CXC chemokine mRNA expression as a potential diagnostic tool in prostate cancer

STEFFEN A. WEDEL, IVAYLO N. RADITCHEV, JON JONES, EVA JUENGEL, TOBIAS ENGL, DIETGER JONAS and ROMAN A. BLAHETA

Klinik für Urologie und Kinderurologie, Zentrum der Chirurgie, Johann Wolfgang Goethe-Universität, D-60590 Frankfurt am Main, Germany

Received November 12, 2007; Accepted December 18, 2007

Abstract. *In vitro* models have suggested that chemokines of the CXC family may regulate prostate cancer cell proliferation and dissemination. In this study, we evaluated the expression of CXC receptors (CXCRs) and CXC ligands (CXCLs) in prostate cancer tissue. CXCL1-16 and CXCR1-6 mRNA were identified by RT-PCR in prostate tumors and adjacent normal tissue specimens. Samples were obtained from 49 patients undergoing radical prostatectomy. mRNA expression was semi-quantitatively scored and correlated with pretreatment prostate-specific antigen (PSA), the Gleason score, early patient follow-up and Kattan postoperative prediction. CXCL12 mRNA expression level was significantly enhanced, whereas CXCL13 was reduced in prostate tumor compared to adjacent 'normal' tissue. No differences were observed in the CXCR4 mRNA level; however, both CXCR3 and CXCR5 were reduced significantly in the tumor tissue. The difference in CXCL12 and CXCL13 ($CXCL_{\Delta}$) correlated significantly with PSA levels and the Gleason score. Furthermore, $CXCL_{\Delta}$ correlated significantly with the Kattan postoperative nomogram. Tumor progression was observed in patients with high $CXCL_{\Delta}$ values, but not in those with low values, in early follow-up. The development and progression of prostate cancer was accompanied by alterations of CXC chemokine expression, in particular CXCL12, CXCL13, CXCR3 and CXCR5. Novel treatment options could therefore be targeted at one or several of these proteins. The practicability of CXC chemokines as potential prognostic markers requires further study.

Correspondence to: Dr Roman Blaheta, J.W. Goethe-Universitätsklinik, Klinik für Urologie und Kinderurologie, Interdisziplinäres Forschungs- und Laborgebäude, Chirurgische Forschung, Haus 25, Zi 204, Theodor-Stern-Kai 7, D-60590 Frankfurt am Main, Germany
E-mail: blaheta@em.uni-frankfurt.de

Key words: chemokines, Kattan, prognosis, prostate cancer, prostate-specific antigen

Introduction

Chemokines are a family of low molecular weight (8-10 kDa) cytokines that bind to G-protein coupled receptors. More than 40 different chemokines have been isolated and classified into four major families based on the relative position of cysteine residues near the NH2-terminus: CC, CXC, C and CX3C. The CXC chemokines have one amino acid residue separating the first two conserved cysteine residues. Their primary function is chemoattraction and the activation of specific leukocytes in diverse immunoinflammatory responses. However, increasing evidence suggests that they also play key roles in neoplastic transformation. In prostate cancer in particular, the chemokine receptor CXCR4 and its ligand CXCL12 are thought to be involved in metastatic progression and tumor malignancy. Taichman *et al* demonstrated that the invasion of prostate cancer cell lines through basement membranes is supported by CXCL12 and inhibited by CXCR4 blockades (1). Xenograft models have shown that prostate tumors that overexpress CXCR4 are 2- to 3-fold larger in volume and weight compared to controls, and that the invasiveness or metastatic activity of these tumors is significantly increased (2). Meanwhile, there is no doubt that, during prostate cancer metastasis, CXCL12/ CXCR4 participates in localizing tumors to the bone marrow (3).

