Angiogenesis, Metastasis, and the Cellular Microenvironment

Anti–IL-20 Monoclonal Antibody Alleviates Inflammation in Oral Cancer and Suppresses Tumor Growth

Yu-Hsiang Hsu, Chi-Chen Wei, Dar-Bin Shieh, Chien-Hui Chan, and Ming-Shi Chang

Abstract

Interleukin-20 (IL-20) is a proinflammatory cytokine involved in rheumatoid arthritis, atherosclerosis, and osteoporosis. However, little is known about the role of IL-20 in oral cancer. We explored the function of IL-20 in the tumor progression of oral cancer. IL-20 expression levels in tumorous and nontumorous oral tissue specimens from 40 patients with four different stages oral cancer were analyzed with immunohistochemistry (IHC) staining and quantitative real-time PCR (qRT-PCR). Expression of IL-20 and its receptor subunits was higher in clinical oral tumor tissue than in nontumorous oral tissue. The role of IL-20 was examined in two oral cancer cell lines (OC-3 and OEC-M1). In vitro, IL-20 promoted TNF-α, IL-1β, MCP-1, CCR4, and CXCR4 and increased proliferation, migration, reactive oxygen species (ROS) production, and colony formation of oral cancer cells via activated STAT3 and AKT/JNK/ERK signals. To evaluate the therapeutic potential of anti–IL-20 monoclonal antibody 7E for treating oral cancer, an ex vivo tumor growth model was used. In vivo, 7E reduced tumor growth and inflammation in oral cancer cells. In conclusion, IL-20 promoted oral tumor growth, migration, and tumor-associated inflammation. Therefore, IL-20 may be a novel target for treating oral cancer, and anti–IL-20 monoclonal antibody 7E may be a feasible therapeutic.

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Introduction

With approximately 500,000 new cases annually, squamous cell carcinomas of the head and neck (HNSCC), are one of the 6 most common cancers in the world (1). Oral cancer comprises nearly 30% of all HNSCC. Of these, approximately 90% of cases are squamous cell carcinomas (2). Oral squamous cell carcinoma (OSCC) is a major health problem worldwide, and patients have a particularly poor 5-year survival rate (3). The staging of oral cancer is an important step in defining the natural history and prognosis of the disease and affects treatment decisions. On the basis of the tumor–node–metastasis (TNM) classification with various modifications, stages are determined according to the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) classification system for oral cancer (3). Each stage is a progression through a primary tumor and progressively nodal, adjacent, and distant metastasis.

Several mechanisms involved in oral cancer have been extensively studied; however, the poor prognosis may reflect an incomplete knowledge of the mechanisms underlying the malignant progression of this cancer type. Oral cancer starts with uncontrolled cell proliferation, and over time becomes able to multiply without limit (immortalization; ref. 4). Next, oral cancer cells acquire the ability to migrate from their original site, invade nearby tissue, and metastasize to distant sites. The accumulation of changes in certain molecules results in these progressive changes from slightly deregulated proliferation to full malignancy.

Prolonged alcohol and tobacco use, betel nut chewing, and infection with the human papillomavirus (HPV) are known risk factors and have suggested the involvement of chronic inflammation and oxidative stress in the development of oral cancer (5, 6). Reactive oxygen species (ROS) are formed in the human oral cavity after they have been exposed to the above stimulants. ROS induce DNA damage and activate intracellular signals and transcription factors (7). These alterations in turn affect the production and function of certain molecules for regulating the cell cycle, growth, differentiation, migration, invasion, and inflammation (8). Inflammation in the tumor microenvironment has many tumor-promoting effects (9). It aids in the proliferation and survival of malignant cells, promotes angiogenesis and metastasis, disrupts adaptive immune responses, and alters responses to hormones and chemotherapeutic agents (10–12). Among these agents, proinflammatory cytokines, such as interleukin-1β (IL-1β), IL-6, and TNF-α, are essential for
regulating the immune response and may have prognostic significance in cancer. The serum levels of IL-1β, IL-6, and TNF-α are higher in patients with oral cancer than in healthy persons, and increased serum levels seem to be correlated with the clinical stage of the disease (13). Leukocyte trafficking, which is critically regulated by chemokines and their receptors, shares many of the characteristics of tumor cell infiltration and metastasis. Increasing evidence suggests a pivotal role for CXCL12/CXCR4 in the establishment of lymph node and distant metastasis in OSCC (14, 15) by inducing epithelial–mesenchymal transition (16).

