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Increased *CXCL8* Expression Is Negatively Correlated with the Overall Survival of Patients with ER-Negative Breast Cancer

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Abstract. *Background: C-X-C motif chemokine ligand 8 (CXCL8) is a multi-functional chemokine and has important roles during tumor formation and development. It was previously reported that increased CXCL8 protein levels occurred in certain patients. Materials and Methods: In the present study, we examined levels of CXCL8 mRNA in breast cancer tissues and analyzed its levels in correlation to patients' clinical data and 10-year overall survival (OS). Results: Our results clearly demonstrated that the level of CXCL8 mRNA was significantly higher in patients without estrogen receptor expression. The receiver operating characteristic curve indicated that the best cut-off value for CXCL8 expression was 3.095 for predicting patient's OS. Conclusion: The present study demonstrated that higher CXCL8 mRNA levels in breast cancer tissues together with estrogen receptor negativity was associated with significantly shorter OS, and could be applied as a negative risk factor for 10-year OS.*

Breast cancer, a most common malignant tumor in women with high biological heterogeneity, is characterized by the diversity of clinical prognosis. Prolonged exposure to excess estrogen has been regarded as one of the important factors for initiation and development of breast cancer (1). Estrogen receptor-alpha (ERα) and -beta (ERβ) are involved in mediating the major biological and pathological effects of estrogen (2, 3). ERα status has been generally used as an important parameter in breast cancer management because ERα-positive breast cancers exhibit less aggressiveness and invasiveness than do ERα-negative breast tumors, which have a worse prognosis. Currently, clinicopathological parameters are still conventionally applied as the practical guidance for clinical treatment and as predictors for patient prognosis (4, 5). However, the role of ERβ in initiation and development of breast cancer and its clinical significance are still not clearly known (6). Based on the status of ERα, progesterone receptor (PR) and human epithelial growth factor receptor-2 (HER2/Neu; ERBB2), breast cancer is divided in five subclasses. Among which, triple-negative breast cancer (TNBC), which is negative for expression of ERα and PR, and with low expression of HER2/Neu, accounts for 20-25% of the breast cancer (7-9). Therefore, in order to identify new prognostic biomarkers useful for defining patients with a specific subclass of breast cancer being at high risk for shorter overall survival (OS) is of great significance for selecting appropriate approaches for therapeutic treatments.

C-X-C motif chemokine ligand 8 (*CXCL8*, also known as interleukin-8), an inflammatory chemokine (chemotaxis cytokine) containing the cysteine (C)-any amino acid (X)-cysteine (C) (CXC) motif, acts through two receptors, namely CXCR1 (IL8RA) and CXCR2 (IL8RB) (10). CXCR2 is known to be involved in mediating the biological and pathological effects of *CXCL8* and other related CXC chemokines. It has been well documented that *CXCL8* contributes to human cancer progression in autocrine and paracrine manners. Multiple mechanisms (11, 12) should be involved in *CXCL8* action, including both direct and indirect effects, *e.g.* angiogenesis, tumor cell growth and migration, attraction and infiltration of inflammatory cells. *CXCL8* is known to possess tumorigenic and proangiogenic properties *in vivo*. Increased expression of *CXCL8* has been detected in

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many human tumor types, including breast cancer. Higher expression of *CXCL8* was reported to be associated with poor prognosis in certain patients with cancer (13, 14). However, it is still unclear whether abnormal overexpression of *CXCL8* is related to the prognosis in Chinese patients with ERα-negative breast cancer as it has been reported that TNBC in Chinese populations exhibits different patterns of relapse (15).

Based on the potential roles of *CXCL8* in ERα-negative breast cancer, the present study aimed to investigate whether *CXCL8* expression together with other clinically available prognostic variables is related to long-term prognosis in Chinese patients with ERα-negative breast cancer and 10-year OS.

Materials and Methods

Patients and tissue samples. A total of 62 patients with primary breast cancer (all females aged between 30 and 84 years, median age of 50 years) who were hospitalized in the Third Affiliated Hospital of Soochow University from January 2003 to December 2003 were recruited in the present study. All the patients underwent modified radical operations and were followed-up to the end of September 2015. All the tumor samples were excised and quickly frozen in liquid nitrogen after resection and preserved at −80˚C until use for nucleotide analyses. All the tissue samples were histopathologically examined. They were all defined as invasive ductal breast cancer. In the present study, no patient was given any treatments before surgery. After surgery, all the patients received common anthracycline-based chemotherapy, but no patient was treated with humanized antibody to HER2 (trastuzumab) although trastuzumab was approved in 2002 in China. All the ERα-positive patients had routinely received a standard endocrinotherapy. However, most patients stopped continuing endocrinotherapy by themselves after 2-3 years and only a few patients with ERα-positive breast cancer continued endocrinotherapy for about 5 years. As several patients were unable to continue or complete the standardized endocrinotherapy, they were excluded from the data analyses.