Still, the role of CXC chemokines in prostate carcinoma might be more complex than initially thought, and the control of cancer progression does not seem to be restricted to the CXCR4-CXCL12 axis. We recently demonstrated *in vitro* that no particular CXC chemokine, but rather alterations of the complete CXC expression profile, may define the malignant properties of prostate cancer (4). In line with our observation, Lu and coworkers speculated that CXCL1, CXCL5, CXCL8 and CXCL16 chemokines released from prostate cancer may form a network that mediates the increased bone destruction associated with prostate tumor growth (5). There are no available patient data dealing with this issue. To illuminate the role of CXC chemokines in prostate cancer, we compared the CXC mRNA expression profile of tumor and adjacent tumor-free tissue taken from patients undergoing radical prostatectomy. To examine whether chemokine expression is secondary to or directly associated with tumor malignancy, we correlated CXC mRNA expression with prostate-specific antigen (PSA) levels, the Gleason score and the Kattan post-operative nomogram, which is used to calculate progression-

Table I. Primer sequences used for RT-PCR.

mRNA	Sense primer sequence	Antisense primer sequence	Size (bp)
CXCL1	ctcttccgctcctctcacag	tcacgttcacactttggatg	232
CXCL2	ctcaagaatgggcagaaagc	tcaaacacattaggcgcaag	214
CXCL3	gcagggaattcacctcaaga	accctgcaggaagtgtcaat	230
CXCL4	gcgctgaagctgaagaagat	gtccggccttgatcacct	105
CXCL5	acccagggaagacaagaagg	cagtgattcctggctcacac	239
CXCL6	gtcctgtctctgctgtgctg	aacttgcttcccgttcttca	158
CXCL7	tcctccaccaaaggacaaac	tttcctcccatccttcagtg	207
CXCL8	caggaattgaatgggtttgc	aaaccaaggcacagtggaac	180
CXCL9	ccaccgagatccttatcgaa	ctaaccgacttggctgcttc	163
CXCL10	aaggatggaccacacagagg	agcagggtcagaacatccac	248
CXCL11	tgcctaaatcccaaatcgaa	gtcctttcacccacctttca	240
CXCL12	ctagtcaagtgcgtccacga	ggacacaccacagcacaaac	221
CXCL13	ctctgcttctcatgctgctg	tgagggtccacacacacaat	220
CXCL14	ggagcaggtctctctgcatc	gttgggaacctcacatgctt	232
CXCL16	ggagtctcgctctgtcatcc	atcaccaggtcaggagttcg	214
CXCR1	tttgtttgtcttggctgctg	agtgtacgcagggtgaatcc	224
CXCR2	acatgggcaacaatacagca	tgaggacgacagcaaagatg	180
CXCR3	acaccttcctgctccaccta	gttcaggtagcggtcaaagc	191
CXCR4	ggtggtctatgttggcgtct	tggagtgtgacagcttggag	227
CXCR5	ggtcttcatcttgccctttg	atgcgtttctgcttggttct	340
CXCR6	ctggtggtgtttgtctgtgg	aggtgaggatgagcatggac	250
GAPDH	atcttccaggagcgagatcc	accactgacacgttggcagt	509

free probabilities. Patient outcome was also analyzed in an early follow-up study.

Materials and methods

Patient selection and specimen processing. Forty-nine patients diagnosed with prostate cancer between 2003 and 2004 who underwent radical prostatectomy as a primary therapy (i.e., no preceding hormonal or radiation therapy) were included in the study. Mean patient age was 65.3±6.1 years. The following tumor-related characteristics were analyzed: pretreatment PSA, the Gleason score, capsule penetration of the tumor, seminal vesicle invasion, surgical margins and lymph node status.

Prostate tumor and adjacent tumor-free specimens were collected following approval by the local ethics committee. All specimens underwent initial H&E staining to localize the cancer cells, and were then reviewed by a pathologist. Prostate sections with tumor cells were used as tumor samples. The same criteria were applied to identify tumor-free 'normal' prostate tissue samples from the same patient. Clinical information and pathological reports accompanied all the samples, without any patient-identifying information. All samples were fresh frozen in liquid nitrogen.

Frozen tissues were homogenized with an Omni GLH Homogenizator (Sued-Laborbedarf GmbH, Gauting, Germany), followed by further homogenization using a QIAshredder Mini Spin Column (Qiagen, Hilden, Germany). Total RNA was prepared using the RNeasy Mini Kit (Qiagen).

