

CCR2 Expression Correlates With Prostate Cancer Progression

Yi Lu,^{1,2} Zhong Cai,^{1,3} Guozhi Xiao,¹ Yulin Liu,⁴ Evan T. Keller,⁵ Zhi Yao,² and Jian Zhang^{1*}

¹Department of Medicine, University of Pittsburgh, Pittsburgh, PA 15240

²Department of Immunology, Tianjin Medical University, Tianjin, China

³Department of Clinical Chemistry, Tianjin Chest Hospital, Tianjin, China

⁴Department of Pathology, Allegheny General Hospital, Pittsburgh, PA 15212

⁵Department of Urology, University of Michigan, Ann Arbor, MI 48109

Abstract Although the primary role of chemokines and their receptors is controlling the trafficking of leukocytes during inflammatory responses, they also play pleiotropic roles in cancer development. There is emerging evidence that cancer cells produce chemokines that induce tumor cell proliferation or chemotaxis in various cancer types. We have previously reported that MCP-1 acts as a paracrine and autocrine factor for prostate cancer (PCa) growth and invasion. As the cellular effects of MCP-1 are mediated by CC chemokine receptor 2 (CCR2), we hypothesized that CCR2 may contribute PCa progression. Accordingly, we first determined CCR2 mRNA and protein expression in various cancer cell lines, including PCa and other cancer types. All cells expressed CCR2 mRNA and protein, but in PCa, more aggressive cancer cells such as C4-2B, DU145, and PC3 expressed a higher amount of CCR2 compared with the less aggressive cancer cells such as LNCaP or non-neoplastic PrEC and RWPE-1 cells. Further, we found a positive correlation between CCR2 expression and PCa progression by analyzing an ONCOMINE gene array database. We confirmed that CCR2 mRNA was highly expressed in PCa metastatic tissues compared with the localized PCa or benign prostate tissues by real-time RT-PCR. Finally, CCR2 protein expression was examined by immunohistochemical staining on tissue microarray specimens from 96 PCa patients and 31 benign tissue controls. We found that CCR2 expression correlated with Gleason score and clinical pathologic stages, whereas lower levels of CCR2 were expressed in normal prostate tissues. These results suggest that CCR2 may contribute to PCa development. *J. Cell. Biochem.* 101: 676–685, 2007. © 2007 Wiley-Liss, Inc.

Key words: CCR2; monocyte chemoattractant protein-1; prostate cancer; metastasis

Bone is the most frequent site for distant metastasis from prostate cancer (PCa). It was reported that skeletal metastases occurred in up to 90% of patients dying from PCa [Abrams et al., 1950; Rana et al., 1993; Landis et al., 1999; Bubendorf et al., 2000]. Skeletal metastases cause significant complications including

severe bone pain, impaired mobility, pathological fracture, spinal cord compression, and hypercalcemia [Coleman, 1997; Moul and Lipo, 1999]. Despite advances in management of PCa, skeletal metastases remain incurable. Strategies to inhibit PCa metastasis include targeting the dynamic and bidirectional interactions between tumor cells and the bone microenvironments. The bone microenvironment is composed of the extracellular matrix, stromal cells, osteoblasts, osteoclasts, and endothelial cells that may produce unique soluble factors, including growth factors and chemotactic factors, to promote PCa progression in bone.

Chemokines are classified into four major families based upon the relative position of cysteine residues near the NH₂-terminus: CC, CXC, C, and CX₃C [Rossi and Zlotnik, 2000]. Chemokines activate receptors, members of large families of seven-transmembrane G-coupled proteins, and play critical roles in cell

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*Correspondence to: Jian Zhang, Department of Medicine, Room 2E110, Pittsburgh VA Healthcare System, Research & Development (151C-U), University Drive, University of Pittsburgh, Pittsburgh, PA 15240.
E-mail: zhangj2@upmc.edu

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migration during inflammatory responses. In addition, they significantly impact normal development, atherosclerosis, and angiogenesis. Increasing knowledge of the effects of chemokines on different aspects of tumor cell biology, including modulation of proliferation, angiogenesis, and immune response to tumor, has begun to emerge [Strieter, 2001].

