CXC chemokines located in 4q21 region are up-regulated in breast cancer

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ABSTRACT

Recent data suggest that chemokines could be essential players in breast carcinogenesis. We previously showed that the CXC chemokine CXCL8 (interleukin-8) was overexpressed in Estrogen Receptor (ER)α-negative breast cell lines. Analysis of CXCL8 chromosomal location showed that several CXC chemokines (CXCL1, CXCL2, CXCL3, CXCL4, CXCL4V1, CXCL5, CXCL6, CXCL7 and CXCL8) were localized in the same narrow region (360 kb in size) of chromosome 4. We thus hypothesized that they could belong to the same cluster. Quantification of these chemokines in breast tumors showed that samples expressing highly CXCL8 also produced elevated levels of CXCL1, 3, 5 and displayed low content of ERα. CXCL1, 2, 3, 5 and 8 were co-regulated both in tumors and in breast cancer cell lines. CXCL5 and 8 were mainly produced by epithelial cells, whereas CXCL1, 2 and 3 had a high expression in blood cells. The overexpression of these chemokines in tumor cells was not the result of gene amplification, but rather of an enhanced gene transcription. Our data suggest that high CXCL8 expression in tumors is mainly correlated to AP-1 pathway and to a minor extent to NF-κB pathway. Interestingly, CXCL1, 2, 3, 5, 6 and 8 chemokines were present at higher levels in metastases compared to grade I and III biopsies. High levels of CXCL8, CXCL1 and CXCL3 accounted for a shorter relapse-free survival of ERα-positive patients treated with tamoxifen. In summary, we present evidences that multiple CXC chemokines are co-expressed in CXCL8 positive breast tumors. In addition, these chemokines could account for the higher aggressiveness of these types of tumors.
INTRODUCTION

The majority of primary breast cancers is hormone dependent and is associated with increased levels of estrogen receptor α (ERα). The lack of ERα expression in breast tumors is associated with a poorer prognosis (Lazennec et al., 2001, Skoog et al., 1987). However, little is known about the possible factors involved in this difference.

Recently, chemokines have emerged as potential factors involved in breast carcinogenesis (Balkwill, 2004, Freund et al., 2003, Walser & Fulton, 2004). It has been reported that chemokines are implicated in many aspects of carcinogenesis like tumor growth, angiogenesis and metastasis development (Balkwill, 2004). Some tumor cells not only regulate their chemokine expression to help recruit inflammatory cells, but also use these factors to stimulate tumor growth and progression (Zhu & Woll, 2005). Chemokines are small heparin-binding proteins that activate and stimulate the infiltration of circulating lymphocytes into tissue from peripheral blood and serve as mediators of inflammation (Luster, 1998, Proudfoot, 2002, Rossi & Zlotnik, 2000). Chemokines can be divided in four groups, namely C, CC, CXC and CX3C, according to cysteine residues in the NH2-terminal part of the protein. We and others have recently shown that CXCL8, a CXC member was expressed in breast cancer cell lines (Freund et al., 2003, Lin et al., 2004). CXC members exhibit two cysteines separated by a non-conserved amino acid residue. CXC chemokine subfamily comprises 16 members, among which CXCL1 (GRO-α), CXCL2 (GRO-β), CXCL3 (GRO-γ), CXCL4 (PF4), CXCL4V1 (PF4V1), CXCL5 (ENA-78), CXCL6 (GCP-2), CXCL7 (NAP-2), CXCL8 (IL-8), CXCL9 (MIG), CXCL10 (IP-10), CXCL11 (I-TAC) and CXCL12 (SDF-1) are the most studied members. The NH2-terminus of several CXC chemokines (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8) contains three amino acid residues (Glu-Leu-Arg; “ELR motif”), which confers them a chemotactic activity for neutrophils and pro-angiogenic properties (Clark-Lewis et al., 1993, Hebert et al., 1991, Strieter et al., 1995, Strieter et al., 2005). CXC chemokines mediate their effects via binding and activating seven trans-membrane receptors, namely CXCR1 to CXCR6.
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(Mantovani, 1999). CXCR1 binds only CXCL6 and CXCL8, while CXCR2 binds all ELR+ CXC chemokines (CXCL1, 2, 3, 5, 6, 7, 8) (Strieter et al., 2005). Recent studies have shown that CXCR1 and CXCR2 were poorly expressed in breast tumors, while CXCR4 was overexpressed in metastatic breast tumors (Muller et al., 2001).