CXC mRNA expression. mRNA expression of CXCR and CXCL was evaluated by reverse transcriptase-polymerase chain reaction (RT-PCR). Complementary DNA was synthesized from 1 μg of total RNA per sample, with a 60-min incubation at 42˚C, using the Moloney murine leukaemia virus reverse transcriptase (Invitrogen, Karlsruhe, Germany) and oligo(dT) priming (Boehringer Mannheim). Amplification was carried out using gene-specific primers and Platinum-Taq polymerase (Invitrogen) in a Mastercycler Gradient Thermocycler (Eppendorf, Hamburg, Germany). Reactions were performed in the presence of 0.5 μl cDNA with an initial incubation step at 94˚C for 5 min. Cycling conditions consisted of denaturation at 94˚C for 60 sec, annealing at 60˚C for 60 sec and extension at 72˚C for 60 sec over a total of 25 cycles. The reaction was completed by another 10-min incubation step at 72˚C. The specific sequences for sense and antisense primers are listed in Table I. The PCR products were subjected to electrophoresis in 1.5% agarose gel and visualized by ethidium bromide. Band intensities were quantified using the Gel Doc 1000 Photo Documentation System (Bio-Rad, München, Germany) and its associated software and expressed in Band Intensity Units (BIU).

Statistical analysis. To interpret the predictive value of CXC alterations, mRNA expression was correlated to pretreatment PSA, the Gleason score, the postoperative probability of tumor progression (Kattan postoperative nomogram) as well as early