A number of downstream signaling events, such as the Ras/Raf/MAPK, the STAT3, and the PI3K/AKT/mTOR pathways, contribute to the malignant growth and metastatic potential of OSCC (17–19). They are often persistently active in oral cancer cells.

IL-20 is a member of the IL-10 family, which includes IL-10, IL-19, IL-20, IL-22, IL-24, and IL-26 (20), which share 18% to 25% amino acid identity with IL-10. IL-20 is expressed in monocytes, epithelial cells, and endothelial cells. It acts on multiple cell types by activating a heterodimer receptor complex of either IL-20R1/IL-20R2 or IL-22R1/IL-20R2 (21). It is also involved in various inflammatory diseases, such as psoriasis (22, 23), rheumatoid arthritis (24, 25), atherosclerosis (26), and renal failure (27).

We previously showed (26, 28) that IL-20 not only promoted angiogenesis but also increased tumor vascularization. Moreover, IL-20 is regulated by hypoxia and contributes to brain injury in an ischemic stroke model, which indicates that IL-20 is a promoting factor in the inflammation-associated disease (29). Most solid tumor cells grow under hypoxic conditions, which may induce IL-20 overexpression. In addition, hypoxia-inducible factor-1α (HIF-1α) is an independent prognostic marker in OSCC and is important in the pathogenesis of oral cancer (30). Little is known about the involvement of IL-20 in the pathogenesis of oral cancer. Therefore, we studied IL-20 expression and its biologic function in oral cancer cells. We also evaluated the therapeutic effects of anti-IL-20 monoclonal antibody (mAb) 7E to alleviate or abrogate key malignant phenotypic characteristics of oral cancer in vitro and in vivo.

Materials and Methods
Participants
Forty patients with oral cancer (stage I, n = 10; stage II, n = 10; stage III, n = 10; and stage IV, n = 10) were enrolled in this study. Tumor and nontumorous tissue from these patients was analyzed. Nontumorous oral tissue was taken after obtaining informed consents for the study from all participants. The study was approved by the Ethics Committee of National Cheng Kung University Hospital (Tainan, Taiwan).

Cell lines
OC-3 cells (31), from a cell line isolated from a Taiwanese patient with OSCC, were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS (Life Technologies), 2 mmol/L of L-glutamine (Life Technologies), 100 μg/mL of streptomycin, and 100 U/mL of penicillin in a humidified 5% CO₂ atmosphere at 37°C. OEC-M1 cells (32), from a cell line derived from the gingival epidermal carcinoma of a Taiwanese patient, were maintained in RPMI-1640 medium.

Immunohistochemistry and immunocytochemistry
Paraffin-embedded tissue samples were used for immunohistochemistry (IHC) staining with anti–IL-20 (7E), anti–IL-20R1, anti–IL-20R2, or anti–IL-22R1 mAb (R&D Systems) at 4°C overnight as previously described (33). Incubating paraffin tissue sections with mouse IgG1 isotype (clone 11711; R&D Systems) instead of primary antibody was the negative control. We used 3 μg/mL as the working concentration for each primary antibody and for the control mouse IgG1. At least 5 sections from each patient’s specimen were analyzed and examined by 2 investigators trained in oral pathology and blinded to patient details. Immunocytochemical staining of IL-20 and its receptors in OC-3 and OEC-M1 cells was done using the same protocol as described earlier.