The median follow-up time was 149.63 months (range=16.83-161.87 months) and terminated at September 30. 2015. The patients lost to follow-up and suspended cases were censored. The characteristics of the patients are listed in the Table I. The protocols of the present study were approved by the Institutional Ethics Committee of the Third Affiliated Hospital of Soochow University [(2001)KENo.1(R01)] and all the patients gave their written informed consent to use of their specimens and data.

Extraction of total RNA, reverse transcription and polymerase chain reaction (RT-PCR). Before RNA extraction, frozen sections of the specimens were examined histologically to ensure that they were representative of the tumor tissue. Total RNA of breast cancer tissue was extracted using total RNA isolation classic kit according to the instructions provided in the kit (SNBC, Shanghai, China) and treated with RNase-free DNase I (TaKaRa, Kyoto, Japan) for 30 min at 37˚C to remove residual DNA. RNA quality and its integrity were verified using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

A standard *CXCL8*-containing plasmid was diluted in a 10-fold series within the range of 10^8-10^1 with Tris-EDTA buffer. Each concentration of standard plasmid DNA sample was amplified by conventional PCR. The PCR-amplified product was separated in 1% agarose electrophoresis and stained with ethidium bromide. The size of polymerase chain reaction-amplified *CXCL8* fragment was 87 bp (Figure 1). This indicated that the maximal resolution of the conventional PCR assay was 10^3 copies per reaction. When the diluted *CXCL8* standard solutions were quantitatively detected by real-time quantitative (q)PCR, the maximal resolution of this method was 10 copies per reaction and there was a linear relationship between the copy number of *CXCL8* standard and the Ct value.

Total RNA (2 μg) was used to synthesize cDNA by reverse transcription. The first-strand cDNAs were synthesized by using Revert Aid first-stand cDNA synthesis kit (MBI Ferments Inc, Ontario, Canada) according to the instructions provided in the kit. The sequences for the primers for *CXCL8* and Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and Taqman probe were designed with primer design software as follows: *CXCL8*: forward primer: 5'-CTCTTGGCAGCCTTCCTGATT-3', reverse primer: 5'-TATGCACTGA CATCTAAGTTCTTTAGCA-3'; *CXCL8* probe: 5'-FAM-CTTGGCAAAACTGCACC TTCACACAGA-TAMRA-3'; *GAPDH*: forward primer: 5'-GGAAGGTGAAGGTCGGAGTC-3', reverse primer: 5'-CGTTCTCAGCCTT GACGGT-3'; *GAPDH* probe: 5'-FAM-TTTGGTCGTATTGGGCGCCTG-TAMRA-3'). These primers and probes were synthesized by Shanghai Shenyou

Table I. *Relationship between mRNA levels of C-X-C motif chemokine ligand 8 (CXCL8) and patient clinical parameters*.

Clinical characteristic	N	*CXCL8/GAPDH* Median (IQR)	Wilcoxon value*	*p*-Value*
Age, years				
≤50	34	2.69 (1.10-8.98)	506	0.677
>50	28	2.48 (1.08-9.24)		
Lymph node metastasis				
No	40	1.30 (1.07-8.15)	500	0.381
Yes	22	2.69 (1.55-9.92)		
Tumor size, cm				
≤2	36	3.16 (1.18-11.60)	555	0.217
>2	26	2.15 (1.02-4.57)		
Estrogen receptor α				
−	22	7.38 (2.33-18.07)	596	0.022
+	40	1.92 (1.08-4.33)		
Progesterone receptor				
−	24	3.14 (1.51-11.45)	501	0.520
+	38	2.35 (1.10-8.96)		
HER2				
−	36	2.59 (1.14-8.61)	434.5	0.638
+	26	2.60 (1.07-17.61)		
P53				
−	40	2.90 (1.14-11.60)	540.5	0.141
+	22	2.34 (1.02-3.67)		
Chemotherapy				
Yes	53	2.54 (1.08-9.16)	244	0.920
No	9	2.65 (1.13-9.58)		

CXCL8: C-X-C Motif Chemokine Ligand 8; IQR: interquartile range. *Wilcoxon rank-sum test.

