Rap1GAP Promotes Invasion via Induction of Matrix Metalloproteinase 9 Secretion, Which Is Associated with Poor Survival in Low N-Stage Squamous Cell Carcinoma

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Abstract

The objective of the current study was to investigate the effects of Rap1GAP on invasion and progression of head and neck squamous cell carcinoma (SCC) and the role of matrix metalloproteinase (MMP) 9 and MMP2 in this process. Rap1GAP functions by switching off Rap1, the Ras-like protein that has been associated with carcinogenesis. Previous findings suggest that Rap1GAP acts as a tumor suppressor protein in SCC by delaying the G1-S transition of the cell cycle. However, cells transfected with Rap1GAP exhibit a more invasive phenotype than corresponding vector-transfected control cells. MMP2 and MMP9 are enzymes that mediate SCC invasion via degradation of the extracellular matrix. Using SCC cells transfected with empty vector or Rap1GAP, cell invasion and MMP secretion were determined by Matrigel assays and gelatin zymography, respectively. Rap1GAP up-regulated transcription and secretion of MMP2 and MMP9, as assayed by quantitative reverse transcription-PCR and zymography. Furthermore, chemical and RNA interference blockade of MMP2/MMP9 inhibited invasion by Rap1GAP-transfected cells. Immunohistochemical staining of a human oropharyngeal SCC tissue microarray showed that Rap1GAP and MMP9 expression and staining intensity are correlated (P < 0.0001) and that, in early N-stage lesions of SCC, high MMP9 is prognostic of poor disease-specific survival (P < 0.05). Furthermore, Rap1GAP staining is correlated with MMP2 (P < 0.03). MMP2 in combination with N stage has a prognostic effect on time to indication of surgery at primary site. MMP2 intensity is also positively correlated with T stage (P < 0.015).

Introduction

More than 90% of head and neck cancers are squamous cell carcinomas (SCC). It is the fifth most common cancer globally and affects ~500,000 individuals (1, 2). The 5-year survival rate is <50%, a prognosis that is poorer than breast cancer or melanoma (3). Current treatment regimens for SCC are selected according to tumor size and the presence of metastasis. For example, early-stage SCC lesions are treated with surgery alone, whereas late-stage lesions are treated with concurrent surgery, radiation, and chemotherapy. However, even tumors at the earliest stage of disease may vary dramatically in treatment response and recurrence. Consequently, a significant number of patients with early-stage SCC who ultimately die of disease would likely have benefited from more aggressive treatment. However, aggressive surgery and radiation therapy are not appropriate for all SCCs because this treatment is physically and emotionally debilitating. Identification of protein biomarkers that are prognostic of tumor progression and their mechanism of regulation will facilitate treatment selection.

Tumor progression is characterized by tumor growth, invasion, and metastasis. Previous findings suggest that Rap1GAP inhibits tumor growth in SCC by delaying the G1-S transition of the cell cycle (4). If Rap1GAP is a tumor suppressor protein, then its expression in SCCs would be prognostic of slower growing lesions and possibly a more favorable prognosis. Rap1GAP promotes inactivation of the small GTPase, Rap1, by enhancing endogenous GTPase activity (5). Rap1 is a critical regulatory protein for cell adhesion and migration (6–10). In Drosophila and in osteosarcoma cell lines, Rap1 inhibits cell invasion by facilitating cell adhesion (7–9). In Drosophila, adherens junctions were uniformly distributed around the circumference of cells with wild-type Rap1 but were clustered on one side of cells with inactivating Rap1 mutations (9). The dominant-negative mutant cell clones randomly invaded surrounding tissues, suggesting a role in cell invasion. Consistent with these findings, osteosarcoma cells with inactive DOCK4, a protein that promotes Rap1 activation, have reduced levels of active Rap1, do not form adherens junctions, and are invasive (10). The effects of Rap1GAP on invasion of SCC cells have not been investigated.

Remodeling of the extracellular matrix is essential for tumor cell invasion and metastasis (11). This matrix degradation requires several matrix metalloproteinases (MMP), which are zinc-dependent proteolytic enzymes. Most MMPs are secreted in a latent form, which is maintained in an inactive state by a cysteine residue in the prodomain that binds to and masks the catalytic zinc ion in the catalytic domain (11). Disruption of this bond, cleavage of the prodomain, and a conformational change result in MMP activation (11–13), which may be modulated by other MMPs. There are 21 MMPs that are classified into several subgroups of metalloproteinases, including gelatinases or type IV collagenases, collagenases, stromelysins, stromelysin-like MMPs, and matrilysins (11). The
in vivo

Permeabilized cells were incubated with 15°C and washed subsequently in the same buffer without Triton X-100. (pH7.3). Cells were permeabilized for 2 min with 0.1% Triton X-100 in TBS-C for 30 min at 37°C. The cells were washed, incubated in streptavidin-HRP for 30 min at room temperature, washed again, and stained with 3,3'-diaminobenzidine (DAB).

