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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Running Title: Downregulation of tristetraprolin promotes invasion in SCCHN

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TRANSLATIONAL RELEVANCE

Invasion, a critical step in tumor progression is induced by concurrent upregulation of multiple cytokines and pro-inflammatory mediators. This may explain why targeted therapy against a single mediator in SCCHN 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 TTP promotes invasion via MMP9, MMP2 and IL-6. Moreover, high IL-6 and/or MMP9 are prognostic for poor outcomes in patients with SCCHN. Rap1B, a critical signaling molecule in SCCHN, 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.
ABSTRACT

Purpose: Invasion is the critical step in progression of a pre-cancerous lesion to squamous cell carcinoma of the head and neck (SCCHN). Invasion is regulated by multiple pro-inflammatory mediators. Tristetraprolin (TTP) is an mRNA degrading protein that regulates multiple pro-inflammatory 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 IL-6 and MMP2/9, which were quantified by ELISA and zymogram. Invasion was evaluated in vitro using the Oral-Cancer-Equivalent (OCE) 3D 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 SCCHN, 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’-UTR. High IL-6 and MMP9 are prognostic for poor clinical outcomes in SCCHN patients.

Conclusions: Targeting the rap1-p38-TTP cascade is an attractive novel treatment strategy in SCCHN to concurrently suppress multiple mediators of invasion.
INTRODUCTION

Novel treatment strategies are required for squamous cell carcinoma of the head and neck (SCCHN) since current regimens have only marginally improved survival in four decades. SCCHN is the 6th most common cancer globally, with ~600,000 new cases a year (1). At ~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 (BM) and invasion of tumor cells into the underlying tissue are required for progression of a precancerous lesion to SCCHN. The BM separates the oral epithelium, the tissue of origin of SCCHN, from the underlying connective tissue. Tumor-derived pro-inflammatory mediators such as MMP9, MMP2 and 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 BM (6). The simultaneous upregulation of these cytokines and pro-inflammatory 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 pro-inflammatory mediators promote invasion, underscoring the importance of identifying and targeting a common regulatory mechanism. Pro-inflammatory mediators are modulated during transcription, post-transcriptionally and post-translationally. RNA-binding proteins (RNA-BPs) that impact the stability of transcripts have a significant role in tumor progression. RNA-BPs regulate mRNA post-transcriptionally by binding the adenylate-uridylate-rich elements (AREs) in the 3’ untranslated region (3’-UTR) of mRNA thereby inducing decay or stabilization of the transcript (8-9). Tristetraprolin (TTP, also known as TISS11, ZFP36 and Nup475) is an RNA-BP that presents transcripts to the decay machinery (9-
10. Since RNA-BPs regulate multiple pro-inflammatory mediators that promote invasion and tumor progression, RNA-BPs may represent potential treatment targets.

We recently showed that TTP expression is inversely correlated with invasion in SCCHN (11). In macrophages, TTP is inactivated by phosphorylation (12). It is unclear if these mechanisms are conserved in cancer. In the current study, we investigate the mechanism by which TTP mediates invasion of SCCHN. We demonstrate that suppression or p-38-mediated phosphorylation of TTP promotes invasion due to increased secretion of IL-6, MMP9 and MMP2.

The BM, the first and most robust structural barrier to invasion, separates the epithelium from the underlying connective tissue (13). A fundamental challenge of invasion studies is the lack of models for human SCCHN that recapitulate the complexity of the BM and underlying connective tissue. Therefore, we developed novel in vitro and in vivo models of invasion of human SCCHN.

In this study, we investigated the mechanism by which TTP mediates invasion of SCCHN. Using novel models of invasion of human SCCHN, we demonstrate that suppression of TTP leads to an invasive phenotype in vitro and in vivo due to increased secretion of IL-6, MMP9 and MMP2. Additionally, we show that rap1 induces p38 MAPK-mediated inactivation of TTP, which enhances transcript stability of IL-6, MMP9 and MMP2 via the 3’-UTR. TTP and its upstream regulators may represent novel therapeutic targets to suppress multiple pro-inflammatory mediators simultaneously with the potential to improve patient survival in SCCHN.
MATERIALS AND METHODS

**Tissue Microarray.** A TMA, described previously (14-15), from stage III/IV SCCHN patients 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 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 two markers in the Cox interaction model. P values represent the results of a Wald Chi-Square test of the interaction.

