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ORIGINAL ARTICLE

# C-X-C Chemokine Receptor Type 7 (CXCR-7) Expression in Invasive Ductal Carcinoma of Breast in Association with Clinicopathological Features

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## Abstract

C-X-C chemokine receptor type 7 (CXCR-7) is an atypical receptor for chemokines whose role in different stages of carcinogenesis has been evaluated in breast cancer cell lines and animal models. Moreover, it has been demonstrated to be a target of regulation by the tumor suppressor microRNA (miR)-100. In the present study, we assessed CXCR-7 expression in 60 breast cancer patients in association with clinicopathological and demographic data of patients. We also extracted the results of our previous work on miR-100 expression in the same cohort of patients to assess the correlation between miR-100 and CXCR-7 expression levels. Transcript levels of *CXCR-7* were significantly higher in tumoral tissues compared with adjacent non-cancerous tissues (ANCTs) (Tumoral vs. ANCTs: $3.64 \pm 1.8$ vs. $0.73 \pm 1.3$, $P = 0.000$). A significant negative correlation was detected between CXCR-7 protein and miR-100 transcript levels ($r = -0.526$, $P < 0.05$). High CXCR-7 mRNA levels were significantly associated with tumor size ($P = 0.01$). Besides, high protein levels were more prevalent in higher TNM stages ($P = 0.000$). Moreover, high CXCR-7 protein levels were significantly associated with ER ($P = 0.005$) and PR ($P = 0.02$) status. The present work provides further evidence for the role of CXCR-7 in breast cancer and proposes the elimination of inhibitory effects of miR-100 on CXCR-7 expression as a mechanism for its up-regulation in breast cancer tissues.

**Keywords** CXCR-7 · C-X-C chemokine receptor type 7 · Breast cancer

## Introduction

C-X-C chemokine receptor type 7 (CXCR-7) is a transmembrane receptor that interacts with chemokines CXCL11/ITAC, CXCL12/SDF-1a, and macrophage migratory inhibitory factor (MIF) [1, 2] and participates in numerous physiological processes including cell survival, adhesion, and kinesis [2]. The alteration in amino acid sequence of the second intracellular loop of this protein has abolished its interaction with the classical G protein which are coupled with receptors. Alternatively, it couples with β-arrestin2 (β-AR2) as an auxiliary protein in a ligand dependent style [3]. Such interaction promotes CXCR7 dependent activation of EGFR following EGF release [3]. Both in vitro and in vivo studies have shown the role of CXCR7 in enhancement of breast tumor growth and metastasis. Besides, the CXCR-7 protein expression has been demonstrated in tumor-associated blood vessels and malignant breast cells but not in normal vasculature [4]. Inhibition of CXCR-7 has suppressed estrogen-induced proliferation of MCF7 cells as well as estrogen and SDF-1-induced tumorigenesis in vivo [5]. Studies in rat mammary adenocarcinoma cell line and animal models has demonstrated that CXCR7 inhibits invasion but increases primary tumor growth via angiogenesis [6]. Studies in human breast cancer MDA-MB-231 cell line have shown the role of CXCR7 inhibition in decreasing breast tumor invasion, adhesion and metastasis [7]. However, only few studies have assessed the expression of CXCR-7 in human breast cancer samples [3]. In the present study, we assessed mRNA and protein levels of CXCR-7 in a cohort of breast cancer patients in association with clinicopathological and demographic data of patients.

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## Material and Methods

### Patients

The current study was conducted in tissue samples obtained from 60 female patients diagnosed with invasive ductal carcinoma of breast in Sina and Farmanieh hospital during 2016–2017. Breast cancer tissues and the matched adjacent non tumoral tissues (ANCTs) were excised during surgery prior to administration of any chemo/radiotherapy and instantly transferred to the department of Medical Genetics in liquid nitrogen. Patients with familial forms of breast cancer and those received any chemo/radiotherapy before surgery were excluded from the study. The study protocol was approved by ethic committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC.1396.152). Informed consent forms were obtained from study participants. All methods were performed in accordance with the relevant guidelines and regulations. Patients classification and TNM staging was carried out according to the American Joint Committee on Cancer (AJCC) staging guidelines (7th edition) [8].