Materials and Methods

Cell culture. Orphparyngeal SCC cell line (21) cells were cultured as described previously in DMEM (Life Technologies) containing 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL 1-glutamine (22).

Stable transfections. FLAG-tagged pcDNA 3.1-Rap1GAP plasmid was a gift from Dr. P. Stork (Oregon Health Sciences University, Portland, OR). The pcDNA 3.1 empty vector was used as a control for transfection effects on endogenous gene expression. SCC cells were transfected with pcDNA and pcDNA-FLAG-Rap1GAP plasmids and mixed clonal populations with high Rap1GAP expression were selected in the presence of G418 (250 μg/mL). The SCC cell line was previously identified as UM-SCC-11A (4, 23), but recent genotyping confirmed that it is instead a UM-SCC-1 derivative, an independent cell line from UM-SCC-11A(24). Two independently transfected mixed clonal populations, one pair (c2 and rg2) transfected by electroporation and the other pair (c1 and rg1) transfected by Lipofectamine, were used, as isolation of multiple single clones was unsuccessful after repeated attempts.

Western blot analysis. Whole-cell lysates were prepared and Western blot analysis was performed as described previously (22). Membranes were incubated in the primary antibody for 1 h at room temperature or overnight at 4°C. Primary antibody concentrations were as follows: mouse anti-FLAG monoclonal antibody M2 (Sigma), 1:10,000 to 1:20,000; mouse anti-Rap1 monoclonal antibody (BD Transduction Laboratories), 1:500 to 1:1,000; and mouse anti-γ-pancreatic secretory protein (Chemicon International), 1:10,000. Affinity-purified secondary antibodies (donkey anti-rabbit IgG, goat anti-mouse IgG, and donkey anti-goat IgG) conjugated with horseradish peroxidase (HRP; 1:10,000 to 1:20,000; Jackson Immunoresearch Laboratories) were used to detect primary antibodies. Immunoreactive proteins were visualized by SuperSignal West Pico Chemiluminescent system (Pierce) and exposed to X-ray film.

Rap1 activation assay. Rap1 activation in whole-cell lysates was assayed with glutathione S-transferase (GST)-tagged ralGDS, the protein that binds specifically to active Rap1(22, 24). The construct for ralGDS was a generous gift from Dr. Johannes J. Bos (University Medical Centre, Utrecht, the Netherlands).

Active Rap1 in intact cells was evaluated using GST-tagged ralGDS, as described, with some modifications (25). Confluent cells grown in an eight-well chamber slide (Lab-Tek) were serum starved and fixed in 4% paraformaldehyde for 20 min at room temperature. After fixation, cells were washed thrice in TBS cytoexclusion buffer [TBS-C; 60 mmol/L PIPES, 25 mmol/L HEPES, 10 mmol/L EGTA, 2 mmol/L MgCl2, 0.12 mol/L sucrose (pH 7.3)]. Cells were permeabilized for 2 min with 0.1% Triton X-100 in TBS-C and washed subsequently in the same buffer without Triton X-100. Permeabilized cells were incubated with 15 μg purified GST-ralGDS diluted in 100 μL 10% heat-inactivated calf serum in TBS-C for 30 min at 37°C in a humidified chamber. The cells were washed thrice in TBS-C buffer followed by incubation with anti-GST monoclonal antibody (B14) in 10% calf serum in TBS-C for 30 min at 37°C. After washing in TBS-C, cells were incubated with biotinylated goat anti-mouse IgG secondary antibody for 25 min at room temperature. Cells were washed, incubated in streptavidin-HRP for 20 min at room temperature, washed again, and stained with 3,3′-diaminobenzidine (DAB).

In vitro proliferation assays. Stably transfected SCC cells were seeded in a 24-well plate and allowed to proliferate for the indicated time. Cells were harvested and the total number of cells was determined in a hemocytometer. Nonviable cells, as determined by trypan blue enumeration assays, were similar in both groups (∼5–10%).

Invasion assay. In vitro cell invasion through Matrigel was determined according to the manufacturer’s instructions (BD Biosciences). DMEM-washed cells were plated on Matrigel-coated inserts at 1.5 × 104 to 2 × 104 in DMEM. For control experiments, cells were plated in DMEM on identical inserts that were not coated with Matrigel. The lower chamber contained DMEM with 5% FBS as a chemoattractant. After 18 to 24 h of incubation, nonmigrating cells were removed from the upper chamber with a cotton swab. Cells that had migrated to the lower surface of the membrane were fixed with methanol and stained with hematoxylin (26). Membranes with migrated cells were mounted on glass microscope slides and cells were counted.

To assess the effects of MMP inhibition on invasion of Matrigel, cells were plated in inserts in serum-free DMEM containing 12.5 μmol/L MMP2/ MMP9 inhibitor 1 (Calbiochem). The same concentration of inhibitor in serum-containing medium was present in the lower chamber. The culture was incubated for 24 h before counting as described.