Monocyte chemotactic protein-1 (MCP-1) is a member of the CC chemokine superfamily that plays a critical role in recruitment and activation of monocytes during acute inflammation and angiogenesis [Galasso et al., 2000; Bernardini G et al., 2003; Charo and Taubman, 2004]. A variety of cancer cells that home to bone, including PCa, breast cancer and myeloma cells, express MCP-1 and its receptor CCR2 [Valkovic et al., 1998; Vande Broek et al., 2003; Lu et al., 2006; Mestdagt et al., 2006]. In patients with breast cancer and ovarian cancer, MCP-1 levels are increased in their serum, which correlating with stage of tumors development [Hefler et al., 1999; Lebrecht et al., 2001]. In squamous cell carcinoma of the esophagus, MCP-1 expression in cancer cells is correlated with venous invasion, distant metastasis, and lymph node metastasis [Koide et al., 2004]. Further, our data indicate that MCP-1 is chemotactic for PCa cells [Lu et al., 2006]. In addition, MCP-1 appears to induce proliferation of PCa cells [Lu et al., 2006], osteoclast fusion and osteoclast formation in vitro [Kim et al., 2006]. MCP-1 is also produced by osteoclasts and is upregulated by RANKL and TNF- α in osteoclasts [Graves et al., 1999; Arendt et al., 2002; Park et al., 2005]. Thus, MCP-1, through its receptor CCR2, may play a critical role in tumor development in bone microenvironment.

Chemokine receptors are expressed by a variety of tumor cell types [Dwinell et al., 1999; Scotton et al., 2001; Soejima and Rollins, 2001]. Specifically, CCR2 is expressed in various cancer types including PCa and breast cancers. Certain types of breast cancer and PCa cell lines also respond to MCP-1 in vitro [Youngs et al., 1997; Lu et al., 2006] indicating presence of functional CCR2 receptors. However, CCR2 expression in different cancer cells and the correlation between CCR2 expression and PCa progression have not been defined. In this study, we first determined CCR2 mRNA expression in various cancer cell lines including PCa, lung cancer, breast cancer, and myeloma. Further, CCR2 mRNA expression in specimens

from PCa patients with bone metastases were examined by analyzing an ONCOMINE gene array database and validated by real-time RT-PCR and compared to localized PCa or benign tissues. Finally, CCR2 protein expression was determined by immunohistochemical staining on tissue microarray specimens from 96 PCa patients and 31 benign controls. The correlation between CCR2 expression with Gleason score and clinical pathologic stages was analyzed.

MATERIALS AND METHODS

Antibodies and Reagents

Goat anti-human CCR2 polyclonal antibody and isotype control antibody (normal goat IgG) were purchased from Calbiochem (San Diego, CA). The CCR2 antibody is human specific and does not cross react with other chemokine receptors according to the manufacture protocol. The anti-human PSA polyclonal antibody was purchased from Ventana Medical Systems (Tucson, AZ). All chemical reagents were purchased from Sigma (St. Louis, MO).

Cell Culture

PCa LNCaP, PC3 and DU145 cells, RWPE-1 (non-tumorigenic human prostate epithelial cells), hFOB (SV 40 large T antigen transfected and immortalized human osteoblasts), and HBME (human bone marrow endothelial cells) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). C4-2B cells derived from its parental LNCaP but with characteristics of skeletal metastasis were obtained from UroCor (Oklahoma City, OK). A549, H1299 (human non-small-cell lung carcinoma cell lines), and BEAS2B (non-neoplastic bronchi epithelial cells) were kindly provided by Dr. Xue Wang, at the University of Pittsburgh, Pittsburgh, PA. MDA-MB-231 and MCF-7 (breast cancer cells) were kindly provided by Dr. Shiyuan Cheng, at the University of Pittsburgh. KAS (interleukin-6-responsive myeloma KAS-6/1 cells) were kindly provided by Dr. G. David Roodman, at the University of Pittsburgh. Prostate epithelial cells (PrEC) were purchased from Cambrex (Walkersville, MD). LNCaP, PC3, DU145, H1299, and KAS cells were cultured in RPMI 1640 media (Invitrogen, Carlsbad, CA). The KAS cells were grown in RPMI media supplemented with recombinant human IL-6 (10 ng/ml). Human FOB cells were grown in media with 50%

DMEM and 50% Ham's F12 media (Invitrogen) at 34°C. At this temperature, the cells exhibit rapid cell division due to the establishment of this cell line by transfection of a temperature sensitive expression vector pUCSVtsA58. C4-2B cells were maintained in T media [80% DMEM, 20% Ham's F12 medium (Invitrogen), 5 µg/ml insulin, 13.6 pg/ml triiodothyronine, 5 µg/ml transferrin, 0.25 µg/ml biotin, and 25 µg/ml adenine]. HBME, A549, BEAS2B, and MDA-MB231 cells were cultured in DMEM media. MCF-7 cells were cultured in DMEM media supplemented with 5 mg/ml insulin. All cell cultures were supplemented with 1% penicillin/streptomycin (Invitrogen) and 10% FBS (HyClone, Pittsburgh, PA). PrEC were maintained in PrEGM BulletKit media (Cambrex). The RWPE-1 cells were grown in Keratinocyte-Serum Free medium supplemented with 5 ng/ml human recombinant EGF and 0.05 mg/ml bovine pituitary extract (Invitrogen). All cells, except hFOB, were maintained in 10 cm tissue culture dishes in a 37°C incubator equilibrated with 5% CO₂ in humidified air.

