Inactivation or Loss of TTP Promotes Invasion in Head and Neck Cancer via Transcript Stabilization and Secretion of MMP9, MMP2, and IL-6

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Abstract

Purpose: Invasion is the critical step in progression of a precancerous lesion to squamous cell carcinoma of the head and neck (HNSCC). Invasion is regulated by multiple proinflammatory mediators. Tristetraprolin (TTP) is an mRNA-degrading protein that regulates multiple proinflammatory mediators. TTP may serve as an excellent treatment target. Rap1 is a ras-like oncoprotein that induces critical signaling pathways. In this study, the role of rap1 in TTP-mediated invasion was investigated.

Experimental Design: Using complementary approaches, we modulated TTP and altered expression of interleukin (IL)-6 and matrix metalloproteinase (MMP) 2/9, which were quantified by ELISA and zymogram. Invasion was evaluated in vitro using the oral-cancer-equivalent (OCE) three-dimensional model and in vivo in the chick chorioallantoic membrane (CAM). The role of rap1 and p38 were established using knockdown strategies.

Results: Downregulation of TTP significantly increased invasion via secretion of MMP9/2 and IL-6. In the novel OCE and CAM invasion models of HNSCC, cells with downregulated TTP destroyed the basement membrane to invade the underlying connective tissue. Rap1 induces p38 mitogen-activated protein kinase (p38)-mediated inactivation of TTP. Inactive TTP enhances transcript stability via binding to the 3'-untranslated region (UTR). High IL-6 and MMP9 are prognostic for poor clinical outcomes in patients with HNSCC.


Introduction

Novel treatment strategies are required for squamous cell carcinoma of the head and neck (HNSCC) because current regimens have only marginally improved survival in 4 decades. HNSCC is the sixth most common cancer globally, with approximately 600,000 new cases a year (1). At approximately 50%, the 5-year survival rate is poorer than breast cancer or melanoma (2). The dismal survival rate is attributed to tumor invasion and metastasis at the time of diagnosis (1). Destruction of the basement membrane and invasion of tumor cells into the underlying tissue are required for the progression of a precancerous lesion to HNSCC. The basement membrane separates the oral epithelium, the tissue of origin of HNSCC, from the underlying connective tissue. Tumor-derived proinflammatory mediators such as matrix metalloproteinase (MMP) 9, MMP2, and interleukin (IL)-6 promote invasion (3, 4), an essential phenotype of cancer progression (5). MMP9 and MMP2 are gelatinases that degrade type IV collagen in the basement membrane (6). The simultaneous upregulation of these cytokines and proinflammatory mediators, each correlated with poor prognosis, may explain why targeted therapy against a single growth factor or its receptor had limited success (7).

Multiple cytokines and proinflammatory mediators promote invasion, underscoring the importance of identifying and targeting a common regulatory mechanism. Proinflammatory mediators are modulated during transcription, posttranscriptionally, and posttranslationally. RNA-binding proteins (RNA-BP) that impact the stability

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Invasion, a critical step in tumor progression, is induced by concurrent upregulation of multiple cytokines and proinflammatory mediators. This may explain why targeted therapy against a single mediator in squamous cell carcinoma of the head and neck (HNSCC) is only marginally successful and underscores the importance of identifying a common regulatory mechanism. Using novel invasion models that recapitulate the oral mucosa, we show that loss or phosphorylation of tristetraprolin (TTP) promotes invasion via matrix metalloproteinase (MMP) 9, MMP2, and interleukin (IL)-6. Moreover, high IL-6 and/or MMP9 are prognostic for poor outcomes in patients with HNSCC. Rap1B, a critical signaling molecule in HNSCC, induces phosphorylation of TTP via p38. These data suggest that targeting TTP expression or phosphorylation may be a novel therapeutic strategy to inhibit invasion and improve patient survival.

Translational Relevance

Invasion, a critical step in tumor progression, is induced by concurrent upregulation of multiple cytokines and proinflammatory mediators. This may explain why targeted therapy against a single mediator in squamous cell carcinoma of the head and neck (HNSCC) is only marginally successful and underscores the importance of identifying a common regulatory mechanism. Using novel invasion models that recapitulate the oral mucosa, we show that loss or phosphorylation of tristetraprolin (TTP) promotes invasion via matrix metalloproteinase (MMP) 9, MMP2, and interleukin (IL)-6. Moreover, high IL-6 and/or MMP9 are prognostic for poor outcomes in patients with HNSCC. Rap1B, a critical signaling molecule in HNSCC, induces phosphorylation of TTP via p38. These data suggest that targeting TTP expression or phosphorylation may be a novel therapeutic strategy to inhibit invasion and improve patient survival.