Gelatin zymography. Cells in 35-mm-diameter culture dishes were washed with DMEM for 8 h, changing medium every 2 h. Finally, the culture was incubated overnight in 1 mL DMEM containing 0.1 mg/mL bovine serum albumin. The conditioned medium was centrifuged to remove cellular debris and used for zymography. Normalization of each sample was performed based on number of cells and the volume of conditioned medium. Substrate-embedded gel electrophoresis with the conditioned medium was performed as reported previously (27). Briefly, the conditioned medium supernatants were mixed with nonreducing sample buffer and electrophoresed in 10% polyacrylamide gels copolymerized with 1 mg/mL of gelatin as substrate. After electrophoresis, the gels were washed twice for 15 min in 2.5% Triton X-100, rinsed briefly with water, and then incubated overnight in activation buffer [50 mmol/L Tris-HCl (pH 7.5), 5 mmol/L CaCl2] at 37°C. Gelatinolytic activity was visualized after staining with 0.1% Coomassie blue R-250 in 40% methanol and 20% acetic acid and destained in the same solution without the dye. The positive control of pro-MMP9 was obtained from Dr. J. Varani (Department of Pathology, University of Michigan, Ann Arbor, MI).

For analysis of gelatinases, the conditioned medium was incubated with 1 mmol/L p-aminophenylmercuric acetate (APMA; Sigma) dissolved in DMSO or DMSO alone at 37°C for 1 h (28) before zymography.

RNA extraction and cDNA synthesis. Total RNA was isolated with the TRNzol reagent (Life Technologies/Invitrogen) according to the manufacturer’s instructions. After RNA isolation, cDNA was synthesized using Taqman Reverse Transcription Reagents (Applied Biosystems). DNsase treatment of the total RNA was performed before cDNA synthesis using RQI RNase-Free DNase (Promega).

Real-time reverse transcription-PCR quantification. The RNA level of each gene was measured by real-time reverse transcription-PCR (RT-PCR) based on Taqman chemistry and quantified using an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems). The reactions were carried out in a 96-well plate. A final reaction volume of 30 μL containing Taqman Universal PCR Master Mix (Applied Biosystems), Taqman Gene Expression Assays (Applied Biosystems) for each forward/reverse primers and probe (Rap1GAP, NM_002885; MMP9, NM_004994; MMP2, NM_004530; GAPDH, NM_002046), and cDNA template (corresponding to 30 ng total RNA) was used. Thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. For all samples, analyses of gene expressions were performed in triplicate in duplicate experiments. To normalize the amount of total RNA present in each reaction, GAPDH, the housekeeping gene, was amplified. To compare the expression levels among different samples, the relative expression level of the genes was calculated using the comparative Cj method and compared with a calibrator according to the manufacturer’s instructions.

RNA interference–mediated knockdown of MMP2 and MMP9. MMP2 and MMP9 were down-regulated by small interfering RNA (siRNA) and short
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Hairpin RNA (shRNA) targeting strategies. For siRNAs, ON-TARGETplus SMARTpool (Dharmacon) was used for MMP2 (L-005959-00-0005) and MMP9 (L-005970-00-0005) knockdown. ON-TARGETplus siCONTROL non-targeting POOL (D-008180-10-05) was used as a negative control. SCC cells were nucleofected with the individual siRNAs using the Nucleofector Device (Ammax, Inc.) and Cell Line Nucleofector Kit V (Ammaxa) according to the manufacturer’s protocol.

For shRNA-mediated knockdown, lentiviral vector packaging was performed by the calcium phosphate–mediated transfection of HEK293 cells. The cells were cotransfected with the appropriate amount of pGIPZ lentiviral shRNAmir vector for MMP2 (V2LHS_48430), MMP9 (V2LHS_249369), or scrambled shRNA (RHS4349: Open Biosystems) for control together with the packaging constructs pSPAX2 and pMD2G (Dr. Didier Trono, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland; all from University of Michigan shRNA Core Facility). The supernatants were harvested and SCC cells were transduced in the presence of polybrene (8 μg/mL; Sigma). Stable cell lines were generated by puromycin (1 μg/mL; Sigma) selection. Knockdown of the gelatinases was verified byzymography of conditioned medium using human MMP2/MMP9 (Chemicon International) as a positive control.

University of Michigan Oral Cavity/Oropharyngeal Cancer Organ Preservation Trial. This is a randomized clinical trial of stage III/IV SCC of the oral cavity and oropharynx that compared concurrent chemotherapy/radiation in tumors that shrank >50% in response to induction chemotherapy (organ preservation) with surgery/radiation in those whose tumors had <50% response to induction chemotherapy (29). After approval by Institutional Review Board approval, a tissue microarray (TMA) was constructed from pretreatment tissue specimens.

