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CXCL12/SDF1 expression by breast cancers is an independent prognostic marker of disease-free and overall survival

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ABSTRACT

The cytokine C-X-C motif chemokine 12 (CXCL12) is synthesised by metastasis target tissues and has been shown to attract tumour cells that express the receptor, C-X-C chemokine receptor type 4 (CXCR4). However, epigenetic silencing of CXCL12 has recently been reported to increase the metastatic potential of breast cancer cells and the reintroduction of the cytokine gene into MDA-MB-231 breast carcinoma cells decreases the number of metastases formed *in vivo*. We therefore wished to know whether CXCL12 expression correlates with relapse-free and overall survival in human breast cancer patients.

The expression of C-X-C motif chemokine 12 (CXCL12) and C-X-C chemokine receptor type 4 (CXCR4) was analysed in 100 archival breast cancer samples by immunohistochemistry and in two breast cancer microarray datasets of 408 cases. Data were analysed by univariate and multivariate COX regression analyses.

CXCL12 and CXCR4 are expressed by epithelial tumour cells and by stromal and endothelial cells. Microarray gene expression analysis and immunohistochemistry revealed that expression of CXCL12 but not of CXCR4 significantly correlates with disease-free and overall survival in oestrogen receptor-positive and -negative cancers. The expression of the oestrogen receptor α and that of CXCL12 do not correlate.

CXCL12 is a strong, independent prognostic marker. We propose that saturation of the receptor through autocrine CXCL12 production reduces chemotaxis towards CXCL12-releasing metastasis target tissues.

1. Introduction

Stromal cell-derived factor 1 (stromal cell-derived factor 1 [SDF1] or cytokine C-X-C motif chemokine 12 [CXCL12]), initially cloned from bone marrow stromal cells,[1] is a widely expressed cytokine that binds to the receptors CXCR4 and C-X-C chemokine receptor type 7 (CXCR7). The CXCL12/CXCR4 axis is involved in haematopoietic, neural, vascular and craniofacial organogenesis and disruption of one of the two components leads to embryonic death (for recent reviews see 2,3). Initially, CXCL12 was described as haematopoietic homing factor.[4] In 2001 Muller and colleagues reported that leucocyte

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homing and cancer metastasis have striking similarities that can be explained by a common cytokine signalling system. They showed that breast cancers express a high level of CXCR4 and that the breast cancer metastasis target tissues lung, liver and bone express peak levels of the ligand, CXCL12.[5]

CXCL12 probably attracts those cells released from the primary tumour that express the CXCL12 receptor, CXCR4, by means of chemotaxis. Inhibition of receptor binding using antibodies against the ligand or the receptor significantly reduces the formation of metastases of the breast cancer cell line MDA-MB-231 in a mouse model.[5] Interruption of CXCR4 function by synthetic peptides,[6] by silencing[7] and by artificial miRNAs[8] also blocks experimental breast cancer metastasis.

In addition to its chemotactic action on breast cancer cells, CXCL12 stimulates proliferation via the ERK and AKT pathways[9] and inhibits apoptosis through the induction of NFκB.[10] CXCL12 also induces angiogenesis[11] and production of matrix metalloproteinases[12] and integrins.[13]

There is strong evidence for the pro-metastatic role of the CXCL12/CXCR4 axis, yet few reports directly address the role of CXCL12 in tumour metastasis. Several studies report blunting of CXCR4 functions by different means[5–8] but neutralisation of CXCL12 function by specific antibodies has been reported only for non-small cell lung cancer where it reduces metastasis.[14]

CXCL12 is expressed by many human tumours and tumour cell lines[2] including several breast cancer cell lines.[15] MCF7, an oestrogen receptor-positive cell line that is tumourigenic in mice but produces metastases only occasionally,[16] expresses CXCL12[17] but the metastatic, oestrogen receptor-negative cell line MDA-MB-231 does not.[18] CXCL12 expression is induced by 17-β-estradiol in MCF7 cells.[17] Hence the absence of the oestrogen receptor α from MDA-MB-231 cells might explain why these cells do not express CXCL12. Kang and colleagues have created CXCL12-expressing MDA-MB-231 cells by transfection of expression plasmid constructs and showed enhanced migration and invasion capacities of these cells. They also performed a gene expression analysis of human breast cancers and reported an inverse correlation between the levels of CXCL12 expression and disease-free and overall survival of breast cancer patients and a positive correlation with recurrence and lymph node metastasis.[19] In contrast with these results, transgene overexpression of CXCL12 in MDA-MB-231 cells has recently been reported to reduce haematogenous metastasis of these cells in the mouse.[18] The authors also report that the endogenous CXCL12 gene is epigenetically silenced.[18] These results attribute an anti-metastatic role to the autocrine production of CXCL12 that is in clear contrast with prevalent hypotheses on the role of this chemokine in tumour progression.

