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ORIGINAL ARTICLE

Elevated CCL20 expression was associated with poor prognosis for breast cancer

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Abstract

Background: The chemokine (C-C motif) ligand 20 (CCL20) exhibits pronounced expression within tumor cells, effectively facilitating tumor progression by modulating the immunosuppressive microenvironment and promoting tumor cell aggressiveness.

Methods: Breast cancer and matched adjacent normal tissues from 113 adult breast cancer patients were collected for immunohistochemical staining of CCL20, E-cadherin, vimentin, and N-cadherin. The assessment evaluated the association between CCL20 expression and clinicopathological factors using Pearson chi-squared test, epithelial-mesenchymal transition (EMT) markers expression using Spearman's rank correlation test, both OS and DFS using Kaplan-Meier survival analysis, and Cox proportional hazards regression modeling.

Results: Cytoplasmic CCL20 expression was stronger in cancer tissues, compared to normal tissue (69.9% vs 23%). Strong correlations were observed between CCL20 expression and many clinicopathological features, including tumor size (p = 0.000), estrogen receptor (ER) status (p = 0.003), Ki67 status (p = 0.000), vascular invasion (p = 0.001), and tumor-node-metastasis stage (p = 0.001). Additionally, CCL20 expression was an independent prognostic predictor for overall survival (OS) (hazard ratio [HR], 3.207; 95% CI, 1.142-9.005, p = 0.027). Furthermore, a significant association between CCL20 expression and EMT markers was observed. CCL20 expression was linked to unfavorable outcomes in all patients (p = 0.000), ER-positive patients (p = 0.001), and node-positive/negative (p = 0.005/0.001) subgroups.

Conclusion: These findings highlighted that elevated CCL20 expression was linked to a more aggressive tumor phenotype and a disappointing OS in breast cancer patients, thus advocating for the consideration of CCL20 expression being a novel independent prognostic biomarker for guiding bespoke treatment strategies.

Keywords: Breast cancer; CCL20; EMT markers; Prognosis

Lay Summary: The protein chemokine (C-C motif) ligand 20 (CCL20) facilitates tumor progression by directly functioning on tumor cells to promote the aggressiveness. In this study, patient tumor samples and paired normal tissues were used to investigate the relationship between the amount of CCL20 and the aggressiveness of breast cancer. The data showed that (1) CCL20 is highly produced in cancer tissues compared to normal tissues; (2) the level of CCL20 is associated with tumor size, tumor cell proliferation, vascular invasion of tumor cells, and stage of metastasis to node; (3) CCL20 expression is significantly linked to unfavorable outcomes in breast cancer patients. These findings highlighted that elevated CCL20 production was linked to a more aggressive tumor status and a disappointing survival in breast cancer patients, thus prompting the consideration of CCL20 expression guiding bespoke treatment strategies.

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1. INTRODUCTION

According to data from the GLOBOCAN database, a project overseen and conducted by the International Agency for Research on Cancer, breast cancer is the predominant type of cancer among women. In the United States in 2015, there were recorded nearly 231 840 incidents of newly diagnosed invasive breast cancer, accompanied by 40 290 breast cancer-related fatalities among women.[1] Recurrence and metastasis emerge as predominant contributors to mortality in breast cancer patients, with an estimated 30% to 40% encountering metastatic progression. The complex interplay of clinicopathological factors profoundly influences the overall survival (OS) outcomes among individuals afflicted with this disease.[2,3]

The tumor microenvironment exerts a pivotal influence on the sustenance and advancement of cancer, orchestrating the recruitment and activation of inflammatory cells within tumor locales, thereby fostering cancer progression. Chemokines are secreted proteins with molecular weights ranging from 8 to 14 kDa, and they were assumed to exhibit crucial roles in mediating the recruitment and modulation of inflammatory cell activity through interactions with their cognate receptors.[4] In immune cells, the involvement of these processes is observed in the growth and maintenance of cells, as well as in the development of tumors. Chemokine (C-C motif) ligand 20 (CCL20), also named macrophage inflammatory protein-3α, acts as the ligand for CCR6. It is produced by both tumor and stromal cells, and plays a crucial function in attracting various populations of immune cells to the

tumor microenvironment. These include macrophages, immature granulocytes, dendritic cells, and tumor-infiltrating lymphocytes (TILs), contributing to the immune milieu within the tumor vicinity. CCL20 enhances the tumor's potential to evade the immune system surveillance and accelerates tumor growth by attracting tumor-promoting immunosuppressive cells to the tumor microenvironment.[5,6] CCL20 and CCR6 are significantly upregulated in many types of malignant tumors[7–10] and have been correlated to poor prognosis, early recurrence, and metastasis in many cancer types, including prostate cancer,[11] pancreatic,[12] breast cancer,[13,14] and colon cancers.[15]

During the process of epithelial-mesenchymal transition (EMT), there is a loss of adhesion between epithelial cells at their cell-cell junctions causing them to transform into stromal-like cells. Consequently, the cells acquire a highly motile, spindle-shaped, mesenchymal fibroblastoid phenotype and lose their epithelial polarity. The alterations increase the potential of tumor cells for invasion and metastasis, via the acquired capability to move through the extracellular matrix (ECM) and basement membrane.[16]

The invasion, metastasis, and progression of many cancer types are significantly influenced by EMT.[17–20] As a consequence of epithelial markers like E-cadherin (E-Cad) being downregulated and mesenchymal-associated proteins like vimentin (Vim), fibronectin, and N-cadherin (N-Cad) being increased, the tumor cells undergoing EMT simultaneously disrupt cell-cell adhesion structures and polarity.[21] EMT can be stimulated by many extrinsic cues originating from the tumor microenvironment, including growth factors, cytokines, chemokines, and components of the ECM.[22] Studies have shown that the PI3K/AKT-ERK1/2 signaling axis is the mechanism by which the CCL20/CCR6 interaction drives the EMT occurrence and subsequent spread of colon cancer.[23]

Breast cancer is a highly heterogeneous tumor. Given the shortage of viable therapeutics, a deeper comprehension of the molecular pathways behind disease progression is essential. In this study, tissue samples were obtained from patients diagnosed with breast cancer to assess the level of CCL20 expression. Furthermore, the correlation between clinicopathologic characteristics and patient survival was analyzed. Subsequently, the connections between EMT markers and CCL20 expression were also investigated.