Quantitative real-time PCR
To examine the oral cancer cell lines and clinical specimens, OC-3 cells, OEC-M1 cells, oral tumor cells, and nontumorous oral tissue specimens from 40 patients were analyzed. Total RNA was isolated (Invitrogen). Reverse transcription was done with reverse transcriptase (Clontech). Human IL-20, IL-20R1, IL-20R2, and IL-22R1 expression was then amplified on a thermocycler (LC 480; Roche Diagnostics), with SYBR Green (Roche Diagnostics) as the interaction agent. Quantification analysis of mRNA was normalized with human glyceraldehyde-3-phosphate dehydrogenase (βGAPDH) used as the housekeeping gene. Relative multiples of change in mRNA expression was determined by calculating 2^ΔΔCt. To examine the expression of TNF-α, IL-1β, and MCP-1, OC-3 and OEC-M1 cells were incubated with IL-20 (200 ng/mL) for the indicated times, and then total mRNA was isolated and analyzed. The expression levels of these genes were analyzed using quantitative real-time PCR (qRT-PCR) with specific primers following the same protocol as described earlier.

Cell proliferation assay
OC-3 cells (3 × 10^5) and OEC-M1 cells (4 × 10^4) were cultured overnight and then exposed to IL-20 (50–200 ng/mL) for 72 hours in medium containing 1% FBS. To show the specific activity of IL-20, 7E (2 μg/mL) was added to the culture system, either alone or together with IL-20 at a 10:1 concentration ratio (7E:IL-20). OC-3 and OEC-M1 cells responses to IL-20 stimulation were assessed using ^3H-thymidine incorporation assays. Briefly,
after 54 hours of incubation, 1 μCi of ³H-thymidine (NEN Life Sciences) was added, and cells were pulse-labeled for 18 hours. The degree of proliferation was determined as counts per minutes, detected using a scintillation counter (Beckman Coulter).

Cell migration assay
OC-3 and OEC-M1 cells were assayed using a 48-well chamber (modified Boyden chamber) housing a polycarbonate filter with 8-μm pores (Nuclepore). The upper wells were loaded with OC-3 (2 × 10⁵) or OEC-M1 (3 × 10⁵) cells. The lower chambers were filled with IL-20 (100 and 200 ng/mL), 7E (2 μg/mL), or IL-20 plus 7E in medium containing 0.1% FBS. Medium with 0.1% FBS was used as a negative control. 7E against IL-20 was used to specifically neutralize the activity of IL-20. The chambers were incubated for 6 hours at 37°C. Cells adhering to the lower side of the filter were fixed with methanol and stained in a Giemsa solution (Diff-Quick; Baxter Healthcare) for counting. The number of OC-3 and OEC-M1 cells on the lower surface of the filter was determined by counting 12 randomly selected fields under a microscope at ×100 magnification. The experiment was done 3 times using quadruplicate wells, and migration was determined as the mean of the total cells counted per field.

Flow-cytometric analysis
We used fluorochromes, 5,6-carboxy-2,7-dichlorofluorescein diacetate (DCFH-DA) to measure the intracellular generation of ROS. OC-3 cells (1 × 10⁶) and OEC-M1 (1.5 × 10⁶) cells were stimulated by the indicated concentrations of IL-20 in serum-free medium for 30 minutes. The OC-3 and OEC-M1 cells were harvested after treatment and stained for 30 minutes at 37°C with 10 μmol/L of DCFH-DA (Molecular Probes). After they had been washed, the cells were kept in the dark and immediately analyzed in a cytometer (FACSCalibur; BD Biosciences). For each analysis, 10,000 events were recorded. The expression of CCR4 and CXCR4 on the surface of IL-20–treated OC-3 and OEC-M1 cells was determined using a flow-cytometric assay. OC-3 (1 × 10⁶) and OEC-M1 (1.5 × 10⁶) cells were treated with IL-20 (100 and 200 ng/mL) for 24 hours. To show the specific activity of IL-20, 7E (2 μg/mL) was added to the culture system, either alone or together with IL-20. The cells were cultured for 10 days, fixed in methanol, and stained with Giemsa. Visible colonies (>50 cells) were counted with the aid of a dissecting microscope.

