Chemokine Receptor 7 (CCR7) Gene Expression Is Regulated by NF-κB and Activator Protein 1 (AP1) in Metastatic Squamous Cell Carcinoma of Head and Neck (SCCHN)*

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Background: The regulation and significance of aberrant CCR7 expression in metastatic SCCHN is poorly understood.

Results: High CCR7 expression correlates with decreased patient survival. CCR7 is regulated by NF-κB and AP1 signaling pathways.

Conclusion: CCR7 is a poor prognosis marker. NF-κB and AP1 signaling pathways co-regulate CCR7 expression.

Significance: Understanding the regulation of CCR7 is crucial for developing therapeutic targets for treating metastatic SCCHN.

The chemokine receptor CCR7 is a seven-transmembrane domain G-protein-coupled receptor that facilitates leukocyte migration to regional lymph nodes. Aberrant CCR7 expression in a number of human malignancies has been linked to prosurvival, -invasive, and -metastatic pathways. We demonstrate here that up-regulation of CCR7 in squamous cell carcinoma of the head and neck (SCCHN) patient tumors correlates with lower survival because of metastatic disease. Because of this important oncogenic phenotype, we investigated the mechanisms that regulate CCR7 expression in these tumors. Interestingly, the inflammatory transcription factor NF-κB has been associated with a more aggressive SCCHN phenotype. Immunohistochemical staining of a SCCHN tumor cohort (n = 47) strongly linked NF-κB staining and CCR7 expression in SCCHN. Thus, we investigated whether NF-κB contributes to metastatic disease by promoting CCR7 expression in SCCHN tumor cells. We characterized four novel, potential NF-κB binding sites in the 1000-bp promoter region upstream of the CCR7 gene, using luciferase, ChIP, and EMSA. However, NF-κB inhibition only resulted in partial reduction in CCR7 expression, prompting consideration of other co-regulators of CCR7. Indeed, cooperation between NF-κB and AP1 transcription factors, which are often co-activated, is crucial to the regulation of CCR7 mRNA expression in metastatic SCCHN cells. Thus, our findings support an important biological role for inflammatory NF-κB and AP1 in the regulation of CCR7 expression in metastatic SCCHN. As such, CCR7, NF-κB, and AP1 could be potentially useful therapeutic targets in controlling the progression and metastasis of SCCHN tumors.

Chemokines are small pro-inflammatory proteins that mediate the selective recruitment and trafficking of leukocytes to inflammatory sites. They signal through seven-transmembrane domain G-protein-coupled chemokine receptors expressed by the migrating cell (1). CCR72 has two known ligands, CCL19 (MIP-3β/ELC) and CCL21 (6Ckine/SLC), that are predominantly expressed in the lymphatic endothelium and the secondary lymphoid organs and provide migratory cues for the homing of CCR7+ cells to these tissues. Among the immune evasive mechanisms used by tumors to facilitate their growth, survival and metastasis is the ability to exploit the tumor microenvironment for inflammatory signals that enhance tumor progression. One such inflammatory gene overexpressed by malignant cells is CCR7. However, the regulation and clinical implications of CCR7 expression are not well understood.

In SCCHN, increased expression of CCR7 in metastatic tumors (2) is associated with autocrine and paracrine CCR7 signaling; contributes to enhanced invasion, survival, and protection from apoptosis; and leads to treatment resistance in SCCHN patients (3, 4). Given these findings and the characteristic nodal metastases observed in SCCHN, CCR7 appears to be a key contributor of SCCHN tumorigenesis, highlighting the need to understand its regulation and contribution to patient outcome.

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The abbreviations used are: CCR, chemokine receptor; SCCHN, squamous cell carcinoma of the head and neck; AP1, activator protein 1; IHC, immunohistochemical; TMA, tissue microarray; HPV, human papilloma virus; PFS, progression-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; TPE, 12-O-tetradecanoylphorbol-13-acetate-responsive element; HBD, human β-defensin; IKK, inhibitor κB kinase.
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Chemokines and their receptors are induced by inflammatory stimuli in the tumor microenvironment, which are often potent activators of NF-κB and activator protein 1 (AP1) transcription factors, thereby linking chronic inflammation to cancer progression (5, 6). In SCCHN, NF-κB is constitutively active and facilitates tumor angiogenesis, growth, and metastasis. On the other hand, the AP1 transcription factor complex consists of Jun and Fos protein families. Typically, inflammatory mediators, hormonal/growth factors, and physical/chemical stressors activate downstream MAPK cascades, which enhance the activation of AP1. AP1 proteins have been linked to oncogenesis and chemokine expression in lymphomas and leukemias (7, 8). In SCCHN, studies have reported dysregulated co-activation of NF-κB and AP1 proteins in tumor cell lines and premalignant and malignant lesions, suggesting that co-activation or cross-talk between NF-κB and AP1 pathways might be crucial in the carcinogenesis of SCCHN (9, 10), but the downstream impact of this co-activation is still undetermined.