2. METHODS

2.1. Patient sample selection

This study included a cohort of 113 adult female patients who were diagnosed with primary invasive breast cancer. Between February 2009 and November 2012, these patients had surgery at Beijing Shijitan Hospital; they had not received neoadjuvant chemotherapy at this point. The inclusion criteria lacked distant metastases, resection tissue availability, and complete follow-up records. All the patients underwent the standard surgical procedure, which included radiation combined with either a wide local excision or a mastectomy. Invasive breast cancer samples (n = 113) and adjacent normal tissues (n = 113) were collected during surgery. Patients with breast cancer who registered with our center were treated during the study in accordance with our center's treatment protocols. Systemic adjuvant treatment was offered to patients with a tumor diameter ≥1 cm or positive for axillary lymph nodes. The primary regimen used was anthracycline and paclitaxel-based chemotherapy. For women who have been diagnosed with hormone receptor-positive breast cancer, they were recommended to undergo adjuvant endocrine therapy spanning a period over 5 years. In the management of premenopausal individuals, the regimen commonly involved the daily administration of tamoxifen at a dosage of 20 mg. Conversely, postmenopausal women were typically advised to undergo treatment with aromatase inhibitors, such as exemestane, letrozole, or anastrozole. Radiotherapy was administered to breast cancer patients when displaying any indication: (1) the size of breast cancer is ≥5 cm; (2) the number of tumor-invaded axillary lymph node is ≥4; (3) the tumor has invaded into the skin or chest wall and behaves as T4 stage; (4) patients received breast-conserving surgery. Histopathological analysis indicated that the breast cancer specimens were either invasive lobular carcinoma (ILC): n = 8 or invasive ductal carcinoma (IDC): n = 105. In accordance with the seventh version of the AJCC Cancer Staging Manual, clinicopathologic classification and staging were used.[24] All participants underwent a follow-up assessment as of January 2017 to ascertain their current status. The study followed the guidelines set by the Institutional Committee for the Protection of Human Subjects and was carried out in conformity with the Declaration of Helsinki. Clinicopathological factors, such as age, histological grade, tumor dimensions, vascular invasion, lymphatic metastasis, and tumor-node-metastasis (TNM) staging, were extracted from the institutional medical record database. Analysis of the outcomes and correlation with clinical variables were conducted based on a cohort comprising 113 patient samples. The expression of the human epidermal growth factor receptor 2 (HER-2), PR, ER, and Ki67 was examined using immunohistochemistry (IHC) on each specimen. Staining over 10% of the nuclei in the tumor region was recognized as positive. Four categories were used to represent the staining intensity for HER-2 expression: negative for grades 0 and 1, undetermined for grades 2, and positive for grades 3. For every grade 2 sample, fluorescence in situ hybridization was conducted. Regarding gene amplification, samples with <2 expression were considered negative, whereas those with >2 expression were considered positive. Lymph node metastases in patients were documented and the TNM stage was established using the AJCC Cancer Staging Manual, 7th edition.[24]

Written informed consent from patients and clearance from the Institutional Study Ethics Committee, was sought to use clinical materials for this study.

2.2. Immunohistochemistry

Tissues that had been embedded in paraffin were sectioned into 4 μm-thick slices. The tissues were heated in citrate buffer to facilitate antigen retrieval after the slides had been deparaffinized and rehydrated. Following a 30-minute blockage of the tissues at room temperature using 3% hydrogen peroxide, the tissues were further incubated with goat serum for an additional 30 minutes at room temperature. Subsequently, each section was incubated with phosphate-buffered saline (PBS) (negative control), rabbit polyclonal anti-human CCL20 (1:50 dilution, ab9829; Abcam, Cambridge, UK), mouse monoclonal anti-human E-Cad (1:400 dilution, ab1416; Abcam), rabbit polyclonal anti-human N-Cad (1:250 dilution, ab18203; Abcam), or mouse monoclonal anti-human Vim (1:100 dilution, ab8978; Abcam) overnight at 4°C in the dark. The tissues were then treated with a secondary antibody (ZSGB-BIO, Beijing, China). The sections were subjected to biotinylation horseradish peroxidase-labeled streptavidin (ZSGB-BIO), followed by 3,3-diaminobenzidine (DAB; ZSGB-BIO) for visualization. Meyer's hematoxylin was used as a counterstain for the sections. The Nikon ECLIPSE E600 microscope, equipped with the Cool SNAP-Procf Color camera was used to assess the slides.

2.3. Evaluation of IHC staining

CCL20, Vim, N-Cad, and E-Cad expression were assessed by analyzing the positive proportion and staining intensity in 10

randomly chosen high-power fields at 400× magnification. The section was considered positive if granules or brown/yellow pigment were present in the tumor cells' plasma. Tumor proportion scoring was as follows: 0: none; 1: <10%; 2: 10% to 50%; 3: 50% to 80%; and 4: 80% to 100%. Another method used for assessment depended on the staining intensity. A score of 0 represented the absence of staining, 1 for a faint light-yellow staining, 2 for a moderate yellow/brown staining, and 3 for a substantial brown staining. The staining was categorized as either low or high expression, based on the staining index (SI). The SI for each area was calculated by multiplying the intensity score by the positive tumor proportion score. When the SI <4, low expression of CCL20, E-Cad, Vim, and N-Cad was assumed, whereas SI >4 indicated high expression. Two pathologists who were blind to the clinical pathological parameters based on Remmele and Stegner assessed the IHC staining score.[25]

2.4. Follow-up

Multiple channels, such as medical records, family visits, or telephone interviews, were utilized for collecting follow-up information from breast cancer patients. With a median follow-up duration of 60 months, ranging from 48 to 70 months, patients initially were followed up every 3 months, then every 6 months, and eventually annually. Clinical and pathological variables were examined, and data about survival and local, regional, and distant recurrences were documented. The follow-up period was defined based on the time from the operation to the date of death or the final follow-up. OS was determined by measuring the time from the operation date until either the date of death or the final follow-up. The period between the surgery and the onset of recurrence or metastasis was used to determine disease-free survival, this is also referred to as DFS.

2.5. Statistical analysis

The statistical software SPSS 21.0 (IBM, Armonk, NY) was utilized for all statistical analyses. The correlation between the expression of CCL20 and clinicopathological features, such as age, histological grade, nodal status, tumor size, tumor stage, ER status, HER-2 positive, and vascular invasion, was examined using Pearson's chi-squared test. While univariate survival association analyses for OS and DFS were conducted utilizing the log-rank test, the relationship between CCL20 and EMT-associated markers was investigated using the Spearman rank correlation test. The Kaplan-Meier method was used to graph survival curves, and the log-rank test was used for comparison. A multivariate Cox proportional hazards regression model was used to identify independent prognostic factors to assess the independent influence of CCL20 expression on DFS and OS. It was considered statistically significant when $p < 0.05$.