Reverse Transcription-PCR

Total RNA was extracted from LNCaP, C4-2B, DU145, PC3, PrEC, RWPE-1, BEAS2B, A549, H1299, MDA-MB-231, MCF-7, KAS, HBME, and hFOB cells; from benign prostate tissue (three cases), localized PCa (three cases), PCa with bone-metastases (seven cases), PCa with liver-metastases (two cases), and PCa with lymph node-metastases (two cases) using TRIzol reagent (Life Technologies, Gaithersburg, MD), then subjected to RT-PCR for detection of CCR2 mRNA. Cases of clinically localized PCa were identified from a radical prostatectomy series at the University of Michigan and cases with PCa metastases were obtained from a Rapid Autopsy Program through the Michigan Prostate Specialized Programs of Research Excellence (SPORE) Tissue Core. PCR primers for CCR2 consisted of sense 5'-CTG TCC ACA TCT CGT TCT CGG TTT A-3' and antisense 5'-CCC AAA GAC CCA CTC ATT TGC AGC-3' resulting in a PCR product of 324 bp [Hanna et al., 2003]. PCR primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, as control) consisted of sense 5'-CCA TGG AGA AGG CTG GGG-3' and antisense 5'-CAA AGT TGT CAT GGA TGA CC-3' resulting in a PCR product of 194 bp [Park et al., 2002]. RT-PCR was performed with 1 µg of total RNA using the

Access RT-PCR system (Promega Corp., Madison, WI) in a thermal cycler (GeneAmp PCR System 2700, Applied Biosystems, CA) under the following conditions: first strand cDNA was synthesized at 48°C for 45 min; then denatured at 94°C for 2 min for the first cycle and at 30 s for additional 35 cycles; annealing was performed at 55°C for 30 s and extension at 72°C for 60 s. Final extension was at 72°C for 7 min. The PCR products were subjected to electrophoreses on a 1.5% agarose gel and stained with ethidium bromide.

Real-Time RT-PCR

Total RNA was extracted as the same as in RT-PCR. Real-time RT-PCR was performed in an iCycler iQ multicolor real-time PCR detection system (Bio-Rad, Hercules, CA) using iScript one-step RT-PCR kit with SYBR Green (Bio-Rad). Primers for CCR2 were sense 5'-GAC CAG GAA AGA ATG TGA AAG TGA-3', and antisense 5'-GCT CTG CCA ATT GAC TTT CCT T-3' [Johrer et al., 2004]. PCR primers for GAPDH were sense 5'-CCA TGG AGA AGG CTG GGG-3', and antisense 5'-CAA AGT TGT CAT GGA TGA CC-3'. Synthesis of cDNA was performed at 50°C for 10 min, then denaturation at 95°C for 5 min, followed by 45 cycles with denaturation at 95°C for 30 s, annealing at 55°C for 15 s, and elongation at 72°C for 1 min. The fluorescence intensity of the double-strand-specific SYBR Green, reflecting the amount of formed PCR product, was monitored at the end of each elongation step. Melting curve analysis was performed to confirm the purity of the PCR products. Relative expression of CCR2 was normalized by GAPDH using the $\Delta\Delta CT$ method [relative expression = $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = C_{T(CCR2)} - C_{T(GAPDH)}$] [Wang et al., 2004]. Data are representative of three separate experiments and are presented as the mean \pm SE from triplicates.

Western Blot Analysis

Cell lysates from LNCaP, C4-2B, DU145, PC3, PrEC, RWPE-1, BEAS2B, A549, H1299, MDA-MB-231, MCF-7, KAS, HBME, and hFOB cells were prepared using standard procedures. All the samples were measured for total protein content using a BCA assay (Pierce, Rockford, IL) to ensure equal loading. Loading buffer was added to 40 µg protein and samples were boiled prior to being resolved on 12% SDS-PAGE gels and then transferred onto PVDF membranes

(Bio-Rad). The blots were blocked using blocking reagents overnight at 4°C with shaking and then incubated for 2 h with primary antibody for CCR2 (diluted 1:1,000 in blocking solution). The blots were washed and incubated for 1 h with donkey anti-goat IgG-HRP (1:5,000). After washing, bands were detected using enhanced chemiluminescence (ECL) (Amersham Biosciences, Piscataway, NJ) and exposed to light-sensitive film. As control for equal loading of the proteins, immunoblots for GAPDH (Santa Cruz, CA) were performed on the stripped membranes. CCR2 was normalized to GAPDH, and the fold change was calculated using the mean value of fold changes from three replicates. The data are representative of three separate experiments and are presented as the mean \pm SE from triplicates.