NF-κB activation has been linked to induction of the CCR7 ligands (CCL19 and CCL21) through a noncanonical NF-κB pathway (11, 12). In breast cancer, NF-κB was reported to promote metastasis by regulating the expression of CXCR4 (13), and in Hodgkin’s lymphoma, CCR7 up-regulation was linked to motile metastasis by regulating the expression of CXCR4 (13). In SCCHN, the CCR7 expression in metastatic SCCHN and the clinical impact of this co-activation is still undetermined.

MATERIALS AND METHODS

Cell Lines—Human metastatic SCCHN cell lines PCI-6B, PCI-15B, and PCI-37B were derived and characterized at the University of Pittsburgh (17). The cells were cultured in DMEM (Invitrogen) containing 10% (v/v) heat-inactivated fetal bovine serum (Equitech-Bio, Ingram, TX), 100 units/ml penicillin G (Invitrogen) containing 10% (v/v) heat-inactivated fetal bovine serum (Equitech-Bio, Ingram, TX), 100 units/ml streptomycin (Invitrogen), and 4 mM l-glutamine (Invitrogen). The cells were regularly tested for mycoplasma to ensure that only mycoplasma-free cell lines were studied in our assays. The cells were also cytogenetically confirmed, and the data remained consistent.

Antibodies and Reagents—Anti-human NF-κB p65 (CT) and normal mouse IgG for the ChIP assays were purchased from Upstate (Temecula, CA). The p50 and p65 antibodies (used for EMSA supershift) and c-Fos antibody (for ChIP assays) were obtained from Santa Cruz (Santa Cruz, CA). Human TNFα was purchased from R & D Systems (Minneapolis, MN). The inhibitors: BAY 11-7082, SB203580, and SP600125 were purchased from Calbiochem, whereas PD98059 was purchased from Invivogen (San Diego, CA). Protease inhibitors were obtained from Roche Applied Science, whereas phosphatase inhibitors were from Sigma-Aldrich.

Human Tissue Samples and Immunohistochemistry—Tumor levels of NF-κB and CCR7 were evaluated by immunohistochemical (IHC) staining of primary tumor and adjacent mucosal specimens arrayed in a previously described tissue microarray (TMA) that was obtained using an institutional review board-approved study and informed consent (18). For the studies presented here, there were 47 evaluable tumor specimens, of which 13 tumors on the TMA had adjacent mucosal tissues available for analysis. A summary of patient and tumor characteristics is provided in Table 1. The human papilloma virus (HPV) status of arrayed tumors was assessed using an HPV pan-specific DNA probe (Dako, wide spectrum HPV DNA probe mixture, biotinylated), which recognizes HPV subtypes 6, 11, 16, 18, 31, 33, 35, 45, 51, and 52, and bright field in situ hybridization. TMA quality assessment and morphologic confirmation of tumor:normal histology, one H&E-stained slide were evaluated every 10 tissue sections. Arrayed tissues were IHC stained for NF-κB p65 and CCR7, and tissue levels were evaluated semi-quantitatively.

Prior to incubation with anti-CCR7 antibody (1:100) (Gene Tex Inc., Irvine, CA), antigen retrieval was performed using Dako citrate pH 6 buffer (Carpinteria, CA) in the Biocare Decloaking chamber. Endogenous peroxidases were quenched with 3% hydrogen peroxide, and slides were blocked with CAS block (Invitrogen). CCR7 staining was developed using rabbit Envision polymer (Dako) followed by incubation with Substrate Chromagen (Dako). The slides were counterstained with Harris hematoxylin. The plasma membrane and cytoplasmic staining intensity (intensity scores of 0–3), as well as percentage of tumor to the nearest 5% were determined by a head and neck cancer pathologist (L. W. and R. R. S.). An IHC score was

| Characteristic | SCCHN case (n = 47) |
|---------------|---------------------|
| Sex           |                     |
| Male          | 33                  |
| Female        | 14                  |
| Ethnicity     |                     |
| Caucasian     | 46                  |
| African American | 1                |
| Smoking status|                     |
| Never smoker  | 5                   |
| Former smoker | 20                  |
| Active smoker | 1                  |
| Unknown       | 1                   |
| Tumor site    |                     |
| Oral cavity   | 20                  |
| Oropharynx    | 8                   |
| Larynx        | 15                  |
| Other         | 2                   |
| Tumor HPV status |             |
| Negative      | 43                  |
| Positive      | 4                   |
| Nodal stage   |                     |
| N0            | 19                  |
| N+            | 24                  |
| Unstaged      | 4                   |