Immunohistochemistry. Immunodetection on tissue sections, including antigen retrieval, was performed as described (22). The sections were stained with affinity-purified anti-Rap1GAP (Santa Cruz Biotechnology) or anti-MMP9 or anti-MMP2 antibodies (Chemicon International).

Statistical analysis. For the in vitro studies, statistical analysis was done by a Student’s t test. A P value of ≤0.05 was considered to be statistically significant.

For analysis of TMA data, interpretation and scoring were performed by a pathologist who was blinded as to the clinical outcomes of the patients. Two variables, intensity and proportion, were quantified for Rap1GAP, MMP9, and MMP2. Each tissue core was scored for intensity of SCC cells staining as follows: 1, undetectable; 2, weak; 3, moderate; 4, strong. Each core was also evaluated for the percentage of tumor cells stained positive (staining proportion). Proportion for Rap1GAP was scored as ≤5%, 5% to 20%, 21% to 50%, and 51% to 100%. Proportion for MMP9 and MMP2 was coded as ordered categorical 10%, 20%, 30%, etc. Intensity and proportion scores were analyzed separately. Multiple TMA core measurements from the same subject were averaged. This average score in its continuous scale was used in all analyses.

The covariates of interest were age, gender, T stage, N stage, and smoking status (never/former/current smoker). T stage, N stage, and smoking status were analyzed as ordinal data. The outcomes of interest were overall survival, disease-specific survival, time to indication of surgery at primary site, and time to recurrence or second primary. To evaluate univariate associations between markers and ordinal variables of interest, the Spearman correlation coefficient was used. For marker associations with nominal variables, the Wilcoxon rank-sum test was used for two-level variables, and the Kruskal-Wallis test was used for variables with three or more levels.

The Kaplan-Meier method and log-rank test were used to test for differences in survival functions between strata defined by clinical variables. Cox proportional hazards model was used to relate time-to-event outcomes to marker levels and other covariates. The significant findings of interaction term between a marker and a clinical variable in the Cox model were presented through Kaplan-Meier survival curves; P values were not generated for such Kaplan-Meier curves to avoid inflating the type I error rate.

Rap1GAP was scored by two raters. Weighted κ statistic was used to assess the interrater variability among all tumor cores. The cores with disagreement from two raters to two or more categories were considered unreliable and were discarded. The cores with disagreement from two raters to within one category were included in the analysis and the average score of two raters was used to derive the subject-wide mean score. The interrater agreement was almost perfect for intensity scores [weighted κ, 0.83; 95% confidence interval (95% CI), 0.75-0.91] and was substantial for proportion scores (weighted κ, 0.78; 95% CI, 0.67-0.89).

All TMA statistical analyses were done using Statistical Analysis System version 9.0 (SAS). A two-tailed P value of ≤0.05 was considered to be statistically significant.

Results

Rap1GAP inhibits Rap1 activation. In previous studies, we observed that Rap1GAP had an inhibitory effect on tumor growth (4). We previously observed that tumors of SCC cells over-expressing Rap1GAP (Fig. 1A) exhibited a greater distance and more extracellular matrix (arrowsheads) between tumor islands (arrows) than control tumors (Fig. 1A). Furthermore, in this tumor, tumor islands extended between skeletal muscle (Fig. 1A, FLAG-Rap1GAP, inset, arrow). These findings are histologically compatible with a more invasive phenotype. In contrast, the tumor islands in control tumors were more cohesive with little intervening extracellular matrix. As observed in this section, the tumor islands lie in proximity to but do not invade skeletal muscle (Fig. 1A, pcDNA, inset, arrow).

Because SCC invasion promotes tumor progression, the objective of this study was to investigate whether Rap1GAP regulates SCC invasion and progression via secretion of MMPs. Rap1GAP is a regulatory protein that inactivates both Rap1 isoforms (30). SCC cells were stably transfected with control vector or FLAG-Rap1GAP, which specifically inactivates Rap1 (31, 32). Enriched mixed clonal populations, generated previously (c1 and rg1; ref. 4) and in a subsequent transfection (c2 and rg2), were validated for Rap1GAP expression by quantitative RT-PCR and immunoblot analysis. In one stably transfected population, Rap1GAP (rg1) mRNA was increased >800-fold (Fig. 1B). A 15-fold increase was observed in the other group of transfected cells (rg2). To confirm functional activity and protein expression of Rap1GAP in transfected cells, assays were performed in which Rap1 was “baited” with the Rap-binding domain of ralGDS, which binds only active, GTP-bound Rap1 (22, 24). Consistent with the functional effects of Rap1GAP in reducing active GTP-bound Rap1, rap1GTP was greater in lysates from control cells than from Rap1GAP-transfected cells (Fig. 1C, c1 and c2 versus rg1 and rg2, respectively). Total Rap1, which includes active GTP-bound and inactive GDP-bound Rap1, was equivalent in the two groups of cells. Exogenous Rap1GAP protein expression by immunoblot analysis was consistent with the quantitative RT-PCR results.