Microarray Analysis From Meta-Analysis of ONCOMINE Database

The expression of CCR2 transcript in PCa tissues was obtained from meta-analysis of cancer gene microarray meta-analysis public database [Rhodes et al., 2004]. Statistical analysis of differences was performed using ONCOMINE algorithms to account for the multiple comparisons among different studies, similar to a meta-analysis, as previously described [Rhodes et al., 2004].

Immunohistochemical Staining and Scoring

Tissue microarray of PCa specimens (83 Asian patients who underwent radical prostatectomy with Gleason score 5 and 6: n=5; Gleason score 7: n=39; Gleason score 8: n=12; Gleason score 9 and 10: n=27; Pathological stage pT2: n=61; pT3 and pT4: n=22) were purchased from ISU Abxis (Seoul, Korea) with corresponding non-neoplastic tissues (42 specimens); Gift specimens (normal and benign tissues 31 cases and carcinoma 13 cases from American men with Gleason score 5 and 6: n=6; Gleason score 7: n=2; Gleason score 8: n=3; Gleason score 9 and 10: n=2; Pathological stage pT2: n=10; pT3: n=3) were kindly provided by Cybrdi, Inc. (Frederick, MD). All slides were stained following a modified protocol [Vakar-Lopez et al., 2004]. Briefly, slides were heated at 55°C for 30 min, deparaffinized and rehydrated, then antigen retrieval was performed with Target Retrieval kit from Dako (Carpinteria, CA). Slides were incubated for 24 h at 4°C with anti-human CCR2 polyclonal antibody (1:400

dilution), or isotype control goat IgG (1:400 dilution, used as a negative control) or anti-human PSA polyclonal antibody (1:400 dilution, used as a positive control). Biotinylated anti-goat or anti-rabbit antibody (Dako) at a dilution of 1:500 was used as the secondary antibody. After incubation in avidin–biotin complex solution (Dako), the staining was developed by the diaminobenzidine method, followed by counterstaining with hematoxylin. Quantitative analysis of the CCR2 expression was determined by scoring protein expression as negative (score = 1), weak (score = 2), moderate (score = 3), or strong (score = 4), which was previously used [Sun et al., 2005]. Differences in CCR2 expression were evaluated statistically using the mean scores from each case. Scoring was performed (by both authors Dr. Jian Zhang at the University of Pittsburgh and Dr. Yulin Liu at the Allegheny General Hospital) without knowledge of overall Gleason score (e.g., tumor grade), tumor size, or clinical outcome [Perrone et al., 2000].

Statistical Analysis

Statistical analysis was performed using Statview software (Abacus Concepts, Berkeley, CA). ANOVA was used for initial analyses, followed by Fisher's protected least significant difference for post hoc analyses. Student's *t*-test was used for analyzing the gene expression data sets from a published cDNA microarray database ONCOMINE [Rhodes et al., 2004]. Differences with a $P < 0.05$ were determined to be statistically significant.

RESULTS

CCR2 Expression in Various Cancer Cells

To determine whether CCR2 mRNA is differentially expressed by different cancer cell lines, we first tested PCa cell lines LNCaP, C4-2B, PC3, and DU145; lung cancer A549 and H1299; breast cancer MCF-7 and MDA-MB-231; myeloma KAS cells by RT-PCR (Fig. 1A) and then quantified by real-time RT-PCR (Fig. 1B). HBME and hFOB cells were used as a comparison for the cells from bone microenvironment; PrEC and RWPE-1 as a control for PCa, and BEAS2B as a control for lung cancer. All cells expressed CCR2 mRNA, however, CCR2 mRNA expressions in PCa LNCaP, C4-2B, PC3, and DU145 cells were significantly greater when compared to PrEC and RWPE-1 cells