3 Wheeler, D. R. Siwak, R. Chai, C. LaValle, R. R. Seethala, L. Wang, K. Cieply, C. Sherer, C. Joy, G. B. Mills, A. Arigiri, J. M. Siegfried, J. R. Grandis, and A. M. Eglolf, unpublished observations.
derived from the product of the intensity and percentage of tumor stained, and IHC scores for each core of a specimen were averaged. For the NF-κB staining, prior to incubation with anti-NF-κB p65 antibody (1:200) (Zymed Laboratories Inc., San Francisco, CA), antigen retrieval was performed using Borger buffer (Biocare Medical, Concord, CA) in a Biocare Decloaking chamber, and quenching and blocking were performed as for CCR7 staining. Staining was developed by incubation with Mach 2 rabbit polymer (Biocare Medical) followed by incubation with Substrate Chromagen. NF-κB nuclear and cytoplasmic staining were evaluated separately, and the IHC scores were derived separately. IHC staining was evaluated and scored as described for CCR7.

Chromatin Immunoprecipitation Assay—The cells were serum-starved for 48 h prior to stimulation. Following treatment, the cells were fixed with 1% formaldehyde (Sigma-Aldrich) for 10 min and then quenched with 0.125 M glycine (Sigma-Aldrich) for 5 min. The cells were then washed twice with ice-cold PBS, scraped, and collected. After centrifugation, the cells were lysed in SDS lysis buffer (Upstate, Temecula, CA) containing protease inhibitors. Chromatin was sheared by sonication 5 times for 10 s each (at 25% of the maximum potency) before the addition of radiolabeled probe. All of the samples were vortexed at 4 °C for at least 80 °C. Protein concentrations were determined by the BCA protein assay (Pierce).

Electromobility Shift Assay—The DNA binding activity of NF-κB was examined using radiolabeled NF-κB oligonucleotide probes in an EMSA. Following the identification of NF-κB binding sites on the CCR7 promoter using promoter scanning techniques, we designed probe sequences that included the 10-bp flanking nucleotides on either side of each κB site as follows: CCR7 κB0, GTGGTTGCGAGGGCTTCTCAGGAG; CCR7 κB1, GGGGATTCGTAGGGACATCTCTTCACACAT; CCR7 κB2, AGGAGCCAGGGCTTTTTTTGTAATGAA; CCR7 κB3, AGGAGCCAGGGCGCTTTTTTTGTAATGAGG; κB consensus, CGACACCCCTCGGGAATTCCCCACTGGCC; CRE motif, GATTCGCTACGGTCATCAAGCGTATGC. 6–10 μg of nuclear extract was added to buffer (20 mM Hepes, 1 mM EDTA, 10% glycerol, 5 mM DTT, 1 μg of poly(dI-dC), 2 μg BSA) and incubated with 0.1 pmol of 32P-labeled oligonucleotide probe for 15 min at room temperature. The DNA binding activity to the “κB consensus” probe was examined as a positive control, whereas that to the “CREB motif” was a loading control. In the competition and antibody supershift analyses, the respective unlabeled oligonucleotides or antibodies were added to the reaction for 10 min before the addition of radiolabeled probe. All of the samples were run on 5% native gels and developed by autoradiography.

Plasmid Constructs and Transient Transfections—The CCR7 promoter regions were amplified by PCR primers designed to encompass each κB region and the 150–200 flanking nucleotides: κB0, 5'-GGGGGTACCTGAAAGAAGCCAGGATGCAGAGGTCG-3' (s) and 5'-GGGAAGCTTTTAAGTTGGCCTAAACTACCCAGAAGCCA-3' (as); κB1, 5'-GGGGGTACCTGAAAGAAGCCAGGATGCAGAGGTCG-3' (s) and 5'-GGGAAGCTTTTAAGTTGGCCTAAACTACCCAGAAGCCA-3' (as); κB2, 5'-GGGGGTACCTGAAAGAAGCCAGGATGCAGAGGTCG-3' (s) and 5'-GGGAAGCTTTTAAGTTGGCCTAAACTACCCAGAAGCCA-3' (as); and κB3, 5'-GGGGGTACCTGAAAGAAGCCAGGATGCAGAGGTCG-3' (s) and 5'-GGGAAGCTTTTAAGTTGGCCTAAACTACCCAGAAGCCA-3' (as).