**Table 1** General demographic and clinical data of patients

| Features | Number of Cases (%) |
|---|---|
| Age | |
| ≤55 | 37(62%) |
| >55 | 23(38%) |
| TNM stage | |
| Low | 39(66%) |
| High | 21(34%) |
| Tumor grade | |
| Low | 40(67%) |
| High | 20(33%) |
| Tumor size | |
| >2 | 12(21%) |
| ≤2 | 48(79%) |
| Lymphatic metastasis | |
| + | 8(36%) |
| - | 14(64%) |
| ER status | |
| + | 48(81%) |
| - | 12(19%) |
| PR status | |
| + | 47(79%) |
| - | 13(21%) |
| Her2 status | |
| + | 43(73%) |
| - | 17(27%) |
| Ki67 | |
| + | 55(92%) |
| - | 5(8%) |

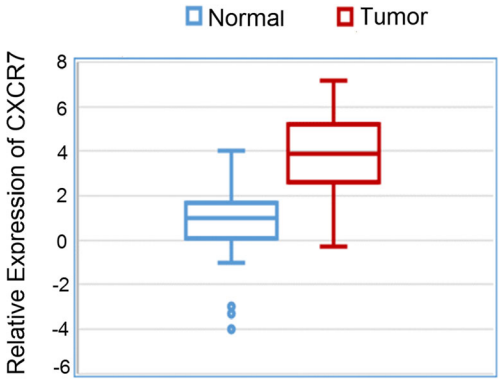


**Fig. 1** Relative expression of *CXCR-7* in tumoral tissues compared with ANCTs (Y axis shows $2^{CT\ B2M-CT\ CXCR7}$ values, Number of tumor sample = 60, Number of normal samples = 60)

### RNA Extraction and Real Time PCR

Total RNA was extracted from tissues using TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase to remove DNA contamination. Afterward, cDNA was produced by RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Lithuania). The relative expression of *CXCR-7* was measured by using SYBR® Premix Ex TaqTM (TaKaRa, Japan) using *B2M* gene as normalizer. The nucleotide sequences of primers are as follows: CXCR7-F: GAAGATGGTACGCCGTGTCG, CXCR7-R: CTGTGCTCGGGGTAGAAGGA, B2M-F: AGATGAGT ATGCCTGCCGTG and B2M-R: GCGGCATCTTCAAA CCTCCA. PCR program included a denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 60 s and a final extension step in 72 °C for 5 min. All experiments were carried out in duplicate in the rotor gene 6000 corbett Real-Time PCR System. We also extracted expression data of microRNA (miR)-100 on the same cohort of patients which was assessed using TaqMan® MicroRNA Reverse Transcription Kit (Invitrogen) and TaqMan® MicroRNA Assays kit (Applied Biosystems) for cDNA synthesis and evaluation of transcript levels respectively. Transcript levels of miR-100 were normalized to *RNU6B* expression levels.

### CXCR-7 Tissue Concentration

CXCR-7 tissue concentration was measured in frozen samples of the same patients using Human Chemokine C-X-C-Motif Receptor 7 (MyBiosource, Southern California, San Diego, USA Cat No: MBS450678) which is used to measure CXCR-7 levels on the basis of the Biotin double antibody sandwich technology. Standards provided in the kit and samples were added to the microtiter plate wells with a biotin-