We wished to clarify whether production of CXCL12 by the tumour itself correlates with the clinical outcome of breast cancers. For this purpose, we investigated CXCL12 and CXCR4 expression in 100 breast cancer tissues by immunohistochemistry and correlated the results with the corresponding clinico-pathological and follow-up data. We also analysed two published microarray gene expression datasets of breast cancers[20,21] for CXCL12 in correlation to disease-free survival. Our results show that CXCL12 expression is positively correlated with disease-free and overall survival in breast cancer in both datasets in a highly significant manner.

2. Materials and methods

2.1. Microarray data analysis

Files containing raw intensity data of Affymetrix HU133A arrays (.cel files) of two independent datasets (GSE1456, GSE3494) were obtained from the Gene Expression Omnibus database. Intensity data were preprocessed using R/Bioconductor. Normalisation was carried out using the quantile algorithm and summarisation was done using GCRMA pmonly. Probe sets used for CXCL12 were 209687_at corresponding to the CXCL12α transcript (NCBI accession number: NM_000609) and 203666_at corresponding to CXCL12β (NCBI accession number: NM_199168). The probe set used for oestrogen receptor α was 205225_at.

2.2. Breast tumour tissue samples

A series of 100 breast cancer tissue samples were taken from the files of the Breast Cancer Registry (Munich-Bogenhausen Academic Clinic). The disease status of the cases was updated by December 2007 (Tumorregister München, TRM) and cases were selected according to outcome forming three different groups as follows: (1) breast cancer cases with 5 years disease-free follow-up, (2) cases with local tumour recurrence within 5 years but without death of disease (DOD) and (3) cases with tumour progression and DOD within 5 years. The cases covered the time period between 2000 and 2002. Basic clinical and histo-pathological information including tumour type, size, nodal status, degree of tumour cell differentiation, proliferative activity and presence/extent of intraductal carcinoma was available. In particular, tumour cell differentiation was determined according to the guidelines by Elston and Ellis.[22] All experiments were conducted following the rules of bioethics issued by the Academic Hospital Bogenhausen, Munich, Germany, which apply to the treatment of pathological specimens.

2.3. Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue samples from the 100 breast cancers were immunohistochemically tested for CXCL12 (DIANOVA, Hamburg, Germany), CXCR4 (Santa Cruz Biotechnology, Santa Cruz, CA), the oestrogen and progesterone receptors (DAKO, Hamburg, Germany), the Her-2-neu oncoprotein and the proliferation antigen Ki-67 (both: DAKO, Hamburg, Germany) according to the manufacturer's protocols. Sections stained with non-specific (pre-immune) serum were run in parallel as negative controls. Positive controls consisted in prostate carcinoma samples with known CXCL12 and CXCR4 expression since the same antibodies had been shown to specifically detect the antigens in prostate carcinomas[23] and breast cancer samples with known expression of hormone receptors (for the oestrogen and the progesterone receptors) or the Her-2-neu oncoprotein and lymph node tissue for the proliferation rate (Ki-67).

The immunostaining was evaluated using a semi-quantitative scoring system similar to that previously applied for the analysis of the hormone receptor status[24] and to the system applied to the analysis of EGFR expression by Cappuzzo and co-workers.[25] The extent of positively labelled cells was ranked into 5 grades, i.e. 0 = 0%, 1 = 1–10%, 2 = 11–50%; 3 = 51–90% and 4 = >90%; staining intensity was graded into 4 steps with 0 = no staining; 1 = low; 2 = moderate and 3 = strong staining. Results were presented as product of the two assessments thus ranging from 0 to 12.

All other immunostainings were evaluated according to established routine protocols.