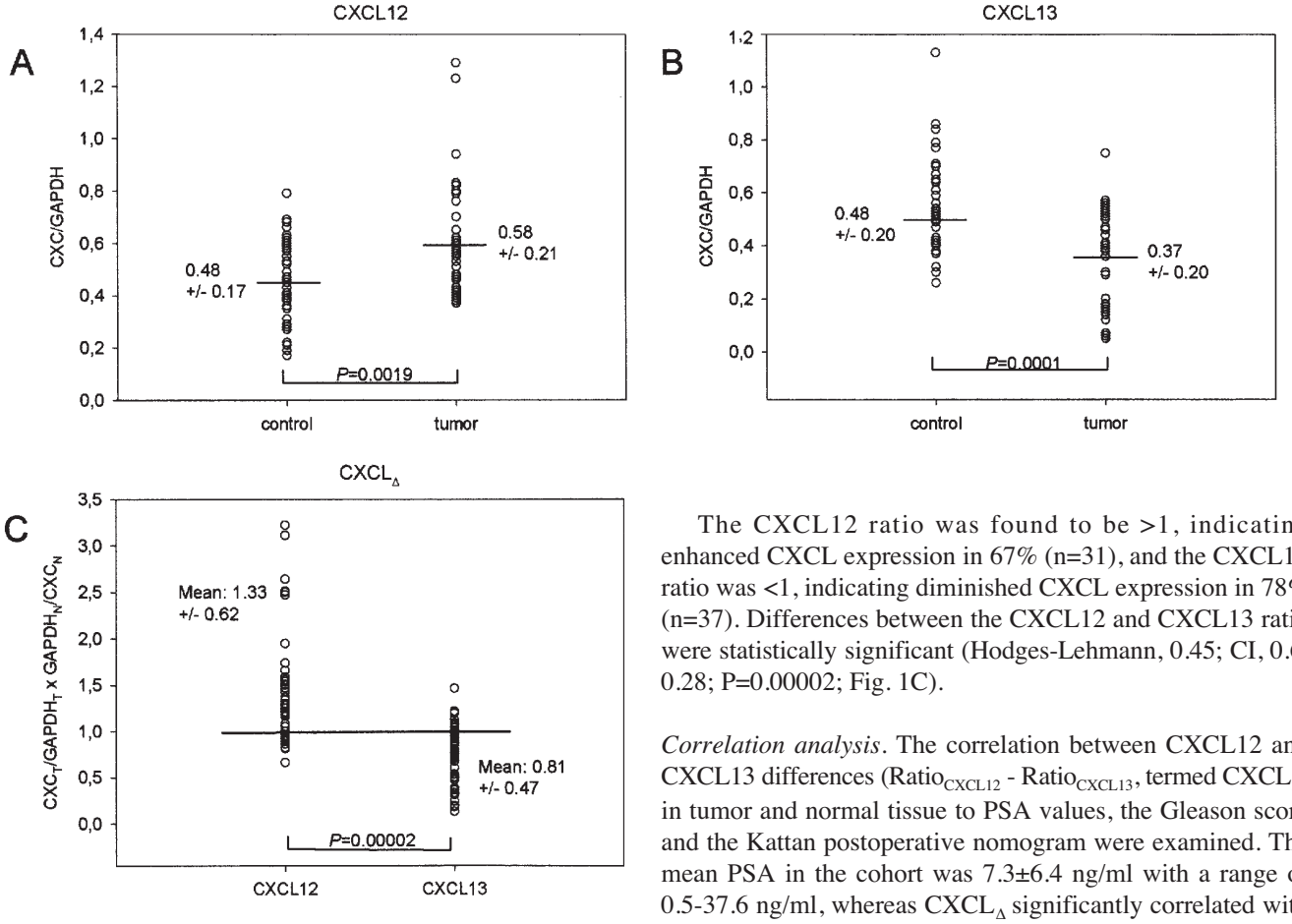

Figure 1. CXCL12 (A) and CXCL13 (B) mRNA expression in normal (control) and tumor tissue. P-values and mean ± SD values are provided in each diagram. (C) CXCL12 - CXCL13 difference values (CXCL$_\Delta$).

patient follow-up. Statistical comparisons of subsets were carried out using the non-parametric Wilcoxon matched pairs test (two groups) or the Kruskal-Wallis test followed by the Dunn's test (more than two groups). Multiple correlation analysis was performed to test correlations among more than two variables, and the Spearman rank correlation was calculated to test correlations between two variables. Hodges-Lehmann estimates and Moses 95% confidence intervals (CIs) were chosen to compare the differences between two parameters.

Results

CXCL expression. Of all the chemokines measured, CXCL12 and CXCR13 mRNA expression levels differed significantly in tumor and normal prostate specimens. Remarkably, CXCL12 was enhanced from 0.48±0.17 to 0.58±0.21 BIU (Hodges-Lehmann, 0.08; CI, 0.0, 0.16; P=0.0019), whereas CXCL13 was reduced from 0.48±0.20 to 0.37±0.20 BIU (Hodges-Lehmann, 0.10; CI, 0.2, 0.01; P=0.0001) in cancer tissues compared to the normal counterparts (Fig. 1A and B). The BIU_{CXCL12} and BIU_{CXCL13} ratios were then calculated for each tissue pair using the formula:

$$CXCLratio = CXCL_T/GAPDH_T \times GAPDH_N/CXCL_N$$

where T is tumor and N is control.

The CXCL12 ratio was found to be >1, indicating enhanced CXCL expression in 67% (n=31), and the CXCL13 ratio was <1, indicating diminished CXCL expression in 78% (n=37). Differences between the CXCL12 and CXCL13 ratio were statistically significant (Hodges-Lehmann, 0.45; CI, 0.6, 0.28; P=0.00002; Fig. 1C).

Correlation analysis. The correlation between CXCL12 and CXCL13 differences ($Ratio_{CXCL12}$ - $Ratio_{CXCL13}$, termed $CXCL_\Delta$) in tumor and normal tissue to PSA values, the Gleason score and the Kattan postoperative nomogram were examined. The mean PSA in the cohort was 7.3±6.4 ng/ml with a range of 0.5-37.6 ng/ml, whereas $CXCL_\Delta$ significantly correlated with the pretreatment PSA (ϱ=0.730; P=0.00002; Fig. 2A). Gleason scores were categorized as ≤5 (n=7), 6 (n=15), 7 (n=13), 8 (n=7) and 9 (n=8). Although there was no significant correlation between $CXCL_\Delta$ and Gleason scores 6/7 compared to 8, significant differences were calculated between CXL_Δ/Gleason score 9 and CXL_Δ/Gleason score 7 (P=0.008), CXL_Δ/Gleason score 6 (P=0.0008) and CXL_Δ/Gleason score ≤5 (P=0.0002) (Fig. 2B). Progression-free probability was divided into four categories: <10%, 10-90%, 91-99% or >99% (Fig. 2C). The non-parametric Kruskal-Wallis test revealed a significant correlation between $CXCL_\Delta$ and the Kattan categories (ϱ=-0.769). Best performance was achieved between $CXCL_\Delta$ and <10% compared to >99% survival probability (P=0.000009).