We demonstrated earlier that CXCL8 expression is negatively linked to ERα status and is associated with higher invasiveness potential of breast cancer cells (Freund et al., 2003). CXCL1 has been also shown to promote growth of tumor cells and to be involved in angiogenesis of colon cancer and melanoma (Li et al., 2004, Loukinova et al., 2000). Since several CXC chemokines, including CXCL1, CXCL2, CXCL3, CXCL4, CXCL4V1, CXCL5, CXCL6, CXCL7 and CXCL8, are located to a short region (360 kb) of the long arm of the chromosome 4, we hypothesized that this gene cluster could exhibit similar regulation in breast cancer. By analyzing the expression of these chemokines in breast tumors, we demonstrate that CXCL1, CXCL3, CXCL5, CXCL6 and CXCL8 are co-regulated and display a higher expression in ERα-negative tumors. These chemokines are produced by tumor cells and also by fibroblasts, endothelial cells or blood cells. At least for CXCL1 and CXCL8, the high expression of these chemokines involved gene regulation events and not gene amplification. We also observed that CXCL1, 2, 3, 5, 6 and 8 chemokines were present at higher levels in metastases compared to grade I and III biopsies. CXCL1, 2, 3, 5, 6 and 8 were then investigated in an independent panel of ERα-positive tumors from 48 postmenopausal breast cancer patients treated with adjuvant tamoxifen alone, of whom 24 relapsed. Overexpression of CXCL1, CXCL3 and CXCL8 was associated with significantly shorter relapse-free survival in univariate analysis. Our data suggest that multiple chemokines, including in particular CXCL8 could be involved in the higher aggressiveness of breast tumors.
MATERIALS AND METHODS

Patients and Samples.

We analyzed samples of 48 primary breast tumors excised from women at our institution from 1980 to 1994. Samples containing more than 70% of tumor cells were considered suitable for this study. Immediately following surgery, the tumor samples were placed in liquid nitrogen until RNA extraction. The patients met the following criteria: primary unilateral non-metastatic postmenopausal breast carcinoma; estrogen receptor alpha positivity [as determined at the protein level by biochemical methods (Dextran-coated charcoal method until 1988 and enzymatic immunoassay thereafter) and confirmed by ERα real-time quantitative RT-PCR assay]; complete clinical, histological and biological information available; no radiotherapy or chemotherapy before surgery; and full follow-up at our institution. The malignancy of infiltrating carcinomas was scored according to Scarff Bloom and Richardson’s histoprognostic system (Bloom & Richardson, 1957). The characteristics of the patients are shown in Table 1. The patients had physical examinations and routine chest radiography every 3 months for 2 years, then annually. Mammograms were done annually. The median follow-up was 7.2 years (range 1.5-10.0 y). All the patients received postoperative adjuvant endocrine therapy (tamoxifen, 20 mg daily for 3-5 years), and no other treatment. Twenty-four patients relapsed. The distribution of first relapse events was as follows: 22 metastases, and 2 local and/or regional recurrences with metastases.

To investigate the inter-relationships between mRNA levels of genes of interest, and the relationship between target mRNA values and ERα expression status, we also analyzed 48 additional primary breast tumors: 24 ERα-negative and 24 ERα-positive tumors. The characteristics of these 48 patients are shown in Table 2.

To evaluate the relationship between mRNA levels of CXCL8 during breast cancer progression, we analyzed tumor RNA of 11 invasive ductal grade I breast tumors, 37 invasive
ductal grade III breast tumors and 24 distant metastasis (10 liver, 7 lung, 4 skin, 2 ovarian and one gastric metastasis).

We analyzed five ERα-positive cell lines (MCF7, T47-D, ZR75-1, MDA-MB361 and HCC1500) and seven ERα-negative cell lines (BT-20, MDA-MB468, SK-BR-3, MDA-MB157, MDA-MB231, MDA-MB435 and MDA-MB436), obtained from the American Tissue Type Culture Collection.

Specimens of adjacent normal breast tissue from six breast cancer patients, and normal breast tissue from three women undergoing cosmetic breast surgery, were used as sources of normal RNA.