2.4. *Statistical methods*

Variables affecting disease-free and overall survival were studied using both univariate and multivariate Cox regression models (stratified by study group when analysing published datasets). Patient samples were classified into two categories that express CXCL12 and CXCR4 higher or lower than the median value. Disease-free survival and overall survival were reported in groups with high and low levels of CXCL12 and CXCR4 expression using a Kaplan–Meier survival analysis and were compared by the log-rank test.

3. Results

CXCL12 is present on Affymetrix HGU133A GeneChips with two probe sets, specific for SDF1α (Affymetyrix probe set ID: 209687_at, NCBI accession number: NM_000609) and SDF1β (Affymetyrix probe set ID: 203666_at, NCBI accession number: NM_199168). The two isoforms are products of alternative splicing and differ in the last four amino acid residues at the carboxy terminus. Biological and functional differences between the two isoforms are not known. We analysed the correlation between the expression values of the two probe sets with disease-free survival in a dataset of 159 breast cancer cases published by Pawitan et al.[21] (Stockholm cohort, GSE1456) and in a similar dataset containing 249 cases published by Miller et al.[20] (Uppsala cohort, GSE3494). The two datasets are derived from un-selected consecutive cases, representative for the breast cancer population. Microarray analyses were performed on RNA extracted from the whole tumours containing prevalently tumour cells but also tumour stroma and infiltrating cells.

CXCL12 is expressed at a greatly variable level in these tumours. In Table 1A the patients' characteristics are given according to the median level of CXCL12 expression. Younger patients, smaller tumours and oestrogen receptor-positive tumours are significantly more frequent in the group expressing the CXCL12 gene above median levels in the Uppsala microarray cohort.

Higher CXCL12 expression level was significantly associated to a better relapse-free survival when evaluated in a Cox regression model stratified by study group: the hazard ratios (HR) were 0.79 (95% confidence interval [CI] = 0.69–0.92, $p = 0.001$) and 0.84 (95% CI = 0.76–0.94, $p = 0.002$) for the two probe sets, respectively. The association remained significant when adjusting for other prognostic factor (age, tumour size, oestrogen receptor (ER) status and lymph node status available on the Uppsala cohort only) in a multivariate model (HR = 0.81, 95% CI = 0.69–0.96, $p = 0.015$ and HR = 0.85, 95% CI = 0.74–0.98, $p = 0.02$).

Fig. 1 shows Kaplan–Meier survival curves for the two probe sets in the two merged datasets, according to CXCL12 levels higher or lower than the median value.

CXCL12 is regulated by ERα.[17] We therefore analysed whether the expression values of the two genes are correlated. This, however, is not the case for both probe sets, since the correlation with ER expression was $r = 0.03$, $p = 0.5$ and $r = -0.06$, $p = 0.3$, respectively (Fig. 2). We also analysed the expression of the receptor CXCR4 and the (co-)receptor CXCR7.[26] Expression of both receptors is not significantly correlated with disease-free survival and none of the probe sets for the receptors adds to the discrimination power in a multiparametric model (data not shown).

We then whished to know whether CXCL12 and CXCR4 proteins are expressed by the epithelial tumour cells or by infiltrating inflammatory cells and local stromal cells and whether the effect observed is reflected at the protein level. We therefore performed immunohistochemical stainings on the sections from 100 breast cancer tissue samples taken from the files of the Breast Cancer Registry (Munich-Bogenhausen Academic Clinic). The choice of immunohistochemical analyses was also guided by the consideration that other techniques such as Western blot and real time PCR do not reveal the cell type of expression and immunohistochemistry is still the method of choice if an application in the routine pathology is envisioned. However, protein expression is more difficult to quantify by immunohistochemical analyses. We therefore applied a highly validated scoring system (see Section 2).

The expression of CXCL12, exclusively observed in the cytoplasm of tumour and stromal cells, is highly variable with respect to extent and intensity. Cytoplasmic staining most probably corresponds to newly synthesised chemokine on its way to secretion since storage of cytokines is uncommon. Strong staining is seen in lobular carcinomas regardless of the tumour cell location (not shown) and in peripheral areas of invasive ductal carcinomas (Fig. 3A, C and D). Similarly, intraductal tumour proliferates within the invasive ductal carcinomas (i.e. ductal carcinoma in situ, DCIS) showed variable, but mostly strong cytoplasmic staining of the tumour cells (data not shown). In comparison, most invasive ductal carcinomas revealed less intense staining in the tumour centre (Fig. 3B), and the staining of stromal cells (Fig. 3D) and endothelia is also less intense. A tumour cell-associated expression is observed in all the 100 tumour samples. The staining for CXCR4 is similarly observed in epithelial tumour cells and stromal and endothelial cells (Fig. 3E–H), again with a stronger and more widespread staining of the tumour periphery (and in intraductal tumour proliferates) when compared to the tumour centre and to stroma and to endothelial cells.