Follow-up analysis. The outcome of the ten patients with the lowest and the ten patients with the highest $CXCL_\Delta$ values ($CXCL_\Delta$-high vs. $CXCL_\Delta$-low) was compared. The mean follow-up in both groups was 42.1±6.8 months (minimum 20 months; maximum 50 months). The mean follow-up in the $CXCL_\Delta$-high group was 41.8±5.2 months (minimum 35 months; maximum 50 months) and, in the $CXCL_\Delta$-low group, 42.4±8.3 months (minimum 20 months; maximum 48 months). All patients in the $CXCL_\Delta$-low group were free of tumor progression. In the $CXCL_\Delta$-high group, one patient died due to tumor relapse and two patients developed disease progression. Radiation therapy was carried out on one patient, and anti-androgen treatment on two.

CXCR expression. The corresponding ligands of CXCL12 and CXCL13, CXCR4 or CXCR5 in tumor and normal tissue

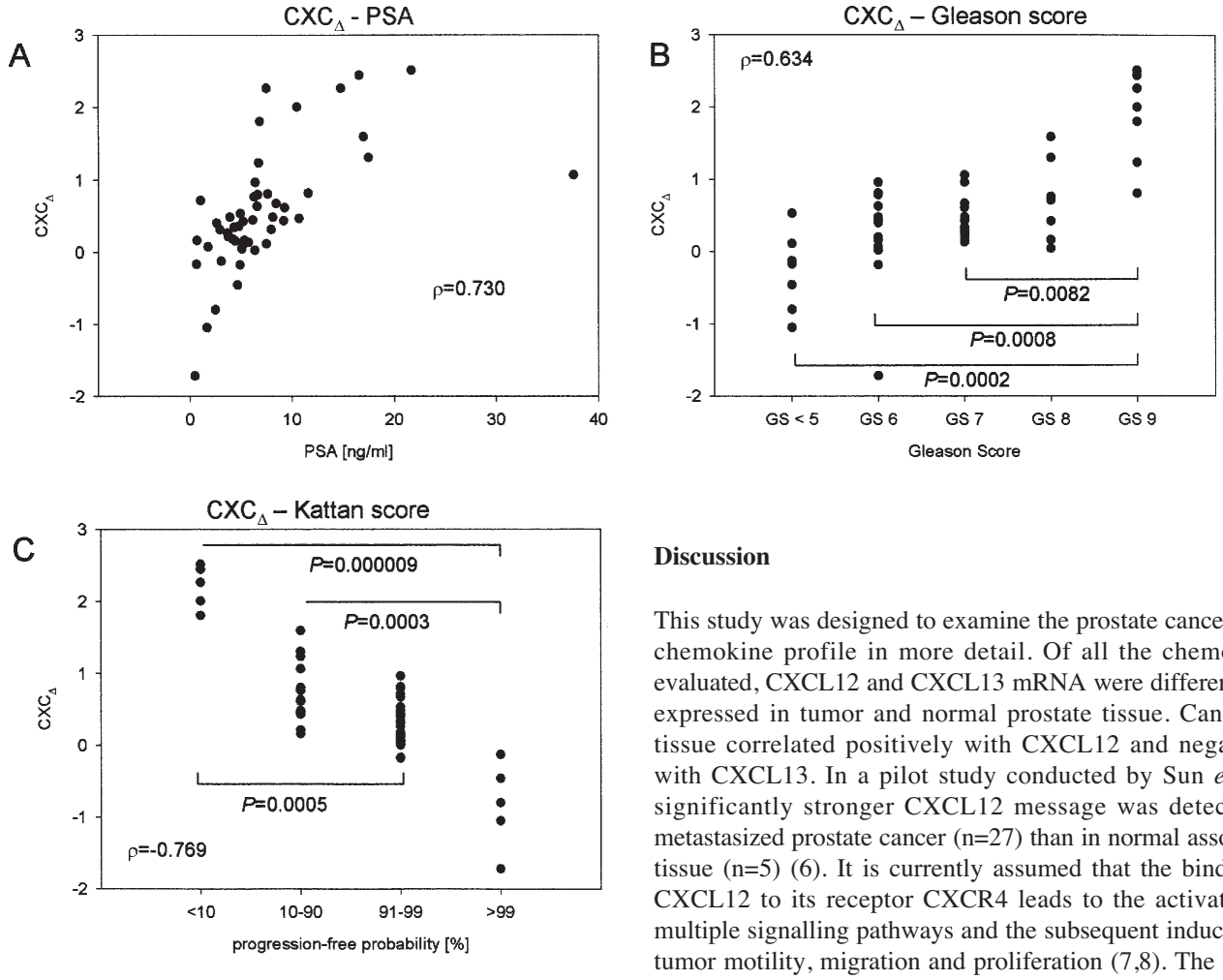

Figure 2. Correlation analysis of $CXCL_\Delta$ - PSA (A), $CXCL_\Delta$ - Gleason score (B) and $CXCL_\Delta$ - Kattan score (C). P-values are included in each diagram.