The constructs were then cloned upstream of the TATA box in the pGL4.26 firefly luciferase vector (Promega, Madison, WI) using KpnI and HindIII restriction enzyme sites. Deletion mutants were obtained by using PCR primers that encompassed successively smaller portions of the κB3 region. To knock out the TPE site using site-directed mutagenesis, a primer sequence containing CAAGTAC to replace TGAGTCA was utilized, and the mutagenesis was performed using the QuikChange II site-directed mutagenesis kit (Agilent Technologies Inc., Santa Clara, CA). Transient co-transfections along with a Renilla luciferase control were performed using Polyfect reagent (Qiagen). After 24 h, the cells were stimulated with relevant cytokine for another 24 h, and the luciferase activity was measured. Where applicable, firefly luciferase activity was normalized to Renilla luciferase.

Quantitative Real Time RT-PCR—RNA was isolated from metastatic SCCHN tumor cell lines using TRizol reagent (Invitrogen) and purified using RNA CleanUp (Qiagen), followed by DNase digestion (Ambion). The concentration and purity of RNA was determined by measuring absorbance at 260 and 280 nm. RNA (2000 ng) was used for first strand cDNA synthesis using random hexamers and MuLV reverse transcriptase (Applied Biosystems, Foster City, CA) as described previously (19). Real time PCR was then performed on the Applied Biosystems 7700 Sequence Detection Instrument, using TaqMan pre-
developed assay reagent for human CCR7 (Applied Biosystems). Cycling parameters were: initial denaturation at 95 °C (12 min), 40 cycles of 95 °C (15 s) and 60 °C (1 min). All of the experiments were performed in triplicate. Relative expression of the CCR7 target gene to endogenous control gene (β-glucuronidase) was calculated using the ΔCT method: relative expression = 2−ΔΔCT, where ΔΔCT = CΔT(CCR7)−CΔT(GUS) (2).

Statistical Analysis—For the IHC analysis, the differences between paired tumor and adjacent mucosa levels of each protein were evaluated using the signed rank test. Correlations between proteins were assessed using Pearson’s correlation coefficient. Progression-free survival (PFS) was defined as time from first treatment to recurrent upper aerodigestive tumor, metastasis, or death. Overall survival (OS) was defined as the time from first treatment to death. Patients who were event-free were censored at last follow-up. Log rank tests and Cox proportional hazards models were implemented to evaluate association between tumor CCR7 levels (high versus low defined as median IHC score) and PFS or OS. The assumption of proportional hazards was tested by interrogation of scaled Schoenfeld residuals. In other experiments, the data are expressed as the means ± S.E. of at least three repeats. An unpaired t test was used to calculate whether observed differences were statistically significant. The threshold for significance was p < 0.05.

RESULTS

CCR7 Overexpression Correlates with Decreased Survival and Constitutive NF-κB Activation in Vivo—To determine the biological and clinical significance of CCR7 expression, we utilized IHC on a TMA from a well-characterized SCCHN cohort (n = 47 patient tumors) (20) to investigate the association of CCR7 expression with PFS and OS. In this TMA, 13 patient tumors had evaluable paired histologically normal, adjacent mucosa. Significantly higher CCR7 staining was found in tumor tissues as compared with surrounding squamous mucosa (p < 0.001). The log rank test indicated that higher tumor CCR7 expression levels were significantly associated with worse median PFS (p = 0.013) and OS (p = 0.026) (Fig. 1). Univariate Cox proportional hazards models confirmed that high tumor CCR7 was associated with decreased PFS (HR = 2.75; 95% CI = 1.20–6.30) and OS (HR = 2.58; 95% CI = 1.09–6.09). When adjusted for other prognostic factors including nodal stage (N0 versus N+), age, and tumor HPV status, high tumor CCR7 levels remained weakly associated with shorter PFS (HR = 2.52; 95% CI = 0.94–6.69, p < 0.06) and OS (HR = 2.24; 95% CI = 0.81–6.21, p < 0.12). These findings indicate that CCR7 is a candidate molecular marker of poor prognosis in SCCHN and a co-variate of nodal metastasis, highlighting the importance of understanding CCR7 regulatory mechanisms.
Given previous evidence that CCR7 is an NF-κB modulated gene (14, 15), and that aberrant activation of NF-κB is observed frequently in SCCHN tumors, we evaluated the relationship between NF-κB activation and CCR7 expression in our SCCHN patient cohort. Using semi-quantitative IHC analysis, we first compared the expression of NF-κB p65 between paired samples of tumor and adjacent mucosa (Fig. 1, C and D). Consistent with previous reports of constitutive NF-κB activation in SCCHN, we observed significantly higher cytoplasmic NF-κB expression in the tumors as compared with adjacent mucosa (n = 13, p < 0.001). When correlated to CCR7 expression, we observed a significant correlation between NF-κB and CCR7 immunostaining (Fig. 1E) (r = 0.508, p < 0.001). These data confirm the constitutive activation of NF-κB in SCCHN. The observed positive correlation between NF-κB and CCR7 expression indicates that NF-κB might be a key transcription factor involved in the regulation of CCR7 expression.

