

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

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33 **ABSTRACT**

34 Recent data suggest that chemokines could be essential players in breast carcinogenesis. We
35 previously showed that the CXC chemokine CXCL8 (interleukin-8) was overexpressed in
36 Estrogen Receptor (ER) α -negative breast cell lines. Analysis of CXCL8 chromosomal location
37 showed that several CXC chemokines (CXCL1, CXCL2, CXCL3, CXCL4, CXCL4V1, CXCL5,
38 CXCL6, CXCL7 and CXCL8) were localized in the same narrow region (360 kb in size) of
39 chromosome 4. We thus hypothesized that they could belong to the same cluster. Quantification
40 of these chemokines in breast tumors showed that samples expressing highly CXCL8 also
41 produced elevated levels of CXCL1, 3, 5 and displayed low content of ER α . CXCL1, 2, 3, 5 and
42 8 were co-regulated both in tumors and in breast cancer cell lines. CXCL5 and 8 were mainly
43 produced by epithelial cells, whereas CXCL1, 2 and 3 had a high expression in blood cells. The
44 overexpression of these chemokines in tumor cells was not the result of gene amplification, but
45 rather of an enhanced gene transcription. Our data suggest that high CXCL8 expression in
46 tumors is mainly correlated to AP-1 pathway and to a minor extent to NF- κ B pathway.
47 Interestingly, CXCL1, 2, 3, 5, 6 and 8 chemokines were present at higher levels in metastases
48 compared to grade I and III biopsies. High levels of CXCL8, CXCL1 and CXCL3 accounted for
49 a shorter relapse-free survival of ER α -positive patients treated with tamoxifen. In summary, we
50 present evidences that multiple CXC chemokines are co-expressed in CXCL8 positive breast
51 tumors. In addition, these chemokines could account for the higher aggressiveness of these types
52 of tumors.

53 INTRODUCTION

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

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

79 (Mantovani, 1999). CXCR1 binds only CXCL6 and CXCL8, while CXCR2 binds all ELR+
80 CXC chemokines (CXCL1, 2, 3, 5, 6, 7, 8) (Strieter *et al.*, 2005). Recent studies have shown
81 that CXCR1 and CXCR2 were poorly expressed in breast tumors, while CXCR4 was
82 overexpressed in metastatic breast tumors (Muller *et al.*, 2001).

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

102

103 **MATERIALS AND METHODS**

104 **Patients and Samples.**

105 We analyzed samples of 48 primary breast tumors excised from women at our institution from
106 1980 to 1994. Samples containing more than 70% of tumor cells were considered suitable for
107 this study. Immediately following surgery, the tumor samples were placed in liquid nitrogen
108 until RNA extraction. The patients met the following criteria: primary unilateral non
109 metastatic postmenopausal breast carcinoma; estrogen receptor alpha positivity [as determined
110 at the protein level by biochemical methods (Dextran-coated charcoal method until 1988 and
111 enzymatic immunoassay thereafter) and confirmed by ER α real-time quantitative RT-PCR
112 assay]; complete clinical, histological and biological information available; no radiotherapy or
113 chemotherapy before surgery; and full follow-up at our institution. The malignancy of
114 infiltrating carcinomas was scored according to Scarff Bloom and Richardson's
115 histoprognostic system (Bloom & Richardson, 1957). The characteristics of the patients are
116 shown in Table 1. The patients had physical examinations and routine chest radiography every
117 3 months for 2 years, then annually. Mammograms were done annually. The median follow-
118 up was 7.2 years (range 1.5-10.0 y). All the patients received postoperative adjuvant endocrine
119 therapy (tamoxifen, 20 mg daily for 3-5 years), and no other treatment. Twenty-four patients
120 relapsed. The distribution of first relapse events was as follows: 22 metastases, and 2 local
121 and/or regional recurrences with metastases.