In the immunohistochemistry cohort, smaller, lymph node-negative tumours were significantly more frequent in the group highly expressing CXCL12. In this cohort, oestrogen receptor status differs significantly in the two groups only when the continuous score is considered and not when the

Table 1 – Patients' characteristics according to CXCL12 expression. (A) Uppsala microarray cohort and (B) Munich immunohistochemistry cohort

Variable (*n*)	Low CXCL12 (124)	High CXCL12 (125)	*p* Value
(a) Microarray Uppsala cohort			
Age			
Mean (SD)	64 (15)	60 (13)	0.02
Tumour size (mm)			
<20	49 (40%)	77 (62%)	0.001
21+	75 (60%)	48(38%)	
Oestrogen receptor			
Negative	21 (17%)	5 (4%)	0.001
Positive	103 (83%)	120 (96%)	
Progesterone receptor			
Negative	51 (41%)	53 (42%)	0.89
Positive	73 (59%)	72 (58%)	
Nodal status			
Negative	79 (64%)	89 (71%)	0.22
Positive	45 (36%)	36 (29%)	
	Low CXCL12 (53)	High CXCL12 (47)	*p* Value
(b) Immunohistochemistry cohort			
Age			
Mean (SD)	61 (13)	58 (13)	0.13
Tumour size (mm)			
<20	27 (51%)	34 (72%)	0.04
21+	26 (49%)	13(28%)	
Oestrogen receptor			
Median (range)	2 (0–9)	4 (0–12)	0.04
Progesterone receptor			
Median (range)	3 (0–12)	4 (0–12)	0.40
Oestrogen receptor			
Negative	28 (53%)	18 (38%)	0.16
Positive	25 (47%)	29 (62%)	
Progesterone receptor			
Negative	26 (49%)	19 (40%)	0.42
Positive	27 (51%)	28(60%)	
Nodal status			
0	24 (45%)	33 (70%)	0.002
1–3	19 (36%)	13 (28%)	
4+	10 (19%)	1 (2%)	
Chemotherapy			
No	23 (45%)	27 (60%)	0.16
Yes	28(55%)	18 (40%)	
Missing	2	2	

oestrogen receptor status indicated by the pathologist (score >2 = ER+, <3 = ER–) is used (Table 1B).

A statistical analysis of the staining results for CXCL12 and CXCR4, along with a correlative analysis of tumour-specific parameters (patient age, tumour size and nodal status) and hormone receptor status reveals a highly significant correlation between disease-free survival and tumour size and nodal status (as expected), but also between disease-free survival and the expression pattern of CXCL12 at the tumour periphery (univariate analysis $p = 0.002$) and at the tumour centre (univariate analysis $p < 0.001$; multivariate analysis $p = 0.002$) and CXCL12 in stromal cells (univariate analysis $p = 0.01$) (Table 2).

Tumour size ($p = 0.001$) nodal status ($p = 0.001$), progesterone receptor ($p = 0.003$) and CXCL12 expression in the tumour centre ($p < 0.001$) and stroma ($p = 0.02$) correlate with overall survival in the univariate analysis and tumour size, progesterone receptor and CXCL12 in the centre and in the stroma resist in a multivariate model (Table 3).

Fig. 4 shows the Kaplan–Meier curves for CXCL12 expression in relation to disease-free survival.

The expression pattern of CXCR4 is not associated with disease-free or overall survival in a statistically significant manner. The expressions of the oestrogen and progesterone receptors are also not correlated with the disease-free survival in this study population (progesterone receptor