3. RESULTS

3.1. Analysis of CCL20 expression in breast cancer and adjacent noncancerous tissues

To understand the expression and distribution of CCL20, we conducted IHC staining of CCL20 in breast cancer tissues (n = 113) and adjacent noncancerous tissues (n = 113). CCL20 protein was predominantly localized in the cytoplasm in both types of tissues (Fig. 1A, B). The staining intensity of CCL20 in breast cancer was stronger than that in normal tissue (Fig. 1). In cancer tissues, 79 of 113 (69.9%) had high cytoplasmic expression of CCL20, and the remaining tissues (34/113, 30.1%) had low cytoplasmic expression. In contrast, only 23.0% (26/113) displayed high CCL20 expression in the matched normal adjacent tissues (Fig. 1C and Table 1). These findings showed that the expression of CCL20 was significantly elevated in malignant tissues compared to the corresponding normal tissues ($p = 0.012$).

3.2. Correlation between CCL 20 expression and clinicopathologic factors

We further evaluated the correlation between clinicopathologic characteristics and CCL20 expression level determined by IHC staining (Table 2). CCL20 expression in the 113 breast cancer cases was significantly correlated with the primary tumor size ($p = 0.000$). In specific, 89.6% (43/48) of tumors >2 cm had high CCL20 expression, meanwhile only 55.4% (36/65) of tumors ≤2 cm had strong CCL20 expression (Fig. 2A). In addition, patients with more advanced clinical stages were more likely to exhibit high CCL20 expression ($p = 0.001$). In detail, positive expression rates of CCL20 were gradually increased from 53.3% (stage I) to 77.4% (stage II), then to 93.3 (stage III) (Fig. 2B). Furthermore, CCL20 expression was significantly higher in ER-negative tumors ($p = 0.003$; Fig. 2C) and triple-negative tumors ($p = 0.006$; Fig. 2D) than that in their counterparts. However, HER-2-positive cases indicated no significant correlation ($p = 0.456$). Moreover, a significant association was observed between CCL20 expression and vascular invasion ($p = 0.001$; Fig. 2E) and Ki67 ($p = 0.000$; Fig. 2F), suggesting the potential role of CCL20 in tumor metastasis and proliferation, respectively. As for other clinicopathologic factors, including patient age, histology, histology differentiation, HER-2-positive status, and positive lymph node status, no correlations were recognized with CCL20 expression. These data supported the association between high CCL20 expression and a more malignant phenotype.

3.3. Association between CCL20 and EMT marker expression in breast cancer specimen

The progression and metastasis of tumors were strongly associated with the occurrence of EMT. To investigate the association between CCL20 and EMT status in breast cancer, IHC was utilized to evaluate the expression level of EMT markers E-Cad, Vim, and N-Cad in patient tissues. Overall, the staining of E-Cad in luminal epithelial cells in normal tissue is stronger than that in breast cancer tissues (strong staining in normal 86/113 vs cancer 49/113) (Fig. 3A–C). In terms of Vim expression, we observed strong Vim signals in normal breast tissue. However, the strong staining was derived from basal myoepithelial cells (red arrows in Fig. 3F) and other stroma cells (yellow arrows in Fig. 3F). As for the luminal epithelial cells (blue arrows in Fig. 3F), there was little expression observed. In cancer tissues, 61 samples showed strong staining of Vim in breast cancer cells (Fig. 3D). In contrast, the rest of the cancer tissues (52 samples) displayed weak/negative staining of Vim in breast cancer cells, although with high/moderate expression of Vim in the stroma cells (yellow arrows in Fig. 3E). Accordingly, when comparing luminal epithelial cells of normal breast tissue and the cancer cells of breast cancer tissue, the expression of Vim in luminal epithelial cells was much weaker than that in breast cancer samples. Similarly, N-Cad was marginally expressed by luminal epithelial cells in normal breast tissue, whilst the breast cancer cells were able to largely upregulate the expression of N-Cad (strong staining in normal 50/113 vs cancer 93/113) (Fig. 3G–I).

Furthermore, to understand the potential role of CCL20 on EMT status in breast cancer, we focused on the cancer tissues to evaluate the relationship between CCL20 and E-Cad, Vim, and N-Cad. Amongst 113 breast cancer specimens, the numbers of samples with positive expression of E-Cad, Vim, and N-Cad

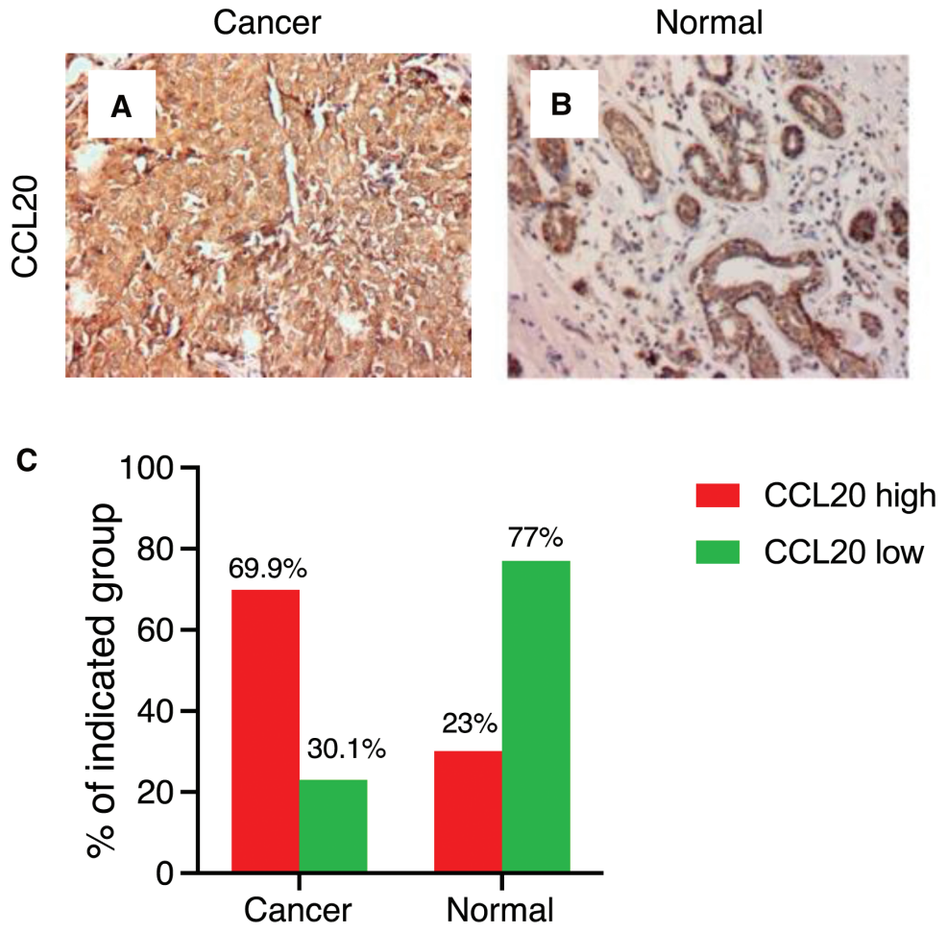

Fig. 1 CCL20 expression is enriched in breast cancer. A, Representative immunohistochemistry staining of CCL20 in breast cancer and matched adjacent normal tissue. B, The percentages of CCL20-high and CCL20-low samples in cancer and adjacent normal tissue. C, The statistical data for proportions of CCL20 expression level in indicated group.

