Targeting Galectin-1 in Carcinoma-Associated Fibroblasts Inhibits Oral Squamous Cell Carcinoma Metastasis by Downregulating MCP-1/CCL2 Expression

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Abstract

Purpose: Carcinoma-associated fibroblasts (CAFs) in tumor stroma play an important role in tumor progression and have been associated with a poor prognosis in oral squamous cell carcinoma (OSCC). However, how CAFs influence OSCC malignancy and whether normalizing CAFs inhibits cancer progression remain unclear.

Experimental Design: The relationship between the expression of Galectin-1 (Gal-1) and alpha-smooth muscle actin (α-SMA, a CAF marker) in OSCC patient samples and primary cultured CAFs was examined by quantitative real-time PCR, Western blotting, and immunofluorescence. To examine the effect of Gal-1 on CAF activation and CAF-mediated tumor invasion and migration in vitro, Gal-1 expression was knocked down by small hairpin RNA. Finally, cancer cells and CAFs were coimplanted into SCID mice to evaluate the effect of Gal-1 on CAF-modulated tumor progression in vivo.

Results: Gal-1 expression is positively associated with α-SMA in the stroma of OSCC specimens. Gal-1 knockdown decreases activated CAF characteristics, resulting in a decrease in α-SMA expression and extracellular matrix protein production. Notably, blocking Gal-1 expression significantly inhibits CAF-conditioned medium-induced tumor cell migration and invasion, possibly by reducing the production of monocyte chemotactic protein-1 (MCP-1/CCL2). MCP-1 induces the migration of OSCC cells by binding to the receptor CCR2; adding an MCP-1 antibody to CAF-conditioned medium that inhibits the interaction between MCP-1 and CCR2 abolishes migration. Finally, we found that Gal-1 knockdown in CAFs significantly reduces CAF-augmented tumor growth and metastasis in vivo.

Conclusions: Our findings demonstrate that Gal-1 regulates CAF activation and indicate that targeting Gal-1 in CAFs inhibits OSCC metastasis by modulating MCP-1 expression.

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colon cancer (8, 9), suggesting that targeting CAFs could provide a feasible therapy for OSCC. Developing approaches to destroy or normalize CAFs has become an important issue in cancer therapy. For example, the genetic knockout of fibroblast-activated protein (FAP), a protein specifically expressed in myofibroblasts, significantly suppressed lung and colon tumor growth in mice (10). The monoclonal antibody against FAP, sibrotuzumab, has been investigated in a Phase I clinical trial (11). In addition, an inhibitor of collagen I synthesis, halofuginone, prevents fibroblast differentiation to myofibroblasts and significantly reduces tumor progression when combined with low-dose chemotherapy (12).

Galectin-1 (Gal-1) belongs to the β-galactoside-binding lectin family and behaves as a monomer of 14.5 kDa that can dimerize under certain circumstances. Each monomer is composed of a carbohydrate-recognition domain that recognizes a wide range of glycoproteins and glycolipids (13). Gal-1 expression in cancer-associated stroma is correlated with poor survival in several types of cancer including breast, prostate, and head and neck cancers (14–17). We previously reported (18) that increased Gal-1 expression in cancer-associated stroma and the tumor-invasive front is significantly correlated with a poor prognosis in OSCC. However, whether Gal-1 affects tumor stroma cell homeostasis, especially in fibroblasts, is still unknown. In this study, we investigated whether Gal-1 affects CAF activation and whether targeting Gal-1 in CAFs could provide a feasible therapy for OSCC.

Materials and Methods

Patients and cell lines

Eighty-two OSCC patients from Chi-Mei Medical Center and National Cheng Kung University Medical Center, Tainan, Taiwan, participated in the study; all signed an informed consent form. The study protocols were approved by the institutional review boards of both hospitals. All patients were staged according to the American Joint Committee on Cancer (5th ACC) 1997 cancer staging guidelines. The information of the 82 patients was listed in Supplementary Table S1. The cell lines (OC-2, OEC-M1, and HSC-3) information was described in our previous paper (19).

Primary culture of HGFs and CAFs

CAFs and normal human gingival fibroblasts (HGFs) were isolated from OSCC tumor tissues and their normal counterparts, as previously described (20). The normal counterparts were defined by the noncancerous region of oral tissue at least 2 cm away from the outer tumor margin. The isolated HGFs and CAFs were further characterized using Western blotting (Fig. 1C). Vimentin was strongly expressed in HGFs and CAFs, cytokeratin was not expressed, and α-SMA was strongly expressed in CAFs but not in HGFs. All HGFs and CAFs used in the experiments were less than 10 passages.