**Differential isolation of epithelial cells and fibroblasts from breast tumors**

Breast tumors were minced with scalpels and incubated overnight with Liberase Blendzyme 2 (Roche Applied Science, Meylan, France) for enzymatic dispersion. Organoids and aggregated cells (epithelial fraction) and isolated cells (fibroblast fraction) were isolated from the digest by differential centrifugation followed by culture in selective media as described earlier (Speirs et al., 1998). Fibroblast fraction was cultured in Ham’s F10 medium containing L-glutamine (3 mM), insulin (5 μg/mL), Thyroid hormone T3 (1 nM), hydrocortisone (1 μg/mL) and 10% serum calf fetal. Epithelial fraction was cultured in the same conditions plus epidermal growth factor (5 ng/mL) and transferrin (5 μg/mL) and with 5% human serum (and without serum calf fetal).

Cells were incubated in a humidified atmosphere of 5% CO2 at 37 °C, and the medium was changed three times a week.
Endothelial and blood cells isolation

HUVEC (endothelial cells) were isolated from human umbilical veins and maintained in EGM-2 medium (Smadja et al., 2005). Total RNA of blood circulating cells was extracted from whole blood of normal healthy donors.

Real-time RT-PCR. Reactions are characterized by the point during cycling when amplification of the PCR product is first detected, rather than the amount of PCR product accumulated after a fixed number of cycles. The larger the starting quantity of the target molecule, the earlier a significant increase in fluorescence is observed. The parameter Ct (threshold cycle) is defined as the fractional cycle number at which the fluorescence generated by SYBR green dye-amplicon complex formation passes a fixed threshold above baseline. The increase in fluorescent signal associated with exponential growth of PCR products is detected by the laser detector of the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA), using PE Biosystems analysis software according to the manufacturer’s manuals.

The precise amount of total RNA added to each reaction mix (based on optical density) and its quality (i.e. lack of extensive degradation) are both difficult to assess. We therefore also quantified transcripts of two endogenous RNA control genes involved in two cellular metabolic pathways, namely TBP (Genbank accession NM_003194), which encodes the TATA binding protein (a component of the DNA-binding protein complex TFIID), and RPLP0 (NM_001002), which encodes human acidic ribosomal phosphoprotein P0. Each sample was normalized on the basis of its TBP (or RPLP0) content.

Results, expressed as N-fold differences in target gene expression relative to the TBP (or RPLP0) gene, and termed "RNA Ntarget", were determined as $N_{\text{target}} = 2^{\Delta C_{\text{t, sample}}}$, where the $\Delta C_t$ value of the sample was determined by subtracting the Ct value of the target gene from the Ct value of the TBP (or RPLP0) gene (Bieche I. et al., 1999, Bieche Ivan et al., 2001). Target genes were considered to be detectable but not quantifiable when the Ct value was above 35.
Primers for the target genes and the endogenous control genes used in the different RT-PCR assays were chosen with the assistance of the computer programs Oligo 5.0 (National Biosciences, Plymouth, MN). We conducted BLASTN searches against dbEST and nr (the non-redundant set of GenBank, EMBL and DDBJ database sequences) to confirm the total gene specificity of the nucleotide sequences chosen for the primers, and the absence of DNA polymorphisms. In particular, the primer pair was selected to be unique relative to the sequences of closely related family member genes or of the corresponding retropseudogenes. To avoid amplification of contaminating genomic DNA for the quantitative RT-PCR, one of the two primers was placed at the junction between two exons, or in a different exon, if possible. The nucleotide sequences of the oligonucleotide primers for real-time RT-PCR are shown in Table 3. Gel electrophoresis was used to verify the specificity of PCR amplicons. For the selected primer pair, we performed no-template control (NTC), which produced negligible signals (usually > 40 in Ct value), suggesting that primer-dimer formation effects were negligible.

RNA extraction, cDNA synthesis and PCR reaction conditions are described in detail elsewhere (Bieche Ivan et al., 2001).

**Statistics.** As the mRNA levels did not fit a Gaussian distribution, (a) the mRNA levels in each subgroup of samples were characterized by their median values and ranges, rather than their mean values and coefficients of variation, and (b) relationships between the molecular markers and clinical and biological parameters were tested by using the non parametric Mann-Whitney \( U \) test (Mann & Whitney, 1947). Differences between two populations were judged significant at confidence levels greater than 95% (\( p<0.05 \)). To visualize the capacity of a given molecular marker to discriminate between two populations (in the absence of an arbitrary cutoff value), we summarized the data in a ROC (receiver
operating characteristics) curve (Hanley & McNeil, 1982). When a molecular marker has no discriminative value, the ROC curve lies close to the diagonal and the AUC is close to 0.5. When a marker has strong discriminative value, the ROC curve moves to the upper left-hand corner (or to the lower right-hand corner) and the AUC is close to 1.0 (or 0).