The functional activity of Rap1GAP was also verified in intact transfected cells (Fig. 1D). Exogenous Rap1GAP was verified with rabbit anti-FLAG antibody and appropriately negative in vector control cells. Consistent with the expected functional activity of Rap1GAP, reduced levels of active, GTP-bound Rap1 were detected in Rap1GAP-transfected cells compared with vector control cells. There was no staining in the absence of ralGDS or in the presence of mouse IgG instead of Rap1 antibody (data not shown).

Rap1GAP promotes invasion via MMP. To investigate the effects of Rap1GAP on invasion, a Matrigel invasion assay was used. Rap1GAP-expressing cells or vector control cells were seeded on a thin layer of Matrigel basement membrane matrix on a membrane
The cells migrating through the matrix were stained. The ratio of cells invading through Matrigel versus cells migrating through the membrane alone, expressed as percent invasion, was significantly higher in Rap1GAP-transfected cells compared with control cells (c1 and rg1; \( P < 0.03 \); Fig. 2A and B). A similar but less prominent trend was observed in the mixed clonal population expressing less Rap1GAP (c2 and rg2; \( P < 0.3 \)). The increase in invasion in Rap1GAP-transfected cells was not due to an increase in cell number because, as reported previously and shown in Fig. 2C, Rap1GAP inhibits proliferation (4).

MMPs, particularly the gelatinases MMP2 and MMP9, mediate degradation of extracellular matrix, thereby facilitating invasion.

Figure 1. Rap1GAP-mediated invasion and active Rap1 in SCC cells. A, Rap1GAP promotes SCC invasion in vivo. In a previous study, we showed that FLAG-tagged Rap1GAP inhibited tumor growth in SCC (4). Another pair of representative control-expressing (pcDNA) and Rap1GAP-expressing (FLAG-Rap1GAP) tumors from the previous study is shown here. Bar, 100 \( \mu \)m. H&E stain. Tumor islands in control tumors (pcDNA) are closely packed (inset) and lie in proximity to but do not invade skeletal muscle (arrowhead). Tumor islands in Rap1GAP-expressing tumors are further apart and exhibit abundant intervening mesenchymal tissue and extracellular matrix (inset, arrow) and skeletal muscle invasion (arrowhead). B, Rap1GAP transcript in SCC cells stably transfected with empty vector (c1 and c2) or Rap1GAP (two independently transfected mixed clonal populations, rg1 and rg2) was measured by quantitative RT-PCR and normalized to GAPDH. Quantitative RT-PCR of Rap1GAP on RNA isolated from pcDNA-transfected (c1 and c2) and Rap1GAP-transfected (rg1 and rg2) cells. mRNAs were standardized and calculated as described in Materials and Methods. Columns, mean within an experiment; bars, SD. For all samples, analyses of gene expressions were performed in triplicate in duplicate experiments. C, the active form of Rap1 in whole-cell lysates (200 \( \mu \)g protein each) of SCC cells stably transfected with empty vector (c1 and c2) or Rap1GAP (rg1 and rg2) was retrieved by the ralGDS pull-down assay, electrophoresed, transferred to nitrocellulose membrane, and blotted with Rap1 antibody. Equivalency of Rap1 expression was verified in whole-cell lysates with Rap1 antibody. Expression of exogenous Rap1GAP was verified by bloting a duplicate set of samples with FLAG antibody. D, immunohistochemical detection of Rap1GAP and active Rap1 in transfected cells. SCC cells stably transfected with pcDNA (left) or FLAG-tagged Rap1GAP (right) were fixed in paraformaldehyde and immunohistochemistry was performed for detection of exogenously expressed Rap1GAP by staining with anti-FLAG M2 monoclonal antibody (top). Middle, fixed cells were stained for active Rap1 (Rap1GTP) by incubating with GST-tagged ralGDS in cytoskeleton buffer with 10% FCS followed by staining with GST mouse monoclonal antibody (B14; Santa Cruz Biotechnology). As control (bottom), fixed cells were incubated only with cytoskeleton buffer with 10% FCS (no ralGDS) followed by GST antibody staining. Bars, 60 \( \mu \)m.
and spread of SCC cells (33). Therefore, MMP secretion was investigated in control- and Rap1GAP-transfected cells. As shown in the gelatin zymogram in Fig. 2D, SCC cells strongly expressing Rap1GAP secreted more MMP9 (∼20-fold increase) than the corresponding control cells (rg1 = 123.5 densitometric units; c1 = 5.8 densitometric units). rg2, which expresses much less exogenous Rap1GAP (Fig. 1B and C) than rg1, showed a slight but insignificant increase (∼1.5-fold) in MMP9 secretion compared with the corresponding control (rg2 = 42.7 densitometric units; c2 = 27.2 densitometric units). Rap1GAP-induced MMP2 secretion was observed in rg1 but was below the limits of detection in rg2. The positive control sample was cells overexpressing MMP9.