Cell invasion and migration assay

Cell invasion and migration assays were done in 24-well Transwell polycarbonate filters (pore size, 8 μm; Corning Costar) coated with or without Matrigel (BD Biosciences) as previously described (19). Briefly, OSCC cells were plated in the upper chamber, and the conditioned media of HGFs, CAFs, CAF sh-Luc cells, or CAF sh-Gal-1 cells were added to the lower chamber. The invaded and migrated cells were counted after the cells had been individually incubated for 24 and 8 hours. Nonpenetrating cells were removed from the upper surface of the filter with a cotton swab. Penetrating cells were fixed and stained with a DiffQuick stain kit (Dade Behring, Inc.) according to the manufacturer’s instructions. For quantification, all of the cells invaded or migrated into the lower surface were stained and counted under a light microscope. All of the experiments were performed in triplicate. Results show the mean ± SD of 3 independent assays.

Quantitative real-time PCR

Total RNA was isolated using a Trizol reagent (Invitrogen Corp.), and then 1 μg was reverse transcribed (ImProm-II Reverse Transcriptase; Promega). Quantitative real-time PCR (qRT-PCR) was used to analyze the expression of different mRNAs, as previously described (21, 22). Briefly, PCR amplifications were performed with SYBR Green PCR Master Mix on a LightCycler 480 real-time PCR detection system (Roche Applied Science). The qRT-PCR was performed as follows: initial denaturation at 95°C for 10 minutes, and 45 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 10 seconds, and extension at 72°C for 10 seconds. Delta-cycle threshold (ΔCT) values were calculated by subtracting the CT value from the corresponding β2-microglobulin CT (internal control) value for each sample. The relative expression level of individual genes were calculated by comparing the ACT values of Gal-1 knockdown cells to those of the control groups and were shown in folds changed.

shRNA lentivirus production

Lentiviral vectors containing luciferase, Gal-1, and CCR2 small hairpin (sh) RNA were obtained from the National RNAi Core Facility (Academia Sinica) and prepared in accordance with standard protocols. Briefly, 3 × 10^6 293T cells were seeded in a 10-cm dish. After 24-hour incubation, cells were cotransfected with 5 μg pLKO.1shRNA, 5 μg pCMVΔR8.91, and 0.5 μg pMD.G plasmids by Lipofectamine 2000 (Invitrogen). After transfection, we changed the medium to remove transfection reagents and virus was collected 48 hours later and filtered through 0.45 μm filters. Cells were infected with lentivirus (multiplicity of infection 5) in the presence of polybrene (8 μg/ml). Forty-eight hours postinfection, the cells were treated with 1 μg/ml of puromycin to select puromycin-resistant clones. The shRNA target sequences were: for CCR2: 5'-GCT GCA AAT GAG TGG GTC TTT-3', and for Gal-1: G1: 5'-GCT GCC AGA TGG ATA CGA ATT-3' and G2: 5'-CGC TAA GAG CTG CCT GCT GAA-3'.
Immunohistochemical analysis
The immunohistochemical analysis was performed as described previously (21).

Immunofluorescence staining
Cells were seeded on the coverslips, incubated for 24 hours, washed twice with PBS, and then fixed on ice in 3.7% paraformaldehyde for 20 minutes. After they had been washed three more times with PBS, the cells were permeabilized on ice in 0.05% Triton X-100 for 5 minutes, washed once more with PBS, and then incubated with α-SMA antibody (MCA1906; Serotec) for 2 hours at room temperature. The cells were then washed and incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies. The α-SMA expression was visualized using a fluorescence microscopy.

Coinjecting mice with OSCC cells and CAFs to measure tumor progression
The animal experiment protocols were approved by the National Cheng Kung University Animal Center ethical committee. Briefly, 1 × 10^5 OC-2 (an OSCC cell line) cells and 3 × 10^5 CAFsh-Luc cells or CAFsh-Gal-1 cells were mixed with Matrigel and subcutaneously (s.c.) co-injected into SCID mice. Tumor size was measured using calipers, and the tumor volume was calculated using the following equation: (length × width × width × 0.45). Tumor metastasis was quantitatively determined by measuring the cancer cells in circulation, as previously described (20). For the detecting circulating cancer cells in mice, peripheral blood samples from mice were collected in heparinized microhematocrit tubes (Assistent). The genomic DNA was extracted using a kit (QIAamp Mini DNA kit; Qiagen). PCR was used to measure the level of circulating tumor cells for the human Alu sequence (23). The PCR results were quantified using ImageJ software, and the relative amount of circulating tumor cells was calculated by dividing the PCR results by those from control mice that had not been implanted with tumor cells.