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Materials and Methods

Tissue microarray

A tissue microarray (TMA), described previously (14, 15), from patients with stage III/IV HNSCC treated with chemotherapy and radiation or surgery and radiation after induction chemotherapy (16), was used for these studies. The TMA was scored for IL-6 (Supplementary Fig. S1) and MMP9 based on no, low, medium, and high staining intensity interpreted as described (11, 14). Representative scoring intensities for IL-6 are shown in Supplementary Fig. S1. Similar reference scoring intensities for MMP9 were previously published (14). Clinical outcomes of the patients analyzed were time to indication of surgery at primary site and time to recurrence, second primary, or death from disease. Marker values were dichotomized into low staining and high staining. Associations were evaluated with Cox proportional hazards models that related time-to-event outcomes to marker levels and other covariates. Models with each marker alone, each marker plus clinical stage, and interaction models with pairs of markers and their interactions were explored. Kaplan–Meier survival curves represent the significant findings of an interaction term between 2 markers in the Cox interaction model. P values represent the results of a Wald χ² test of the interaction.

Cell culture

HNSCC cell lines from the University of Michigan (Ann Arbor, MI) were validated (genotyped) from frozen stock cultures and maintained via continuous passages. Normal human oral keratinocytes were from frozen stocks purchased from ScienCell Research Laboratories and were cultured as described (14, 17). Individual siRNAs were used to knockdown TTP (J-01789-13), IL-6 (J-007993-07), and rap1B (J-010364-06 and -07; Dharmacon). For MMP9, MMP2 (14), and p38 knockdown, ON-TARGETplus siRNA SMART-pools and control NonTargeting SMART-pools were used. Transfections were conducted as described (11). For stable knockdown of TTP, HNSCC cells were transduced with short hairpin RNA [shRNA (shTTP and shscramble control)] in lentiviral particles containing a GFP (Open Biosystems) and were selected as described (11).

Western blot analysis

Whole-cell lysates were immunoblotted with phospho-p38 (p-p38), p38, rap1B, actin, and MMP2 (Cell Signaling), MMP9, TTP, and phosphoserine (Abcam) IL-6 (R&D) primary and horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson Laboratories). For immunoprecipitation of TTP (IP-TTP) detection, HRP-conjugated Rbi-TrueBlot anti-immunoglobulin G (IgG; eBioscience) secondary antibody was used.
Organotypic oral cancer equivalent

AlloDerm (LifeCell) is an acellular dermal matrix used to generate normal oral mucosa with human oral keratinocytes (18). We took advantage of this tissue matrix to develop a three-dimensional (3D) model of invasion of human HNSCC. AlloDerm sections were rehydrated in PBS. The rehydrated tissue with the epidermal side up was coated with human type IV collagen (50 μg/mL; Sigma-Aldrich) overnight at 4°C. UM-SCC-1-shTTP or -shControl cells were trypsinized and seeded on the tissue matrix. Cells proliferated for 2 days on tissue submerged in Dulbecco’s modified Eagle’s medium (DMEM)/FBS. This oral cancer equivalent (OCE) was raised to the air/liquid interface for another 2 days to allow cellular stratification. The harvested, formalin-fixed tissue was divided into 3 parallel segments at 2 to 3 mm intervals before paraffin embedding. Tissue sections were stained with hematoxylin and eosin. Invasive islands and the total number of invasive cells were quantified in 3 separate sections from each OCE, each section with all 3 segments of tissue.

Chick choorioallantoic membrane

The chick choorioallantoic membrane (CAM) consists of surface chorionic epithelium separated by connective tissue from the endodermal allantoic membrane (19, 20). The chorionic epithelium is separated from the underlying connective tissue by an intact epithelial-derived basement membrane that contains type IV collagen (19). UM-SCC-1-shTTP or UM-SCC-1-shControl cells were seeded on the CAM. Day 11 chick embryos were used, as described (19, 21). After 2 days, the CAM was harvested, frozen, sectioned, and stained with hematoxylin and eosin or immunostained for collagen IV and diamidino-2-phenylindole (DAPI; Invitrogen) to highlight the basement membrane and nuclei, respectively (19). Images for GFP, collagen IV, and DAPI were merged in 50% ratios with DP Manager. Invasive islands were quantified in multiple fields and graphed after normalization to control.