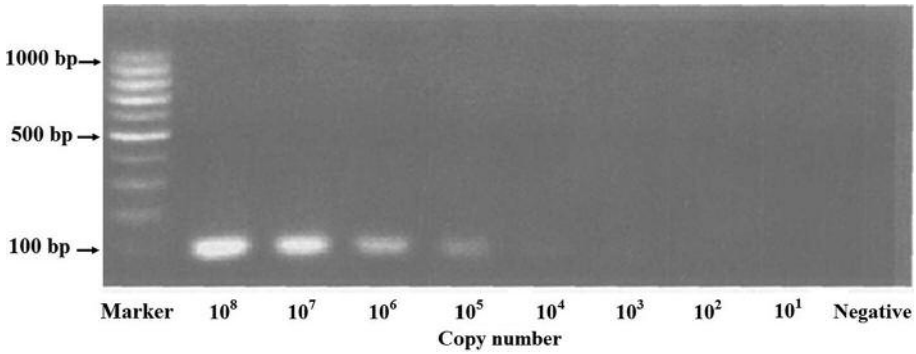

Figure 1. *The agarose electrophoresis image of conventional polymerase chain reaction-amplified C-X-C motif chemokine ligand 8 (CXCL8) fragment.*

Biotechnology, Ltd. (Shanghai, China). The PCR reaction for each target fragment was performed in total volume of 25 μl containing 2 μl of cDNA template, 2.5 μl of 10×buffer, 1.5 μl of $MgCl_2$ (25 mM), 0.5 μl of 4×dNTPs (10 mmol/l), 0.1 μl of 100 μM primers and probes and 0.5 μl of DNA polymerase. The thermal cycling conditions included the following steps: 180 s at 50˚C, 300 s at 95˚C, followed by 40 cycles of 2-step PCR consisting of 20 s at 95˚C, 60 s at 60˚C (collecting fluorescence signal). All PCRs were performed on a LightCycler PCR system (Roche Company, Indianapolis, IN, USA). The mRNA levels of *CXCR8* and *GAPDH* were quantitated based on their standard curves.

CXCL8 standard curve was established by plotting the known copy numbers of the positive *CXCL8* template against the corresponding Ct values. For each sample, the PCR assay was repeated three times and the mean value was taken as the expression of CXCL8 in that sample. The RT-qPCR reaction system for *GAPDH* was the same as that for *CXCL8*. The relative expression levels of *CXCL8* gene were calculated by normalizing *CXCL8* levels with the corresponding levels of *GAPDH* (CXCL8/GAPDH) and calculated based on *CXCL8* standard curve to obtain the relative expression of *CXCL8* gene.

Immunohistochemistry. Formalin-fixed and paraffin-embedded tissues were cut into 3-μm-thick consecutive sections, dewaxed in xylene and rehydrated in graded ethanol solutions. Polyclonal rabbit antibody against human HER2 and P53 (DAKO Company, Fuzhou, Fujian, China) and corresponding polyclonal mouse antibodies against human ERα and PR (MXB Biotechnologies, Fuzhou, PR China) were used. There were two steps in EnVision IHC staining and color development, 3,3'-diaminobenzidine (DAB) was used as the color reagent, and phosphate-buffered saline was used as substitute for the primary antibodies and taken as the negative control. Tumors were considered positive when there were at least 1% positively stained tumor nuclei in the sample on testing in the presence of expected reactivity of internal (normal epithelial elements) and external controls (16, 17).

Immunostaining was scored by two pathologists. The intensity (I) of staining was graded on a scale of 0-3+ with 0 representing no detectable staining and 3+ representing the strongest staining. The four strongest staining regions were randomly selected under a 400× field. In each of the four regions, the rate of positive cell staining (R) under a 400× field was calculated and defined as follows: 0. no staining; 1, ≤10% tumor cells with staining; 2, 11-50% tumor cells with staining; 3, 51-75% tumor cells with staining; and 4, >75% tumor cells with staining. Samples with scores <3 were considered negative, while those with scores ≥3 were considered positive. Histochemistry score=I × R (18).