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

126 To evaluate the relationship between mRNA levels of CXCL8 during breast cancer
127 progression, we analyzed tumor RNA of 11 invasive ductal grade I breast tumors, 37 invasive

128 ductal grade III breast tumors and 24 distant metastasis (10 liver, 7 lung, 4 skin, 2 ovarian and
129 one gastric metastasis).

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

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

137

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

139 Breast tumors were minced with scalpels and incubated overnight with Liberase Blendzyme 2
140 (Roche Applied Science, Meylan, France) for enzymatic dispersion. Organoids and aggregated
141 cells (epithelial fraction) and isolated cells (fibroblast fraction) were isolated from the digest by
142 differential centrifugation followed by culture in selective media as described earlier (Speirs *et*
143 *al.*, 1998). Fibroblast fraction was cultured in Ham's F10 medium containing L-glutamine (3
144 mM), insulin (5 μ g/mL), Thyroid hormone T3 (1 nM), hydrocortisone (1 μ g/mL) and 10% serum
145 calf fetal. Epithelial fraction was cultured in the same conditions plus epidermal growth factor
146 (5 ng/mL) and transferrin (5 μ g/mL) and with 5% human serum (and without serum calf fetal).
147 Cells were incubated in a humidified atmosphere of 5% CO₂ at 37 °C, and the medium was
148 changed three times a week.

149

150 **Endothelial and blood cells isolation**

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

154

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

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

171 Results, expressed as N-fold differences in target gene expression relative to the *TBP* (or
172 *RPLP0*) gene, and termed "RNA N_{target} ", were determined as $N_{target} = 2^{\Delta C_t}_{sample}$, where
173 the ΔC_t value of the sample was determined by subtracting the C_t value of the target gene from
174 the C_t value of the *TBP* (or *RPLP0*) gene (Bieche I. *et al.*, 1999, Bieche Ivan *et al.*, 2001). Target
175 genes were considered to be detectable but not quantifiable when the C_t value was above 35.

176 Primers for the target genes and the endogenous control genes used in the different RT-PCR
177 assays were chosen with the assistance of the computer programs Oligo 5.0 (National
178 Biosciences, Plymouth, MN). We conducted BLASTN searches against dbEST and nr (the non
179 redundant set of GenBank, EMBL and DDBJ database sequences) to confirm the total gene
180 specificity of the nucleotide sequences chosen for the primers, and the absence of DNA
181 polymorphisms. In particular, the primer pair was selected to be unique relative to the sequences
182 of closely related family member genes or of the corresponding retropseudogenes. To avoid
183 amplification of contaminating genomic DNA for the quantitative RT-PCR, one of the two
184 primers was placed at the junction between two exons, or in a different exon, if possible. The
185 nucleotide sequences of the oligonucleotide primers for real-time RT-PCR are shown in Table 3.
186 Gel electrophoresis was used to verify the specificity of PCR amplicons. For the selected primer
187 pair, we performed no-template control (NTC), which produced negligible signals (usually > 40
188 in Ct value), suggesting that primer-dimer formation effects were negligible.
189 RNA extraction, cDNA synthesis and PCR reaction conditions are described in detail elsewhere
190 (Bieche Ivan *et al.*, 2001).

191

192 **Elisa.** CXCL8 and CXCL1 concentration in culture supernatants was determined by ELISA
193 using DuoSet kits (R&D Systems, Minneapolis, MN) as recommended by the manufacturer.

194

195 **Statistics.** As the mRNA levels did not fit a Gaussian distribution, (a) the mRNA levels in each
196 subgroup of samples were characterized by their median values and ranges, rather than their
197 mean values and coefficients of variation, and (b) relationships between the molecular markers
198 and clinical and biological parameters were tested by using the non parametric Mann-Whitney *U*
199 test (Mann & Whitney, 1947). Differences between two populations were judged significant at
200 confidence levels greater than 95% ($p < 0.05$).