**FIGURE 2. The CCR7 promoter contains NF-κB binding sites.** A, schematic representation of the CCR7 promoter, highlighting the four potential NF-κB binding sites and the AP1 site; the TATA box is underlined, and the translation start site is indicated by an arrow. B, PCI-37B cells were stimulated with TNFα (20 ng/ml, 10 min), and the nuclear extracts were incubated with 32P-radiolabeled CCR7 κB probes or a control CRE motif probe and assayed by EMSA. Binding was competed away using excess unlabeled κB consensus probe. C, PCI-37B cells were stimulated with TNFα (20 ng/ml, 10 min), CCL19 (100 ng/ml, 10 min), or CCL21 (100 ng/ml, 10 min). Nuclear extracts were prepared, incubated with radiolabeled κB consensus probe in the presence of p50 or p65 supershift antibodies, and assayed by EMSA.

**CCR7 Promoter Contains κB Sites**—To determine whether NF-κB binding sites are encoded in the CCR7 promoter, we used promoter scanning techniques to analyze the 1000-bp upstream promoter region. At least four potential κB sites were identified by virtue of their homology to the κB consensus binding sequence motif, albeit with some deviation from the canonical motif (Fig. 2A). Of the four sites, two (κB1 and κB2) were previously identified (15) but not confirmed functionally, and two were novel (κB0 and κB3). Interestingly, we also identified a canonical AP1 transcription factor binding site (12-O-tetradecanoylphorbol-13-acetate-responsive element (TPE)) directly adjacent to the κB3 site (Fig. 2A). To determine whether the κB sites could bind to activated NF-κB in nuclear extracts, oligonucleotide probes were constructed encompassing each CCR7 κB site and the 10 flanking nucleotides on either side. Nuclear extracts were prepared from TNFα-stimulated cells, incubated with 32P radiolabeled κB sequence probes, and
binding was analyzed by gel shift assay (Fig. 2B). The κB0, κB1, and κB2 probes bound nuclear proteins from the TNFα-stimulated cells. This binding was comparable with that observed on a consensus κB probe. However, there was undetectable binding at the κB3 site. Nevertheless, the binding observed on the other CCR7 probes was specific as evidenced by the ability to compete away nearly all of the NF-κB binding from each probe using increasing amounts of unlabeled κB consensus probe. The inability to completely compete away κB1 binding likely reflected the high affinity of co-binding of other factors at this site. As a control for sequence specificity, protein binding to a CRE motif-containing probe was not competed away under these conditions.

To study whether the NF-κB p50-p65 heterodimer complex was present and responsible for the observed binding in these nuclear extracts, we performed EMSA supershift analysis using antibodies to p50 and p65 (Fig. 2C). We found that NF-κB p50-p65 was the predominant heterodimer bound to the consensus κB probe. Furthermore, there was no detectable increase in the activation of NF-κB following stimulation with CCL19 or CCL21, suggesting that autocrine secretion of ligands was not responsible for basal NF-κB activation. This finding is consistent with other results showing that the use of a neutralizing anti-CCR7 antibody does not decrease basal NF-κB activation in these cells (not shown). Together, the results suggest that other factors are responsible for the constitutive activation of NF-κB observed in these cells.

NF-κB and AP1 Co-regulate CCR7 Promoter Activity—To directly test the functional ability of each κB containing promoter region to induce CCR7 gene expression, we performed luciferase reporter assays. Reporter vectors consisting of each κB site (and its flanking 150–200 bp sequences) inserted upstream of a TATA box-containing luciferase gene were transfected into a metastatic CCR7 SCCHN cell line. Reporter activity was measured in the presence or absence of TNFα, a potent NF-κB inducing cytokine. Although the vectors demonstrated varying degrees of basal activation, TNFα induced approximately a 2-fold induction in the promoter activity of each vector (Fig. 3A). The observed TNFα-mediated NF-κB activation of the promoter was specific because it could be inhibited by an IκBAA super-repressor (not shown). Interestingly, contrary to the undetectable NF-κB binding in the gel shift assays, the κB3 region vector had the highest basal promoter activation, prompting us to investigate the role of the AP1 binding site contained in this region in the basal regulation of the CCR7 promoter.