Hierarchical clustering was performed using the GenANOVA software (Didier et al., 2002).

Relapse-free survival (RFS) was determined as the interval between diagnosis and detection of the first relapse (local and/or regional recurrence, and/or metastasis). Survival distributions were estimated by the Kaplan-Meier method (Kaplan & Meier, 1958), and the significance of differences between survival rates was ascertained by using the log-rank test (Peto et al., 1977).
RESULTS

ER-negative breast cancers display a higher aggressiveness in terms of metastasis development and worse prognosis compared to ER\(\alpha\)-positive breast tumors. (Osborne, 1998, Sheikh et al., 1994). We have demonstrated recently an inverse correlation between expression of the CXC chemokine CXCL8 and ER\(\alpha\) levels in human breast cancer cell lines (Freund et al., 2003), which could account for their higher aggressiveness. Interestingly, CXCL8 gene is located with a number of other CXC chemokines in a short region (360 kb) of chromosome 4 (4q21) (Fig. 1 and Table 4). The goal of this study was to determine whether the chemokines of this cluster were co-regulated and what could be the factors controlling their expression.

CXCL1, CXCL2, CXCL3, CXCL5, CXCL6 and CXCL8 are co-regulated in breast cancer.

We first analyzed by real-time PCR the expression in breast cancer biopsies of a number of genes located on 4q21 (Table 4). This included in particular CXC chemokines CXCL1, CXCL2, CXCL3, CXCL4, CXCL4V1, CXCL5, CXCL6, CXCL7 and CXCL8. The 48 samples (Table 2) were divided in two groups according to their low (-/+) or high (++) CXCL8 content (see statistics section in Materials and Methods). In the two groups, the RNA levels of the genes located in 4q21 location, as well as the expression of ER\(\alpha\), KI67, and CXCR1 and CXCR2 genes were determined (Table 5). We observed that CXCL8 ++ tumors expressed significantly lower levels of ER\(\alpha\) (Table 5), which is in agreement with our and other group data on breast cancer cell lines and breast cancer biopsies (Freund et al., 2003, Lin et al., 2004). CXCL4, CXCL4V1, CXCL7 and Albumin genes were not significantly expressed. In addition, CXCL1, CXCL3, CXCL5 and CXCL6 were overexpressed in CXCL8 ++ tumors. CXCL2 displayed also a trend of overexpression in CXCL8 tumors. All the other genes of the 4q21 region exhibited a similar expression in both types of tumors (Table 5). CXCR1 and CXCR2, the receptors mediating the
effects of the chemokines present on chromosome 4, as well as KI67, displayed a similar expression in the two CXCL8 groups.

Breast cancer cells produce multiple chemokines.

To determine which cells of the tumor could produce chemokines, we measured the RNA levels of the 4q chemokines cluster in breast cancer cell lines. By comparing the levels of chemokines in cell lines producing low or high levels of CXCL8 (Table 6), we observed that CXCL6, CXCR1 and CXCR2 were poorly expressed. In contrast, CXCL1, CXCL2, CXCL3 and CXCL5 were more expressed in CXCL8 positive cell lines compared to CXCL8 low or negative cell lines. Interestingly, with the exception of ZR-75-1 cells, all cell lines which expressed high levels of CXCL8 were ERα-negative. Low producers of CXCL8 were mostly composed of ERα-positive cell lines and a few ERα-negative cells (MDA-MB468 and SKBR-3), which is in agreement with our previous data (Freund et al., 2003).

As tumors are composed not only of tumor cells but also of blood cells, endothelial cells, and fibroblasts, we also measured the chemokine content of these cells (Table 7). CXCL8 was mainly a component of tumoral epithelial cells and to a lesser extent of endothelial and intra-tumor fibroblasts (Table 7). CXCL5 and CXCL6 were also mainly produced by epithelial cells. On the other hand, CXCL1 was made mainly by blood cells and epithelial cells, CXCL2 by circulating and endothelial cells, CXCL3 by blood cells and to a lower extent by epithelial and endothelial cells. Blood cells were also the main sites of synthesis of CXCL4, CXCL4V1, CXCL7, CXCR1 and CXCR2 (Table 7).