201 To visualize the capacity of a given molecular marker to discriminate between two populations
202 (in the absence of an arbitrary cutoff value), we summarized the data in a ROC (*receiver*

203 *operating characteristics*) curve (Hanley & McNeil, 1982). When a molecular marker has no
204 discriminative value, the ROC curve lies close to the diagonal and the AUC is close to 0.5. When
205 a marker has strong discriminative value, the ROC curve moves to the upper left-hand corner (or
206 to the lower right-hand corner) and the AUC is close to 1.0 (or 0).

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

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

212

213

214

215 **RESULTS**

216

217 ER-negative breast cancers display a higher aggressiveness in terms of metastasis development
218 and worse prognosis compared to ER α -positive breast tumors. (Osborne, 1998, Sheikh *et al.*,
219 1994). We have demonstrated recently an inverse correlation between expression of the CXC
220 chemokine CXCL8 and ER α levels in human breast cancer cell lines (Freund *et al.*, 2003), which
221 could account for their higher aggressiveness. Interestingly, CXCL8 gene is located with a
222 number of other CXC chemokines in a short region (360 kb) of chromosome 4 (4q21) (Fig. 1
223 and Table 4). The goal of this study was to determine whether the chemokines of this cluster
224 were co-regulated and what could be the factors controlling their expression.

225

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

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

240 effects of the chemokines present on chromosome 4, as well as KI67, displayed a similar
241 expression in the two CXCL8 groups.

242

243 **Breast cancer cells produce multiple chemokines.**

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

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

261

262 **Mechanisms controlling chemokine expression in tumors.**

263 To address the question of the coregulation of these chemokines, we first hypothesized that some
264 of the overexpressed chemokines could be the result of genomic DNA amplification. To test this
265 hypothesis, we analyzed five genomic DNA samples from normal patients, 4 breast tumors with

266 low CXCL8 mRNA expression and 4 breast tumors with high CXCL8 mRNA overexpression.
267 Among the 8 tumors tested, no tumor showed 4q21 DNA amplification when looking at CXCL1
268 and CXCL8 genes (data not shown).

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

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

285

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

288 Many CXC chemokines are strong inducers of chemotaxy. We thus hypothesized that they could
289 also be involved in tumor progression and that they could increase metastase formation. To
290 evaluate this, we analyzed CXCL8 expression in 11 grade I tumors, 37 grade III tumors and 24
291 metastases in primary tumors and distant metastases (Table 10). We observed that CXCL8

292 expression raised from grade I to grade III primary tumors and exhibited a strong increase (8-
293 fold increase) when looking at distant metastases, suggesting that this chemokine could be a key
294 player in metastasis. CXCL1, CXCL2, CXCL3, CXCL5 and CXCL6 showed also increased
295 levels from grade III to metastases but not between grade I and grade III patients (Table 10).

296

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

298 ER α -negative tumors are generally more aggressive than ER α -positive tumors. However,
299 some ER α -positive tumors display also aggressive features. It is of great interest to
300 understand why this subpopulation of ER α -positive tumors are more metastatic. To address
301 this issue, we measured the expression levels of CXCL1, CXCL2, CXCL3, CXCL5, CXCL6
302 and CXCL8, as well as HER2 by real-time RT-PCR in a cohort of 24 ER α -positive breast
303 tumor patients who relapsed and 24 ER α -positive breast tumor patients who did not relapse
304 (Table 1). All these 48 ER α -positive tumors were from postmenopausal patients treated with
305 primary surgery followed by adjuvant tamoxifen alone. We used univariate analysis (log-
306 rank test) to further study the prognostic value of these seven genes. For each gene, the 48
307 ER α -positive breast tumors were divided into two groups of 24 tumors with “low” and
308 “high” CXC chemokine mRNA levels. Univariate analysis showed that a high expression
309 level of CXCL1, CXCL3 and CXCL8 correlated with significantly shorter relapse free
310 survival (RFS) (Fig. 3 and Fig. 4). The outcome of the 24 patients with high mRNA levels of
311 these 3 genes was significantly worse than those of the 24 patients with low CXCL1, CXCL3
312 and CXCL8 mRNA levels. No significant prognostic value was associated with the four
313 other gene, CXCL2 (P=0.59), CXCL5 (P=0.47), CXCL6 (P=0.16) and HER2 (P=0.83).
314 Moreover, using a Cox proportional hazards model, we also assessed the prognostic value,
315 for RFS, of parameters that were significant or near-significant (P<0.1) in univariate
316 analysis, i.e. SBR grade, lymph-node status (Table 1) and the genes CXCL1, CXCL3 and