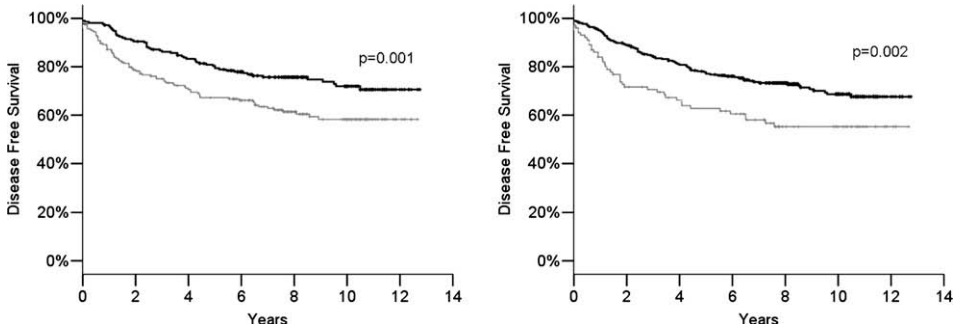

Fig. 1 – Kaplan–Meier disease-free survival curves for CXCL12 on the combined datasets GSE1456[21] and GSE 3494[20] for the expression values of CXCL12α (A) and CXCL12β (B). Datasets were obtained from Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/). Curves were calculated using R software on GCRMA[31] normalised microarray data. Patients were divided into two groups based on the level of CXCL12 expression (black line = expression levels above the median value, grey line = expression levels below the median).

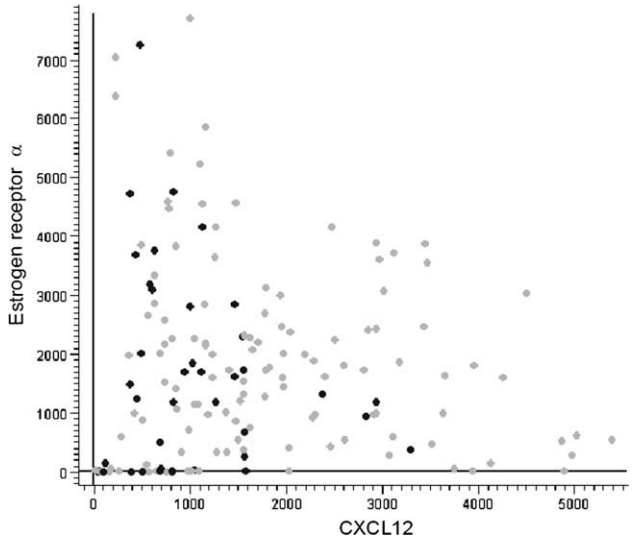

Fig. 2 – (A) Correlation of ERα and CXCL12 expression in human breast cancer (dataset GSE1456). Expression values were obtained from GCRMA[31] normalised microarray data. Logarithmic expression values were transformed into natural numbers. Clinical outcome of the patients is indicated (black = relapse, grey = no relapse).

$p = 0.06$). Chemotherapy status was only weakly correlated with disease-free survival ($p = 0.06$) and overall survival ($p = 0.15$).

4. Discussion

Despite compelling evidence for the pro-metastatic function of the CXCR4/CXCL12 axis[2,5] little attention has been devoted to the precise role of CXCL12/SDF1. CXCL12 has clear pro-tumoural activities inasmuch as it promotes proliferation, inhibits apoptosis and induces angiogenesis[11] in synergy with the vascular endothelial growth factor (VEGF).[27] However, pro-tumoural not necessarily means pro-metastatic since tumour dimension is only one of many determinants of tumour progression. The main indication of a pro-metastatic role of CXCL12 comes from studies where its receptor, CXCR4, is blocked or silenced. CXCL12 apparently is the exclusive ligand for CXCR4, hence its pro-metastatic function appears most likely. But the evidence for such a role is thin. Blunting of CXCL12 function by specific antibodies has been reported for non-small lung cancer cells where it reduces metastasis.[14] Kang and co-workers reported an inverse correlation of CXCL12 expression with disease-free and overall survival in breast cancer patients.[19] This work also claims a pro-invasive effect of CXCL12 when transfected into the mammary carcinoma cell line MDA-MB-231 that does not express endogenous CXCL12. Increased invasiveness is in contrast to reduced metastasis *in vivo* that has been reported for CXCL12-expressing MDA-MB-231 cells in a more recent study.[18] The two studies also show contrasting results on CXCL12 expression in normal breast tissue, absent for Kang et al.[19] and strong for Wendt et al.[18] The presence of highly divergent subclones within populations of MDA-MB-231 cells has been described[28] and it cannot be excluded that during the selection of stably transfected cells, specific subclones with different invasive behaviour have been selected. The invasion assay in the study by Kang et al. was run for 96 hours. The number of invading cells can thereby also be affected by pro-proliferative effects of the autocrine expression of the transfected chemokine.