**Cell culture:** SCCHN cell lines from the University of Michigan were validated (genotyped) from frozen stock cultures and maintained via continuous passages. 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 target plus siRNA SMART-pools and control NonTargeting SMART-pools were used. Transfections were performed as described (11). For stable knockdown of TTP, SCCHN cells were transduced with short hairpin RNA (shTTP and shscramble control) in
lentiviral particles containing a green fluorescent protein (GFP) (Open Biosystems) and were selected as described (11).

**Western Blot Analysis:** Whole cell lysates were immunoblotted with phospho-p38 (pp38), p38, rap1B, actin, and MMP2 (Cell Signaling), MMP9, TTP and phosphoserine (Abcam) IL-6 (R&D) primary and HRP-conjugated secondary antibodies (Jackson Laboratories). For TTP-IP detection HRP conjugated Rbt-TrueBlot anti-IgG (eBioscience) secondary antibody was used.

**Organotypic Oral Cancer Equivalent (OCE):** 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 3D model of invasion of human SCCHN. AlloDerm sections were rehydrated in PBS. The rehydrated tissue with the epidermal side up was coated with human type IV collagen (50μg/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 DMEM/FBS. This OCE was raised to the air/liquid interface for another two days to allow cellular stratification. The harvested, formalin-fixed tissue was divided into 3 parallel segments at 2-3 mm intervals prior to paraffin-embedding. Tissue sections were stained with Hematoxylin-Eosin. Invasive islands and the total number of invasive cells were quantified in three separate sections from each OCE, each section with all three segments of tissue.

**Chick Chorioallantoic Membrane (CAM):** The CAM consists of surface chorial 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 BM 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 two days, the CAM was harvested, frozen, sectioned and stained with hematoxylin and eosin or
immunostained for collagen IV and DAPI (Invitrogen) to highlight the BM 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.

**Enzyme Link Immunosorbent Assay (ELISA) and Gelatin zymography:** Conditioned media (CM) 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 Q-RT-PCR:** QIAzol (Qiagen) was used to isolate total RNA, and cDNA was synthesized (11). Quantitative Real time PCR was performed with SYBR Green Master mix on an Applied Biosystems 7600HT Real Time PCR machine. Forward and reverse primers are listed in Supplementary Table T1.

**mRNA stability:** 10nM of actinomycin D (Sigma) was used to halt transcription for 0-3h. RNA was collected and processed (11). Cycle threshold values for IL-6, MMP9 and MMP2 were normalized to GAPDH 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 non-target control siRNA (NT). A promoter driven firefly luciferase reporter construct containing the 3’untranslated region (UTR) of IL-6, MMP9 or MMP2 (SwitchGear) and a Renilla reporter construct (normalization control) were co-transfected with Lipofectomine 2000 (Invitrogen). Luciferase and Renilla were quantified in cell extracts using the DualGlo Reporter Luciferase System (Promega) on LMaxx^{384} (Molecular Devices). Firefly luciferase activity was normalized to Renilla activity and expressed as relative luminescence units (RLU).
**Immunoprecipitation of TTP:** Immunoprecipitation of TTP was performed 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 performed using a Student’s *t*-test. A p-value of < 0.05 was considered to be significant.

**RESULTS**

**IL-6 and MMP9 are predictive of poor outcomes in SCCHN.** The interaction between IL-6 and MMP9 was explored in terms of negative sequelae for SCCHN 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 SCCHN progression.