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317 CXCL8 (Fig 3 and 4). Only the prognostic significance of CXCL8 mRNA status shows a
318 trend toward significance (P=0.068).

319

320

321 **DISCUSSION**

322 The aim of this study was to determine whether breast cancer samples expressing high levels of
323 CXCL8 could also produce other CXC chemokines, with a particular interest to the chemokines
324 localized to a narrow region of chromosome 4. Indeed, by analyzing chromosome 4 sequences,
325 we observed that 9 CXC chemokines (CXCL1, CXCL2, CXCL3, CXCL4, CXCL4V1, CXCL5,
326 CXCL6, CXCL7 and CXCL8) were mapped to a short region of 356 kb, which could suggest
327 that they belong to an inflamed cluster. By measuring the RNA levels of these chemokines in
328 breast tumors, we reported that tumors expressing high levels of CXCL8 were also producing
329 elevated levels of CXCL1, CXCL3, CXCL5, CXCL6 and to a minor extent of CXCL2. CXCL4,
330 CXCL4V1 and CXCL7 were not significantly expressed in both types of tumors. Concomitant
331 expression of CXCL8 with other cytokines such as IL-6 has already been reported in serum for
332 breast and ovarian cancer (Benoy I. *et al.*, 2002, Jiang *et al.*, 2000, Kozlowski *et al.*, 2003,
333 Penson *et al.*, 2000). In addition, we observed that CXCL8 ++ tumors were expressing low levels
334 of ER α , which is in agreement with previous work (Freund *et al.*, 2003, Lin *et al.*, 2004). It is
335 interesting to note that, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6 and CXCL8 were all ELR
336 (glutamate-leucine-arginine)-positive chemokines (Moser *et al.*, 2004). This appears quite
337 important as the NH₂-terminal motif has been shown to be responsible for the ability of
338 chemokines to attract neutrophils as well as a necessary motif involved in angiogenic properties
339 of these chemokines (Strieter *et al.*, 2005). The coregulation of CXCL1, CXCL3, CXCL5,
340 CXCL6 and CXCL8 in tumors was puzzling. Our data suggest that this high expression does not
341 arise from genomic amplification, but rather from an increased transcriptional activity of these
342 genes. In addition, we showed that CXCL1, 2, 3 and 8 were induced by TNF α in breast cancer
343 cells, demonstrating the genes were functional. Our previous work on CXCL8 gene regulation in
344 breast cancer cells had shown that CXCL8 overexpression in ER α -negative breast cancer cell
345 lines was mainly mediated through an overexpression of NF- κ B and AP-1 transcription factors,
346 which in turn could induce CXCL8 promoter activity (Freund *et al.*, 2004). To date, only a few

347 data are available concerning the regulation of CXCL1, 3, 5 and 6 gene promoters. Of particular
348 note is the fact that CXCL1 promoter displays many features common to CXCL8, with in
349 particular also a crucial NF- κ B site involved in constitutive expression of CXCL1 gene in
350 melanoma (Wood & Richmond, 1995, Yang & Richmond, 2001). So, we hypothesized that
351 tumors producing high amounts of CXCL8 could also have deregulated NF- κ B and AP-1
352 pathways. To test this hypothesis, we measured the expression levels of a series of NF- κ B and
353 AP-1 transcription factors or target genes. Our data showed that CXCL8 expression was
354 correlated primarily to AP-1 transcription factors such as ATF3, c-Jun or JunB and typical AP-1
355 target genes, but to a lesser extent to NF- κ B pathway.