Cytokine array analysis
The cytokines secreted from HGFs, CAFs, CAFsh-Luc cells, and CAFsh-Gal-1 cells were analyzed using a kit.
Proteome Profiler Array; R&D Systems) according to the manufacturer’s instructions. Briefly, supernatant from different cells (1×10^6) was collected. The conditioned medium was incubated with the array immobilized with different antibodies. The relative expression level of each cytokine was analyzed by comparing signal intensities (ImageJ software; NIH).

**Statistical analysis**

The association between Gal-1 and α-SMA expression was analyzed using Fisher’s exact test (Prism 5; Graphpad Software, Inc.). The other experiments were assessed using an unpaired Student’s t test or 1-way ANOVA, and shown as mean ± SD. Statistical significance was set at P < 0.05.

**Results**

**Gal-1 overexpression is associated with fibroblast activation**

To determine whether Gal-1 expression is associated with CAFs, we first investigated the localization of Gal-1 and α-SMA in clinical OSCC samples. Gal-1 colocalizes...
(Fig. S2), which indicates a dominant role for Gal-1 in CAFs. CAFs were at least 10 times higher than in OSCC cells and in CAFs. Both Gal-1 and α-SMA were expressed in cancer cells, we analyzed Gal-1 and α-SMA expression both in the OSCC cell lines and in CAFs. Both Gal-1 and α-SMA expression levels in CAFs were at least 10 times higher than in OSCC cells (Fig. S2), which indicates a dominant role for Gal-1 in CAFs. The data in Figure 1 show that Gal-1 expression closely correlates with CAF activation and suggests that Gal-1 may regulate CAF homeostasis.

Gal-1 modulates fibroblasts transdifferentiation to myofibroblasts

Because CAFs promote tumor invasion and migration through paracrine stimulation (1), we investigated whether silencing Gal-1 inhibits CAF-augmented tumor cell invasion and migration. HGFs and CAFs were infected with lentivirus carrying luciferase shRNA (sh-Luc) or Gal-1 shRNA (shGal-1). The conditioned medium was collected as a chemoattractant. Cell migration and invasion assays were performed as described in the Materials and Methods. All of the experiments were performed in triplicate. Results show the mean ± SD of 3 independent assays. *P < 0.05, **P < 0.01, and ***P < 0.001.

with α-SMA in spindle-like cells (Figs. 1A and S1), suggesting that Gal-1 overexpression might be associated with CAF activation. To verify the correlation of Gal-1 and α-SMA expression, we used qRT-PCR. Eighty percent of the tumors with high Gal-1 expression had high α-SMA expression, and 63% of the tumors with low Gal-1 expression had low α-SMA expression (Fig. 1B, P < 0.0001). To further examine this correlation, we directly isolated CAFs and HGFs from 2 OSCC tissue samples. The absence of Pan-CK expression in HGFs and CAFs indicated that the samples were not contaminated by epithelial cells. Vimentin (a fibroblast marker) is strongly expressed in HGFs and CAFs. Both Gal-1 and α-SMA expression are higher in CAFs than in paired HGFs (Fig. 1C), which is consistent with the results shown in Figure 1B. To rule out the possibility that Gal-1 and α-SMA were expressed in cancer cells, we analyzed Gal-1 and α-SMA mRNA expression both in the OSCC cell lines and in CAFs. Both Gal-1 and α-SMA expression levels in CAFs were at least 10 times higher than in OSCC cells (Fig. S2), which indicates a dominant role for Gal-1 in CAFs.

Because Gal-1 expression is associated with CAF activation, we next examined whether Gal-1 regulates the transdifferentiation of fibroblasts to myofibroblasts. Treatment of HGFs with recombinant Gal-1 protein significantly induced α-SMA expression in a dose-dependent manner, indicating that Gal-1 stimulates HGF transdifferentiation (Fig. 2A). RNA interference was used to knockdown Gal-1 expression in CAFs. Two different Gal-1 shRNAs were used to exclude off-target effects. Strikingly, Gal-1 knockdown in CAFs significantly reduced the expression of α-SMA (Fig. 2B). Another CAF marker (FAP, fibroblast activation protein) was also reduced upon knockdown of Gal-1 in CAFs (Fig. 2C). The expression of extracellular matrix proteins such as fibronectin and collagen I expression was also suppressed (Fig. 2B and C). α-SMA is reorganized into stress fibers in activated fibroblasts (1). Immunofluorescence staining showed that the knockdown of Gal-1 not only reduces α-SMA expression but also disrupts the stress fiber structure in CAFs (Fig. 2D). These data strongly support the notion that Gal-1 controls CAF activation.