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

To recapitulate the BM of the oral epithelium and the complexity of human connective tissue, we developed a three-dimensional (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 to corresponding control cells (Fig. 2D, left panels; arrowheads show tumor islands completely detached from surface epithelium in shTTP). There were significantly more invasive tumor islands (Fig. 2D middle panel) as well as invasive cells (Fig. 2D, right panel) in OCE-shTTP compared to control OCE-shC (*p<0.01).

The oral mucosa consists of surface epithelium separated from the underlying connective tissue by BM. SCCHN 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 BM (19, 22). The human SCCHN cells are placed on top of the surface epithelium and must destroy the BM to invade the connective tissue, thereby simulating human SCCHN. Tumors with UM-SCC-1-shTTP showed destruction of the BM and more GFP-tagged tumor cells invading the connective tissue compared to corresponding control tumors (Fig. 2E, left panel, arrows on merged shTTP panel). There were significantly more invasive islands per field in shTTP compared to control (Fig. 2E, right panel; *p<0.01). In tumors with shTTP, the BM was completely disrupted (Fig. 2F, lower panel; and Fig. S3, type
IV collagen staining; white arrows highlight invasive tumor cells) while in control tumors the BM appeared intact (Fig. 2F, top panel, yellow arrows show intact BM), supporting that low TTP in SCCHN promotes invasion.

**Downregulation of TTP promotes invasion via upregulation of IL-6, MMP9 and MMP2.** Previously we showed that TTP inhibits IL-6, but its effect on gelatinases (MMP2, MMP9), which promote invasion in SCCHN, is unknown (11, 14). The impact of TTP expression on MMP9 and MMP2 secretion was evaluated by gelatin zymography in conditioned medium from SCCHN cells transfected with NT and siTTP (Fig. 3A). In UM-SCC-1 cells, suppression of TTP increased MMP9 secretion by 89% and MMP2 secretion by 42% compared to cells transfected with NT (Fig 3A middle panel). TTP knockdown was verified (Fig. 3A right panel). Similar findings were observed in UM-SCC-81B, another independent SCCHN cell line (Fig. S4A).

To determine whether downregulation of TTP induces invasion via overexpression of IL-6, MMP9 and MMP2, we investigated whether knockdown of these pro-inflammatory 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 (Fig. S4B, S4C). Taken together these results suggest that low TTP in SCCHN 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 AU-rich elements (AREs) in the 3’-UTR of mRNAs to promote degradation (9, 23). AREs are present in multiple cytokines and pro-inflammatory 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-siTTP cells compared to control cells (Fig. 4A, *p*<0.01). Similar results were observed in UM-SCC-81B (Fig. S5A).

To determine if TTP destabilizes transcripts of IL-6, MMP9 and MMP2, UM-SCC-1-shTTP or UM-SCC-1-shC cells were treated with actinomycin D, which inhibits transcription. Loss of TTP enhanced stability of IL-6, MMP9 and MMP2 transcripts compared to control cells (Fig. 4B, left three panels). IL-6 mRNA stability was shown previously in an independent experiment (11) (*p*<0.01). Downregulation of TTP was verified (Fig. 4B, right panel). This data implies that TTP promotes mRNA degradation of IL-6, MMP9 and MMP2.

To verify that loss of TTP stabilizes the transcripts for IL-6, MMP9 and MMP2 via the 3’-UTR, UM-SCC-1-siTTP and control were co-transfected with a firefly luciferase reporter construct containing the 3’-UTR of IL-6 (schematic in Fig. 4C), MMP9 and MMP2 and a *Renilla* construct. Suppression of TTP significantly increased the relative luminescence of IL-6, MMP9 and MMP2 after normalization to *Renilla* compared to control cells (Fig. 4C, left three panels) (*p*<0.01). TTP knockdown was verified (Fig. 4C, right panel). Similar results were observed in UM-SCC-81B (Fig. S5B). Taken together, these studies show that TTP promotes degradation of mRNA for IL-6, MMP9 and MMP2 via the 3’-UTR.
Rap1B activates p38 in SCCHN. We previously showed that rap1B is a critical signaling molecule in SCCHN and induces ERK/MAPK activation (14, 17, 25-26). To determine if rap1B mediates p38 activation in SCCHN, UM-SCC-1 cells were induced with IL-1β (1ng/ml). An increase in active, GTP-bound rap1B was observed prior to p38 phosphorylation, suggesting that rap1B is upstream of p38 (Fig. 5A). Similar results were observed in UM-SCC-81B (Fig. S6A).