were 78 (69%), 61 (54%), and 79 (70%), respectively (Table 3). In the E-Cad staining group, 64.1% (50/78) of E-Cad$^+$ samples were showing CCL20 high expression, meanwhile 82.9% (29/35) of E-Cad$^-$ samples were displaying CCL20 high expression (Fig. 4 and Table 3). This result revealed an inverse correlation between CCL20 expression and E-Cad expression in breast cancer. In contrast, CCL20 was positively associated with Vim and N-Cad expression (Fig. 4 and Table 3). In detail, 82% (50/61) of Vim$^+$ samples and 78.5% (62/79) of N-Cad$^+$ samples were stained with CCL20 high expression, while only 55.8% (29/52) of Vim$^-$ and 50% (17/24) of N-Cad$^-$ samples were showing CCL20 high expression (Fig. 4 and Table 3). These findings from staining analysis were consistent with classical change for these markers when EMT is undergoing downregulated E-Cad and upregulated Vim and N-Cad, thus indicating the strong potential role of CCL20 in EMT promotion in breast cancer.

3.4. CCL20 is linked to decreased survival rates among individuals diagnosed with breast cancer

In order to explore the correlation between CCL20 expression and the survival of breast cancer patients, we conducted univariate Cox proportional hazard regression analyses. These analyses identified that in addition to associations between other prognostic clinicopathological factors and survival, CCL20 expression levels showed a significantly strong relationship with clinical outcomes (Table 4). The median follow-up period for the study encompassed 60 months, ranging from 51

Table 1

Expression of CCL20 in breast cancer and adjacent noncancerous tissues

		CCL20				
		High expression		Low expression		
	Total	n	Rate	n	Rate	*p*
Cancer	113	79	69.9%	34	30.1%	0.012
Noncancerous	113	26	23.0%	87	77.0%	

CCL20 = chemokine (C-C motif) ligand 20.

Table 2

Relationship between CCL20 expression and clinicopathologic parameters of breast cancer patients

Variables	Case no.	CCL20 expression		*p*
		High	Low	
Age, y				0.356
≤50	37	28 (75.7%)	9 (24.3%)	
>50	76	51 (67.1%)	25 (32.9%)	
Histology				0.055
IDC	105	71 (67.6%)	34 (32.4%)	
ILC	8	8 (100%)	0 (0%)	
Histological grade				0.071
I	15	8 (53.3%)	7 (46.7%)	
II	85	60 (70.6%)	25 (29.4%)	
III	13	11 (84.6%)	2 (15.4%)	
Tumor size				0.000*
≤2 cm	65	36 (55.4%)	29 (44.6%)	
>2 cm	48	43 (89.6%)	5 (10.4%)	
Estrogen receptor				0.003*
Positive	78	48 (61.5%)	30 (38.5%)	
Negative	35	31 (88.6%)	4 (11.4%)	
Progesterone receptor				0.529
Positive	58	39 (67.2%)	19 (32.8%)	
Negative	55	40 (72.7%)	15 (27.3%)	
HER-2				0.456
Positive	25	19 (76.0%)	6 (24.0%)	
Negative	88	60 (68.2%)	28 (31.8%)	
Ki67				0.000*
≤15%	33	16 (48.5%)	17 (51.5%)	
15%-50%	48	32 (66.7%)	16 (33.3%)	
>50%	32	31 (96.9%)	1 (0.3%)	
Stage (TNM)				0.001*
I	45	24 (53.3%)	21 (46.7%)	
II	53	41 (77.4%)	12 (22.6%)	
III	15	14 (93.3%)	1 (0.6%)	
Nodal involvement				0.087
None	72	47 (65.3%)	25 (34.7%)	
1-3	26	18 (69.2%)	8 (30.8%)	
4-9	5	5 (100%)	0 (0%)	
≥10	10	9 (90.0%)	1 (10%)	
Vascular invasion				0.001*
Present	35	32 (91.4%)	3 (8.6%)	
Absent	78	47 (60.3%)	31 (39.7%)	
Triple-negative tumor				0.006*
Yes	15	15 (100%)	0 (0%)	
No	98	64 (65.3%)	34 (34.7%)	

* indicated statistically significant.

CCL20 = chemokine (C-C motif) ligand 20; HER-2 = human epidermal growth factor receptor 2; IDC = invasive ductal carcinoma; ILC = invasive lobular carcinoma; TNM = tumor-node-metastasis.

to 70 months. There were 52 cancer-related deaths, 5 (14.7%) and 47 (59.5%) of which had low CCL20-expressing and high CCL20-expressing phenotype, respectively, further supporting that CCL20 could be strongly associated with the patient's survival in breast cancer.

In the group with low CCL20 expression, the cumulative 5-year cancer-related survival rate was 85.3%, while the survival rate in the group with high CCL20 expression was 40.5% (Table 4). The log-rank test revealed a significant disparity in survival time between the two groups (p = 0.000) (Table 4). Furthermore, we examined the relationship between CCL20 expression and DFS. In general, the median DFS was 48 months. The DFS was 45 months for patients with high CCL20 expression and 60 months for those with low expression. At 5 years, patients with high expression of CCL20 had DFS rates of 35.40%, while patients with low expression of CCL20 had DFS rates of 79.40% (p = 0.000) (Table 4). In all patients, those with high CCL20-expressing tumors exhibited significantly lower survival rates than those with low-expressing tumors (p = 0.000) (Fig. 5A). Furthermore, we conducted a multivariate Cox regression analysis to assess the independent influence of CCL20 expression on prognosis (Table 5). The findings indicated that elevated CCL20 expression in individuals with invasive breast cancer was an independent predictive factor for unfavorable OS (hazard ratio, 3.207; 95% CI, 1.142-9.005, p = 0.027). Given that a poorer prognosis was linked to increased CCL20 expression, the findings indicate that CCL20 can serve as a prognostic marker for individuals diagnosed with breast cancer. Furthermore, ER status, Ki67, and histology were independent predictors