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

370 We next focused our attention to CXCL8 to evaluate its potential prognosis value. Our data
371 showed that CXCL8 content in the primary tumor was increased in grade III patients compared
372 to grade I patients. This is in agreement with other reports showing that CXCL8 is overexpressed

373 in breast tumors compared to normal tissues and that breast cancer patients display higher seric
374 levels of CXCL8 (Green *et al.*, 1997, Kozlowski *et al.*, 2003). Moreover, distant metastases were
375 extensively producing CXCL8, suggesting that CXCL8 could be one of the factors leading to
376 metastasis. Indeed, our previous work showed that CXCL8 could increase the invasion potential
377 of breast cancer cells, one of the first step before metastasis (Freund *et al.*, 2003). Benoy et al.
378 have also shown that patients with metastatic breast tumors displayed high seric amounts of
379 CXCL8 (Benoy I. H. *et al.*, 2004). In vivo studies in athymic mouse models also corroborate
380 these data, suggesting that breast tumors secreting high levels of CXCL8 metastasize more easily
381 to bone (Bendre *et al.*, 2002, De Larco *et al.*, 2001). We then wondered whether the other
382 chemokines of the cluster were following the same trend. We observed that CXCL1, 2, 3, 5 and
383 6 were also more expressed in metastasis compared to grade I and grade III patients, suggesting
384 that they could also be involved in metastasis. If CXCL1 has been shown to increase breast
385 cancer cell invasion in vitro (Youngs *et al.*, 1997), so far, this is the first report of the
386 involvement of CXCL1, CXCL2, CXCL3, CXCL5 and CXCL6 in breast cancer metastasis.
387 From this point, we knew that CXCL8, 1, 2, 3, 5 and 6 chemokines were mainly produced by
388 ER α -negative tumors, and were globally overexpressed in breast cancer metastases. But, one
389 crucial question was to look at the case of patients with ER α -positive tumors who were treated
390 with tamoxifen and had an impaired survival. Indeed, it is quite difficult to understand why a
391 subpopulation of ER α -positive breast cancer patients will still have a bad outcome. So, our goal
392 was to determine whether CXCL1, CXCL2, CXCL3, CXCL5, CXCL6 or CXCL8 could
393 discriminate among ER α -positive breast cancer patients, those who will develop rapidly
394 metastasis. Univariate analysis showed that, among these six genes (as well as HER2), only
395 CXCL1, CXCL3 and CXCL8 correlated with RFS. In multivariate analysis, only CXCL8 shows
396 a trend toward significance to be an independant predictor of response to tamoxifen therapy.
397 These data, which need to be confirmed on a larger set of patients, suggest that CXCL8 could
398 help to improve the prognosis of ER α -positive patients who are treated with tamoxifen.

399 In conclusion, our work suggest that breast tumors expressing high levels of CXCL8 have a bad
400 prognosis evolution which could be the reflect of their higher content of ELR-positive CXC
401 chemokines such as CXCL1, CXCL3, CXCL5 and CXCL6. These chemokines belong to an
402 hyperactive gene cluster, which is likely to be regulated by AP-1 and to a lesser extent by NF- κ B
403 pathways. Moreover, ER α -positive breast cancer patients with high levels of CXCL1, CXCL3 or
404 CXCL8 display a shorter relapse free survival. It will be interesting to dissect further the
405 mechanisms of regulation, as a potential therapeutic approach could be to identify the common
406 factors up-regulating these chemokines in the same subset of tumors.

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581

582 **LEGENDS TO FIGURES**

583

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

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

588

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

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

594

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

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

599

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

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

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