ELISA and gelatin zymography

Conditioned media was collected as described (11) and concentrated in Centrifugal Filter Units (Millipore). Gelatin enzyme activity for MMP9 and MMP2 was evaluated as described (14). IL-6 was measured by ELISA (R&D).

cDNA synthesis and qRT-PCR

QIAzol (Qiagen) was used to isolate total RNA, and cDNA was synthesized (11). Quantitative real-time PCR was conducted with SYBR Green Master mix on an Applied Biosystems 7600HT Real-Time PCR machine. Forward and reverse primers are listed in Supplementary Table S1.

mRNA stability

A total of 10 nmol/L of actinomycin D (Sigma) was used to halt transcription for 0 to 3 hours. RNA was collected and processed (11). Cycle threshold values for IL-6, MMP9, and MMP2 were normalized to glyceraldehyde-3-phosphate dehydrogenase and expressed as a percentage of time 0.

3’UTR luciferase assay

UM-SCC-1 and -81B cells were transfected with siRNA targeting TTP (siTTP) or nontarget control siRNA (NT). A promoter-driven firefly luciferase reporter construct containing the 3’UTR of IL-6, MMP9, or MMP2 (SwitchGear) and a Renilla reporter construct (normalization control) were cotransfected with Lipofectomine 2000 (Invitrogen). Luciferase and Renilla were quantified in cell extracts using the DualGlo Reporter Luciferase System (Promega) on LMaxx (Molecular Devices). Firefly luciferase activity was normalized to Renilla activity and expressed as relative luminescence units.

Immunoprecipitation of TTP

IP-TTP was conducted with TTP antibody (Santa Cruz) crosslinked to Amino-Link Plus Coupling Resin beads (Pierce) in the presence of protease (Roche) and phosphatase (Sigma) inhibitors.

Data analysis

Statistical analysis was conducted using a Student t test. A P value of < 0.05 was considered to be significant.

Results

IL-6 and MMP9 are predictive of poor outcomes in HNSCC

The interaction between IL-6 and MMP9 was explored in terms of negative sequelae for HNSCC including short time to recurrence, second primary tumor, or surgery or death from disease (Fig. 1). Patients with low IL-6 and high MMP9 or with high IL-6 and low MMP9 had the poorest outcomes (P = 0.020) followed by patients with both high IL-6 and high MMP9 (P = 0.020). Patients with low IL-6 and low MMP9 had the best outcome with respect to tumor recurrence, second primary tumor, surgery or death from disease. These findings suggest that IL-6 and MMP9, individually or together, have a significant role in HNSCC progression.

Downregulation of TTP promotes invasion through the basement membrane

Invasion, an essential step in tumor progression, is required for transformation of precancerous lesions to HNSCC. In initial in vitro and in vivo studies, we investigated whether TTP modulates invasion. Transient suppression of TTP was optimized with 4 individual siRNAs and the siRNA that had the best sustained knockdown was used for subsequent studies (Supplementary Fig. S2). When TTP was suppressed in UM-SCC-1, a HNSCC cell line, invasion was significantly increased compared with nontarget controls, as well as migration (Fig. 2A, left and right, respectively; *P < 0.01). TTP suppression was verified by immunoblot analysis (Fig. 2B).

To recapitulate the basement membrane of the oral epithelium and the complexity of human connective tissue, we developed a 3D OCE model of invasion to evaluate whether TTP suppression promotes invasion. To facilitate sustained knockdown of TTP for the OCE model, UM-SCC-1 cells were stably transduced with...
control shRNA (UM-SCC-1-shC or shC) or shRNA targeting TTP (UM-SCC-1-shTTP or shTTP). Knockdown was verified by immunoblot analysis (Fig. 2C). In the OCE model, loss of TTP significantly increased invasion compared with corresponding control cells (Fig. 2D, left; arrowheads show tumor islands completely detached from surface epithelium in shTTP). There were significantly more invasive tumor islands (Fig. 2D middle) as well as invasive cells (Fig. 2D, right) in OCE-shTTP compared with control OCE-shC (\[P < 0.01\]).