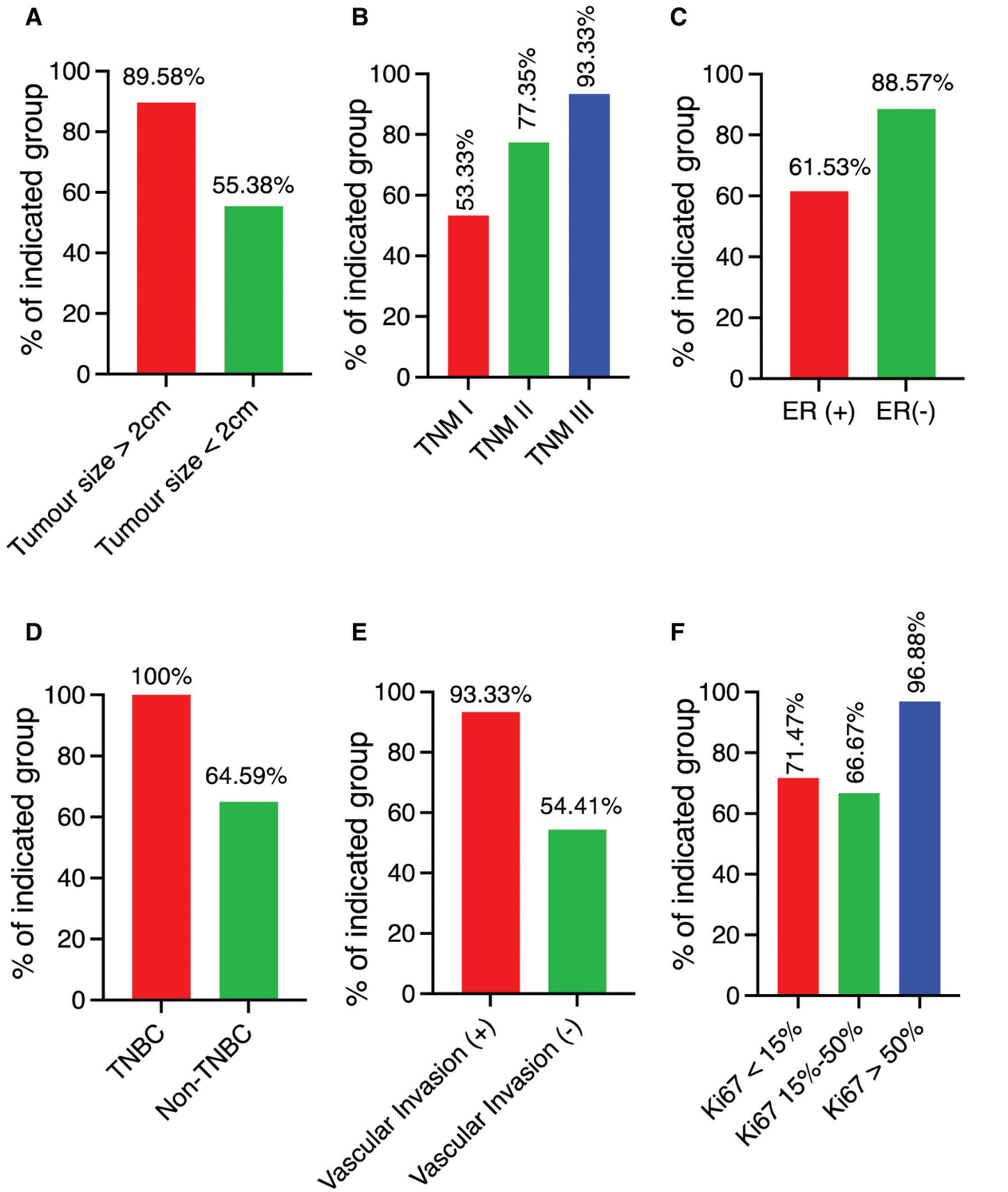

Fig. 2 Correlation between CCL 20 expression in cancer tissues and clinicopathologic factors. The numbers indicated the percentage of cancer tissues displaying CCL20-high expression in indicated subgroups. A, tumour size; B, TNM stage; C, ER status; D, TNBC status; E, invasion status; F, Ki67 expression. ER = estrogen receptor; TNM = tumor-node-metastasis.

of outcome. Because of such observation, we examined the prognostic importance of CCL20 expression in specific stratified subgroups based on the status of lymph nodes and the ER. A significant correlation (p = 0.001) was found between high CCL20-expressing tumors and a poorer prognosis for women (n = 78) with ER-positive tumors (OS: p = 0.001;