Tumors were considered as HER2-positive when cells exhibited strong membranous staining (3+). Tumors exhibiting 0 or 1+ staining for HER2 protein expression were considered to be negative. In cases of equivocal membrane staining (score 2+) for HER2, fluorescence *in situ* hybridization was used to evaluate *HER2/neu* gene amplification (16, 17).

Statistical analyses. Statistical analyses were performed with the R software, version 3.0.3 (http://journal.r-project.org/). Breast carcinoma-specific OS was defined as the time from the date of diagnosis to death if the patient died from breast carcinoma. Wilcoxon rank-sum test was used to examine the distribution of quantitative *CXCL8* levels according to clinicopathological parameters. Survival curves were estimated by the Kaplan–Meier method, and the curves were compared with the log-rank test in univariate analyses. To examine the association of *CXCL8* and clinicopathological parameters with OS, the Cox proportional hazards regression model was applied for calculating the hazard ratios (HR) and for corresponding 95% confidence interval (95% CI). Forward stepwise regression was used to calculate the multivariate HRs. A *p*-value of less than 0.05 was considered as statistically significant. The receiver operating characteristic (ROC) curve was defined as a plot of test sensitivity as the Y-axis versus its 1-specificity as the X-axis, which was conducted to determine the cut-off value of *CXCL8* expression level for predicting 10-year OS of breast cancer patients. Then, the area under the curve (AUC) of the ROC was used to assess the predictive validity.

Results

Clinical characteristics and distribution of relative expression of CXCL8. The relative mRNA levels of *CXCL8* (*i.e.* the ratio of *CXCL8*/*GAPDH*) in breast cancer tissues of all 62 patients were examined. The patient's clinical parameters and the distribution of relative expression of *CXCL8* are shown in Table I. There were no significant differences (Wilcoxon rank-sum test) in mRNA expression

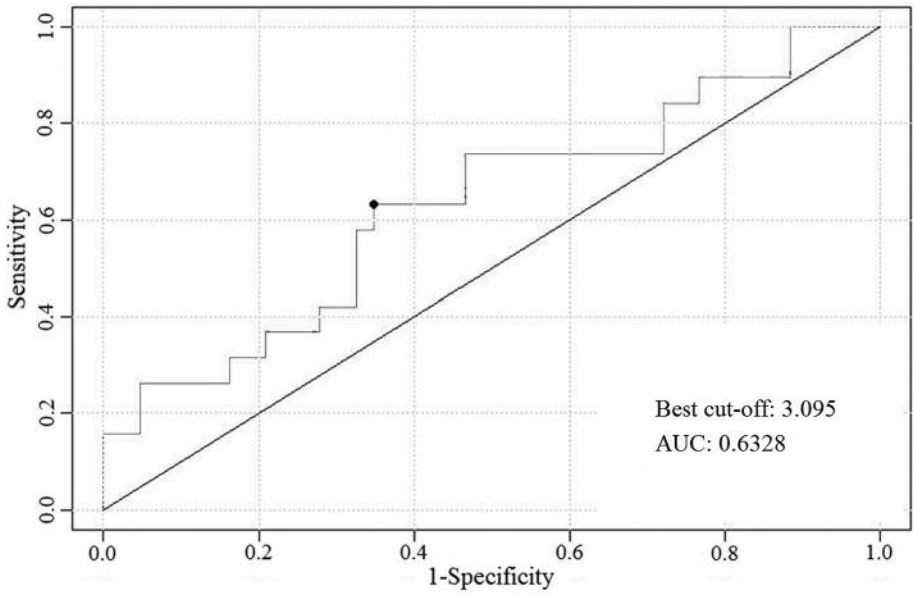

Figure 2. *Receiver operating characteristic curve based on the relative mRNA level of C-X-C motif chemokine ligand 8 (CXCL8). AUC: Area under the ROC curve.*

of *CXCL8* in terms of patient's age, lymph node metastatic status, tumor size, PR status, HER2, p53 and chemotherapy. The relative mRNA levels of *CXCL8* in ERα-negative breast cancer tissues were significantly higher than those in ERα-positive breast cancer tissues (Wilcoxon rank-sum test, p=0.022). Thereafter we performed an interaction test between paired variables on clinicopathological characteristics but their interaction showed no statistical significance (data not shown).