were also investigated. No differences were found in CXCR4 mRNA level. However, CXCR5 was diminished from 0.42±0.21 to 0.17±0.09 BIU (Hodges-Lehmann, 0.22; CI, 0.34, 0.11; P=0.0007; Fig. 3A). CXCR3 was also significantly reduced (P=0.017; Fig. 3B). Expression levels of CXCR1, CXCR2 and CXCR6 were similar in tumor and normal prostate tissue.

Discussion

This study was designed to examine the prostate cancer CXC chemokine profile in more detail. Of all the chemokines evaluated, CXCL12 and CXCL13 mRNA were differentially-expressed in tumor and normal prostate tissue. Cancerous tissue correlated positively with CXCL12 and negatively with CXCL13. In a pilot study conducted by Sun *et al*, a significantly stronger CXCL12 message was detected in metastasized prostate cancer (n=27) than in normal associated tissue (n=5) (6). It is currently assumed that the binding of CXCL12 to its receptor CXCR4 leads to the activation of multiple signalling pathways and the subsequent induction of tumor motility, migration and proliferation (7,8). The role of CXCL13 is not clear in this context. Very recently, CXCL13 has been linked to prostate-associated lymphoid tissue (PALT), whose structural and functional features may enable the gland to mount a local immune response against infections and tumors (9). We were unable to localize the cell type(s) responsible for reduced CXCL13 production. Speculatively, CXCL13 down-regulation may be caused by destruction, quantitative or morphological changes in the PALT compartment in the course of malignant lesions.

Since both CXCL12 and CXCL13 are modified during tumor development and/or progression, these parameters in

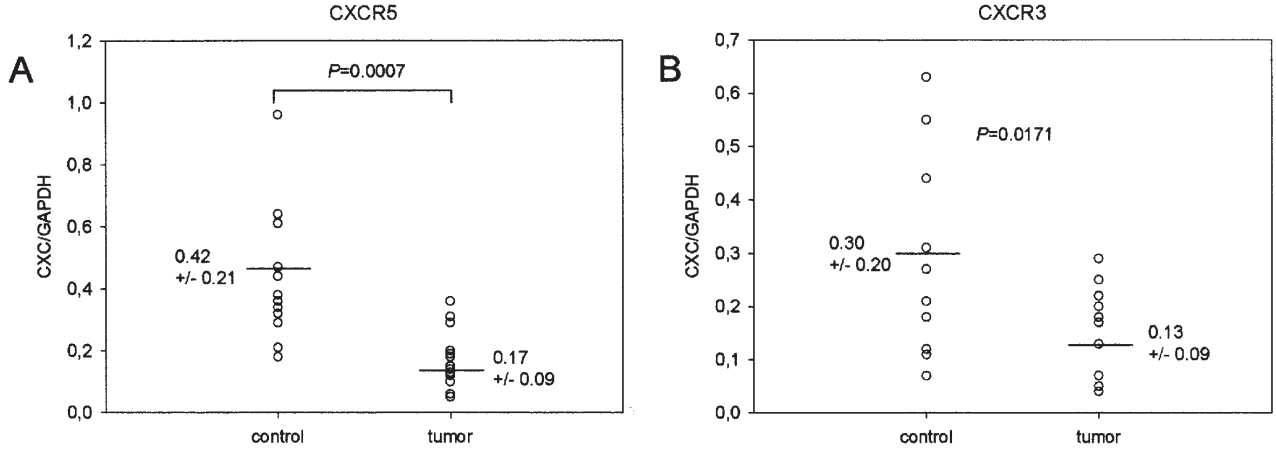

Figure 3. CXCR5 (A) and CXCR3 (B) mRNA expression in normal (control) and tumor tissue. P-values and mean ± SD values are provided in each diagram.

concert (rather than singly) may be a critical biomarker providing additional diagnostic information. Indeed, $CXCL_{\Delta}$ values, which include both chemokines, were clearly associated with PSA values and the Gleason score. Notably, differences between high and low PSA levels and between high and low Gleason scores were underlined by distinct changes in $CXCL_{\Delta}$ values. This is important since PSA and Gleason score alone may not sufficiently distinguish low-risk from high-risk patients. Detailed chemokine analysis may provide valuable information regarding not only tumor characteristics, but also the optimization of therapeutic protocol (10,11).