The oral mucosa consists of surface epithelium separated from the underlying connective tissue by basement membrane. HNSCC occurs when transformed surface oral epithelial cells invade the connective tissue. The effect of TTP on invasion was verified in the CAM in vivo model. Similar to the oral mucosa, the CAM consists of surface epithelium separated from the underlying connective tissue by an intact epithelial-derived basement membrane (19, 22). The human HNSCC cells are placed on top of the surface epithelium and must destroy the basement membrane to invade the connective tissue, thereby simulating human HNSCC. Tumors with UM-SCC-1-shTTP showed degradation (9, 23). AREs are present in multiple cytokines and proinflammatory factors including MMP2 and MMP9 (24). However, TTP-mediated regulation of MMPs via the 3'-UTR has not been investigated in initial studies. The effect of downregulation of TTP on transcripts for IL-6, MMP9, and MMP2 was investigated. The steady state mRNA levels of IL-6, MMP9, and MMP2 were significantly increased in UM-SCC-1-shTTP cells compared with control cells (Fig. 4A, \(*, P < 0.01\)). Similar results were observed in UM-SCC-81B (Supplementary Fig. S4B and S4C). Taken together, these results suggest that low TTP in HNSCC favors secretion of IL-6, MMP9, and MMP2 to promote invasion.

**TTP regulates the stability of transcripts for IL-6, MMP2, and MMP9 via the 3'-UTR**

TTP binds AREs in the 3'-UTR of mRNAs to promote degradation (9, 23). AREs are present in multiple cytokines and proinflammatory factors including MMP2 and MMP9 (24). However, TTP-mediated regulation of MMPs via the 3'-UTR has not been investigated. In initial studies, the effect of downregulation of TTP on transcripts for IL-6, MMP9, and MMP2 was investigated. The steady state mRNA levels of IL-6, MMP9, and MMP2 were significantly increased in UM-SCC-1-shTTP cells compared with control cells (Fig. 4A, \(*, P < 0.01\)). Similar results were observed in UM-SCC-81B (Supplementary Fig. S4A).

To determine whether downregulation of TTP induces invasion via overexpression of IL-6, MMP9, and MMP2, we investigated whether knockdown of these proinflammatory mediators suppresses the shTTP-induced invasion. UM-SCC-1-shTTP cells were transfected with siRNA targeting IL-6, MMP9, or MMP2. Knockdown of IL-6, MMP9, and MMP2 was verified by immunoblot analysis (Fig. 3B). Invasion was evaluated in a FluoroBlok invasion assay (Fig. 3C). UM-SCC-1-shTTP cells exhibit an invasive phenotype (Fig. 3C, NT), which was suppressed when IL-6, MMP2, or MMP9 were downregulated with siRNA (Fig. 3C). Suppression of IL-6, MMP9, and MMP2, all significantly decreased invasion (\(*, P < 0.01\)). No differences in migration were observed. Similar results were observed in UM-SCC-81B (Supplementary Fig. S4B and S4C). Taken together, these results suggest that low TTP in HNSCC favors secretion of IL-6, MMP9, and MMP2 to promote invasion.
Downregulation of TTP promotes invasion. A, UM-SCC-1 cells transfected with NT or siTTP were analyzed for invasion and migration. B, whole-cell lysates were immunoblotted with TTP and actin. Signal intensity was quantified as arbitrary densitometric unit (DU) by Image J software, normalized to actin, and presented as the percentage of NT. C, whole-cell lysates from UM-SCC-1 cells stably transduced with shControl (shC) and shTTP were immunoblotted with TTP and actin, as a loading control. Signal intensity (DU) was quantified, normalized to actin, and presented as the percentage of shC. D, left, hematoxylin and eosin staining of UM-SCC-1-shC and -shTTP cells grown as an OCE. Black arrows depict invasive islands, which are more visible at the higher magnification (right). Middle, quantification of total number of invasive islands. Right, quantification of total number of invaded cells, normalized to total cell number. Data are representative of 2 independent experiments with 2 replicates in each experiment. E, left, UM-SCC-1-shC and -shTTP CAM sections were stained with hematoxylin and eosin. Images were taken under bright-field and fluorescence (GFP) at ×20 and merged to visualize cells invading past the basement membrane (basement membrane, white arrows). Right, quantification of invasive islands. Results are representative of 3 independent experiments. F, CAM sections were immunostained for collagen IV to identify the basement membrane (yellow arrows). Images were taken at ×20 under fluorescence and merged (green-tumor cells, red-Col IV, blue-DAPI). White arrows show invading tumor cells (also Supplementary Fig. S3). *P < 0.01.