Association of CXCL8 level and clinical parameters in relation to patient prognosis. In the present study, the patients were followed-up for 10 years and the 10-year OS rates were analyzed. Based on the receiver operating characteristic (ROC) curve analysis (Figure 2), the optimal cut-off point of *CXCL8* expression was found to be 3.095 for 10-year OS rates. The sensitivity, specificity, area under the ROC curve (AUC) and the 95% CIs were 63.16%, 65.12%, 0.6328 and 0.4758-0.7898, respectively. At a median follow-up of 149.63 months (range=16.83-161.87 months), 70.97% (44/62) of all patients were alive.

The univariate prognostic factors for OS are shown in Table II. Patients with ERα-negative tumor (HR=3.11, p=0.015) and relative *CXCL8* expression value of 3.095 or more (HR=2.58, p=0.047) had a significantly lower rate of 10-year OS than did patients with the ERα-positive tumor with relative CXCL8 expression value of <3.095.

We further divided patients into four different subgroups according to their ERα status and the expression level of *CXCL8* (as shown in Table III). Compared other subgroups, the ERα-negative group with higher expression of *CXCL8* had a significantly poorer 10-year OS rate of 35.7% (p<0.001). After adjusting for age, tumor size, PR status, HER2, p53, chemotherapy and lymph node metastatic status, the multi-adjusted HR for ERα negativity together with high expression of *CXCL8* inversed to 16.19 (95% CI=3.32-78.96).

The results of log-rank testing shown in Figure 3 demonstrate that the ERα status and *CXCL8* mRNA level significantly influenced 10-year OS (p=0.001 and p=0.039, respectively). When the *CXCL8* level was used as a continuous variable, the HR was 1.01 (95% CI=1.00-1.01; p=0.027). Multiple stepwise linear regression analysis revealed that tumor size >2 cm), ERα negativity and high mRNA level of *CXCL8* were negative risk factors for 10-year OS (p=0.009, p=0.028 and p=0.022, respectively) (Table IV).

Discussion

It is well documented that elevated expression of *CXCL8* can contribute to cancer progression. A number of studies have highlighted the prognostic and predictive significance of *CXCL8* in different types of cancers, including ovarian (19), colon (20, 21), pancreatic (22), bladder (23), and prostate cancer (24), leukemia (25) and melanoma (26). However, the association of *CXCL8* level and clinical parameters with long-term OS rates of patients with breast cancer has yet to be reported as far as we are aware. In the present study, we