$CXCL_{\Delta}$ correlated well with the Kattan postoperative prediction, a nomogram for prostate cancer recurrence after radical prostatectomy that shows good statistical concordance with actual survival rates (concordance index, 0.79-0.81) (12,13). Besides the predictive involvement of the Kattan model, we also concentrated on early follow-up. In this context, follow-up analysis revealed a higher risk of cancer progression in patients with high $CXCL_{\Delta}$ values than in those with low values. This finding should be interpreted with great caution due to the relatively small number of participants and the short follow-up period of <5 years. Furthermore, all data are from the same institution, and we have no outside validation datasets. However, while keeping these limitations in mind, the results presented here do point to significant alterations in the chemokine expression profile of cancerous tissue. This may allow patient assignment of a favorable or unfavorable prognosis. Still, confirmatory studies are necessary to assess whether $CXCL_{\Delta}$ fulfills all the criteria to become a prognostic and monitoring tool.

In close analogy to CXCL13, CXCR5 mRNA - which encodes for the corresponding receptor - was significantly reduced in cancerous tissue. This finding favors our hypothesis that PALT destruction during tumorigenesis ultimately leading to a diminished CXCL13-CXCR5 mRNA level. Surprisingly, mRNA encoding for the CXCL12 ligand CXCR4 was not altered in tumor specimens. This may contradict earlier reports presenting evidence closely associating CXCR4 with a more aggressive phenotype of prostate cancer cells (2). However, clinical sample analyses carried out by Sun *et al* and Arya *et al* also revealed a similar mRNA message in localized, compared to metastasized, tumors or benign tissue (6,14). It was concluded by the authors that post-transcriptional CXCR4 regulation is responsible for tumor cell differentiation. Based on *in vitro* experiments, it has been suggested that the concentration of CXCL12, rather than the amount of CXCR4 at the surface level, drives the tumor cell to become highly motile and invasive (8).

Concerning additional CXCRs, we also found diminished CXCR3 mRNA expression in tumor tissue. No further patient data are available so far. However, tumors in TRAMP/$CXCR3^{-/-}$ mice were palpable much earlier than those in TRAMP/$CXCR3^{+/+}$ mice, and had greatly increased angiogenesis (15). *In vitro*, CXCR3 expression correlated inversely with tumor growth and adhesion (4), and up-regulation of CXCR3 inhibited proliferation and decreased PSA production (16). Presumably, CXCR3 down-regulation contributes directly to accelerated prostate tumor growth. Ongoing studies are necessary for confirmation.

In conclusion, distinct modifications of the CXC mRNA expression profile were found in prostate tumor and 'normal' tissue. This is particularly true of CXCL12, CXCL13, CXCR3 and CXCR5. Consequently, evaluating these parameters may provide important diagnostic information. $CXCL_{\Delta}$, which combines both the CXCL12 and CXCL13 alterations, may possibly become a prognostic tool. Further studies would be of value in investigating its applicability. Since chemokines are modified in prostate cancer, novel treatment options targeted at one or several of these proteins may be developed.

Acknowledgements

We would like to thank Karen Nelson for critically reading the manuscript. The help of Dr Ackermann from the Department of Informatics is deeply appreciated. This work was supported by the 'Horst Müggenburg-Stiftung', 'Jung-Stiftung', 'Ebert-Stiftung' and 'Held-Hecker-Stiftung'.