To determine the minimal region of promoter activity necessary to maintain basal activation of the CCR7 promoter, we
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FIGURE 4. Activated NF-κB and c-Fos/AP1 is bound to the CCR7 promoter in vivo. A, ChIP assay of PCI-37B induced with TNFα (20 ng/ml, 30 min). Cell extracts were fixed and incubated with a p65 antibody, and the immunoprecipitated genomic regions were assayed in a PCR using primers that encompassed overlapping sections of the CCR7 promoter. PCR results from control primers for the GAPDH gene promoter are also shown. B, PCI-6B cells were induced with TNFα as shown and the timeline of NF-κB recruitment to the CCR7 promoter examined by ChIP assay. C, Western blot showing HBD3-mediated activation of c-Fos/AP1. The γ-tubulin antibody is used as a loading control. D, PCI-37B cells were induced with HBD3 as shown, and the timeline of c-Fos/AP1 recruitment to the CCR7 promoter was examined by ChIP assay. IB, immunoblot.

used deletional analysis to sequentially eliminate successive 5′-sequences from the κB3 region. The κB3 region encompassed in Fig. 3A was labeled κB3(1) with each sequentially deleted region labeled κB3(2) and then κB3(3) as depicted in Fig. 3B. The AP1 binding (TPE) site on κB3(3) was knocked out using site-directed mutagenesis to create κB3(3)AP1mut. As before, these sequences were cloned upstream of a luciferase gene, the vectors transfected into CCR7 before, these sequences were cloned upstream of a luciferase gene. Either the wild type promoter (CCR7-luc) or an AP1 mutant (CCR7-lucAP1mut) was co-transfected along with an empty or IκBaAA super-repressor containing plasmid (Fig. 3, B and C). Knocking out the TPE site gave the most significant reduction in promoter activity (30–50%, \( p < 0.01 \)), suggesting that AP1 binding to the TPE site was important for maintaining CCR7 promoter activation.

We then tested the relative importance of NF-κB and AP1 signaling within the full CCR7 promoter cloned upstream of a luciferase gene. Either the wild type promoter (CCR7-luc) or an AP1 mutant (CCR7-lucAP1mut) was co-transfected along with an empty or IκBaAA super-repressor containing plasmid (Fig. 3, B and E). In each metastatic SCCHN cell line examined, inhibition of NF-κB activity using the IκBaAA super-repressor resulted in \( \sim 20–50\% \) reduction in promoter activity (\( p < 0.02 \)). Mutating the TPE site resulted in greater than 50% reduction in promoter activity (\( p < 0.01 \)). Together, the inhibition of NF-κB combined with TPE mutation resulted in a significantly greater reduction in promoter activity (60–70%, \( p < 0.03 \)) compared with NF-κB or AP1 inhibition alone. These findings demonstrate that NF-κB and AP1 each contribute to basal CCR7 expression and that both transcription factors are major determinants of full CCR7 expression in metastatic SCCHN cells.

Activated NF-κB and AP1 Transcription Factors Are Bound to the CCR7 Promoter in Vivo—To analyze whether activated NF-κB and AP1 is directly recruited and bound to the CCR7 promoter in vivo, ChIP assays were performed using a CCR7+ metastatic SCCHN cell line that was either left untreated or induced with TNFα or the human β-defensin 3 (HBD3), which activates CCR7 expression via NF-κB and c-Fos/AP1-dependent pathways (21). A p65-specific mAb was used to immunoprecipitate genomic regions that were bound by NF-κB, whereas a c-Fos-specific mAb immunoprecipitated genomic regions bound by c-Fos/AP1. Because the κB regions are located closely together and could not be individually resolved using PCR, the genomic regions were amplified in groups using primers encompassing the κB0–κB1, κB1–κB2, and κB2–κB3 regions. Although all of the sites showed constitutive binding of NF-κB p65, the κB0–κB1 and κB2–κB3 regions demonstrated inducible recruitment of p65 after TNFα stimulation (Fig. 4A). Importantly, the housekeeping gene GAPDH, which is not regulated by NF-κB, was not immunoprecipitated by a p65 mAb, demonstrating specificity of these findings. Time course experiments show that additional NF-κB complexes were recruited to the CCR7 promoter within 30 min and remained bound to the promoter for up to 60 min after TNFα stimulation (Fig. 4B). As shown, the kinetics of recruitment to the CCR7 promoter were similar to IκBa, another well described NF-κB target gene. Furthermore, treatment with HBD3, which stimulates c-Fos/AP1 activation (Fig. 4C), resulted in increased recruitment of c-Fos to the CCR7 promoter within 60 min (Fig. 4D). As a control, the IκBa promoter, which is not under AP1 regulation, did
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FIGURE 5. NF-κB and AP1 activation pathways are important for CCR7 gene expression. A and B, PCI-15B (A) and PCI-37B (B) cells were treated with BAY 11-7082 (10 μM) (IKK-NF-κB inhibitor), SB203580 (20 μM) (MAPK/p38 inhibitor), PD98059 (100 μM) (MAPK/ERK inhibitor), or SP600125 (20 μM) (MAPK/JNK inhibitor) for 3 or 6 h. Cellular mRNA was extracted, and semi-quantitative as well as quantitative real time RT-PCR was performed to examine the changes in expression of CCR7 mRNA.