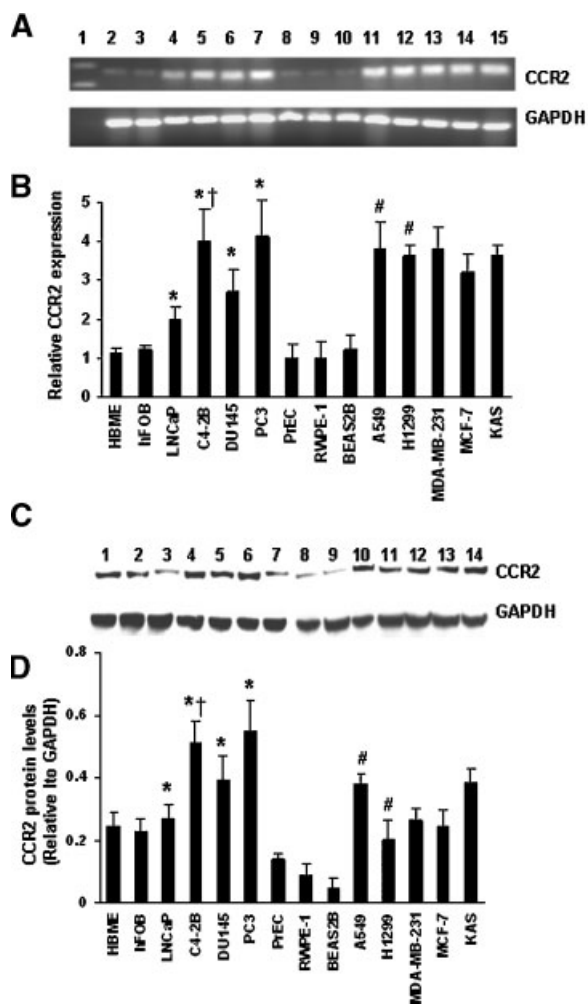


Fig. 1. CCR2 mRNA is highly expressed by aggressive cancer cells. CCR2 mRNA and protein expression in different cell lines were determined by RT-PCR, real-time PCR, and Western blot analysis. **A:** Total RNA was extracted from the indicated cells and then subjected to RT-PCR. Lane 1: DNA marker; lane 2: HBME; lane 3: hFOB; lane 4: LNCaP; lane 5: C4-2B; lane 6: DU145; lane 7: PC3; lane 8: PrEC; lane 9: RWPE-1; lane 10: BEAS2B; lane 11: A549; lane 12: H1299; lane 13: MDA-MB-231; lane 14: MCF-7; and lane 15: KAS. **B:** Real time RT-PCR was performed and relative CCR2 mRNA expression was calculated. * $P < 0.001$ compared to PrEC or RWPE-1. † $P < 0.001$ compared to LNCaP. # $P < 0.001$ compared to BEAS2B. **C:** Cell lysates were prepared and Western blot analysis was performed. Lane 1: HBME; lane 2: hFOB; lane 3: LNCaP; lane 4: C4-2B; lane 5: DU145; lane 6: PC3; lane 7: PrEC; lane 8: RWPE-1; lane 9: BEAS2B; lane 10: A549; lane 11: H1299; lane 12: MDA-MB-231; lane 13: MCF-7; and lane 14: KAS. **D:** Quantitative data of Western blot analysis. * $P < 0.001$ compared to PrEC or RWPE-1. † $P < 0.001$ compared to LNCaP. # $P < 0.001$ compared to BEAS2B.

(Fig. 1A (lanes 4–9) and Fig. 1B). Specifically, there was about a onefold higher expression observed in C4-2B as compared to LNCaP (Fig. 1A (lanes 4 and 5) and Fig. 1B). CCR2 mRNA expressions in lung cancer A549 and

H1299 cells were significantly greater compared with BEAS2B (Fig. 1A (lanes 11, 12, and 10) and Fig. 1B). Breast cancer, MCF-7 and MDA-MB-231 cells, and myeloma KAS cells expressed similar levels of CCR2 mRNA compared with PCa cells (Fig. 1A (lanes 13–15) and Fig. 1B). HBME and hFOB cells also expressed CCR2 mRNA, but at significantly lower levels (Fig. 1A (lanes 2 and 3) and Fig. 1B).

To determine whether the CCR2 protein expression is also differentially expressed by different cancer cells, CCR2 protein levels in the cell lysates were examined by Western blot analysis (Fig. 1C,D). Consistent with mRNA levels, the more aggressive cancer cells such as C4-2B, PC3, and DU145 expressed higher levels of CCR2 protein compared with the less aggressive cancer cells LNCaP or non-neoplastic PrEC or RWPE-1 cells. CCR2 protein expressions in lung cancer A549 and H1299 cells were significantly higher compared with that in BEAS2B. MCF-7, MDA-MB-231, and KAS expressed similar levels of CCR2 protein compared to PCa cells, whereas HBME and hFOB expressed low levels of CCR2.

CCR2 mRNA Expression Correlates With PCa Progressiveness

Analysis of gene expression data sets from cancer gene microarray meta-analysis public database [Rhodes et al., 2004] revealed that CCR2 mRNA expression levels increased with PCa progressiveness (Fig. 2). Yu et al. [2004] originally determined the gene expression patterns in benign prostate tissues (23 cases), localized PCa tissues (64 cases), and metastatic PCa tissues (25 cases). CCR2 mRNA expression was significantly higher in metastatic PCa compared to both benign prostate tissues ($P = 4.6 \times 10^{-6}$) and localized PCa ($P = 0.002$). Furthermore, CCR2 mRNA expression was significantly higher in the localized PCa compared with the benign prostate ($P = 0.012$).

