MCP1-Induced Epithelial–Mesenchymal Transition in Head and Neck Cancer by AKT Activation

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Abstract. Aim: To explore whether monocyte chemotactic protein-1 (MCP1) is associated with the epithelial–mesenchymal transition (EMT) and neck metastases in head and neck cancer (HNC). Materials and Methods: MCP1 and its related protein were evaluated using western blotting, and a migration assay for HNC cell lines. Thirty-five patients with HNC were recruited for the evaluation of MCP1 expression and pathologically-proven neck metastases from their tissue specimens. Results: MCP1 changed the phenotype of OML-1 cells to a spindle shape, with increased mobility. In OML3 cells, MCP1 knockdown with siRNA blocked EMT. Activation of protein kinase B (AKT) was positively associated with the EMT phenotype, and this transition was abrogated with a phosphoinositide 3 kinase (PI3K) inhibitor. By comparing clinical outcomes, the histological MCP1 score was associated with pathological neck metastases (p=0.027). Conclusion: The overexpression of MCP1 in HNC cells may partially induce EMT through the AKT pathway. A high cellular expression of MCP1 was associated with pathological neck metastases.

Head and neck cancer (HNC) is the fifth most commonly diagnosed cancer in men and the fifth leading cause of cancer death for men and accounts for 650,000 new cases and 350,000 deaths annually worldwide (1, 2). Smoking, alcohol consumption, human papilloma virus infection, especially of serotype 16, and betel quid chewing are known risk factors for head and neck squamous cell carcinoma (HNSCC) (3, 4). Long-term survival rates for patients with HNC have not greatly improved over the past several decades (5, 6). Reliable prognostic factors may be used to improve the present TNM staging system and to identify high-risk groups.

Inflammatory processes are highly associated with cancer progression (7-9). Chemokines such as monocyte chemotactic protein-1 (MCP1) have recently received consideration. Elevated MCP1 expression levels in the tumor microenvironment are crucial for cancer growth, dissemination, and metastasis (10-13). MCP1/chemokine (C-C motif) ligand 2 (CCL2) is a 76-amino-acid peptide which can recruit monocytes and macrophages to inflammatory sites and regulate their activities (14). MCP1 has also been associated with survival in HNC (15, 16). Certain evidence suggested that MCP1 may influence cancer metastasis (17). However, the impact of MCP1 in cancer metastasis, as well as in the epithelial–mesenchymal transition (EMT), has not yet been well-explored; the EMT is a process involving tumor development, migration, invasion, and metastasis during which epithelial cells lose their polarity (18).

The aim of the present study was to determine whether MCP1 expression is related to the EMT in HNC. We also investigated the relationship between MCP1 expression and pathological neck metastases.

Materials and Methods

Ethics statement. This study was initiated after receiving approval from the Institutional review board at the Dalin Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Taiwan, ROC (B09904004-1 and B10101014). Review board requirements for written informed consent regarding immunohistochemical staining and a survival analysis were waived because all personal identifying information was removed from the database prior to data analysis.

Cell culture. OML1, a buccal mucosa squamous cell carcinoma cell line, and OML3, a tongue mucosa squamous cell carcinoma cell line, were obtained from the Department of Dentistry, Veterans General Hospital, Taichung, Taiwan, ROC. All cell lines were maintained in an RPMI medium supplemented with 10% fetal
IQR, Interquartile-range; NA: not available; SD, standard deviation.

3300

routinely kept in an incubator at 37˚C supplied with 5% CO₂, and bovine serum (FBS) and 1% penicillin/streptomycin. The cells were seeded, for the EMT tests and western blot analysis, the cells in 6-well dishes, grown for 16 h, and transfected with siRNA specifically targeting MCP1 (70 nM SASI_Hs01_00024134; Sigma) using TurboFect reagent, (Thermo Scientific, Thermo Fisher Scientific Inc., Waltham, MA, USA). The cells were grown for 72 h prior to western blot studies.