**Rap1B activates p38 in HNSCC**

We previously showed that rap1B is a critical signaling molecule in HNSCC and induces extracellular signal-regulated kinase (ERK)/MAPK activation (14, 17, 25, 26). To determine whether rap1B mediates p38 activation in HNSCC, UM-SCC-1 cells were induced with IL-1β (1 ng/ml). An increase in active, GTP-bound rap1B was observed before p38 phosphorylation, suggesting that rap1B is
upstream of p38 (Fig. 5A). Similar results were observed in UM-SCC-81B (Supplementary Fig. S6A).

To verify that rap1B activates p38 in HNSCC, 2 siRNAs targeting rap1B were used. Downregulation of rap1B inhibited IL-1β–induced p38 phosphorylation by 40% in UM-SCC-1 cells compared with corresponding stimulated cells transfected with NT (Fig. 5B). Similar findings were observed in UM-SCC-81B (Supplementary Fig. S6B). Thus, rap1B induces p38 in HNSCC.

**p38-mediated phosphorylation of TTP promotes secretion of proinflammatory mediators**

In macrophages, p38 inactivates TTP via MK2-mediated phosphorylation at 2 serine sites (12, 27). However, the role of p38 in TTP phosphorylation in other cells, including cancer cells, is unknown. Moreover, signaling mechanisms vary between cell types, even in different types of cancer cells. Initial studies focused on constitutive p38 activity in HNSCC. Active, p-p38 was evaluated in whole-cell lysates from a panel of 6 HNSCC cell lines (Fig. 5C). All 6 cell lines exhibited an increase of more than 200% in p-p38 expression compared with normal human oral keratinocytes after normalization to total p38 and actin. Subsequently, TTP phosphorylation was evaluated in 3 HNSCC cell lines that express variable levels of TTP. Because a phospho-TTP antibody was not commercially available, TTP was immunoprecipitated from cell lysates and blotted with phosphoserine and TTP antibodies to detect phospho-TTP and as a loading control, respectively. IL-1β–induced phosphorylation of TTP (Fig. 5E, lane 4) was significantly inhibited with SB203580 (Fig. 5E, lane 6).

In a complementary approach, we inhibited TTP phosphorylation by siRNA-mediated knockdown of p38 and evaluated phospho-TTP (29). UM-SCC-1 cells were transiently transfected with siIL-6, siMMP9, siMMP2, or NT before stimulation with IL-1β. TTP was immunoprecipitated from cell lysates and blotted with phosphoserine and TTP antibodies to detect phospho-TTP and as a loading control, respectively. IL-1β–induced phosphorylation of TTP (Fig. 5F, lane 4) was significantly inhibited with SB203580 (Fig. 5E, lane 6).

To verify that TTP phosphorylation is mediated by p38, we used the chemical inhibitor SB203580, which inhibits the catalytic activity of p38. SB203580 binds to the ATP-binding pocket of p38, thereby inhibiting downstream effector molecules without altering phosphorylation of p38 itself (28). UM-SCC-1 cells were serum starved for 4 hours followed by 2 hours preincubation with 10 μmol/L of SB203580 before stimulation with IL-1β. TTP was immunoprecipitated from cell lysates and blotted with phosphoserine and TTP antibodies to detect phospho-TTP and as a loading control, respectively. IL-1β–induced phosphorylation of TTP (Fig. 5E, lane 4) was significantly inhibited with SB203580 (Fig. 5E, lane 6).

In a complementary approach, we inhibited TTP phosphorylation by siRNA-mediated knockdown of p38 and evaluated phospho-TTP (29). UM-SCC-1 cells were transfected with nontarget or sip38 before stimulation with IL-1β. TTP was immunoprecipitated from cell lysates and blotted with phosphoserine and TTP antibodies to detect phospho-TTP and as a loading control, respectively. IL-1β–induced phosphorylation of TTP (Fig. 5F, lane 4) was significantly inhibited with SB203580 (Fig. 5E, lane 6).
p38-mediated phosphorylation of TTP promotes secretion of proinflammatory mediators.