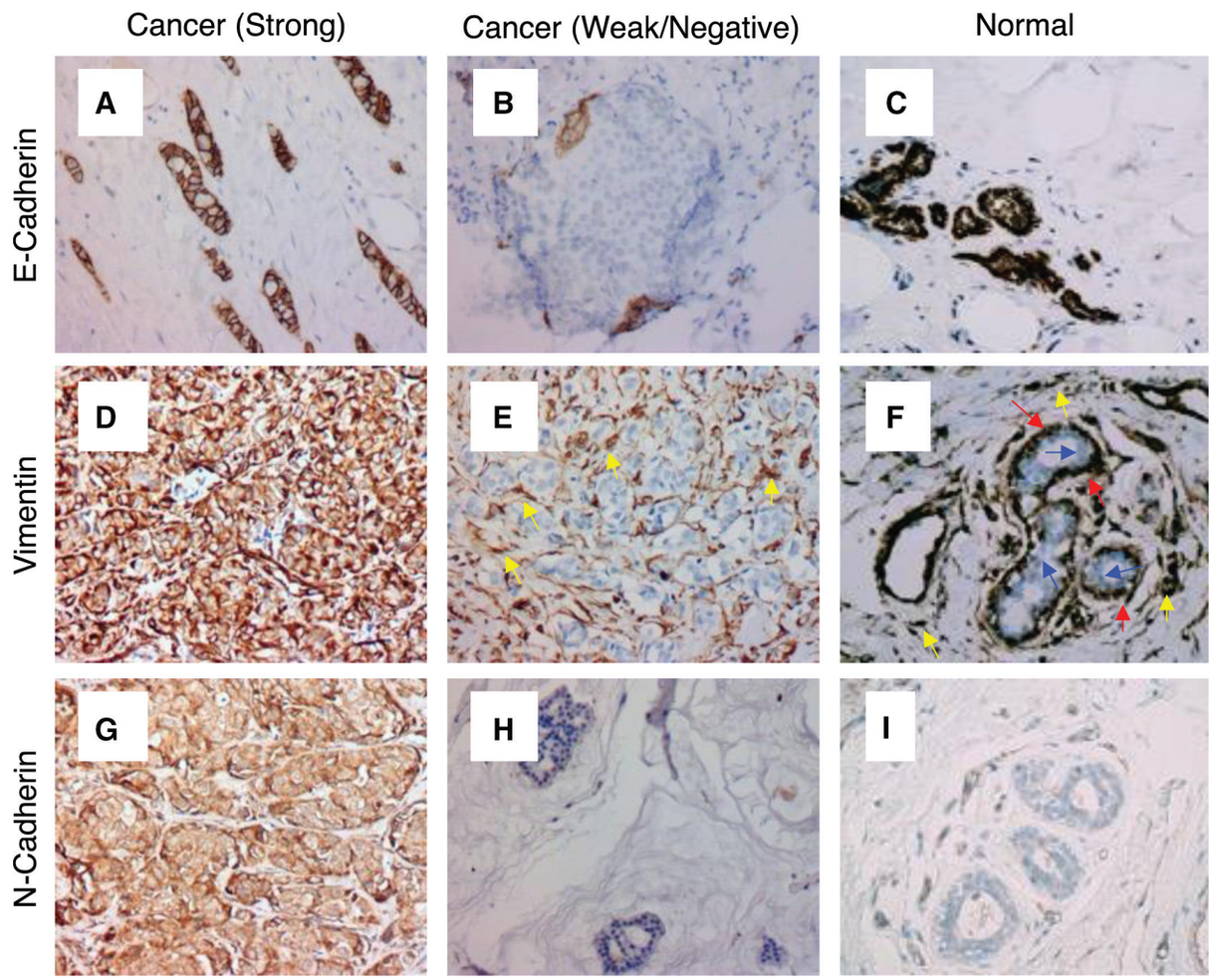

Fig. 3 Representative IHC images of E-cadherin (A, B, and C), vimentin (D, E, and F), and N-cadherin (G, H, and I) in breast cancer tissues with strong cancer cell-related expression (A, D, and G), with weak/negative cancer cell-related expression (B, E, and H), and paired adjacent normal tissues (C, F, and I) under 200× magnification. Blue, yellow, and red arrows in E and F indicate myoepithelial cells, stroma cells, and luminal epithelial cells, respectively. IHC = immunohistochemistry.

Table 3

Relationship between CCL20 and EMT markers in breast cancer tissues

			CCL20		
		n (113)	High	Low	*p*
E-Cad	+	78	50 (64.1%)	28 (35.9%)	0.044
	–	35	29 (82.9%)	6 (17.1%)	
Vim	+	61	50 (82.0%)	11 (18.0%)	0.002
	–	52	29 (55.8%)	23 (44.2%)	
N-Cad	+	79	62 (78.5%)	17 (21.5%)	0.002
	–	34	17 (50.0%)	17 (50.0%)	

CCL20 = chemokine (C-C motif) ligand 20; E-Cad = E-cadherin; EMT = epithelial-mesenchymal transition; N-Cad = N-cadherin; Vim = vimentin.

Fig. 5B). Among patients (n = 41) with positive lymph nodes, those with high CCL20 expression had a significantly poorer OS compared to those with low expression (OS: $p = 0.005$, Fig. 5C). Patients exhibiting higher levels of CCL20 expression in comparison to those with lower expression had a significantly poorer OS among lymph node-negative patients (n = 41) (OS: $p = 0.001$; Fig. 5D).

4. DISCUSSION

The CCR6–CCL20 axis plays pivotal roles in several inflammatory and autoimmune diseases, such as inflammatory bowel disease,[26] psoriasis,[27] and rheumatoid arthritis.[28] Over a prolonged duration, it has been proposed that chronic inflammation can promote neoplastic transformation. In tumor environments, CCL20 enhances the chemotaxis of effector/

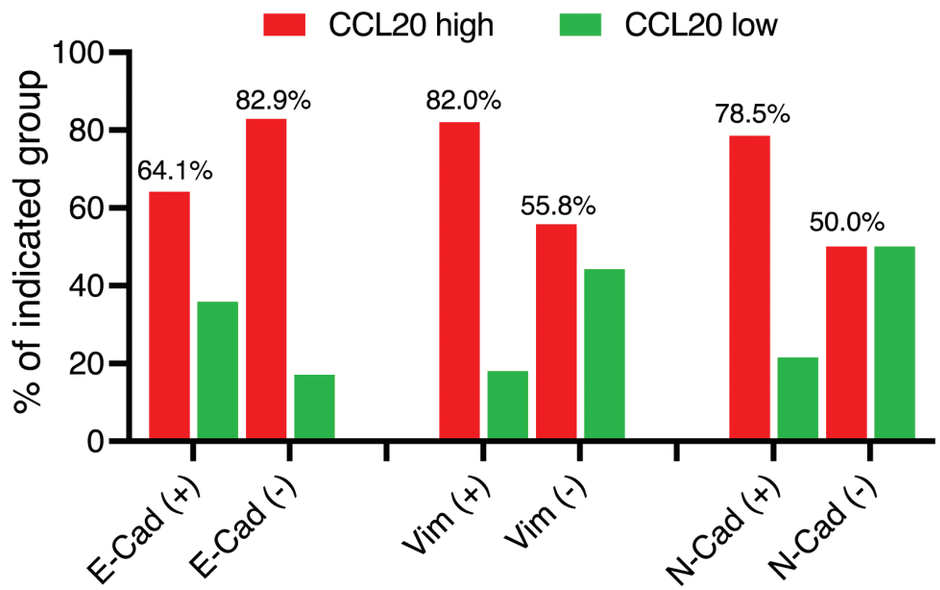

Fig. 4 The correlation of CCL20 and EMT markers in cancer tissue: E-Cad, Vim, and N-Cad. The graph shows the percentages of samples with CCL20-high (red bar) and CCL20-low (green bar) expression based on E-Cad expression (left), Vim expression (middle), and N-Cad expression (right). The numbers indicate the percentage of CCL20-high expression cancer tissues in the indicated group. E-Cad = E-cadherin; EMT = epithelial-mesenchymal transition; N-Cad = N-cadherin; Vim = vimentin.

memory T cells and B cells along with dendritic cells.[29] Tumor growth and invasion are accelerated through the process of chronic inflammation, the body releases chemokines, antigenic growth factors, and matrix-degrading enzymes.[30] However, it is still uncertain what chemical pathways underlie this transformation.