We show here that CXCL12 expression is associated with disease-free and overall survival in a highly significant manner in two independent microarray gene expression datasets as well as in 100 breast cancer cases that we have analysed by means of immunohistochemistry. Multivariate analysis reveals that CXCL12 expression is a strong independent prognostic marker. CXCL12 is strongly expressed by many breast cancers and its expression is observed for tumour epithelial cells as well as for stromal fibroblasts and vascular cells. In our immunohistochemical analysis we found a strong staining for CXCL12 predominantly in the cytoplasm of epithelial tumour cells that appears to be stronger in the tumour periphery than in the tumour centre.

Both oestrogen receptor α-positive and -negative cancers that express CXCL12 have a better clinical outcome than those that have lost expression of the chemokine. CXCL12 is strongly oestrogen regulated in MCF7 cells[17] but expression of the oestrogen receptor α and that of CXCL12 are not corre-

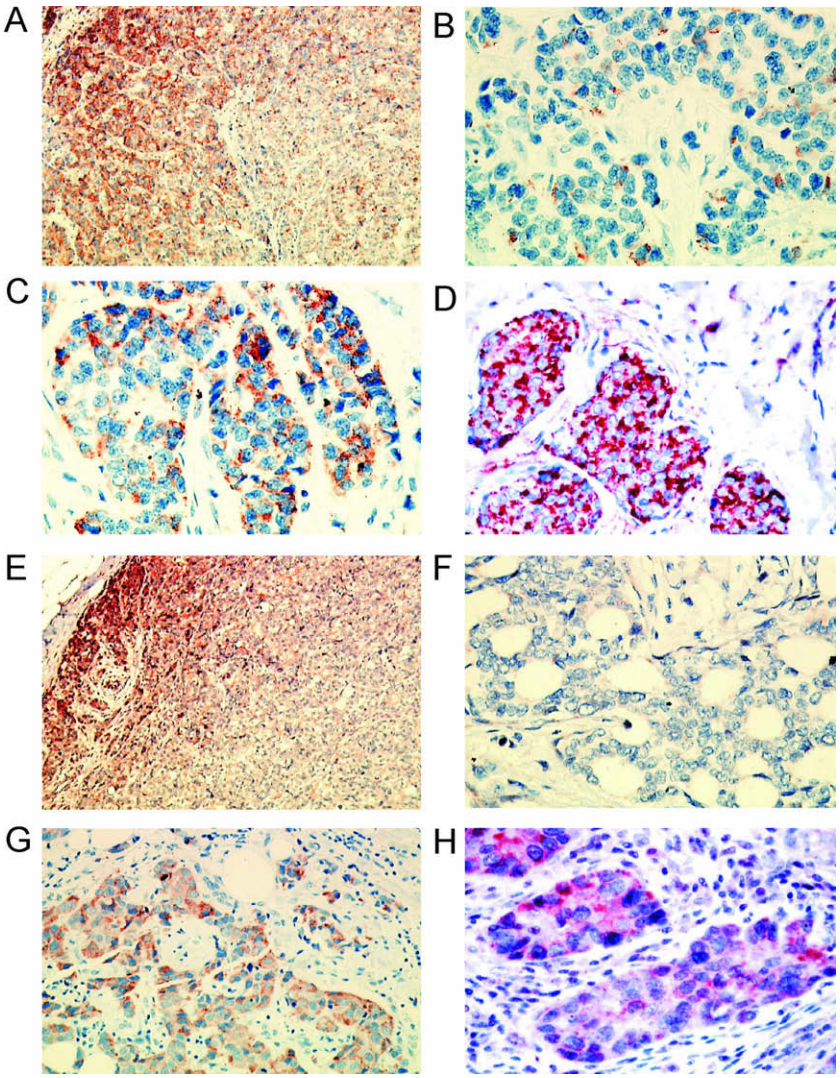

Fig. 3 – Immunohistochemical expression analysis of CXCL12 (A–D) and CXCR4 (E–H) in human invasive ductal breast cancer tissues. The panel shows a significant staining of tumour cells – more prominent at the tumour periphery (A) when compared to the centre (B) – with typical cytoplasmic staining (C, D) and positive labelling of adjacent stromal cells (D). The staining pattern for CXCR4 is similar with a stronger staining at the tumour periphery (E, G) when compared to the tumour centre (F), again a cytoplasmic labelling (G) and positive stromal cells (H). (A–D: anti-CXCL12; E–H: anti-CXCR4; original magnification A: ×100; B, C, F, G: ×400; D and H: ×600).

lated. Wendt and colleagues reported strong expression of CXCL12 in normal breast tissue that is silenced through DNA methylation in CXCL12-negative cancers.

