver [2]. However, other studies have found that CXCR4 contributes to tumor growth not through a direct role in adhesion and migration but rather through CXCL12-triggered signaling and modulation of cell adhesion through integrin receptors [25,26]. Wang et al. [9] have found that expression of CXCR4 and αvβ6 was strongly associated with colorectal cancer (CRC) metastasis to the liver. They showed

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that CXCR4–CXCL12 interactions upregulated the expression of αvβ6 via the extracellular signal-regulated kinase–Est-1 pathway, and induced the directional migration of CRC cells to the liver via αvβ6. Similarly, CXCR4–CXCL12 induces ovarian cancer cell invasion through αvβ6-mediated p38 mitogen-activated protein kinase and phosphoinositide 3-kinase/protein kinase B signaling and urokinase expression [27]. However, little was known in the past about the relationship between CXCR4 and αvβ6 in BC. Here, we found that co-overexpression of CXCR4 and αvβ6 in tumor tissue was associated with larger tumor size, lymph node metastasis, and shorter DFS and OS. Moreover, co-overexpression of CXCR4 and αvβ6 was an independent prognostic factor for unfavorable OS in BC. We also detected a positive correlation between CXCR4 and αvβ6 expression in BC tissues; therefore, we hypothesize that the 2 proteins may functionally interact. However, our study sample was small, and further investigation is warranted of the co-expression and possible functional associations between CXCR4 and αvβ6 in cell lines and animal models. Despite this limitation, the results of our study suggest the possibility of crosstalk between CXCR4 and αvβ6 in BC.

Given that CXCR4 and αvβ6 play important roles in the malignant progression of multiple cancers, numerous studies have investigated the anticancer effects of therapy targeted to CXCR4 or αvβ6. In a phase I trial, balixafortide, a potent, selective CXCR4 antagonist, in combination with eribulin showed encouraging signs of anticancer activity in women with HER-2 negative, CXCR4-positive metastatic BC [28]. Similarly, 264RAD, a potent human monoclonal antibody against αvβ6, has shown promise in significantly reducing tumor growth and metastasis of BC [29]. 264RAD can also enhance the effect of trastuzumab, thereby offering a potential alternative approach to the treatment of high-risk and trastuzumab-resistant BC patients [30]. Based on these results, we hypothesize that patients with CXCR4 and αvβ6 co-overexpressing tumors might benefit from co-targeted treatment. This possibility will be tested in our future work.

In summary, we found that CXCR4 and αvβ6 are highly expressed in BC, and that their overexpression correlates with several clinicopathological characteristics and clinical outcomes in BC patients. Most importantly, CXCR4 expression and αvβ6 expression were positively correlated, and patients co-overexpressing the 2 proteins showed a poorer prognosis. These findings suggest not only that a functional interaction between the 2 proteins may play an important role in the development of BC, but also that co-targeting of CXCR4 and αvβ6 may represent a new strategy for the prevention and treatment of BC.

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