Western blotting
OC-3 cells (5 × 10⁵) were stimulated with IL-20 (200 ng/mL; R&D Systems) for the indicated time periods. Western blotting used antibodies specific for phosphorylated AKT, c-jun-NH₂ kinase (JNK), extracellular signal–regulated kinase (ERK), and STAT3 (Cell Signaling Technology). α-Tubulin was detected using a specific antibody (Cell Signaling Technology) as an internal control.

Colony formation
OC-3 (2,500/well) and OEC-M1 (3,500/well) cells were suspended in 0.5% Bacto Agar and plated onto a layer of 1% Bacto Agar in medium containing 5% FBS in 6-well tissue-culture plates (Corning). The agar-containing cells were allowed to solidify overnight at 37°C in a 5% CO₂-humidified atmosphere. Additional medium with 5% FBS with IL-20 (200 ng/mL) was overlaid on the agar, and the cells were allowed to grow undisturbed. The culture medium was changed every 2 days. To show the specific activity of IL-20, 7E (2 μg/mL) was added to the culture system, either alone or together with IL-20. The cells were cultured for 10 days, fixed in methanol, and stained with Giemsa. Visible colonies (>50 cells) were counted with the aid of a dissecting microscope.

Oral cancer-bearing tumor model
All animal experiments were carried out according to the protocols based on the Taiwan National Institutes of Health (Taipei, Taiwan) standards and guidelines for the care and use of experimental animals. The research procedures were approved by the Animal Ethics Committee of National Cheng Kung University. Eight-week-old female severe combined immunodeficient (SCID) mice were used in all experiments. The left mammary fat pad of each mouse was subcutaneously injected with OC-3 cells (2 × 10⁶ cells) or OEC-M1 cells (4 × 10⁶ cells). The mice were then randomly assigned to 3 groups (n = 6 per group), and treated with PBS, 7E (5 mg/kg; s.c.), or mouse immunoglobulin G (1gG; 5 mg/kg; s.c.) every 3 days for the duration of the treatment regimen. The antibody was injected (s.c.) into the periphery of the growing tumor in tumor-bearing SCID mice. Healthy controls were not injected with tumor cells. Fifty days after the tumor cells had been injected, the mice were killed and their tumor tissue was harvested and weighted. The tumor tissues isolated from the 6 mice in each group were fixed in 3.7% formaldehyde for hematoxylin and eosin (H&E) staining. To analyze the expression levels of IL-20, TNF-α, IL-1β, and MCP-1, the tumor tissue was placed in PBS solution and homogenized, and then RNA was extracted. Levels of cytokines were detected using qRT-PCR with specific primers as described earlier.

Statistical analysis
Prism 5.0 (GraphPad Software) was used for the statistical analysis. A one-way ANOVA nonparametric test (Kruskal–Wallis test) was used to compare the data between groups. Post hoc comparisons were done using Dunn multiple comparison test. Results are expressed as mean ± SD. Significance was set at P < 0.05.
Results

Higher expression of IL-20 and its receptors in oral cancer

To examine whether IL-20 is involved in the pathogenesis of oral cancer, IHC staining was used to analyze the expression levels of IL-20 in tumorous and nontumorous oral tissue specimens from 40 patients with oral cancer. IL-20 and its receptors were strongly stained on tumor cells of oral tumor tissue but only slightly stained on endothelial cells in nontumorous oral tissue samples (Fig. 1A). Furthermore, qRT-PCR showed that transcript levels of IL-20 (Fig. 1B) and its receptors (Fig. 1C–E) in oral tumor tissue were significantly higher than in nontumorous oral tissue samples. Their expression levels also correlated well with the stages of the oral cancer (Fig. 1B–E). The expression levels of IL-20 and receptor subunits were the highest in stage IV oral tumor tissue. These data suggested that IL-20 is involved in the pathogenesis of oral cancer.