not show any observable increase in c-Fos recruitment. Interestingly, c-Fos recruitment was observed on the κB0-κB1, κB1-κB2, and κB2-κB3 regions of the CCR7 promoter, suggesting that other noncanonical AP1 sites may exist in these upstream promoter regions. Together, these data show the requirement for NF-κB and AP-1 activation for CCR7 induction and confirm the in vivo recruitment of NF-κB and c-Fos/AP1 to the CCR7 promoter, thereby providing a direct mechanism for the modulation of CCR7 expression by constitutive and inducible NF-κB and AP1 activation. The constitutive expression and recruitment of these transcription factors suggests that they may contribute to the overexpression of CCR7 on these metastatic SCCHN cell lines.

**CCR7 Expression Is Regulated by Both NF-κB and AP1-mediated Pathways**—To evaluate whether activation and recruitment of NF-κB to the CCR7 promoter was functionally related to CCR7 gene transcription, we treated CCR7 + SCCHN tumor cells with BAY 11-7082, an established inhibitor of inhibitor κB kinase (IKK)-mediated NF-κB activation (22), and examined CCR7 mRNA levels using RT-PCR. Furthermore, to determine the role of AP1 signaling, we used several MAPK pathway inhibitors and again assayed changes in CCR7 mRNA using RT-PCR analysis. The inhibition of IKK-NF-κB or MAPK (p38, ERK, and JNK) pathways resulted in a significant decrease in CCR7 mRNA expression. Real time RT-PCR was used to confirm and quantify these differences (Fig. 5). These data show that the basal CCR7 gene transcription in metastatic SCCHN cells, which was found to be a strong prognostic factor for poor outcome, is dependent upon both IKK-NF-κB and MAPK-AP1 activation.

**DISCUSSION**

We and others have shown that CCR7 is expressed by metastatic tumor cells and that higher levels of CCR7 expression correlate with a highly metastatic phenotype in SCCHN (2). CCR7 signaling activates pro-survival pathways in tumors (3), dendritic cells (23) and T cells (24) through PI3K/Akt activation, as well as pro-invasive characteristics in tumors (3). Furthermore, the expression of CCR7 on tumors is sufficient to induce lymph node metastasis in a B16 melanoma model (25), suggesting that CCR7 is involved in facilitating the observed organ-specific metastases. As such, the expression of CCR7 correlates with more aggressive and metastatic tumors, thereby highlighting the need to understand its signaling and regulation. However, these studies utilize murine models and do not specifically address regulatory control of CCR7 expression and function, nor has the clinical significance of CCR7 as a pathologic biomarker been determined as it relates to inflammatory NF-κB signals in the tumor microenvironment.

CCR7 expression has been linked with poor prognosis in esophageal and other squamous malignancies, and we now provide an important biological mechanism to support these
observations. Following IHC analysis of CCR7 expression in a cohort of SCCHN patients, tumor CCR7 expression level was found to be a reliable indicator of poor prognosis. High tumor CCR7 expression was associated with lower PFS and OS, likely because of increased tumor aggressiveness, chemotherapeutic resistance, and metastasis (2, 3). Interestingly, when adjusted for nodal stage (N0 versus N+), age and HPV status, high tumor CCR7 levels retained an association with shorter PFS (p < 0.06), although this did not reach statistical significance. It is remarkable, however, that CCR7 levels retain some prognostic value even when adjusted for nodal metastasis, and it suggests that metastasis, as well as other CCR7-mediated functions such as pro-survival and pro-invasive behavior, are indeed important contributors of CCR7 expression on disease outcome. A larger homogenously treated cohort may help address the value of CCR7 expression as a discriminator to guide the need for surgical management of cervical nodal disease. Nevertheless, for the first time, our data directly link inflammatory microenvironment signals with CCR7 oncogene expression.