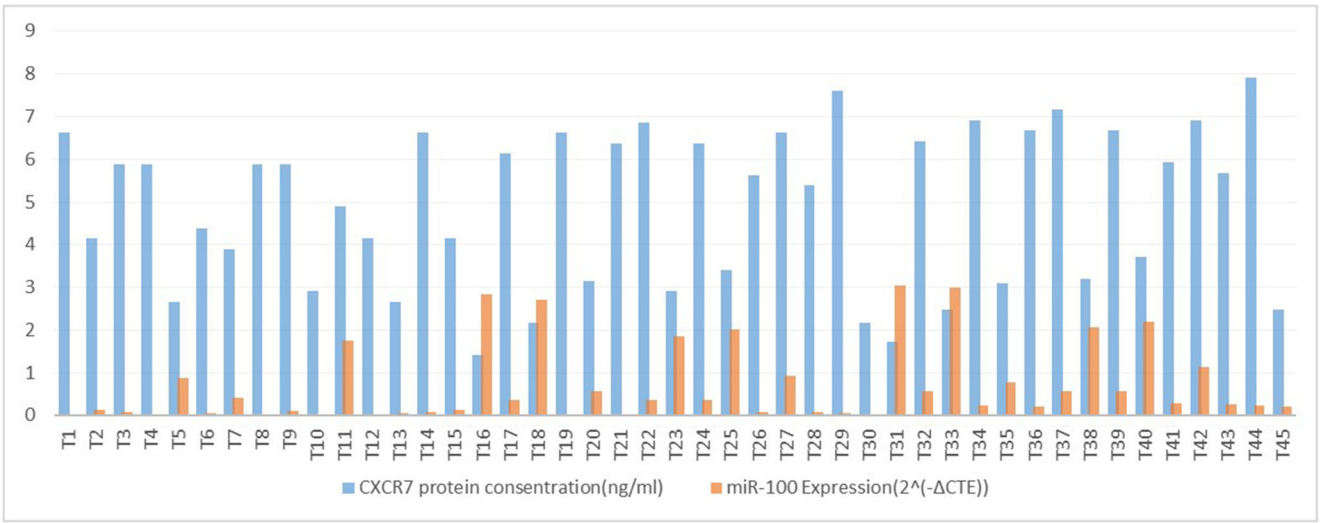


**Fig. 2** Comparative presentation of CXCR-7 protein concentration and miR-100 relative expression

conjugated antibody preparation specific to CXCR7. Subsequently, Avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated. After adding TMB substrate solution, only those wells that had CXCR7, biotin-conjugated antibody and enzyme-conjugated Avidin demonstrated a change in color. The enzyme-substrate reaction was finished by the addition of sulphuric acid solution and the color alteration was evaluated spectrophotometrically at a wavelength of 450 nm ± 10 nm. The concentration of CXCR7 in each sample was calculated by comparing the O.D. of the sample to the standard curve. We calculated average values of the duplicate readings for each standard, control and sample, then subtracted the average zero standard optical density. The best fit curve was depicted through the points on the graph with CXCR7 concentration on the y-axis and absorbance on the x-axis. The values of CXCR-7 were shown in pg/ml.

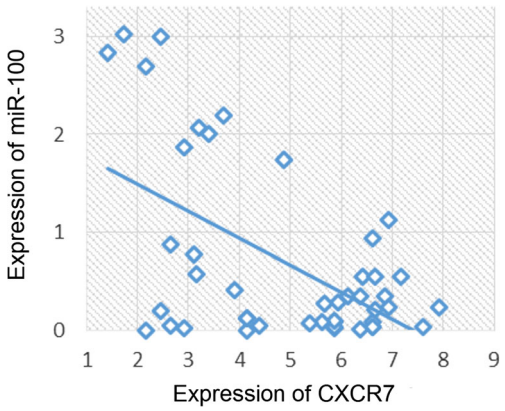


**Fig. 3** Correlation between CXCR-7 protein levels and miR-100 transcript levels

## Extraction of Microarray Data

We used the Gene Expression Omnibus (GEO) source at the National Center for Biotechnology Information (NCBI) [9] to acquire GSE41228, GSE59248 and GSE29044 microarray gene expression data which include data from both in situ and invasive breast cancer samples. Expression of *CXCR-7* gene was compared between in situ and invasive samples using GEO2R web tool (https://www.ncbi.nlm.nih.gov/geo/info/geo2r.html). In addition, the associations between expression levels of *CXCR-7* and estrogen receptor (ER) have been assessed in the GSE59248 and GSE29044 microarray datasets.

## Statistical Analysis

Statistical analysis was performed in R software (version 3.3.1). Data were presented as mean ± standard deviation (SD). Differences in gene expression between paired and unpaired samples were evaluated using Student's paired and un-paired t-tests. Chi-square test was used for assessment of the association between clinicopathological characteristics and CXCR-7 expression levels. The expression fold change was calculated using 2^-ΔΔCt method. The median values of fold changes were used as cut-offs to determine CXCR-7 up−/down-regulation. The correlations between relative expressions of CXCR-7 and miR-100 genes were evaluated using Spearman's rank test. For all statistical tests, the level of significance was set at $P < 0.05$.

# Results

## General Demographic and Clinical Data of Patients

The general characteristics of the 60 patients included in the study are shown in Table 1.