Targeting Gal-1 suppresses CAF-augmented cancer cell invasion and migration

Because CAFs promote tumor invasion and migration through paracrine stimulation (1), we investigated whether silencing Gal-1 inhibits CAF-augmented tumor cell invasion and migration. CAFs were infected with lentivirus carrying luciferase shRNA (sh-Luc) or Gal-1 shRNA (shGal-1). The conditioned medium was collected as a chemoattractant. Cell migration and invasion assays were performed as described in the Materials and Methods. All of the experiments were performed in triplicate. Results show the mean ± SD of 3 independent assays. *P < 0.05, **P < 0.01, and ***P < 0.001.

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Gal-1 regulates CAF-augmented tumor cell invasion and migration by increasing MCP-1/CCL2 expression

Because cytokines secreted from stromal cells influence cancer cell invasion and migration in many types of cancer (1), we examined the secretion of cytokines in the conditioned media of HGFs and CAFs (CAF-CM and HGF-CM) using cytokine array analyses. The secretion levels of several cytokines are higher in CAF-CM than in HGF-CM (Fig. 4A); in particular, MCP-1 expression is 14 times higher (Fig. 4A). Surprisingly, when we compared cytokine...
expression in the conditioned media of CAFs with and without Gal-1 expression, MCP-1 expression was strongly down-regulated in CAF sh-Gal-1 CM than in CAF sh-Luc CM (Fig. 4B), which was confirmed by Western blotting and ELISA assays in other primary culture CAFs (Figs. 4C and S3). These results suggest that MCP-1 may be a critical regulator in CAF-mediated tumor invasion and metastasis and that it is an important target of Gal-1.

MCP-1 was originally identified as a chemotactic protein for monocytes and macrophages, but its effect on OSCC cell migration and invasion is not clear. Therefore, we determined whether MCP-1 induces OSCC cell migration...
by treating cells with recombinant MCP-1 protein. MCP-1 treatment significantly increased tumor cell migration in a dose-dependent manner (Fig. 5A). We next examined whether MCP-1 is a critical regulator in CAF-mediated tumor invasion and metastasis. Adding an anti-MCP-1 inhibitory antibody in CAF-CM inhibited cancer cell migration but not in HGF-CM (Fig. 5B), suggesting a pivotal role for MCP-1 in tumor–stroma interaction.

To determine whether Gal-1 regulates CAF-CM–induced cell migration by modulating MCP-1 expression, we treated CAF sh-Luc CM and CAF sh-Gal-1 CM with MCP-1 inhibiting antibody. This treatment reduced CAF sh-Luc CM–induced cell migration but had no effect on sh-Gal-1 CM–induced cell migration (Fig. 5C). Because MCP-1 induces signal transduction primarily by binding to CCR2, we investigated the effect of MCP-1 on cancer cell migration by knocking down CCR2. The knockdown efficiency in OEC-M1 cells was about 80% (Fig. S4). Down-regulating CCR2 inhibited CAF sh-Luc CM–induced cell migration but did not affect CAF sh-Gal-1 CM–induced cell migration (Fig. 5D). These results show that Gal-1 modulates MCP-1 expression in CAFs, which promotes cancer cell migration by interacting with CCR2.

Gal-1 knockdown in CAFs inhibits OSCC progression in vivo

To determine whether targeting Gal-1 in CAFs efficiently inhibits cancer progression and metastasis in vivo, we mixed the CAFs (infected with sh-Luc or sh-Gal-1 shRNA lentivirus) and OC-2 cells in a 3:1 ratio and injected (s.c.) the mixtures into SCID mice as previously reported (8). Tumors were visible as early as 20 days postinjection in the CAFs (sh-Luc) mixed with OC-2 cells; however, it took 30 days for injections of OC-2 cells alone to generate tumors (Fig. 6A). CAFs (sh-Luc) mixed with OC-2 cells also generated larger tumors than OC-2 cells alone (Fig. 6A). Notably, we found that Gal-1 knockdown in CAFs significantly inhibited CAF-promoted tumor growth (Fig. 6A). Circulating tumor cells have been reported as a prognostic factor for distant metastases and survival in...
in cancer patients (24, 25). In this study, we found that OC-2 cells mixed with sh-Gal-1-transduced CAFs resulted in fewer cancer cells in circulation than OC-2 cells mixed with CAFs (sh-Luc), and Group-3 mice were injected with OC-2 mixed with CAFs (sh-Gal-1). Tumor size was measured as described in the Materials and Methods; **, P < 0.01. B, Group-3 (n = 4) and Group-1 (n = 5) mice had fewer circulating cancer cells than did Group-2 mice (n = 5). The index shows the relative amount of circulating tumor cells measured 42 days after tumor inoculation and was calculated as described in the Materials and Methods; *, P < 0.05. C, Group-2 mice died earlier than Group-1 and -3 mice. The survival curve was computed using the Kaplan-Meier product-limit method. The log-rank test was used to compare the survival curves. D, a summary of how Gal-1 activates stromal fibroblasts and promotes cancer metastasis.