To further confirm the higher expression of CCR2 mRNA in the specimens from metastatic PCa patients, we extracted total RNA from benign prostate tissues, localized PCa, PCa with bone-metastases, PCa with liver-metastases, and PCa with lymph node-metastases. Both RT-PCR and real-time RT-PCR analyses were performed on these RNAs (Fig. 3A,B). Specimens from PCa with metastasis, specifically PCa with bone-metastases, but not liver-metastases and lymph node-metastases,

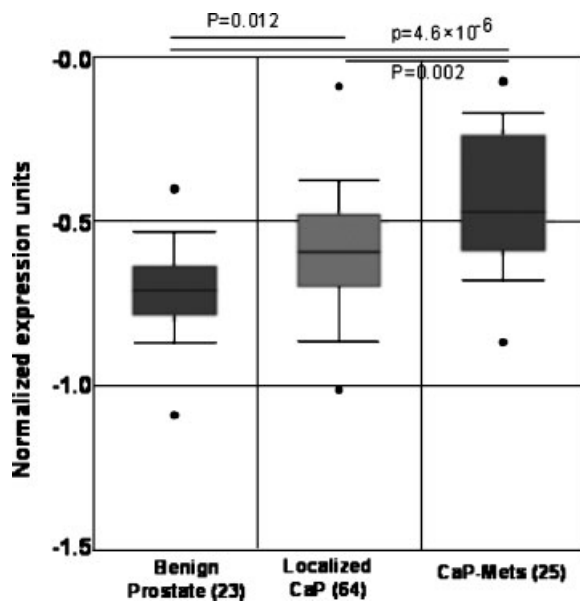


Fig. 2. CCR2 mRNA expression correlates with PCa progressiveness. CCR2 mRNA levels were analyzed by using gene expression data sets from cancer gene microarray meta-analysis public database [Rhodes et al., 2004] that was originally reported by Yu et al. [2004]. Twenty-three cases of benign prostate, 64 cases of localized PCa, and 25 cases of metastatic PCa were included in this study. Y-axis represents normalized expression units. Shaded boxes represent interquartile range making the 25th–75th percentile; whiskers represent 10th–90th percent range; bars represent median. The *P*-value was calculated by using the Student's *t*-test.

expressed significantly higher level of CCR2 mRNA compared to the localized PCa or benign prostate specimens.

CCR2 Protein Expression Correlates With Gleason Score and Pathologic Stages

To test whether the CCR2 protein is differentially expressed in PCa tissues compared to benign tissues, immunohistochemical staining was performed on tissue microarray specimens from 96 PCa patients and 31 normal controls. All specimens were graded using the Gleason score system and pathologic stage [Babaian et al., 1995; Gleason and Mellinger, 2002]. The immunohistochemical staining revealed that CCR2 was differentially expressed in PCa specimens and non-neoplastic tissues (Fig. 4A), and 81 out of 96 (84%) PCa tissue samples expressed CCR2 at heterogeneous levels. In contrast, 19 out of 73 (26%) non-neoplastic tissue samples expressed CCR2, although at lower levels. The CCR2 positive staining was located mostly in epithelial and fibromuscular

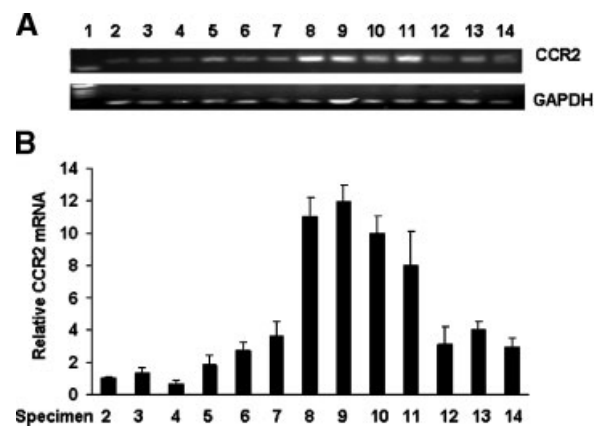


Fig. 3. CCR2 mRNA was highly expressed in metastatic PCa tissues. Total RNA was extracted from benign prostate tissue, localized PCa, PCa with bone-metastases, PCa with liver-metastases, and PCa with lymph node-metastases. RT-PCR and real-time RT-PCR were performed. **A:** PCR products for CCR2 were detected in benign prostate tissues, localized PCa tissues and PCa with metastasis tissues. GAPDH served as an internal control. **Lane 1:** DNA marker; **lanes 2–4:** benign prostate tissues; **lanes 5–7:** Localized PCa; **lanes 8–11:** PCa bone-metastases; **lanes 12–13:** PCa liver-metastases; **lane 14:** PCa lymph node-metastases. **B:** Quantitative data for relative mRNA expression (for specimen corresponding to subpart A) by real-time RT-PCR. Data are representative of three separate experiments and is presented as mean \pm SE from triplicates. GAPDH served as an internal control.