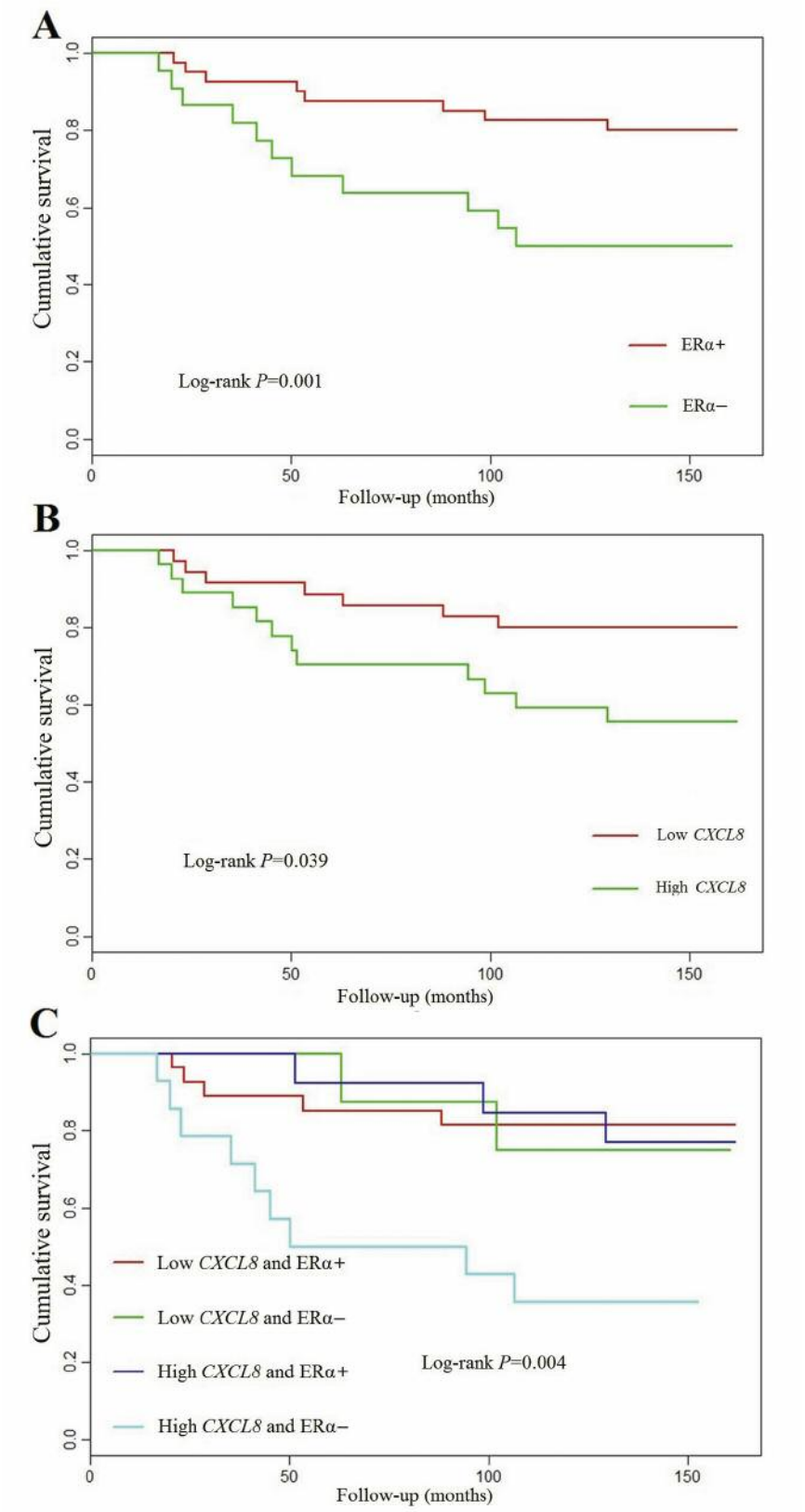

Figure 3. *Comparison of survival curves according to estrogen receptor (ER) status (A), C-X-C motif chemokine ligand 8 (CXCL8) mRNA level (B), and ER status with CXCL8 mRNA level (C).*

Table II. *Univariate analysis of patient overall survival.*

Clinical parameter		10-Year OS	Univariate	
			HR (95% CI)	*p*-Value
Age, years	>50 *vs.* ≤50	68% *vs.* 71%	1.12 (0.45-2.75)	0.811
Lymph node metastasis	No *vs.* yes	73% *vs.* 68%	0.79 (0.30-2.08)	0.637
Tumor size, cm	>2 *vs.* ≤2	58% vs.78%	2.32 (0.93-5.78)	0.070
Estrogen receptor α	− *vs.* +	50% *vs.* 80%	3.11 (1.25-7.74)	0.015
Progesterone receptor	+ *vs.* −	74% *vs.* 63%	0.63 (0.25-1.54)	0.307
HER2	+ *vs.* −	69% *vs.* 69%	0.90 (0.36-2.25)	0.826
P53	+ *vs.* −	73% *vs.* 68%	0.82 (0.31-2.16)	0.523
CXCL8	High *vs.* low	56% *vs.* 80%	2.58 (1.01-6.55)	0.047
Chemotherapy	Yes *vs.* no	72% *vs.* 56%	0.55 (0.18-1.66)	0.289

HER2: Human epidermal growth factor receptor 2; *CXCL8*: C-X-C motif chemokine ligand 8; CI: confidence interval; HR: hazard ratio.

Table III. *Patient overall survival (OS) in relation to Estrogen receptor-alpha (ERα) status and mRNA level of C-X-C motif chemokine ligand 8 (CXCL8).*

CXCL8	ERα	10-Year OS	HR (95% CI)*	*p*-Value	HR (95% CI)&	*p*-Value
Low	+	81.5%	1.00	-	1.00	-
Low	−	75.0%	1.27 (0.25-6.56)	0.773	2.70 (0.35-21.04)	0.344
High	+	76.9%	1.16 (0.28-4.86)	0.838	1.40 (0.30-6.48)	0.663
High	−	35.7%	5.08 (1.69-15.25)	0.004	16.19 (3.32-78.96)	<0.001

*Univariate analysis. &Adjusted for age, tumor size, progesterone receptor, human epidermal growth factor receptor 2, P53, chemotherapy and lymph node metastatic status.