To determine whether MMP9 is secreted as pro-MMP9 or in its active form, conditioned medium samples were treated with APMA, which activates the proenzyme. MMP9 migration was altered in the presence of APMA, consistent with it being secreted as a proenzyme (Fig. 3A, rg1 and rg2 in right panel versus left panel). In contrast, MMP2 showed no change in molecular mass, consistent with its presence in an active form in conditioned medium. A faint MMP9 band was also detected in rg2 after APMA treatment, and as noted above, MMP2 was not detected. Hence, SCC cells strongly expressing Rap1GAP secrete MMP9, which can be activated on secretion.

To determine whether steady-state levels of MMP9 mRNA are regulated by Rap1GAP, quantitative RT-PCR was performed on control vector–transfected and Rap1GAP-transfected cells. In rg1, which strongly overexpresses exogenous Rap1GAP (Fig. 1B and C), mRNA of both MMP2 (Fig. 3B) and MMP9 (Fig. 3C) was up-regulated. In contrast, rg2 that has weak exogenously expressed Rap1GAP showed a slight reduction in MMP9 transcript and essentially undetectable MMP2. Thus, overexpression of Rap1GAP up-regulates mRNA steady-state levels of MMP2 and MMP9.

To investigate whether Rap1GAP facilitates invasion via MMP2 and MMP9, invasion assays were performed in the presence or absence of a MMP2/MMP9 inhibitor (Fig. 3D). As observed previously (Fig. 2B), the percent invasion was higher in Rap1GAP-transfected cells compared with control cells (c1 and rg1; \( P < 0.02 \); Fig. 3D) but was blocked in the presence of the inhibitor. A similar trend was observed in the mixed clonal population

![Figure 2](image-url)
expressing less Rap1GAP, but the change was less prominent (c2 and rg2; \( P < 0.3 \)).

To determine whether either or both MMP2 and MMP9 regulate Rap1GAP-mediated invasion, these gelatinases were down-regulated in SCC cells stably transfected with Rap1GAP by RNA interference (RNAi) strategies. RNAi-mediated down-regulation was verified by zymography. As shown in Fig. 4A, MMP9 and MMP2 were appropriately decreased regardless of whether the cells were targeted with shRNA or siRNA. In stably transfected cells, MMP2 knockdown was greater than that of MMP9 (Fig. 4A, shRNA, lanes 2 and 9, respectively), which is secreted at higher levels in the parent Rap1GAP-overexpressing cells (lane U). MMP2 serves as an internal loading control when MMP9 is down-regulated and vice versa (Fig. 4A, lanes 9 and 2 compared with lane S, scrambled control, or lane NT for shRNA and siRNA, respectively).\( \frac{\text{percent invasion}}{\text{percent invasion}} \)

Invasion assays were performed on triplicate in duplicate experiments. Columns, mean within an experiment except for MMP2 in c2 and rg2, which was unreliably detected and represents only one reading; bars, SE. *\( P < 0.02 \) for c1 versus rg1, \( P < 0.04 \) for c1 versus rg2 in the presence of inhibitor; \( P < 0.3 \) for c1 versus rg1, \( P < 0.3 \) for c2 versus rg2 in the presence of inhibitor.

Taken together, these studies show that Rap1GAP inhibits proliferation (4) but promotes invasion of SCC via release of MMPs.

Rap1GAP/MMP9 and Rap1GAP/MMP2 expression is correlated in human SCC. As Rap1GAP up-regulates MMP9 and MMP2 expression in vitro, we investigated whether MMP9 and MMP2 expression was correlated with Rap1GAP expression in SCC tissues. Rap1GAP was strongly or moderately expressed in the cytoplasm of some SCCs (Fig. 4C, bottom right and bottom left, respectively) and weakly or not expressed in other SCC specimens (Fig. 4C, top right and top left, respectively). IgG controls were appropriately negative (data not shown).

MMP9 was also variably expressed in biopsies from oropharyngeal SCC (data not shown). In some biopsies, it was primarily membranous, whereas in others it was cytoplasmic and membranous. The expression intensity of Rap1GAP and MMP9 strongly correlated with that of MMP9 (Spearman’s rho = 0.7; \( P < 0.0001 \); Fig. 4D).
The relationship of Rap1GAP and MMP9 expression and patient outcome was determined using a TMA from a completed clinical study. Multivariate analysis (Cox regression model) showed that the effects of MMP9 expression on disease-specific survival depend on N-stage lesions. Subjects with lower N-stage lesions coupled with lower expression of MMP9 have better disease-specific survival than subjects with the same N stage but higher expression of MMP9 (Fig. 5A). In contrast, subjects with higher N-stage lesions coupled with higher MMP9 expression have better disease-specific survival than subjects with the same N stage but lower MMP9 expression (N stage $P = 0.12$; MMP9 $P = 0.04$; interaction $P = 0.025$; Fig. 5A). Similar results were found between time to recurrence or second primary and MMP9 and N-stage lesion (Fig. 5B). Early N-stage lesions with high MMP9 had a shorter time to recurrence than early N-stage lesions with low MMP9 (N stage $P = 0.13$; MMP9 $P = 0.02$; interaction $P = 0.02$).