References

1. Taichman RS, Cooper C, Keller ET, Pienta KJ, Taichman NS and McCauley LK: Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. Cancer Res 62: 1832-1837, 2002.
2. Darash-Yahana M, Pikarsky E, Abramovitch R, Zeira E, Pal B, Karplus R, Beider K, Avniel S, Kasem S, Galun E and Peled A: Role of high expression levels of CXCR4 in tumor growth, vascularization, and metastasis. FASEB J 18: 1240-1242, 2004.
3. Sun YX, Schneider A, Jung Y, Wang J, Dai J, Wang J, Cook K, Osman NI, Koh-Paige AJ, Shim H, Pienta KJ, Keller ET, McCauley LK and Taichman RS: Skeletal localization and neutralization of the SDF-1 (CXCL12)/CXCR4 axis blocks prostate cancer metastasis and growth in osseous sites *in vivo*. J Bone Miner Res 20: 318-329, 2005.
4. Engl T, Relja B, Blumenberg C, Muller I, Ringel EM, Beecken WD, Jonas D and Blaheta RA: Prostate tumor CXC-chemokine profile correlates with cell adhesion to endothelium and extracellular matrix. Life Sci 78: 1784-1793, 2006.
5. Lu Y, Cai Z, Xiao G, Keller ET, Mizokami A, Yao Z, Roodman GD and Zhang J: Monocyte chemotactic protein-1 mediates prostate cancer-induced bone resorption. Cancer Res 67: 3646-3653, 2007.
6. Sun YX, Wang J, Shelburne CE, Lopatin DE, Chinnaiyan AM, Rubin MA, Pienta KJ and Taichman RS: Expression of CXCR4 and CXCL12 (SDF-1) in human prostate cancers (PCa) *in vivo*. J Cell Biochem 89: 462-473, 2003.
7. Chinni SR, Sivalogan S, Dong Z, Filho JC, Deng X, Bonfil RD and Cher ML: CXCL12/CXCR4 signaling activates Akt-1 and MMP-9 expression in prostate cancer cells: the role of bone microenvironment-associated CXCL12. Prostate 66: 32-48, 2006.
8. Engl T, Relja B, Marian D, Blumenberg C, Muller I, Beecken WD, Jones J, Ringel EM, Bereiter-Hahn J, Jonas D and Blaheta RA: CXCR4 chemokine receptor mediates prostate tumor cell adhesion through alpha5 and beta3 integrins. Neoplasia 8: 290-301, 2006.
9. Carlo ED, Magnasco S, D'Antuono T, Tenaglia R and Sorrentino C: The prostate-associated lymphoid tissue (PALT) is linked to the expression of homing chemokines CXCL13 and CCL21. Prostate 67: 1070-1080, 2007.
10. Cuzick J, Fisher G, Kattan MW, Berney D, Oliver T, Foster CS, Moller H, Reuter V, Fearn P, Eastham J and Scardino P; Transatlantic Prostate Group: Long-term outcome among men with conservatively treated localised prostate cancer. Br J Cancer 95: 1186-1194, 2006.
11. Fall K, Garmo H, Andren O, Bill-Axelson A, Adolfsson J, Adami HO, Johansson JE and Holmberg L; Scandinavian Prostate Cancer Group Study No. 4: Prostate-specific antigen levels as a predictor of lethal prostate cancer. J Natl Cancer Inst 99: 526-532, 2007.

12. Chun FK, Karakiewicz PI, Briganti A, Walz J, Kattan MW, Huland H and Graefen M: A critical appraisal of logistic regression-based nomograms, artificial neural networks, classification and regression-tree models, look-up tables and risk-group stratification models for prostate cancer. BJU Int 99: 794-800, 2007.
13. Graefen M, Karakiewicz PI, Cagiannos I, Hammerer PG, Haese A, Palisaar J, Fernandez S, Noldus J, Erbersdobler A, Huland H, Scardino PT and Kattan MW: A validation of two preoperative nomograms predicting recurrence following radical prostatectomy in a cohort of European men. Urol Oncol 7: 141-146, 2002.
14. Arya M, Patel HR, McGurk C, Tatoud R, Klocker H, Masters J and Williamson M: The importance of the CXCL12-CXCR4 chemokine ligand-receptor interaction in prostate cancer metastasis. J Exp Ther Oncol 4: 291-303, 2004.
15. Shen H, Schuster R, Lu B, Waltz SE and Lentsch AB: Critical and opposing roles of the chemokine receptors CXCR2 and CXCR3 in prostate tumor growth. Prostate 66: 1721-1728, 2006.
16. Nagpal ML, Davis J and Lin T: Overexpression of CXCL10 in human prostate LNCaP cells activates its receptor (CXCR3) expression and inhibits cell proliferation. Biochim Biophys Acta 1762: 811-818, 2006.