Variable pN −, n (%) pN +, n (%) p-Value

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Reagents and antibodies. Antibody against cleaved vimentin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against phosphorylated protein kinase B (AKT) (473) and phosphorylated transcription 3 (STAT3) (Y705) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against SNAIL and SLUG were purchased from AbCam (Cambridge, UK). Antibodies against β-Actin and E-Cadherin were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against phosphorylated protein kinase B (AKT) (473) and phosphorylated transcription 3 (STAT3) (Y705) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against cleaved vimentin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against phosphorylated protein kinase B (AKT) (473) and phosphorylated transcription 3 (STAT3) (Y705) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against SNAIL and SLUG were purchased from AbCam (Cambridge, UK). Antibodies against β-Actin and E-Cadherin were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against phosphorylated protein kinase B (AKT) (473) and phosphorylated transcription 3 (STAT3) (Y705) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against cleaved vimentin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against phosphorylated protein kinase B (AKT) (473) and phosphorylated transcription 3 (STAT3) (Y705) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against SNAIL and SLUG were purchased from AbCam (Cambridge, UK). Antibodies against β-Actin and E-Cadherin were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against phosphorylated protein kinase B (AKT) (473) and phosphorylated transcription 3 (STAT3) (Y705) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell migration assay. The cells were grown to 95% confluence, and the monolayers were then scratched with a pipette tip. The cells were washed twice with PBS, and a medium containing 1% FBS and MCP1, which was purchased from AbCam (Cambridge, UK), (100 ng/ml) was added and incubated for 16 h. Three randomly selected fields were photographed at the beginning and end of the treatment. Subsequently, the wound healing was analyzed using Image J software (National Institutes of Health, Bethesda, Maryland, USA).

siRNA transfection. OML3 cells were plated in 6-well (2×10⁵/well) dishes, grown for 16 h, and transfected with siRNA specifically targeting MCP1 (70 nM SASI_Hs01_00024134; Sigma) using TurboFect reagent, (Thermo Scientific, Thermo Fisher Scientific Inc., Waltham, MA, USA). The cells were grown for 72 h prior to western blot studies.

Tumor immunohistochemical staining. This study included 35 patients with HNC, but without distant metastases, who were newly diagnosed between 2000 and 2009. All patients initially underwent surgical curative treatment and with the approval of our IRB, tissue samples from these patients were available for immunohistochemical staining. The following data were collected: patient age, gender, pathological TNM stage, treatment modality, and outcomes (local/regional recurrence, distant metastasis, and death). All tumors were staged according to the American Joint Committee on Cancer cancer staging system, modified in 2007 (19).

Sample processing and immunohistochemical staining were performed as previously described (20). MCP1 expression was detected immunohistochemically for the 35 HNC tissues. Paraffin sections were deparaffinized with xylene, rehydrated, submerged in citrate buffer, microwaved for antigen retrieval, and treated with 3% hydrogen peroxide in methanol to quench endogenous peroxidase activity. Sections were washed in 1% Bovine serum albumin, treated with mouse monoclonal antibody to MCP1 (ab9858, 1:100; AbCam) overnight at 4°C, incubated with a secondary antibody from the Ultra Vision Quanto Detection System HRP DAB system (Thermo Fisher Scientific, Waltham, MA, USA) at room temperature, and subsequently visualized with a diaminobenzidine (DAB; used as a chromogen substrate) reaction. Negative controls were obtained by omitting the primary antibody. Two independent observers assessed the expression of MCP1 protein. Scoring resulted from the summation of the percentage and intensity score and was performed as previously described (21).

Statistical analysis. All data were analyzed using the SPSS system (version 15; SPSS Inc, Chicago, IL, USA). The association between categorical variables was tested using Pearson’s χ² test or Fisher’s exact test. Continuous variables were compared with a two-sample test or Mann–Whitney U-test, according to the distribution of data.
Results

MCP1 promotes EMT phenotype and HNC cell migration. Exogenous recombinant MCP1 was administrated to OML1 HNC cells to determine whether MCP1 induces EMT. With MCP1 treatment, we observed that the OML1 cells had undergone morphological changes from what could be described as a cobblestone-like to fibroblast-like appearance (Figure 1). There were no significant changes found in the control cells. Recombinant MCP1 was used to treat OML1 cells, and the MCP1 treatment induced cell migration, as shown in Figure 1B and C. Quantification was conducted by measuring the migration distance, as compared with the control (Figure 1C). The MCP1 treatment increased OML1 cell motility, compared to the control, as shown in Figure 1B and C.