Discussion

Poor survival is correlated with secretion of proinflammatory mediators (11, 14). Targeted therapy against individual mediators was only marginally successful in treating HNSCC (7), likely due to the concurrent upregulation of multiple mediators with overlapping functions in tumor progression. Targeting each factor may improve prognosis but is impractical, emphasizing the importance of identifying a common regulatory mechanism for multiple mediators. The complementary approaches presented here, conclusively show that downregulation or inactivation of TTP in HNSCC promotes invasion via secretion of multiple proinflammatory mediators. We show that IL-1β stimulates rap1B, which in turn induces p38-mediated phosphorylation of TTP to promote secretion of multiple proinflammatory mediators. Downregulation of or p38-mediated inactivation of TTP promotes invasion via stabilization of mRNAs of MMP9/2 and IL-6. Active TTP promotes transcript degradation via the 3′UTR. Secretion of MMP9/2 and IL-6 promote invasion and progression of HNSCC. This rap1B-p38-induced signaling cascade represents a potential therapeutic target for HNSCC because p38 regulates multiple mediators of invasion. Moreover, p38 inhibitors are orally bioavailable and are already in clinical trials for inflammatory diseases (30).

Figure 4. TTP regulates the stability of transcripts of IL-6, MMP9, and MMP2 via the 3′-UTR. A, left three panels, IL-6, MMP9, and MMP2 mRNA levels were quantified by qRT-PCR from UM-SCC-1 cells transfected with NT and siTTP. Data are representative of 2 independent experiments with 3 replicates in each experiment. Right, whole-cell lysates were immunoblotted with TTP and actin. Signal intensity (DU) was quantified, normalized to actin, and expressed as the percentage of NT. B, left 3, UM-SCC-1shC or shTTP cells were treated with actinomycin D followed by RNA isolation and quantification via qRT-PCR. Right, whole-cell lysates were immunoblotted with TTP and actin. Signal intensity (DU) was quantified, normalized to actin, and expressed as percent of shC. C, left 3, UM-SCC-1 cells transfected with NT or siTTP were cotransfected with a luciferase reporter construct containing the 3′-UTR of IL-6, MMP9, or MMP2 and a Renilla construct for normalization. Data are representative of 3 independent experiments with 3 replicates in each experiment. Right, whole-cell lysates were immunoblotted with TTP and actin antibodies. Signal intensity (DU) was quantified, normalized to actin, and expressed as the percentage of NT. *P < 0.01.
TMA findings in this study, based on a small number of patients, show that either high IL-6 or high MMP9 are correlated with poor outcomes, suggesting that IL-6 and MMP-9 may also be regulated by a mechanism independent of TTP. In fact, expression of proinflammatory cytokines is impacted by transcriptional regulation of genes as well as RNA stability (24). The variation in gene transcription with each HNSCC and redundancy in function between IL-6 and MMP9 may be regulated by a mechanism independent of TTP. In fact, expression of proinflammatory cytokines is impacted by transcriptional regulation of genes as well as RNA stability (24). The variation in gene transcription with each HNSCC and redundancy in function between
proinflammatory mediators underscores the relevance of targeting a common regulatory mechanism of transcript stability, to effectively inhibit adverse effects.

Other RNA-binding proteins that bind transcripts of proinflammatory mediators have been identified but are mostly uncharacterized in HNSCC. HuR (human antigen–related protein), which is overexpressed in HNSCC, increases stability of COX2 and VEGF transcripts (24, 31). ARE/poly(U)-binding and degradation factor (AUF-1) stabilizes granulocyte-macrophage colony-stimulating factor (32), whereas CUG binding protein 1, destabilizes transcripts of TNF-α (33), but neither has been investigated in HNSCC.

MMPs have a critical role in remodeling the extracellular matrix. While remodeling is important for wound healing, excessive MMP production is associated with invasion (34). Invasion is an essential event in tumor progression. We previously showed that MMP9/2 promote invasion and are prognostic of poor outcomes in HNSCC (14). Although we and others showed that the loss of TTP promotes invasion in cancer (11, 35) and MMP9/2 have AREs in the 3’UTR, the role of MMP2 and MMP9 in TTP-mediated invasion has not been investigated. Knockdown of TTP promotes invasion of HNSCC, which was significantly suppressed when IL-6, MMP2, or MMP9 was downregulated with siRNA. Concurrent knockdown of IL-6 and MMP9 lead to such severe apoptosis that the remaining cells were insufficient to conduct invasion assays (not shown). These findings are consistent with an overlap in function between multiple proinvasive mediators. In support of the possibility that loss of TTP promotes invasion via multiple proteins, loss of TTP enhances MMP1, urokinase plasminogen activator, and IL-8 in other cancers (35, 36), all of which promote tumor progression.