**Table 2** The associations between CXCR-7 expression (both mRNA and protein levels) and patients' clinicopathological and demographic data (Median value of fold changes in tumoral tissues vs. the paired ANCTs and median value of protein concentration in tumoral tissues have been set as cut-off levels for mRNA and protein expressions)

| Features | High *CXCR-7* mRNA expression | *P* value | High CXCR-7 Protein expression | *P* value |
|---|---|---|---|---|
| Age | | | | |
| ≤55 | 21 | NS | 21 | NS |
| >55 | 12 | | 9 | |
| TNM stage | | | | |
| Low | 19 | NS | 11 | 0.000 |
| High | 14 | | 19 | |
| Tumor grade | | | | |
| Low | 20 | NS | 17 | NS |
| High | 13 | | 13 | |
| Tumor size | | | | |
| ≥2 | 10 | 0.01 | 11 | NS |
| <2 | 23 | | 19 | |
| Lymphatic metastasis | | | | |
| + | 6 | NS | 6 | NS |
| - | 15 | | 12 | |
| ER | | | | |
| + | 25 | NS | 28 | 0.005 |
| - | 8 | | 2 | |
| PR | | | | |
| + | 26 | NS | 27 | 0.02 |
| - | 7 | | 3 | |
| HER2 | | | | |
| + | 23 | NS | 20 | NS |
| - | 10 | | 8 | |
| Ki67 | | | | |
| + | 29 | NS | 27 | NS |
| - | 4 | | 3 | |

## CXCR-7 Expression in Breast Cancer

Transcript levels of *CXCR-7* were significantly higher in tumoral tissues compared with ANCTs (Tumoral vs. ANCTs: $3.64 \pm 1.8$ vs. $0.73 \pm 1.3$, $P = 0.000$) (Fig. 1).

We also assessed the concentration of CXCR-7 proteins in tumoral tissues and extracted miR-100 expression data of the same cohort of patients (Transcript levels of miR-100 were normalized to *RNU6B* expression levels) (Fig. 2).

## Correlation between CXCR-7 and miR-100 Expression

No significant correlation was found between mRNA levels of *CXCR-7* and miR-100 ($r = -0.114$, $P = 0.12$). However, a significant negative correlation was detected between CXCR-7 protein and miR-100 transcript levels ($r = -0.526$, $P < 0.05$) (Fig. 3). No significant correlation was demonstrated between mRNA and protein levels of CXCR-7 ($r = 0.178$, $P = 0.1$).

## Association between CXCR-7 Expression and Clinicopathological Features

We assessed the associations between CXCR-7 expression (both mRNA and protein levels) and patients' clinicopathological and demographic data (Table 2). High *CXCR-7* mRNA expression was significantly associated with tumor size ($P = 0.01$). Besides, high CXCR-7 protein levels were more prevalent in higher TNM stages ($P = 0.000$). Moreover, high CXCR-7 protein levels were significantly associated with ER ($P = 0.005$) and PR ($P = 0.02$) status. However, no association was found between its mRNA or protein levels and other clinicopathological data.

## Assessment of *CXCR-7* Expression in Invasive Samples Vs. In Situ Samples Using Microarray Datasets

*CXCR-7* was significantly up-regulated in invasive samples compared with in situ samples in all mentioned microarray datasets (Table 3).

**Table 3** Relative expression of *CXCR-7* in invasive samples vs. in situ samples

| Dataset accession number | Samples | Platform | Expression of *CXCR-7* (log Fold Change) | *P* value |
|---|---|---|---|---|
| GSE29044 | In situ:6<br>Invasive:67 | GPL570 | 0.573 | 0.02 |
| GSE41228 | In situ:22<br>Invasive:22 | GPL1352 | 0.625 | 0.03 |
| GSE59248 | In situ:46<br>Invasive:56 | GPL13607 | 0.384 | 0.03 |

## Assessment of Association between *CXCR-7* Expression and ER/PR Status Using Microarray Datasets

In GSE59248 dataset, *CXCR-7* expression was significantly higher in ER+ samples compared with ER- samples ($P = 0.04$). However, there was no significant difference in *CXCR-7* expression between ER+ and ER- samples in the GSE29044 dataset. No association was found between *CXCR-7* expression and PR status in either of datasets. The GSE41228 dataset did not contain data regarding ER/ PR status of samples (Table 4).