Similarly, the relationship of Rap1GAP and MMP2 expression and patient outcome was determined on the same TMA. MMP2 was variably expressed in SCC tissue sections and was scored as 1 to 4 representing none, low, medium, and high, respectively. The expression intensity score of Rap1GAP correlated with that of MMP2 (Spearman's rho $= 0.35$; $P < 0.038$; data not shown). The Cox regression model showed that the interaction between MMP2 and N stage was significant for time to indication of surgery at primary site (N stage $P = 0.015$; MMP2 $P = 0.085$; interaction $P = 0.034$). Early N-stage lesions with low MMP2 had a shorter time to indication of surgery, whereas late N-stage lesions with low MMP2 had a longer time to indication for surgery (Fig. 5C). Furthermore, the expression intensity of MMP2 is positively correlated with T stage (Spearman's rho $= 0.398$; $P < 0.015$; Fig. 5D). MMP2 did not have a significant interaction with N stage and disease-specific survival.

**Discussion**

In SCC, proliferation and invasion of malignant keratinocytes lead to tumor growth, spread to regional lymph nodes, and spread to distant sites, which are incompatible with patient survival (34). We have previously shown that Rap1GAP has an inhibitory effect on tumor growth due to a delay in the G1-S transition of the cell cycle (4). Rap1GAP is a regulatory protein that down-regulates GTP-bound, active Rap1. In the current study, Rap1GAP promoted invasion of SCC cells via secretion of MMP9 and MMP2. Taken together, our proliferation and invasion results suggest that growth suppression by Rap1GAP is accompanied by enhanced invasive potential via MMP9 and MMP2 secretion and, consequently, more
Figure 5. MMP9 and MMP2 and clinical outcome in SCC. A, MMP9 intensity, N stage, and disease-specific survival. High MMP9 is prognostic of poor clinical outcome in lower N-stage lesions. Patient groups: red line, N0 N1, low MMP9 (n = 9); yellow line, N0 N1, high MMP9 (n = 7); blue line, N2 N3, low MMP9 (n = 15); green line, N2 N3, high MMP9 (n = 8). Events (drop in the graph lines) were deaths from SCC of the head and neck. Subjects who did not experience the events, such as death from unrelated causes, were censored. B, MMP9 intensity, N stage, and tumor recurrence or second primary tumor. Patient groups: red line, N0 N1, low MMP9 (n = 9); yellow line, N0 N1, high MMP9 (n = 7); blue line, N2 N3, low MMP9 (n = 15); green line, N2 N3, high MMP9 (n = 8). Events were recurrence of SCC or second primary. Subjects who did not experience the events were censored. C, MMP2 intensity, N stage, and failure of local control. MMP2 and N stage have an effect on failure of local control. Patient groups: red line, N0 N1, low MMP2 (n = 8); yellow line, N0 N1, high MMP2 (n = 5); blue line, N2 N3, low MMP2 (n = 20); green line, N2 N3, high MMP2 (n = 4). Events (drop in the graph lines) were failure of local control that required surgery at the primary site. Subjects who did not experience the event were censored. D, MMP2 intensity and T stage. Patient groups: T1, tumor size <2 cm (n = 4); T2, tumor size 2 to 4 cm (n = 9); T3, tumor size >4 cm (n = 15); T4, tumor invades through cortical bone, inferior alveolar nerve, floor of mouth, or skin of face (n = 9).
aggressive lesions (Fig. 6). These findings are consistent with a proposed model for wound healing and SCC, in which proliferation is inhibited at the wound edge/invasive front so as to facilitate cell invasion, thereby promoting wound closure and tumor progression, respectively (35). In that study, concurrent expression of p16 and the γ2 subunit of laminin 5 was associated with growth arrest and migration in neoplastic cells (35).

Degradation of the extracellular matrix, including the basement membrane, is critical for invasion into the adjacent tissues and regional and distant metastases (34, 36). SCC cells secrete several matrix-degrading enzymes, such as MMPs, serine proteinases, plasminogen activator, and cysteine proteinases, of which the MMPs are the most important for tumor progression (34, 37–41). MMPs, particularly the gelatinases MMP9 and MMP2, degrade type IV collagen, a significant component of the extracellular matrix, to facilitate SCC invasion (11, 42). Although overexpression of both MMP9 and MMP2 has been associated with tumor progression in SCC and short disease-free survival (16, 33, 43, 44), the role of these MMPs is controversial (34). Therefore, in the present study, we investigated whether Rap1GAP regulates invasion via secretion of gelatinases, whether Rap1GAP and MMP9/MMP2 expression is correlated in human SCC, and whether MMP9 and MMP2 expression is prognostic of patient survival. Consistent with its role in promoting invasion, Rap1GAP promoted MMP9 and MMP2 secretion in SCC cells. Furthermore, Rap1GAP expression was significantly correlated with MMP9 and MMP2 expression in human SCC tissues. Importantly, in low N-stage disease, high MMP9 expression in pretreatment biopsies is prognostic of poor disease-specific survival and shorter duration to tumor recurrence and second primary SCCs. The latter two factors have been independently correlated with poor disease-specific survival. Thus, high MMP9 expression correlates with locally aggressive tumors. These results from a small group of patients strongly suggest that pretreatment screening for Rap1GAP and MMP9 in larger trials may identify those patients with early N-stage disease that are likely to benefit from aggressive initial treatment.