Mechanisms controlling chemokine expression in tumors.

To address the question of the coregulation of these chemokines, we first hypothesized that some of the overexpressed chemokines could be the result of genomic DNA amplification. To test this hypothesis, we analyzed five genomic DNA samples from normal patients, 4 breast tumors with
low CXCL8 mRNA expression and 4 breast tumors with high CXCL8 mRNA overexpression. Among the 8 tumors tested, no tumor showed 4q21 DNA amplification when looking at CXCL1 and CXCL8 genes (data not shown).

We then designed an assay to evaluate the functionality of 4q21 chemokine genes in cell lines. Cells were treated with TNF-α, a general activator of cytokine expression and we first monitored at the RNA levels the expression of CXCL1, CXCL2, CXCL3 and CXCL8 in MCF-7 (low CXCL8) and MDA-MB231 (high CXCL8) cells. TNF-α strongly induced the expression of the chemokines tested, suggesting that all four genes were fully active in the two cell lines (Table 8). Genes surrounding the chemokine cluster were only modestly (AREG, EREG and ADAMTS3) or not (RASSF6) regulated by TNF-α in the two cell lines. Finally, we also observed that CXCL8 and CXCL1 protein secretion were up-regulated by TNF-α (Fig. 2).

As CXCL8 gene is mainly regulated in breast cancer cell lines through an AP-1 and a NF-κB site at the promoter level (Freund et al., 2004), we undertook to analyze whether CXCL8 RNA levels found in breast tumors could be correlated with the amounts of AP-1 and NF-κB members or known target genes (Table 9 and Supplemental Data Table 1S). We observed that CXCL8 RNA levels were strongly correlated to those of AP-1 members such as ATF3, c-Jun or JunB, and to their target genes such as MMP1, MMP9 or TNC (Table 9). CXCL8 expression was also correlated GADD45B, a typical NF-κB target gene, and also to TNC and MMP9, but not with most of NF-κB members or target genes tested.

CXCL1, CXCL2, CXCL3, CXCL5, CXCL6 and CXCL8 are present at high levels in metastases

Many CXC chemokines are strong inducers of chemotaxy. We thus hypothesized that they could also be involved in tumor progression and that they could increase metastase formation. To evaluate this, we analyzed CXCL8 expression in 11 grade I tumors, 37 grade III tumors and 24 metastases in primary tumors and distant metastases (Table 10). We observed that CXCL8
expression raised from grade I to grade III primary tumors and exhibited a strong increase (8-fold increase) when looking at distant metastases, suggesting that this chemokine could be a key player in metastasis. CXCL1, CXCL2, CXCL3, CXCL5 and CXCL6 showed also increased levels from grade III to metastases but not between grade I and grade III patients (Table 10).

**Prognostic value of CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL8 and HER2.**

ERα-negative tumors are generally more aggressive than ERα-positive tumors. However, some ERα-positive tumors display also aggressive features. It is of great interest to understand why this subpopulation of ERα-positive tumors are more metastatic. To address this issue, we measured the expression levels of CXCL1, CXCL2, CXCL3, CXCL5, CXCL6 and CXCL8, as well as HER2 by real-time RT-PCR in a cohort of 24 ERα-positive breast tumor patients who relapsed and 24 ERα-positive breast tumor patients who did not relapse (Table 1). All these 48 ERα-positive tumors were from postmenopausal patients treated with primary surgery followed by adjuvant tamoxifen alone. We used univariate analysis (log-rank test) to further study the prognostic value of these seven genes. For each gene, the 48 ERα-positive breast tumors were divided into two groups of 24 tumors with “low” and “high” CXC chemokine mRNA levels. Univariate analysis showed that a high expression level of CXCL1, CXCL3 and CXCL8 correlated with significantly shorter relapse free survival (RFS) (Fig. 3 and Fig. 4). The outcome of the 24 patients with high mRNA levels of these 3 genes was significantly worse than those of the 24 patients with low CXCL1, CXCL3 and CXCL8 mRNA levels. No significant prognostic value was associated with the four other gene, CXCL2 (P=0.59), CXCL5 (P=0.47), CXCL6 (P=0.16) and HER2 (P=0.83).