Table IV. *Multiple stepwise linear regression analysis of patient overall survival (OS).*

Clinicopathlogical parameter		10-Year OS	Multivariate*	
			HR (95% CI)	*p*-Value
Tumor size, cm	>2 *vs.* ≤2	58% *vs.* 78%	3.61 (1.37-9.52)	0.009
ERα	− *vs.* +	50% *vs.* 80%	2.85 (1.12-7.24)	0.028
CXCL8	High *vs.* low	56% *vs.* 80%	3.19 (1.18-8.64)	0.022

ERα: Estrogen receptor-alpha; *CXCL8*: C-X-C motif chemokine ligand 8; CI: Confidence interval; HR: hazard ratio.*Adjusted for tumor size, ERα and *CXCL8*.

investigated the important prognostic role of *CXCL8* in Chinese patients with ERα-negative breast cancer. The statistical analysis (as shown in Figure 2) demonstrated the sensitivity and specificity of *CXCL8*, the best cut-off point being at 3.095 for relative mRNA expression of *CXCL8*. mRNA expression of *CXCL8* in breast cancer tissues had both high sensitivity and specificity for predicting 10-year OS rates. A positive correlation between the serum protein level of *CXCL8* and progression of disease, and a negative correlation with survival rate of patients with breast cancer have been demonstrated (27-29). Zuccari *et al.* reported an inverse correlation between the *CXCL8* protein levels determined by immunohistochemical staining and metastasis and/or local recurrence in patients with breast cancer (30). Herein, our results further confirmed that higher mRNA levels of *CXCL8* were the negative prognostic significance for 10-year OS rates in Chinese breast cancer patients with ERα-negative expression.

Breast cancer is one of the most common carcinomas in females with increasing morbidity worldwide. About 40-70% of breast cancer growth is related to estrogen (31) and ERα status is an important index in breast cancer management. It is well-known that patients with ERα-positive breast cancer have a much better prognosis than those with ERα-negative tumors (32). This difference is essentially due to the higher aggressiveness and invasiveness of ERα-negative tumors. In the present study, we demonstrated that Chinese patients with ERα-negative breast cancer had a relatively shorter OS. Furthermore, we found that the *CXCL8* mRNA levels were significantly higher in patients with ERα-negative than in those with ERα-positive disease, which could suggest that the mRNA level of *CXCL8* in patient's breast cancer tissues may be negatively linked to their ERα status. Moreover, we found that *CXCL8* was overexpressed at the transcriptional level in Chinese patients with ERα-negative cancer. This observation is consistent with those reported in previous studies showing potential significance of *CXCL8* mRNA levels in relation to patient's ERα status, *i.e*. *CXCL8* expression level is negatively linked to ERα status of breast and ovarian cancer cells (33-35). A study with a human cytokine antibody array confirmed that *CXCL8* was inversely associated with ERα status, but positively associated with the metastatic potential of ERα-negative breast cancer cells (36). Moreover, *CXCL8* expression was negatively linked to ERα status of breast and ovarian cancer cells and associated with a higher invasiveness potential of cancer cells *in vitro* (37). A subgroup of patients with TNBC with a low *CXCL8* expression level was characterized by a relatively low risk of recurrence, even in the absence of systemic therapy, and had a recurrence-free survival rate of 84% at 5 years (33). Our results demonstrate that patients with ERα-negative breast cancer with increased expression of *CXCL8* had only a survival rate of 35.7% at a median follow-up of 149.63 months (as shown in Table III).

A more recent study indicated that ERβ1 expression in archival TNBC specimens was related to significantly worse 5-year OS and that silencing ERβ expression significantly reduced TNBC proliferation and inhibition of ERβ expression with ERβ-specific antagonist also reduced TNBC growth (38). Although in the present study we did not examine the ERβ status of the breast cancer tissues, it is likely that different ERβ isoforms may exist and play a role in these patients with breast cancer. Further study is needed to address this possibility.

In conclusion, this study has suggested that the mRNA level of *CXCL8* in breast cancer tissue can be a promising prognostic biomarker for 10-year OS, especially for patients with the ERα-negative type cancer, and that increased *CXCL8* expression may contribute to a worse patient's progress probably *via* increasing the invasiveness of cancer cells. The detailed mechanism underlying this, needs further investigation.

Conflicts of Interest

The Authors declare that they have no competing interests.

Acknowledgements

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