CXCR4 expression occurs in tumour epithelial cells and in stromal cells of the vast majority of breast cancers. CXCR4 expression does not correlate with disease-free survival (data not shown).

Our data are consistent with those reported by Wendt et al.[18] who have shown that CXCL12 transgene overexpression reduces metastasis of breast cancer cells *in vivo*. CXCL12 is silenced by DNA methylation. Two independent clones of CXCL12-transfected MDA-MB231 cells showed increased proliferation but not apoptosis. Intracellular calcium flux in response to exogenous CXCL12, a measure of CXCR4 receptor activity, was reduced in CXCL12-transfected cells as compared to mock transfected controls.[18] These data indicate that reduced metastasis of CXCL12-overexpressing cells is most likely due to the lack of response to the ligand released from metastasis target tissues.

Kang et al., however, obtained discordant results when analysing CXCL12 expression in human breast cancers.[19] They found an inverse correlation between the levels of CXCL12 expression and overall and disease-free survival of breast cancer patients and a positive correlation with recurrence and lymph node metastasis. The differences in the CXCL12 expression analysis in breast tumours performed by Kang et al. could be due to the PCR primers: sequence alignment of the primers published reveals that the sense primer is highly unspecific and that the antisense primer is not in the transcribed sequence of CXCL12.

We are therefore convinced that the data presented here, together with the study of Wendt et al.,[18] establish a protec-

Table 2 – Univariate and multivariate disease-free survival Cox analyses.

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p* Value	HR	95% CI	*p* Value
Age (yrs)	1.01	0.99–1.03	0.32			
Tumour size (mm)[a]						
<20	1	Ref.				
21+	2.01	1.24–3.39	0.005			
Nodal status[a]						
0	1	Ref.		1		
1–3	2.56	1.48–4.46	<0.001	2.28	1.31–3.99	0.005
4+	3.58	1.71–7.50		2.62	1.23–5.55	
Oestrogen rceptor	0.95	0.88–1.02	0.18			
Progesterone receptor[a]	0.93	0.86–1.00	0.06			
Chemotherapy[a]	1.62	0.98–2.69	0.06			
CXCR4 (peri)	0.98	0.89–1.08	0.67			
CXCR4 (centre)	0.99	0.89–1.11	0.86			
CXCR4 (CIS)	1.08	0.79–1.48	0.64			
CXCR4 (Stroma)	0.96	0.80–1.14	0.63			
CXCL12 (peri)[a]	0.84	0.77–0.94	0.002			
CXCL12 (centre)[a]	0.77	0.68–0.89	<0.001	0.78	0.68–0.89	0.002
CXCL12 (CIS)	0.78	0.59–1.02	0.07			
CXCL12 (Stroma)[a]	0.85	0.75–0.96	0.01			

CXCL12 (CIS) was not included in the multivariate model because of a high number of missing values.
a Included in multivariate analysis.

Table 3 – Univariate and multivariate overall survival Cox analyses.

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p* Value	HR	95% CI	*p* Value
Age (years)	1.02	0.99–1.05	0.14			
Tumour size (mm)[a]						
<20	1	Ref.		1		0.02
21+	4.9	2.22–10.6	<0.001	2.62	1.15–5.98	
Nodal status[a]						
0	1	Ref.				
1–3	3.93	1.47–7.85	<0.001			
4+	6.72	2.49–18.16				
Oestrogen receptor[a]	0.86	0.76–0.97	0.016			
Progesterone receptor[a]	0.83	0.74–0.94	0.003	0.83	0.72–0.94	0.004
Chemotherapy	1.72	0.82–3.59	0.15			
CXCR4 (peri)	1.03	0.90–1.18	0.65			
CXCR4 (centre)	0.99	0.85–1.16	0.92			
CXCR4 (CIS)	0.86	0.51–1.45	0.57			
CXCR4 (stroma)	1.06	0.84–1.36	0.60			
CXCL12 (peri)[a]	0.75	0.63–0.89	0.001			
CXCL12 (centre)[a]	0.55	0.41–0.74	<0.001	0.52	0.38–0.72	<0.001
CXCL12 (CIS)[a]	0.47	0.21–1.04	0.07			
CXCL12 (stroma)[a]	0.70	0.56–0.87	0.02	0.72	0.58–0.91	0.005

a Included in multivariate analysis.