## Discussion

In the current study we demonstrated over-expression of CXCR-7 in breast tumor samples compared with their corresponding ANCTs which is in accordance with the results of in silico analysis of microarray data regarding its up-regulation in invasive samples compared with in situ samples. Previous reports have assigned CXCR-7 over-expression to different mechanisms such as expression of IL-8, down-regulation of *hypermethylated in cancer-1* (*HIC1*) gene, over-expression of *hypoxia-inducible factor-1* (*HIF-1α*), and induction of nuclear factor kappa B (NF-kB) [3]. We have previously detected up-regulation of *HIF-1α* in Iranian breast cancer patients [10]. An alternative mechanism for up-regulation of CXCR-7 expression in breast cancer might be elimination of inhibitory effects of miR-100 as we demonstrated a negative correlation between expression levels of these two genes in our cohort of patients. A recent study has reported an inverse correlation between miR-100 and CXCR-7 levels in gastric cancer cell lines in accordance with the results of in silico analysis which predicted miR-100 binding to the 3′-untranslated region of CXCR7 to preclude its protein translation [11]. In addition, CXCR-7 has been validated as a target of miR-100 in esophageal squamous cancer cells through application of luciferase target gene reporter assay [12].

Moreover, we have shown a significant association between CXCR-7 protein levels and ER/PR status. The association between CXCR-7 expression and ER status has been confirmed in our in silico analysis of a selected microarray dataset and is in line with Salazar et al.'s study which assessed human breast samples for colocalization of CXCR7 and EGFR and reported a prominent enhancement of receptor colocalization in human ER+ breast cancer tissues compared to normal breast tissues implying a critical function for the CXCR7-EGFR interaction in promotion of proliferation of these cells [3]. In vitro study of endometrial stroma cells has shown the stimulatory effect of both estrogen and progesterone on expression of CXCR-7 [13]. However, another study has shown the inhibitory effect of estrogen on CXCR-7 expression in breast cancer cells by disrupting the interaction of NFκB transcription factor with its promoter which is exerted through ERα [14]. On the other hand, CXCR7 up-regulation has enhanced the basal MCF-7 cell growth rate and reduced the stimulatory effect of estrogen on these cells [15]. Taken

**Table 4** Relative expression of *CXCR-7* in association with ER/ PR status in two microarray datasets

| Dataset accession number | Samples | Expression of CXCR7 (log Fold Change) | *P* value |
|---|---|---|---|
| GSE59248 | ER+:53<br>ER-:27 | 0.495 | 0.04 |
| | PR+:46<br>PR-:34 | 0.250 | 0.2 |
| GSE29044 | ER+:69<br>ER-:29 | 0.0689 | 0.5 |
| | PR+:38<br>PR-:28 | 0.186 | 0.08 |

together, these data suggest the presence of two-sided interaction network between estrogen and CXCR-7 in the contexts of breast cancer. Future studies are needed to elaborate the mechanism of such tissue-dependent effect of estrogen on CXCR-7 expression as well as the underlying mechanism of association between CXCR-7 expression and ER status.

We also detected a significant association between high *CXCR-7* mRNA expression and tumor size. However, such association has not been confirmed at protein level. On the other hand, the associations between CXCR-7 protein levels and ER/PR status were not observed at mRNA level. These data are in line with the observed lack of correlation between mRNA and protein levels of this gene and provide further evidence for Liu et al. suggestion that mRNA levels by themselves are not adequate to foretell protein levels in many situations [16]. Considering the need for quantifying gene expression at different levels for the comprehensive assessment of biological processes [16], in the present study we assessed CXCR-7 levels at both mRNA and protein levels and suggested that assessment of protein levels might better reflect the clinical relevance of gene expression. The present work provides further evidence for the role of CXCR-7 in breast cancer and proposes the elimination of inhibitory effects of miR-100 on CXCR-7 expression as a mechanism for its up-regulation in breast cancer tissues.

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**Authors' Contributions** MSA and RS performed the experiments. MB supervised the protein experiments. MDO provided the technical support. SGH designed the study, supervised it and wrote the manuscript.

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## Compliance with Ethical Standards

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

**Ethical Approval** Ethical approval was obtained from the ethical committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC.1396.152). Informed consent was obtained from the patients.

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