For high N-stage lesions, high MMP9 was linked to better survival. If MMP9 is inversely related to proliferation as our studies and previous studies suggest (45), then once a lesion has spread local growth is likely to be a factor in morbidity and mortality. Hence, lesions that have high MMP9 and possibly slower growth will have a better prognosis than faster-growing, low MMP9 lesions.

Previous studies in SCC cell lines suggest that low MMP2 was associated with better response to neoadjuvant chemotherapy (46). In contrast to these observations, our studies in human tissue show that early N-stage lesions with low MMP2 had a shorter time to indication of surgery. The latter is selected for patients whose tumors do not shrink (>50% reduction in size) in response to chemotherapy or who exhibit disease progression after chemo-therapy/radiation combination therapy. Furthermore, although the overall effect of Rap1GAP is to inhibit tumor growth (4), MMP2 expression was correlated with advanced T stage (i.e., larger tumors). This disparity may be related to downstream regulatory molecules that are processed by MMP2. Consistent with this notion, a recent study showed that the N-terminal and COOH-terminal domains of heparin affinity regulatory peptide, a target of MMP2, increased or decreased cell proliferation, respectively (47).

The catalytic activity of MMPs may be regulated by gene expression (transcription and translation) or secretion (34, 44). The secreted enzyme activity is further regulated by cleavage of the propeptide or by the naturally occurring inhibitors tissue inhibitors of metalloproteinases (TIMP), which mask the zinc-binding site by forming a noncovalent stoichiometric complex (11, 48, 49). MMPs may be activated in vitro by compounds such as APMA, an organomercurial that reacts with the cysteine on the prodomain, thereby preventing it from interacting with the zinc ion (11). In the present study, Rap1GAP up-regulated secretion of MMP9 in its propeptide form, as evidenced by its APMA-dependent activation. In conditioned medium from rgl1, although MMP9 was activated by APMA whereas MMP2 was not, this does not entirely exclude the possibility that secreted MMP2 is in its active form because it may have been cleaved on secretion and the catalytic activity contained by binding to members of the TIMP family.

Using inactive Rap1GAP, a catalytically inactive mutant with RXR to LIG mutations in amino acids 284 to 286 rendering it unable to inactivate GTP-bound Rap1 (50), we previously showed

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**Figure 6.** Proposed model for interaction between Rap1GAP and MMP9 in regulation of tumor progression of early N-stage SCC. Rap1GAP inhibits tumor growth and promotes MMP9 secretion via Rap1-mediated effects on MMP9 protein and mRNA. Lower N-stage lesions expressing MMP9 are associated with poor survival.
that the growth-inhibitory effects of Rap1GAP are dependent on inactivation of Rap1 (4). In the current study, efficient inactivation of Rap1 and inhibition of proliferation were observed with both rg1 and rg2 (Figs. 1C and 2C, respectively). In contrast, invasion and MMP9/MMP2 secretion were highly up-regulated in rg1, which strongly expresses Rap1GAP, compared with rg2, in which exogenous Rap1GAP overexpression was not as prominent. Our findings show that Rap1GAP also up-regulates mRNA steady-state levels of MMP9 and MMP2. Together, these data suggest that Rap1GAP promotes invasion via MMP9 and MMP2 secretion and possibly via direct effects on steady-state levels of MMP9 and MMP2 mRNA. The specific mechanism via which Rap1GAP regulates MMP9 and MMP2 secretion is currently under investigation.

Surprisingly, although staining intensity of Rap1GAP and MMP9/MMP2 on TMAs was correlated, the prognostic value of Rap1GAP as an independent biomarker of tumor progression was not validated. This may be due to the smaller sample size for Rap1GAP staining; more tissue cores are lost as the TMA is sectioned and Rap1GAP sections were obtained after those for MMP9. Studies with larger sample sizes will help address this issue.

In summary, our studies suggest that Rap1GAP, which inhibits tumor growth, up-regulates MMP9 secretion in head and neck SCCs. MMP9 promotes invasion and progression of SCC. Taken together, these results suggest that high MMP9 expression in early-stage lesions is prognostic of tumor progression. These findings are the first link between Rap1GAP and MMP9, which plays a major role in tumor progression.

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References


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