IL-20 increased cell proliferation, migration, and ROS production in oral cancer cells

To study the effects of IL-20 on oral cancer cells in vitro, the expression of IL-20 and its receptors in OC-3 and OEC-M1 cells was analyzed. OC-3 cells were established from an OSCC of a nonsmoking long-term areca (betel nut) chewer (31), whereas OEC-M1 is a cell line derived from gingival epidermal carcinoma of a Taiwanese patient (32). RT-PCR and immunocytochemical staining showed that IL-20, IL-20R1, IL-20R2, and IL-22R1 were all expressed on OC-3 and OEC-M1 cells (Fig. 2A and B). In addition, ELISA was used to detect the endogenous IL-20 protein secreted in the conditioned media of OC-3 (373.1 pg/mL) and OEC-M1
This indicated that IL-20 might target cancer cells using a paracrine or autocrine mechanism. A3H-thymidine incorporation assay was used to analyze the proliferation of oral cancer cells treated with IL-20. IL-20 dose dependently increased OC-3 and OEC-M1 cell proliferation after 72 hours of treatment ($P < 0.05$ compared with the untreated controls; Fig. 2C). To confirm the specificity of IL-20–induced proliferation of OC-3 and OEC-M1 cells, anti–IL-20 mAb 7E was used to neutralize the IL-20–induced proliferation of OC-3 and OEC-M1 cells. 7E inhibited IL-20–induced cell proliferation (Fig. 2C). To determine whether IL-20 affected cell migration in OC-3 and OEC-M1 cells, a Boyden chamber assay was used to evaluate the migration ability (Fig. 2D). IL-20 significantly promoted OC-3 and OEC-M1 cell migration ($P < 0.05$ compared with the untreated controls), which was inhibited by 7E (Fig. 2D). Because oxidative stress is vital in the malignant transformation of oral cancer, we also

Figure 2. IL-20 increased proliferation, migration, and ROS production in oral cancer cells. A, the expression of IL-20 and its receptors in OC-3 and OEC-M1 cells was analyzed using RT-PCR with specific primers. B, IHC staining showed that IL-20 and its receptors, IL-20R1, IL-20R2, and IL-22R1, were expressed in OC-3 and OEC-M1 cells. C, OC-3 (3 x 10⁴) and OEC-M1 (4 x 10⁴) cells were cultured for 72 hours in 1% FBS with or without IL-20 protein (50–200 ng/mL). Cell proliferation was determined using a3H-thymidine incorporation assay. 7E against IL-20 (2 μg/mL) was used to specifically neutralize the proliferative activity of IL-20. Values are mean ± SD of triplicate experiments. D, OC-3 (2 x 10⁵) and OEC-M1 (3 x 10⁵) cells were incubated for 6 hours in medium containing 0.1% FBS with IL-20 (100–200 ng/mL), 7E (2 μg/mL), or IL-20 plus 7E. Medium alone was used as a negative control. 7E against IL-20 (2 μg/mL) was used to inhibit the activity of IL-20. Cell migration was evaluated using a modified Boyden chamber assay. $*, P < 0.05$ versus untreated controls. #, $P < 0.05$ versus treatment with IL-20 alone. Values are mean ± SD of triplicate experiments. E, OC-3 and OEC-M1 cells were treated with IL-20 for the indicated concentrations in serum-free medium for 30 minutes, and ROS generation was measured using flow cytometry. $*, P < 0.05$ versus untreated controls. #, $P < 0.05$ versus treatment with IL-20 alone. Values are mean ± SD of triplicate experiments.
analyzed the effect of IL-20 on ROS generation in OC-3 and OEC-M1 cells. IL-20 significantly and dose dependently increased intracellular ROS production in OC-3 and OEC-M1 cells (P < 0.05 compared with the untreated controls), which was inhibited by 7E (Fig. 2E). These results suggested that IL-20 increased proliferation, migration, and ROS production in oral cancer cells, and was critical in the progression of oral tumor growth.