Moreover, using a Cox proportional hazards model, we also assessed the prognostic value, for RFS, of parameters that were significant or near-significant (P<0.1) in univariate analysis, i.e. SBR grade, lymph-node status (Table 1) and the genes CXCL1, CXCL3 and...
CXCL8 (Fig 3 and 4). Only the prognostic significance of CXCL8 mRNA status shows a trend toward significance (P=0.068).
DISCUSSION

The aim of this study was to determine whether breast cancer samples expressing high levels of CXCL8 could also produce other CXC chemokines, with a particular interest to the chemokines localized to a narrow region of chromosome 4. Indeed, by analyzing chromosome 4 sequences, we observed that 9 CXC chemokines (CXCL1, CXCL2, CXCL3, CXCL4, CXCL4V1, CXCL5, CXCL6, CXCL7 and CXCL8) were mapped to a short region of 356 kb, which could suggest that they belong to an inflamed cluster. By measuring the RNA levels of these chemokines in breast tumors, we reported that tumors expressing high levels of CXCL8 were also producing elevated levels of CXCL1, CXCL3, CXCL5, CXCL6 and to a minor extent of CXCL2. CXCL4, CXCL4V1 and CXCL7 were not significantly expressed in both types of tumors. Concomitant expression of CXCL8 with other cytokines such as IL-6 has already been reported in serum for breast and ovarian cancer (Benoy I. et al., 2002, Jiang et al., 2000, Kozlowski et al., 2003, Penson et al., 2000). In addition, we observed that CXCL8 ++ tumors were expressing low levels of ERα, which is in agreement with previous work (Freund et al., 2003, Lin et al., 2004). It is interesting to note that, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6 and CXCL8 were all ELR (glutamate-leucine-arginine)-positive chemokines (Moser et al., 2004). This appears quite important as the NH2-terminal motif has been shown to be responsible for the ability of chemokines to attract neutrophiles as well as a necessary motif involved in angiogenic properties of these chemokines (Strieter et al., 2005). The coregulation of CXCL1, CXCL3, CXCL5, CXCL6 and CXCL8 in tumors was puzzling. Our data suggest that this high expression does not arise from genomic amplification, but rather from an increased transcriptional activity of these genes. In addition, we showed that CXCL1, 2, 3 and 8 were induced by TNFα in breast cancer cells, demonstrating the genes were functional. Our previous work on CXCL8 gene regulation in breast cancer cells had shown that CXCL8 overexpression in ERα-negative breast cancer cell lines was mainly mediated through an overexpression of NF-kB and AP-1 transcription factors, which in turn could induce CXCL8 promoter activity (Freund et al., 2004). To date, only a few
data are available concerning the regulation of CXCL1, 3, 5 and 6 gene promoters. Of particular note is the fact that CXCL1 promoter displays many features common to CXCL8, with in particular also a crucial NF-κB site involved in constitutive expression of CXCL1 gene in melanoma (Wood & Richmond, 1995, Yang & Richmond, 2001). So, we hypothesized that tumors producing high amounts of CXCL8 could also have deregulated NF-κB and AP-1 pathways. To test this hypothesis, we measured the expression levels of a series of NF-κB and AP-1 transcription factors or target genes. Our data showed that CXCL8 expression was correlated primarily to AP-1 transcription factors such as ATF3, c-Jun or JunB and typical AP-1 target genes, but to a lesser extent to NF-κB pathway.

To determine whether tumor cells themselves were producing these chemokines, we first analyzed breast cancer cell lines. Our data confirmed that cell lines overexpressing CXCL8 were producing CXCL1, CXCL2, CXCL3 and CXCL5, which is in good agreement with the overall expression of breast tumors. Of particular note, CXCL1, CXCL2, CXCL3, CXCL5 and CXCL8 were also produced mostly by ERα-negative cell lines (Table 6), which constitutes the first report of such a coregulation. To go further, we next measured the chemokine content of intratumoral fibroblast and epithelial cells, as well as the one of blood cells and endothelial cells. Interestingly, we found that CXCL5, 6 and 8 were mostly produced by intratumoral epithelial cells, whereas CXCL1, CXCL2 and CXCL3 were synthetized in priority by blood cells. But other types of cells could also produce CXCL8. Indeed, CXCL8 was also produced at relatively high levels by endothelial cells and intratumoral fibroblasts. In addition, CXCR1 and CXCR2 were mainly detected in endothelial cells, even though CXCR2 is considered to be the primary receptor responsible for endothelial cell chemotaxis (Addison et al., 2000, Salcedo et al., 2000).