Up-regulation of p-AKT, SNAIL and SLUG by MCP1. To determine the MCP1 downstream signaling pathway that led to EMT, recombinant MCP1 was used to treat the OML1 cells. MCP1 induced the expression of transcription factors SNAIL and SLUG, and mesenchymal cell-type markers vimentin and p-AKT, and reduced the expression of epithelial cell marker E-cadherin in a time-dependent manner (Figure 2).

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**Figure 1. The Impact of monocyte chemotactic protein-1 (MCP1) on OML1 morphology and migratory ability.** A: MCP1 induced a fibroblast-like change. B and C: With MCP1 treatment, the mobility of OML1 cells was significantly increased.

**Figure 2. Up-regulation of p-protein kinase B (AKT), SNAIL and epithelial–mesenchymal transition (EMT) phenotype induced by monocyte chemotactic protein-1 (MCP1).** MCP1 treatment enhanced the phosphorylation of AKT, transcription 3 (STAT3) and SNAIL and induced an EMT phenotype in a time-dependent manner.
Inhibiting MCP1 expression reduced EMT. In order to determine the role of MCP1 in EMT, we used MCP1 siRNA to treat mesenchymal-type OML3 cells. MCP1-knockdown suppressed an EMT which the OML3 cells with siRNA transfection had the cobblestone-like appearance and caused a significant increase in E-Cadherin and a decrease in vimentin (Figure 3A and B).

Blocking the PI3K pathway reversed MCP1-induced EMT. Although MCP1 induced p-AKT expression, we hoped to determine whether MCP1-induced EMT occurred through the PI3K-AKT pathway. Cells were pretreated for 2 h with PI3K inhibitor before MCP1 treatment, and we found that the PI3K inhibitor abrogated MCP1-induced EMT in the morphology (Figure 4A). In western blot analysis, the PI3K
inhibitor also inhibited MCP1-induced expression of mesenchymal cell-type marker vimentin, as well as that of SNAIL and SLUG (Figure 4B).

Clinical outcomes. Thirty-five patients with HNC were enrolled, and their tissue samples were IHC stained. Table I shows the association of pathological neck metastases with various clinical variables. In patients with HNC, an advanced T classification and MCP1 staining were positively associated with pathological neck metastases (Figure 5A and B).

Discussion

In HNC cell lines such as OML1, exogenous MCP1 induced EMT. In OML3 cells, the knockdown of MCP1 with siRNA blocked EMT. Activation of AKT was positively associated...
with EMT, and the phenomenon was abrogated with a PI3K inhibitor. By comparing the clinical outcomes it was found that the histological MCP1 score was positively associated with pathological neck metastases. MCP1 may be regarded as a candidate for target therapy for HNC in the future.

Despite apoptosis and autophagy, the EMT is an important issue for cancer progression, especially as it is associated with tumor cell metastasis. The EMT is a process involving tumor development, migration, invasion, and cancer metastasis by which epithelial cells lose their polarity and are converted to a mesenchymal phenotype. Phenotypic changes in EMT include the down-regulation of epithelial markers (E-Cadherin) and transcription factors (SNAIL,Slug, or TWIST) and the up-regulation of mesenchymal markers (vimentin or N-cadherin) (18). The expression of an EMT marker is known to be associated with cancer recurrence and poor survival (22-24).

Many factors induce EMT, including MCP1, SLUG (Snail 2), Smad interacting protein 1 (SIP1), TWIST1, forkhead box C2 (FOXC2), E47, and goosecoid, and have been reported to be associated with tumor migration, invasion, and metastasis (25-28). Generally, EMT can be induced by hyperpermetabolism, hypoxia, impaired differentiation, and stromal reactions (28). Inflammatory responses, a stromal reaction, can initiate secretion of activating factors and nuclear factor-κB (NFκB) that regulate SLUG, SNAIL, and TWIST (29). MCP1 is associated with monocyte cell trafficking and memory T-cells in inflammatory processes (14). Many direct or indirect data have indicated that MCP1 can activate the upstream regulators of EMT. Previous studies showed that MCP1 had pro-sclerotic effects in human mesangial cells. MCP1 was positively associated with fibronectin deposition in glomeruli in mice with diabetes (30, 31). Lee et al. reported that MCP1 activated SNAIL and resulted in the EMT in both in vivo and in vitro studies (32). They suggested that MCP1 was involved in the peritoneal EMT process and extracellular matrix synthesis and that this phenomenon may be mediated by transforming growth factor-β1 (32). In prostate cancer cell lines, MCP1 altered actin morphology, resulted in lamellipodia formation, and increased cancer cell migration through the activation of a small RAC GTPase (33). Recently, MCP1, CCL5, tumor necrosis factor-alpha (TNFα) and Interleukin-1 beta (IL1β) were detected with immunohistochemistry and were associated with the EMT in breast cancer (34).