stromal cells, but was also noted in some extracellular areas surrounding neoplastic glands and epithelial cells (Fig. 4A). Quantitative analysis showed that levels of CCR2 expression in the malignant epithelia were greater than in the non-neoplastic epithelia, which correlated with Gleason score and pathologic stage (Fig. 4B). Prostate specific antigen (PSA), expressed by prostate epithelium, was immunohistochemically stained and used as a positive control.

DISCUSSION

Chemokines are produced by various types of human tumors, however, only limited data have been reported on their receptors that mediate the cancer pathobiology. In this study, we first reported that CCR2 mRNA and protein are differentially expressed by various cancer cell lines including PCa, lung cancer, breast cancer, and myeloma. All cells expressed CCR2 mRNA and proteins, however, their expression levels in PCa cell lines LNCaP, C4-2B, PC3, and DU145 were significantly higher than that of PrEC and RWPE-1. The aggressive cancer cells,

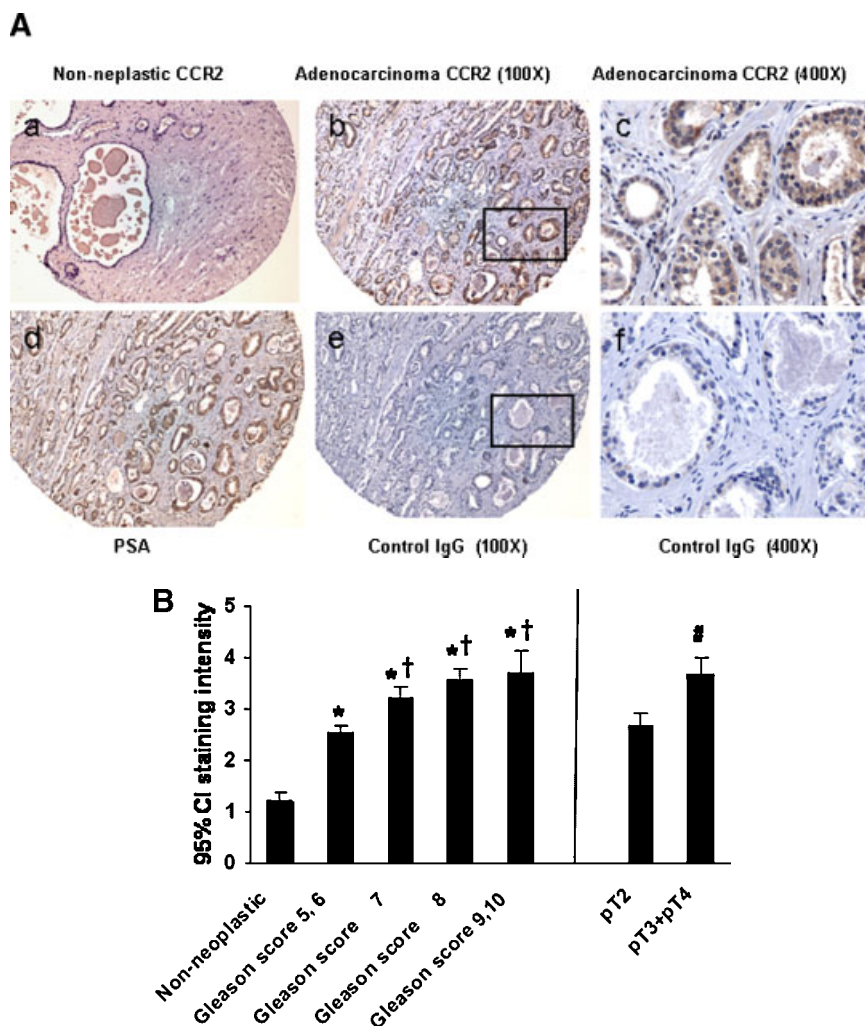


Fig. 4. CCR2 protein expression correlates with Gleason scores and clinical pathologic stages. Immunohistochemical staining was performed for detection of CCR2 in human PCa and non-neoplastic tissues. **A:** Representatives of staining. **a:** CCR2 negative staining in non-neoplastic prostate tissue. **b** and **c:** CCR2 positive staining in PCa tissues. **d:** PSA positive staining as positive control. **e** and **f:** Negative staining of CCR2 isotype control IgG antibody. Different sections of **b**, **c**, and **d** are from the same patient. Original magnification: 100 \times . **c** and **f** magnifica-

tion: 400 \times . **B:** Quantitative evaluation of CCR2 expression in PCa. Immunostaining was scored and analyzed as described in Materials and Methods section. The mean expression scores \pm SD for all PCa and non-neoplastic tissues examined cases are presented in a graphical format using error bars with 95% confidence intervals (CI). * $P < 0.001$ compared to non-neoplastic tissues. † $P < 0.01$ compared to Gleason score 5 and 6 specimens. # $P < 0.001$ compared to pathological stage T2 specimens.

both in PCa and lung cancer cell lines, expressed higher levels of CCR2 compared with the less aggressive cancer cells or benign cells.