We next focused our attention to CXCL8 to evaluate its potential prognosis value. Our data showed that CXCL8 content in the primary tumor was increased in grade III patients compared to grade I patients. This is in agreement with other reports showing that CXCL8 is overexpressed
in breast tumors compared to normal tissues and that breast cancer patients display higher seric levels of CXCL8 (Green et al., 1997, Kozlowski et al., 2003). Moreover, distant metastases were extensively producing CXCL8, suggesting that CXCL8 could be one of the factors leading to metastasis. Indeed, our previous work showed that CXCL8 could increase the invasion potential of breast cancer cells, one of the first step before metastasis (Freund et al., 2003). Benoy et al. have also shown that patients with metastatic breast tumors displayed high seric amounts of CXCL8 (Benoy I. H. et al., 2004). In vivo studies in athymic mouse models also corroborate these data, suggesting that breast tumors secreting high levels of CXCL8 metastasize more easily to bone (Bendre et al., 2002, De Larco et al., 2001). We then wondered whether the other chemokines of the cluster were following the same trend. We observed that CXCL1, 2, 3, 5 and 6 were also more expressed in metastasis compared to grade I and grade III patients, suggesting that they could also be involved in metastasis. If CXCL1 has been shown to increase breast cancer cell invasion in vitro (Youngs et al., 1997), so far, this is the first report of the involvement of CXCL1, CXCL2, CXCL3, CXCL5 and CXCL6 in breast cancer metastasis. From this point, we knew that CXCL8, 1, 2, 3, 5 and 6 chemokines were mainly produced by ERα-negative tumors, and were globally overexpressed in breast cancer metastases. But, one crucial question was to look at the case of patients with ERα-positive tumors who were treated with tamoxifen and had an impaired survival. Indeed, it is quite difficult to understand why a subpopulation of ERα-positive breast cancer patients will still have a bad outcome. So, our goal was to determine whether CXCL1, CXCL2, CXCL3, CXCL5, CXCL6 or CXCL8 could discriminate among ERα-positive breast cancer patients, those who will develop rapidly metastasis. Univariate analysis showed that, among these six genes (as well as HER2), only CXCL1, CXCL3 and CXCL8 correlated with RFS. In multivariate analysis, only CXCL8 shows a trend toward significance to be an independant predictor of response to tamoxifen therapy. These data, which need to be confirmed on a larger set of patients, suggest that CXCL8 could help to improve the prognosis of ERα-positive patients who are treated with tamoxifen.
In conclusion, our work suggest that breast tumors expressing high levels of CXCL8 have a bad prognosis evolution which could be the reflect of their higher content of ELR-positive CXC chemokines such as CXCL1, CXCL3, CXCL5 and CXCL6. These chemokines belong to an hyperactive gene cluster, which is likely to be regulated by AP-1 and to a lesser extent by NF-kB pathways. Moreover, ERα-positive breast cancer patients with high levels of CXCL1, CXCL3 or CXCL8 display a shorter relapse free survival. It will be interesting to dissect further the mechanisms of regulation, as a potential therapeutic approach could be to identify the common factors up-regulating these chemokines in the same subset of tumors.
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LEGENDS TO FIGURES

**Fig. 1.** CXCL1, CXCL2, CXCL3, CXCL5, CXCL6 and CXCL8 belong to the same sub-cluster.

Schematic representation of long arm of chromosome 4 showing that multiple CXC chemokines are present on this chromosome.

**Fig. 2.** CXCL8 and CXCL1 protein expression is induced by TNF-α in cell lines.

MCF-7 and MDA-MB231 cells were treated with TNF-α (50 ng/ml). Media were then collected after 20 min, 1h, 6h or 18h of treatment to evaluate CXCL8 and CXCL1 protein levels by ELISA. Results are expressed as fold of control levels and represent the mean of two independent experiments.

**Fig. 3.** CXCL8 is involved in relapse free survival

We used univariate analysis (log-rank test) to study the prognostic value of CXCL8. The 48 ERα-positive breast tumors were divided into two equal groups of 24 tumors with “low” and “high” CXCL8 mRNA levels.

**Fig. 4.** CXCL1 and CXCL3 predict relapse free survival

We used univariate analysis (log-rank test) to study the prognostic value of CXCL1, CXCL2, CXCL3, CXCL5, CXCL6 and HER2. The 48 ERα-positive breast tumors were divided into two equal groups of 24 tumors with “low” and “high” CXC chemokine mRNA levels of these genes.