In OML1 cell line, a 100-ng/ml concentration of MCP1 induced the strong activation of p-AKT 4 h after treatment and that of p-STAT3, and EMT 16 h after treatment (Figure 2). After adding MCP1, the morphology of OML1 became spindle shaped; adding the PI3K inhibitor Ly 294002 abrogated this phenomenon (Figure 3). In western blot analysis, a PI3K inhibitor down-regulated SNAIL and reversed the EMT phenotype (Figure 4).

For pathways associated with MCP1 and EMT, the MCP1–STAT3 axis and MCP1–GTPase pathway had been postulated in prostate cancer (33, 35). In endometrial stromal cells, Li et al. concluded that MCP1 increased cell invasiveness partially through AKT and Mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase-1/2 signal pathways (36). Our series indicates that MCP1 might partially modulate the EMT through the PI3K/AKT/SNAIL pathway.

In evaluation of the clinical significance of MCP1, we observed that a high cellular expression of MCP1 was associated with pathological neck metastases. Our result was in agreement with previous studies. Yoshimua et al. reported that stromal cell-derived MCP1 in primary tumors promoted metastasis of breast cancer cells to the lungs (37). MCP1-dependent STAT3 activation, along with that of the EMT pathway, was revealed in an in vitro co-culture model with prostate cancer cells and macrophages (35). Furthermore, increased MCP1 expression in tissue sections was associated with poor prognosis for patients with prostate cancer (35, 38). In addition to MCP1, several inflammatory markers such as TNFα, Interleukin-8 (IL8), or IL1β were also found to be increased in cancer tissues and were demonstrated to be associated with poor prognosis in various types of cancers (34, 38). Some of these proteins were controlled by NF-κB, while others were not (39). MCP1 and other inflammatory markers could act synergistically to promote tumor progression through multiple mechanisms. Thus, MCP1 might be considered a surrogate marker of all these inflammatory markers, and its immunohistochemistry may be viewed as a marker of inflammatory severity, thus representing the aggressiveness of cancer.

Our findings may have some clinical implications in the treatment of HNC. Besides its impact on pro-survival signals in HNC, MCP1 was associated with EMT and neck metastases. Monoclonal antibodies against MCP1 have been used alone or in combination with chemotherapy in patients with solid tumors (40-42). Targeting MCP1 could be regarded as an additional treatment strategy in the future. For patients with HNC, opinions regarding the treatment of cN0 disease (clinically negative for neck metastases) remain unclear. Relying on tumor depth, tumor location, differentiation, clinical T classification, and perineural invasion, we decided whether a selective neck dissection should be performed (43, 44). However, at least 60% of patients with HNC underwent an unnecessary neck dissection, as the final pathological findings did not demonstrate any positive lymph nodes. Moreover, in addition to clinical features, biological markers could help identify high-risk groups for pN+, and MCP1 may be regarded as a predictor of pN+ status (45).

There existed certain limitations to this study. Firstly, whether upstream SNAIL is regulated by MCP1 is not clear,
and the role of STAT3 in EMT has not been established. Secondly, semi-quantitative methods, such as western blot and immunohistochemistry, may be improved upon by using real-time polymerase chain reaction. Thirdly, this was an in vitro study, and co-culture with macrophages or cancer-associated fibroblasts was not performed. Therefore, the interactions among HNC cells, cancer-associated fibroblasts, and macrophages was not clearly explored. Lastly, the clinical data available for analysis in this study were limited.

Our study found that recombinant MCP1 enhanced HNC cell mobility and induced EMT phenotype, with the increased phosphorylation of AKT, overexpression of STAT3, SNAI1, and vimentin; and repression of E-cadherin. Knockdown of MCP1 with siRNA reverted the EMT phenotype of HNC cell lines. Pathological neck metastases were positively associated with MCP1 histological score. The aforementioned findings indicate that MCP1 is a potential marker for HNC metastasis.

Conflicts of Interest
The Authors declare that they have no competing interests and no financial interests in the publication of this article.

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References


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