Numerous human malignancies, including liver, colon, prostate, pancreas, and breast, have been found to involve the CCR6/CCL20 axis, which has been associated to the pathogenesis, progression, and metastasis. Studies investigating the correlation between the expression of CCR6/CCL20 and the prognosis of liver cancer have demonstrated that CCR6 is inversely linked to the OS rate in liver cancer, which may be a result of the increased likelihood of CCR6-mediated metastasis. CCL20, produced from tumors, can spread to adjacent breast tissues to stimulate NF-κB activation and enhance the expression of matrix metalloproteinase 9 (MMP9). These processes promote the growth and mobility of tumor cells.[31]

Previous investigations have revealed that, from clinical samples, CCL20 is upregulated in breast cancer at the mRNA level,[32,33] and has been associated with the aggressiveness of breast cancer and poor prognosis.[32,34] The first objective of this investigation was to examine the expression and distribution of CCL20 in breast cancer and matched noncancerous tissues. In our study, when compared to corresponding adjacent normal tissues, malignant tissues had considerably higher levels of CCL20 expression in the cytoplasm, determined at the protein level by IHC. We further examined the relationship between the expression of CCL20 and clinicopathologic variables. We identified that CCL20 expression was associated with the primary tumor size (p = 0.000) and clinical stage (p = 0.001). Patients with stage III cancer had a considerably higher positive expression of CCL20 (93.3%) than patients at stage II (77.4%) and stage I (53.3%). In addition, tumors with high expression of CCL20 were more likely to be triple-negative (p = 0.006) and ER-negative (p = 0.003), which is in line with the finding in a previous study that CCL20 mRNA is gradually increased from luminal type to HER-2$^+$ then to TNBC.[32]

From the perspective of tumor progression mechanism exploration, CCL20 has been reported to be involved in (1) EMT-related migration and invasion of both breast epithelial cells and TNBC cells via increased MMP2/9 production[33,35,36]; (2) proliferation of TNBC cells[35,36]; (3) VEGF induction for angiogenesis mediated progression[34]; (4) chemotherapy resistance development via improved self-renewal of breast cancer stem cells to taxanes.[37] Our study observed a strong correlation between CCL20 and vascular invasion (p = 0.001) and proliferation marker Ki67 (p = 0.000), which in turn provided the clinical evidence for those in vitro mechanism conclusions.

In accordance with Kaplan-Meier curves, women with high tumor-expressing CCL20 had a poorer OS (p = 0.000) and DFS (p = 0.005) than women with low tumor-expressing CCL20. Women with ER-positive, node-positive, and node-negative tumors identically exhibited a strong correlation (p = 0.001) between increased CCL20 expression and a poorer prognosis. The relationship between the expression of CCL20 and a more malignant phenotype is supported by these findings.

Breast cancer arises from the normal cells in the breast epithelium, and this process is complex and is impacted by both the tumor cells and the tumor microenvironment. Even before invading the stroma, tumor cells have the ability to educate the tumor microenvironment to facilitate tumor spread. Furthermore, in the early stages of carcinogenesis, the peritumoral region might alter its functional characteristics.[38] Although breast cancer patients can be successively treated if diagnosed in the early stage, poor survival rates are associated with metastasis and rapid progression. The process of cancer metastasis comprises several stages, starting from the dispersal of initial malignant cells and their morphological transformation from a transition to a more migratory phenotype, and ending with distant homing. The complex process known as EMT, the process of metastasis and progression in various types of solid tumors involves a transformation, from a less mobile epithelial form to a greater mobile spindle-shaped mesenchymal form. EMT is distinguished by the downregulation of genes such as E-Cad that maintain the distinctiveness of epithelial cells, while simultaneously

Table 4

Univariate analysis of clinicopathologic parameters in breast cancer patients

		5 y OS			5 y DFS		
	Total	**n**	**(Rate %)**	***p***	**n**	**(Rate %)**	***p***
Age				0.108			0.104
≤50	37	17	45.90%		14	37.80%	
>50	76	44	57.90%		41	53.90%	
Histology				0.203			0.036*
IDC	105	58	55.20%		52	50.50%	
ILC	8	3	37.50%		2	25.00%	
Histological grade				0.006*			0.001*
I	15	10	66.70%		10	66.70%	
II	85	48	56.50%		43	50.60%	
III	13	3	23.10%		2	15.40%	
Tumor size				0.004*			0.045*
≤2 cm	65	42	64.60%		36	55.40%	
>2 cm	48	19	39.60%		19	39.60%	
ER				0*			0*
Positive	78	55	70.50%		52	66.70%	
Negative	35	6	17.10%		3	8.60%	
PR				0.006*			0.004*
Positive	58	38	65.50%		35	60.30%	
Negative	55	23	41.80%		20	36.40%	
HER-2				0.001*			0.000*
Positive	25	7	28.00%		4	16.00%	
Negative	88	54	61.40%		51	58.00%	
Ki67				0*			0*
≤15%	33	26	78.80%		25	75.80%	
15%-50%	48	28	58.30%		28	58.30%	
>50%	32	7	21.90%		2	6.30%	
Stage TNM				0*			0*
I	45	32	71.10%		27	60.00%	
II	53	27	50.90%		26	49.10%	
III	15	2	13.30%		2	13.30%	
Nodal involvement				0*			0*
None	72	45	62.50%		40	55.80%	
N1	26	14	53.80%		13	50.00%	
N2	5	1	20.00%		1	20.00%	
N3	10	1	10.00%		1	10.00%	
Vascular invasion				0.011*			0.017*
Present	35	14	40.00%		12	34.30%	
Absent	78	47	60.30%		43	55.10%	
Triple-negative				0*			0*
Yes	15	1	6.70%		1	6.70%	
No	98	60	61.20%		54	55.1%%	
CCL20				0*			0*
Low	34	29	85.30%		27	79.40%	
High	79	32	40.50%		28	35.40%	
Luminal				0*			0*
Yes	79	55	69.60%		52	65.80%	
No	34	6	17.60%		3	8.80%	

* indicated statistically significant.

CCL20 = chemokine (C-C motif) ligand 20; ER = estrogen receptor; HER-2 = human epidermal growth factor receptor 2; IDC = invasive ductal carcinoma; ILC = invasive lobular carcinoma; TNM = tumor-node-metastasis.

upregulating the expression of genes that promote invasion and migration, such as Vim and N-Cad.[39]

CCR6 expression is high in node-positive colon cancer cases, with the highest expression occurring in metastatic cases. In response to CCL20, EMT markers alter significantly, and there is a decrease in proliferation as well as an increase in migration and invasive potential.[40] In accordance with recent studies, CCL20 induced the decrease of E-Cad and ZO-1 in breast cancer patients, while also causing the expression of Vim and N-Cad that are involved in the mesenchymal process. These changes are strongly associated with poor prognosis and tumor aggressiveness.[33]

In the final stages, mammary epithelial cells exhibit increasingly invasive and migrating phenotypes through the induction of the EMT. Moreover, the release of CCL20 may have a function in a paracrine loop between malignant and normal cells during the development of breast carcinoma. IHC was utilized in our investigation to identify the levels of EMT markers,