To directly study the molecular mechanism(s) regulating CCR7 expression in tumors, we began with an analysis of the CCR7 promoter. Using promoter scanning techniques, we identified four κB elements whose activity was confirmed in EMSA and luciferase assays and a consensus AP1 binding TPE site whose activity was confirmed using luciferase assays. Although two of these sites (κB1 and κB2) have been previously identified (15), they have not been extensively studied. Our study here has identified and characterized other novel and important binding sites (κB0, κB3, and AP1) that appear to have significant CCR7 regulatory activity. Notably, when tested using luciferase assays, the “κB3 region” of the CCR7 promoter contained most of the gene regulatory activity. Upon further examination, we demonstrated that the AP1 binding site located in this region was crucial for the observed basal promoter activation. The lack of AP1 binding onto the CCR7 κB3 probe on the EMSA (Fig. 2B) is likely due to the absence of any flanking sequences on the 3’ end of this oligonucleotide that would enable AP1 to bind effectively. Nevertheless, the subsequent studies provide direct evidence for the regulation of CCR7 by both NF-κB and AP1 transcription factors.

In previous studies, we reported that metastatic CCR7+ tumor cells secrete CCL19 and CCL21, which are responsible for maintaining basal PI3K/Akt activation (4). This pathway can result in the activation of NF-κB, which is found basally active in many SCCHN cell lines. Other studies support the regulation of CCL19 and CCL21 by alternative NF-κB complexes (12). As such, our observed autocrine secretion of ligands by CCR7+ cells prompted us to investigate whether NF-κB was a downstream molecule mediating autocrine signaling in these cells. Our findings show that TNFα, but not the CCR7 ligands, stimulate NF-κB activation in these cells. TNFα as well as other inflammatory factors have been implicated in paracrine and autocrine activation of NF-κB and AP1-induced gene expression in SCCHN (26). Indeed, we recently demonstrated that innate immune signals, such as α-defensin-induced NF-κB-mediated signals strongly up-regulated CCR7 in primary nonmetastatic SCCHN cells (21). Previously, TNFα was shown to mediate co-activation of IKK-NF-κB- and MAPK-AP1-induced cytokine IL-8 activation (27). IL-1α has been demonstrated to be an autocrine factor contributing to co-activation of NF-κB, AP1, and cytokine IL-8 (28). Together, these studies implicate inflammatory cytokines and innate immune signals that induce NF-κB and AP1 co-activation in the expression of CCR7 and other genes promoting the malignant phenotype.

Using ChIP assays, we further confirmed the in vivo recruiting of NF-κB and AP1 to the CCR7 promoter following stimulation of cells with TNFα or HBD3. Our findings show that within 30–60 min of TNFα treatment, NF-κB was recruited to the CCR7 promoter in a similar fashion to that described for the well studied NF-κB target gene, 1kBα. Interestingly, increased promoter recruitment was observed in the κB0−1 and κB2−3 regions following NF-κB activation. Any recruitment to the κB1−2 region was below the limit of detection by this assay. Furthermore, HBD3 stimulation for 60 min resulted in significant recruitment of c-Fos/AP1 to the CCR7 promoter. The observed increase in recruitment of c-Fos/AP1 to the κB0−κB1, κB1−κB2, and κB2−κB3 regions of the CCR7 promoter suggests that several noncanonical AP1 sites may exist in these upstream promoter regions.

Finally, to ascertain the involvement of NF-κB and AP1 activation pathways in the regulation of CCR7, we performed RT-PCR analysis of CCR7 mRNA extracted from metastatic SCCHN cells treated with various inhibitors of these pathways. The inhibition of NF-κB or MAPK/AP1 activation resulted in decreased expression of CCR7 mRNA, confirming a role for these pathways in CCR7 expression. In summary, the data presented here show that CCR7 expression is dependent on NF-κB and AP1 activation pathways. Our studies suggest that both NFκB and AP1 are important and likely co-operate to increase transcription. If one factor is removed, then the required entire complex of factors needed for transcription does not form, thereby resulting in decreased gene expression. The precise signals or injury responses initiating these pathways in squamous malignancies are an important topic of investigation. The anti-inflammatory targeting of downstream molecules along these activation cascades may provide potential benefit in the control of metastatic carcinoma by directly suppressing CCR7 expression on tumor cells.

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