Discussion

In this study, we demonstrated that Gal-1 plays a critical role in stimulating the transdifferentiation of fibroblasts to myofibroblasts and increases MCP-1 secretion, which promotes tumor progression and metastasis by interacting with CCR2 on cancer cells (Fig. 6D). Silencing Gal-1 in CAFs suppresses CAF-augmented tumor cell migration and invasion in vitro as well as tumor formation and intravasation in vivo, which suggests that targeting Gal-1 in CAFs may provide a viable antimetastasis strategy. Our findings also suggest that targeting Gal-1 in CAFs may be more efficient in advanced-stage cancer because myofibroblast accumulation in tumor stroma is often associated with late-stage cancer (7).

There are no published studies on the role of Gal-1 in CAFs extracted from clinical tissues. Our results show that the knockdown of Gal-1 reverses activated CAFs to quiescent fibroblasts and highlights the role of Gal-1 in CAF homeostasis. Notably, we found that Gal-1 expression is
CCR2 signals may attenuate OSCC metastasis. This strategy implies a critical role for MCP-1/CCR2 signaling in cell invasion and migration support our findings. Our results show that MCP-1 directly induces prostate and hepatic tumor cell motility and OSCC cell invasion and migration by binding to its receptor, CCRL2. We found that MCP-1 secreted by CAFs promotes cancer cell invasion and migration in OSCC. We have also shown that Gal-1 may activate myofibroblast activation by modulating NOX4 expression. Conversely, Gal-1 could be the downstream mediator of factors that induce myofibroblast transdifferentiation such as transforming growth factor (TGF-β1; ref. 34), endothelin-1 (ET-1; ref. 35), and platelet derived growth factor (PDGF; ref. 34). However, we did not observe a change in Gal-1 expression upon TGF-β1 or endothelin-1 treatment (Fig. S5), indicating that Gal-1 may be regulated by other factors. For example, PDGF-induced myofibroblast transdifferentiation was associated with Gal-1 upregulation (34).

It has been reported that MCP-1 promotes cancer progression by indirectly recruiting monocytes or macrophages to tumor sites (36, 37); however, its direct function in cancer cell invasion and migration is not clear in OSCC. We found that MCP-1 secreted by CAFs promotes OSCC cell invasion and migration by binding to its receptor, CCRL2, indicating a direct effect of MCP-1 on tumor malignancy. Recent studies (38, 39) showing that MCP-1 directly induces prostate and hepatic tumor cell invasion and migration support our findings. Our results imply a critical role for MCP-1/CCRL2 signaling in cell invasion and migration; therefore, blocking MCP-1/CCRL2 signals may attenuate OSCC metastasis. This strategy has proved effective in prostate cancer: systemic administration of MCP-1 neutralizing antibody efficiently inhibited prostate cancer metastasis (40).

Our findings suggest that targeting Gal-1 may be effective in different types of epithelial tumors for the following reasons: (a) myofibroblasts are associated with a poor prognosis in different types of cancer such as OSCC, prostate, breast, and lung carcinoma (7, 41–43); (b) Gal-1 is overexpressed in many types of epithelial tumor stroma (Fig. S6) and increased Gal-1 in cancer-associated stroma is associated with a poor outcome in breast and prostate cancer (16–18); (c) the downstream target gene, MCP-1, which is up-regulated in the stroma of prostate and breast cancer, is associated with more advanced cancer stages and poor disease survival (36, 44, 45); (d) it has been reported that Gal-1 directly induces MCP-1 production in pancreatic stellate cells (27, 46). Taken together, these facts support the hypothesis that targeting Gal-1 in CAFs inhibits tumor progression and metastasis by downregulating MCP-1 expression. Therefore, developing agents, such as siRNAs or anginex (47), that target Gal-1 in CAFs may open a new avenue in cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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