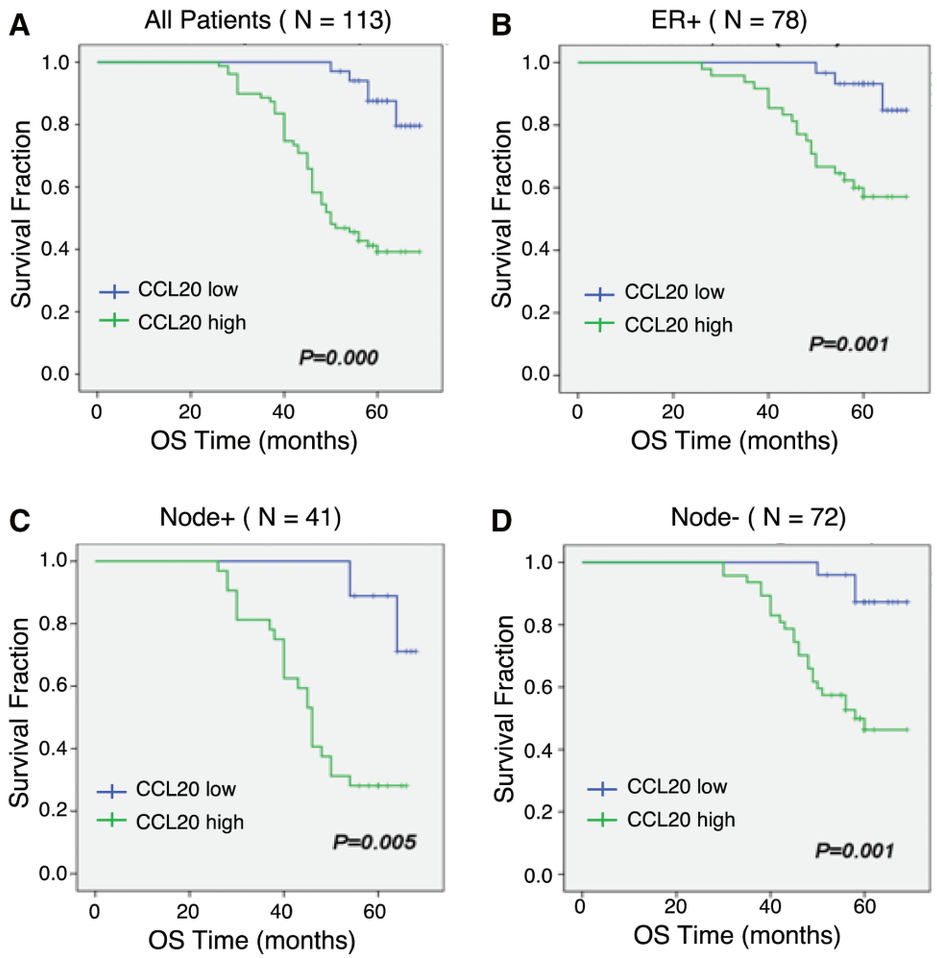

Fig. 5 The overall survival rates in all breast cancer patients (A), ER+ breast cancer patients (B), lymph node involvement positive patients (C), and lymph node involvement negative patients (D), based on CCL20 expression. ER = estrogen receptor; OS = overall survival.

Table 5

Multivariate analysis of contributory factors to the prognosis among 113 invasive breast cancer patients

	DFS			OS		
Variables	**Hazard ratio**	**95% CI**	***p***	**Hazard ratio**	**95% CI**	***p***
Age	1.114	0.584-2.128	0.743	1.182	0.593-2.354	0.634
Histology	0.77	0.218-2.712	0.684	0.222	0.051-0.969	0.045*
CCL20	1.985	0.800-4.921	0.139	3.207	1.142-9.005	0.027
Vascular invasion	0.762	0.385-1.508	0.435	0.793	0.397-1.585	0.511
HER-2 positive vs negative	1.058	0.514-2.178	0.879	0.857	0.412-1.784	0.68
TNM stage	1.853	0.515-6.669	0.345	2.081	0.538-8.049	0.288
ALND-N0N1N2N3	1.524	0.700-3.317	0.288	1.733	0.756-3.973	0.194
Ki67 stage	1.683	1.078-2.627	0.022	1.798	1.096-2.951	0.020*
ER-positive vs negative	0.122	0.042-0.354	0	0.143	0.049-0.421	0.000*
PR positive vs negative	1.388	0.535-3.601	0.501	1.078	0.398-2.921	0.883
Differentiation	1.193	0.596-2.390	0.618	0.958	0.476-1.928	0.904

* indicated statistically significant.

CCL20 = chemokine (C-C motif) ligand 20; DFS = disease-free survival; ER = estrogen receptor; HER-2 = human epidermal growth factor receptor 2; OS = overall survival; TNM = tumor-node-metastasis.

including E-Cad, Vim, and N-Cad. The analysis demonstrated a positive correlation between elevated levels of CCL20 expression and the expression of Vim and N-Cad. Conversely, a negative correlation was detected with E-Cad expression. Our research indicated that the existence of CCL20 may lead to a decreased OS rate in individuals with breast cancer by facilitating the process of EMT, another strong evidence supporting the in vitro mechanism findings.[33,35,36] These results together can highly inspire a promising practice of interfering with the function of the CCL20/CCR6 axis to delay breast cancer progression and metastasis. A study delivered a promising therapeutic efficacy that functionally blocked CCL20 using neutralizing antibodies and was able to inhibit the MDA-MB-231 cell bone metastasis in an in vivo mouse model.[35]

The current study relied substantially on the clinical samples from our center that were not collected specifically for a designed topic. To more comprehensively show the association between CCL20 and the EMT status and the survival of patients, we tried to include as many samples as possible. However, we were aware that most of the breast cancer samples were IDC (94.7%, 107/113), meanwhile, ILC only accounted for 5.3% (6/113). This unbalanced percentage between IDC and ILC could make some findings masked. By increasing the size of ILC samples until reaching a sensible number, the association between CCL20 and various clinicopathological parameters can be compared between ILC and IDC, which may provide exciting results. In the meantime, cross-validation using samples from multicenter would significantly lead to more convincing conclusions.

To focus on the primary site of breast cancer, we excluded the patients with distant metastasis. Previous studies have shown a significant correlation between CCL20 and metastasis-free survival.[32] The distant metastasis samples should be utilized to better understand the roles of CCL20 in migration/invasion/metastasis. The conclusion will indicate the significance of targeting CCL20 for blocking metastasis of breast cancer. Furthermore, we would assume that the patients with relapse had accumulated greater resistance to treatment. These samples are invaluable for the mechanism exploration for CCL20-mediated treatment resistance development.

Overall, this study further raised a promising beneficial strategy by investigating how the tumor microenvironment's secreted components controlling tumor growth and metastasis. Understanding and characterizing the molecular mechanisms in breast cancer that control the metastatic process and the aggressiveness of the disease is essential to improve the therapy design and subsequent prognosis. The CCL20 expression, EMT status, and properties of tumors and adjacent tissues may provide crucial new insights into the manageability of cancer emergence and metastasis. All the results above, together with previous studies from other groups, indicated that CCL20 is highly involved in the progression of breast cancer and the EMT-metastasis process. Thus, targeting the CCL20-CCR6 axis in breast cancer could be an exciting therapy strategy for breast cancer patients.

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