**IL-20 upregulated TNF-α, IL-1β, MCP-1, CCR4, and CXCR4 in oral cancer cells**

The molecular pathways of cancer-related inflammation were revealed, which allowed us to identify new target molecules that might lead to improved diagnosis and treatment. Therefore, we investigated whether IL-20 is involved in tumor-associated inflammation. We incubated OC-3 and OEC-M1 cells with IL-20 and used qRT-PCR to analyze the expression of proinflammatory cytokines, chemokines, and chemokine receptors. The TNF-α, IL-1β, and MCP-1 expression significantly increased in IL-20–treated OC-3 (Fig. 3A–C) and OEC-M1 cells (data not shown); however, the expression of other inflammatory mediators, namely, IL-6, IL-8, matrix metalloproteinase-2 (MMP-2), MMP-7, and MMP-9, was not affected (data not shown). IL-20 treatment significantly induced CCR4 and CXCR4 expression on the surface of OC-3 cells (Fig. 3D–E). Although flow-cytometric analysis showed that IL-20 increased CCR4 and CXCR4 expression on the surface of OEC-M1 cells, they showed no significant difference when the data were analyzed using ANOVA.

To confirm that IL-20 specifically induced their expression, the OC-3 cells were treated with IL-20 plus 7E. Flow-cytometric analysis showed that 7E had significantly neutralized CCR4 and CXCR4 expression in cotreated OC-3 cells (Fig. 3D–E). In addition, we found that IL-20 expression was significantly correlated with CXCR4 expression in the oral tumor tissue of patients with stage IV cancer (data not shown). To clarify the mechanism that IL-20 uses to...
promote tumor progression, the signal molecules of JNK, STAT3, ERK, and AKT were assessed in IL-20–stimulated OC-3 cells. Western blotting showed that IL-20 promoted the activation of JNK, STAT3, ERK, and AKT (Fig. 3F). These results indicated that IL-20 induced inflammatory mediators in oral cancer, triggered proliferation-associated signals, and was a promoting factor for tumor growth.

**IL-20 promoted colony formation of OC-3 and OEC-M1 cells**

Neoplastically transformed cells show reduced requirements for extracellular growth-promoting factors, and they are not restricted by cell–cell contact. Anchorage-independent growth is one of the hallmarks of the malignant transformation of cells. Therefore, we used a soft agar colony-formation assay to monitor the ability of IL-20 in the anchorage-independent growth of OC-3 and OEC-M1 cells. IL-20 significantly stimulated OC-3 and OEC-M1 cells to form more colonies than the controls formed, which was inhibited by 7E (Fig. 4A and B). OC-3 cells cotreated with IL-20 and 7E formed significantly fewer and smaller (Fig. 4C) colonies in soft agar than did cells treated with IL-20 alone. These results suggest a significant role for IL-20 in the malignant transformation of oral cancer cells.

**7E suppressed in vivo tumor growth and reduced inflammation within the tumor microenvironment**

IL-20 was highly expressed in oral cancer tissue, and it increased oral tumor growth and inflammation in vitro. 7E has shown specificity and neutralization activity in vitro and in vivo (24, 28, 33). Therefore, we analyzed whether 7E reduced tumor growth in vivo. OC-3 cells were injected into the left mammary fat pads of SCID mice. One day later, the mice were injected (s.c.) with PBS, 7E (5 mg/kg), or mlgG (5 mg/kg; n = 6 per group) every 3 days for 50 days. Fifty days after they had been injected with OC-3 cells, all the mice were killed and the tumors were isolated. Tumors were significantly smaller (Fig. 5A) and weighed significantly less (Fig. 5B) in the 7E-treated group than in the control mlgG- and PBS-treated groups. H&E staining showed that immune cell infiltration was reduced in the tumor mass of the 7E-treated group compared with the mlgG-treated control group (Fig. 5C). Tumor tissue from each group was then isolated and homogenized to extract RNA. qRT-PCR assays showed that IL-20 levels in the tumor were lower in the 7E-treated group than in the mlgG-treated group (Fig. 5D). In addition, the expression of the inflammation mediators TNF-α, IL-1β, and MCP-1 was significantly lower in the 7E-treated group than in the mlgG-treated group (Fig. 5E–G). Furthermore, we used OEC-M1 cells to confirm the effect of 7E in tumor-bearing SCID mice and obtained similar results (Supplementary Fig. S1). These results indicated that 7E suppressed tumor growth in vivo by reducing IL-20 expression, decreasing other proinflammatory cytokines, and inhibiting immune cell infiltration.