tive role for CXCL12 expression in primary breast carcinomas. Its use as an additional prognostic marker, eventually useful for the selection of small, lymph node-negative, oestrogen receptor-positive cancers of grade two for chemotherapy, would depend on the prospective validation of these results. Similarly, plasma levels have been shown to predict distant metastasis[29] since they are positively correlated with disease-free survival. In this study, CXCL12 plasma levels were measured before and after the removal of the primary tumour, and no major differences were found. The contribution of tumour-derived CXCL12 to plasma levels is apparently negligible. The prognostic power of plasma and intratumoural levels is likely to be independent.

We suggest that the chemotactic pro-metastatic action of CXCL12 released from the metastasis target tissues is limited to cancer cells that do not express CXCL12. Autocrine CXCL12

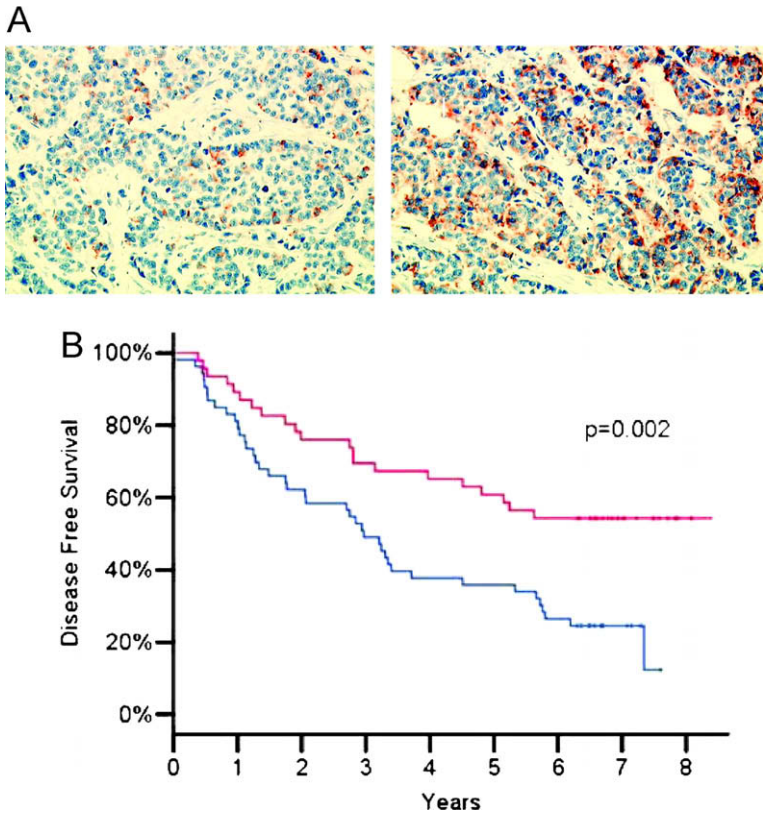

Fig. 4 – (A) Representative samples with anti-CXCL12 staining below (left) and above (right) the median. (B) Kaplan–Meier disease-free survival curves for CXCL12 immunohistochemistry in 100 breast cancer samples. Patients were divided into two groups based on the level of CXCL12 expression (red line = expression levels above the median value, blue line = expression levels below the median). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expression might saturate the receptor thereby making the cell insensitive to the chemotactic stimulus. It is likely that CXCL12 exerts a pro-invasive stimulus on breast carcinoma cells yet overexpressing tumours do not follow an external chemokine gradient. The expression of CXCL12 by the tumour surrounding stroma could determine paracrine effects that would also interfere with the response to the chemotactic signal released from target tissues.

Finally, targeted therapies directed against CXCR4[30] should consider the CXCL12 expression status of the tumours to be treated, since autocrine overexpression might make the tumour less sensitive to CXCR4 antagonists that compete with CXCL12 for binding to the receptor.

Conflict of interest statement

None declared.

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