A positive correlation between CCR2 mRNA expression and PCa progression was further demonstrated in this study through analyzing an ONCOMINE Gene Array Database. Consistent with the findings on the differential CCR2 mRNA expressions in aggressive cancer cells compared to less aggressive cancer cells, higher mRNA expression in PCa metastatic tissues was validated in bone metastatic tissues

compared to the localized PCa or benign prostate tissues by real-time RT-PCR. In addition, the findings that tissues from PCa with bone-metastases, but not liver-metastases and lymph node-metastases, expressed significantly higher level of CCR2 mRNA compared to the localized PCa or benign prostate tissues, which suggest that CCR2 may be one of the bone specific mediators that plays a critical role in the crosstalk between PCa cells and the bone microenvironment. Although we were unable to distinguish exactly what cell type(s) might

overexpress CCR2 transcripts in the metastatic tissues, the fact that CCR2 is differentially expressed in both PCa cell lines and tissues led us to examine its expression in situ on PCa specimens. Thus, we examined the CCR2 protein expressions using immunohistochemical staining in specimens from PCa patients and normal control tissues. CCR2 expressions correlated with Gleason score as well as clinical pathologic stages, while lower levels of CCR2 were expressed in normal prostate tissues. CCR2 positive stainings were located mostly in epithelial, fibromuscular stromal cells, and in the extracellular areas surrounding neoplastic glands and epithelial cells. These positive stainings suggested that CCR2 was overexpressed not only in PrECs, but also in the bone microenvironment. To cover broader specimens, we included specimens from both Asian and American men in this study. These results suggested that CCR2 contributes to PCa progression.

Chemokines regulate metastatic behavior of cancer cells through their functional receptors. It has been reported, for example, the chemokine stromal-derived factor (SDF-1; CXCL12) and its receptor CXCR4 play important role in various tumor cells, including PCa [Aiuti et al., 1999; Kim and Broxmeyer, 1999] cells, homing to bone. Tumor cells that frequently metastasize to bone (myeloma, breast cancer, PCa, and neuroblastoma) all express CXCR4 [Darash-Yahana et al., 2004; Gazitt and Akay, 2004]. However, blocking CXCR4 only partially inhibits bone metastasis [Marchesi et al., 2004]. These data indicate that other factors, produced by cancer cells or cells in the bone microenvironment, may also contribute to the pathogenesis of cancer bone metastases. Those factors may attract and stimulate cancer cell growth in bone.

Recent studies have shown that the expression of CC chemokine CCL5 (RANTES) is important in the progression of breast cancer [Manes et al., 2003]. A chemokine receptor antagonist of CCR5 (one of the CCL5 receptors) inhibited experimental breast tumor growth [Robinson et al., 2003]. Vaday et al. [2006] recently demonstrated that PCa cells expressed functional CCR5 receptors that may mediate pro-malignant activities. Together with our findings, in this study, it is shown that CCR2 is highly expressed by metastatic or aggressive PCa cells compared to less aggressive cancer

cells, and chemokines and chemokines receptors, including MCP-1/CCR2, may have profound effects on tumor progression. Although there is no published data on testing a conceivable hypothesis that CCR2 blockage may prevent tumor growth in vivo, there is evidence that MCP-1 deficient mice may be protected in models of endogenous mammary carcinoma development [Conti and Rollins, 2004], which supports and promotes researchers to pursue further studies in vivo.

When PCa cells metastasize to bone, they interact with the bone microenvironment. These interactions result in growth of PCa cells and osteolysis followed by osteoblastic response. There are bidirectional interactions between PCa cells and the bone microenvironment that increase production and expression of unique cytokines/chemokines and adhesion molecules on both marrow stromal cells and PCa cells, which drive the bone destruction process. Among the factors, we have demonstrated in this study that CCR2, the functional receptor for MCP-1, may mediate PCa development in bone. Although further studies are needed to delineate the factors regulating CCR2 expression in PCa cells and bone cells, our data from this study suggest that CCR2 may be a novel therapeutic target for PCa treatment.

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