**Discussion**

We found that both IL-20 and its receptors were highly expressed in primary, nodal-involved, and distantly metastatic oral tumor tissue. IL-20 expression was correlated with the tumor stages in patients with oral cancer. All IL-20 receptor subunits were expressed in our clinical specimens, which suggested that both autocrine and paracrine effects of IL-20 on oral tumors and contributed to the pathogenesis of oral cancer.
IL-20 directly affected oral cancer cell proliferation by activating proliferation-associated signals, such as JNK, ERK, and STAT3, and migration by activating migration-associated signals, such as JNK and ERK. IL-20 also provided a microenvironment for tumor growth and metastasis by inducing ROS and proinflammatory cytokines, which are essential for the malignant process from normal epithelium to invasive oral cancer (34). ROS may increase the inflammatory responses mediated by activating signaling molecules, such as ERK and JNK, and transcription factors, such as NF-κB. NF-κB regulates many proteins involved in tumor initiation, tumor expansion, and metastasis promotion in many types of cancers, including oral cancer (35, 36).

A variety of cytokines, such as IL-1, IL-6, and TNF-α, as well as chemokines, such as IL-8, CXCL5, CXCL6, CXCL7, CCL5, and CCL20, were upregulated in progressive oral cancer cells (6, 37, 38) and in patients with oral cancer (13). The effects of CCL and CXCL chemokines are mediated via their CCR and CXCR receptors. IL-20–treated OC-3 and OEC-M1 cells increased TNF-α and IL-1β production, which are potent proinflammatory cytokines for the growth, metastasis, and immune evasion of oral tumors. We also found that IL-20 induced MCP-1 and CCR4 expression in OC-3 cells. MCP-1 (39, 40) and CCR4 (41, 42) have been associated with several cancers. Therefore, IL-20 may promote leukocyte trafficking, which augments inflammation, tumor cell infiltration, and metastasis in oral tumors, by such a novel, undefined mechanism. Several studies (43, 44) have associated CXCR4 and oral cancer. IL-20 induced more CXCR4 expression on the surface of IL-20–treated OC-3 cells than of the untreated control group. The involvement of CXCR4 signaling in OSCC was mediated by the activation of both the ERK and the AKT/PKB pathways (45). Therefore, IL-20–induced activation of ERK and AKT may cross-link to amplify the CXCR4 signaling for the chemotaxis of oral cancer cells. 7E treatment to inhibit IL-20–induced CXCR4 expression may be a potent antimetastatic therapy against lymph node and distant metastases in cases of CXCR4-related OSCC. IL-20 increased the anchorage-independent growth of OC-3 and OEC-M1 cells, which

Figure 5. Anti–IL-20 mAb treatment suppressed tumor growth in vivo. OC-3 cells (2 × 10^6) were injected (s.c.) into the mammary fat pads of SCID mice. One day later, the mice were injected (s.c.) with PBS, 7E (5 mg/kg), and mIgG (5 mg/kg; n = 6 per group) every 3 days for 50 days. A, tumor size was measured using a vernier caliper. *, P < 0.05 versus mIgG controls. ** P < 0.01 versus mIgG controls. B, mice were killed 50 days after they had been injected with OC-3 cells, and their tumors were collected and weighed. *, P < 0.05 versus mIgG controls. C, tumor sections from each group were stained with H&E (magnification: ×200). Representative photos are shown for each group. D–G, tumor tissue samples from each group (n = 6) were isolated at the end of the experiment. The expression of IL-20, TNF-α, IL-1β, and MCP-1 in the tumor tissue was analyzed using qRT-PCR with specific primers. *, P < 0.05 versus mIgG controls. Results are from a representative experiment. All experiments were done 3 times. Values are means ± SEM.
confirmed that IL-20 was potentially involved in the malignant transformation of oral cells.