While suppression of TTP leads to a greater increase in MMP9 than MMP2 secretion (Figs. 3A and S4A), the smaller increase in MMP2 has a significant effect on invasion given that downregulation of MMP2 had a similar impact on invasion as knockdown of MMP9.

Although p38 mediates TTP phosphorylation in macrophages and endothelial cells (37, 38), this mechanism has not been investigated in cancer, which is important given that each cell type has a unique signaling repertoire. Our data from 2 different HNSCC cell lines using biochemical and siRNA approaches show that p38 phosphorylates and inactivates TTP, and increases secretion of proinflammatory mediators.

Rap1, a critical signaling mediator in HNSCC, facilitates adhesion, proliferation, survival, migration, and invasion in HNSCC (17, 39). Two isoforms exist in HNSCC, rap1A and rap1B. Although active, GTP-bound rap1B has a critical role in ERK/MAPK activation in HNSCC (39), its role in modulating p38 has not been investigated in any cancer. Our findings show a novel mechanism of p38-mediated TTP regulation in cancer via rap1B (Fig. 6). Although we established that IL-1β rapidly activates rap1B in HNSCC, this does not eliminate the possibility that other secreted proteins activate p38 via rap1B in HNSCC.

Together, the novel OCE and CAM models of invasion of human HNSCC supported the in vitro findings that loss of TTP facilitates invasion. In precancerous oral lesions (epithelial dysplasia or carcinoma-in-situ), epithelial changes are restricted to the surface epithelium, that is, above the basement membrane (40). Destruction of the basement membrane and invasion of tumor cells into the underlying connective tissue are required for progression of a precancerous lesion to HNSCC. Thus, the basement membrane is the first structural barrier to invasion (13), but current models for human HNSCC do not recapitulate this structure. Two dimensional invasion assays lack the basement membrane and the complexity of the connective tissue. Alternative 3D assays that investigate invasion on a collagen

**Figure 6. Proposed model for role of TTP in invasion.** TTP, downregulated or functionally inactivated by rap1B and p38, promotes tumor invasion and progression via mRNA stabilization and increased secretion of IL-6, MMP9, and MMP2.
matrix lack basement membrane and connective tissue. The novel in vitro OCE 3D invasion model described here, recapitulates invasion through a basement membrane barrier into human connective tissue. This model was based on the organotypic oral mucosa model that is used for tissue regeneration of normal oral mucosa (18).

Given the structural complexity of the basement membrane and the cellular and structural complexity of the connective tissue itself (13), we developed an in vivo model for invasion of human HNSCC using the chick embryo CAM. Mouse models of human HNSCC are inadequate for invasion because tumor cells are injected directly into the connective tissue thereby bypassing the basement membrane of the surface epithelium. The CAM has been used for breast, brain, and ovarian cancers (19, 22, 41) but not HNSCC. The CAM consists of surface chorionic epithelium separated from the underlying connective tissue by an intact epithelial-derived basement membrane that contains type IV collagen (13, 19). Tissues underlying the CAM contain types I and III collagen and blood vessels (42). Human HNSCC cells are seeded on top of the chorionic epithelium. To invade the underlying connective tissue, the HNSCC cells must destroy the basement membrane thereby recapitulating the process that occurs in human HNSCC. Invasion is easily visualized and quantified. Thus, the CAM model of invasion simulates the basement membrane, the complexity of the connective tissue, and the microscopic features that simulate invasion of human HNSCC. Studies in the OCE and CAM models highlight the role of TTP-mediated invasion in HNSCC.

A recent study further supported the role of low TTP in cancer progression; low TTP mRNA level is a negative prognostic indicator in breast cancer (43). Overexpression of TTP decreases tumor growth and VEGF secretion in colon cancer (44) and decreases invasion in breast cancer (35). Moreover, when treating patients with deficient TTP, a synonymous polymorphism in TTP genes predicted failure to respond to Herceptin/Trastuzumab in patients with HER2-positive breast cancer (45).

Taken together, our findings provide insight into a master regulatory mechanism that promotes invasion in HNSCC. To establish this mechanism, we developed in vitro and in vivo models for HNSCC that recapitulate the complexity of invasion in human oral mucosa. By elucidating TTP-mediated invasion, we uncovered a possible explanation for failure of HNSCC to respond favorably to inhibitors targeting a single proinflammatory mediator. The studies presented here suggest that targeting the p38-TTP cascade is an attractive treatment strategy in HNSCC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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