IL-20 was upregulated in the tumor tissue of patients with oral cancer. In our mouse tumor model using OC-3 and OEC-M1 cells, we confirmed that IL-20 was highly expressed in the tumor mass, which is consistent with our clinical findings. IL-20 is a hypoxia-inducible gene and the hypoxia response element (HRE) has been identified in IL-20 promoter (29). Areae (betel) nut extract modulates a signaling cascade that induces HIF-1α expression in oral cancer cells (46). HIF-1α is an independent prognostic marker in OSCC (30). Therefore, one possible mechanism upregulating IL-20 in the tumor may be the response of oral cancer cells to hypoxia. HIF-1α binds to the HRE on IL-20 promoter and activates IL-20 expression. HPV infection is a known risk factor in the development of oral cancer (1, 5). Whether HPV regulates IL-20 expression in the pathogenesis of oral cancer is not well understood. Additional investigations of the regulation between IL-20 and HPV infection are required.

7E suppressed OC-3 and OEC-M1 tumor growth and tumor weight in vivo. The inhibition of tumor growth and tumor size in 7E-treated mice correlated with lower IL-20 expression, which indicated that IL-20 was involved in the progression of oral cancer by regulating tumor cell proliferation in vivo.

We hypothesize a working model of IL-20 in oral cancer progression. Oral cancer cells or infiltrated immune cells secrete a large amount of IL-20, which promotes tumor cell proliferation by phosphorylating ERK, JNK, AKT, and STAT3 in an autocrine/paracrine manner. The overexpressed IL-20 stimulates ROS production in tumor cells. IL-20–induced production of proinflammatory cytokines and ROS by tumor cells amplify local inflammation within the tumor microenvironment. Eventually, all of these tumor-promoting effects contribute to the proliferation and survival of malignant cells, angiogenesis, metastasis, and the impairment of immune responses. In addition, the chemotactant activity of IL-20 further increases immune cell infiltration, which results in IL-20 overexpression, which, in turn, forms a positive loop to oral cancer progression. 7E suppresses IL-20–induced tumor growth and local inflammation in vitro and in vivo, which supports our hypothesis.

We previously showed that IL-20 was induced in response to hypoxia; promoted angiogenesis in endothelial cells; induced IL-6, IL-8, and MCP-1 expression in several types of cells; and was a chemoattractant for neutrophils. In the present study, we showed that IL-20 increased the proliferation and migration of oral cancer cells. IL-20 also induced the expression of TNF-α, IL-1β, and MCP-1, as well as the production of CCR4 and CXCR4, on oral cancer cells. These properties of IL-20 provide evidence that IL-20 is involved in many phases of tumor progression and nurtures a microenvironment for tumor cells.

In summary, our findings indicate that IL-20 is involved in oral cancer progression. IL-20 not only directly increased the proliferation, anchorage-independent growth, and migration of cancer cells but also generated a microenvironment that facilitated tumor progression by inducing the production of ROS and of several proinflammatory cytokines and chemokine receptors. We conclude that IL-20 is a novel target for pharmacologic intervention for inhibiting the tumor growth and metastasis of oral cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: Y.-H. Hsu, M.-S. Chang
Development of methodology: Y.-H. Hsu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y.-H. Hsu, D.-B. Shieh
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y.-H. Hsu, D.-B. Shieh, C.-H. Chan
Writing, review, and/or revision of the manuscript: Y.-H. Hsu, C.-C. Wei, M.-S. Chang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y.-H. Hsu, C.-H. Chan
Study supervision: M.-S. Chang

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