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

p38–mediated phosphorylation of TTP promotes secretion of pro-inflammatory mediators. In macrophages, p38 inactivates TTP via MK2-mediated phosphorylation at two 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 different types of cancer cells. Initial studies focused on constitutive p38 activity in SCCHN. Active, phospho-p38 was evaluated in whole cell lysates from a panel of 6 SCCHN cell lines (Fig. 5C). All 6 cell lines exhibited an increase of greater than 200% in phospho-p38 expression compared to normal human keratinocytes (HOK) after normalization to total p38 and actin. Subsequently, TTP phosphorylation was evaluated in 3 SCCHN cell lines that express variable levels of TTP. Since a phospho-TTP antibody was not commercially available, TTP was immunoprecipitated with TTP antibody and immunoblotted with phosphoserine antibody. Input and IgG controls are shown for each cell lines. After normalization to TTP eluted after immunoprecipitation, UM-SCC-1 had the lowest expression of phospho-TTP of the three SCCHN cell lines (Fig. 5D).
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 pre-incubation with 10μM of SB203580 prior to stimulation with IL1β. TTP was immunoprecipitated from cell lysates and blotted with phosphoserine and TTP antibodies to detect phospho-TTP and as a loading control, respectively. IL1β-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 non-target or sip38 prior to stimulation with IL1β followed by immunoprecipitation of TTP. IL-1β induced phosphorylation of TTP was abrogated by knockdown of p38 (Fig. 5F, top panel, lanes 2 and 4). Knockdown of p38 was verified by immunoblot analysis (Fig. 5F, lower panels, lanes 3 and 4). Similar results were observed in UM-SCC-81B (Fig S6). Taken together, our findings with a chemical inhibitor of p38 and with siRNA show that p38 mediates IL1β-induced phosphorylation of TTP.

To determine if phosphorylation of TTP promotes secretion of pro-inflammatory mediators, p38 was suppressed by siRNA. Knockdown of p38 reduced IL-6 secretion by 45% in UM-SCC-1 (Fig. 5G) and by 75% in UM-SCC-81B, (Fig. S6D) compared to control cells (p<0.01). Together these findings show that p38–mediated phosphorylation of TTP promotes secretion of pro-inflammatory mediators.
DISCUSSION

Poor survival is correlated with secretion of pro-inflammatory mediators (11, 14). Targeted therapy against individual mediators was only marginally successful in treating SCCHN (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 SCCHN promotes invasion via secretion of multiple pro-inflammatory mediators. We demonstrate that IL-1β stimulates rap1B, which in turn induces p38-mediated phosphorylation of TTP to promote secretion of multiple pro-inflammatory mediators. Downregulation 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 SCCHN. This rap1B-p38-induced signaling cascade represents a potential therapeutic target for SCCHN, since p38 regulates multiple mediators of invasion. Moreover, p38 inhibitors are orally bio-available and are already in clinical trials for inflammatory diseases (30).

Tissue microarray 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 pro-inflammatory cytokines is impacted by transcriptional regulation of genes as well as RNA stability (31). The variation in gene transcription with each SCCHN and redundancy in function between pro-inflammatory 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 pro-inflammatory mediators have been identified but are mostly uncharacterized in SCCHN. HuR (human antigen-related protein), which is overexpressed in SCCHN increases stability of COX2 and VEGF transcripts (31-32). ARE/poly(U)-binding and degradation factor (AUF-1) stabilizes granulocyte-macrophage colony-stimulating factor (GM-CSF) (33) whereas CUG binding protein 1 (CUGBP1), destabilizes transcripts of TNFα (34), but neither has been investigated in SCCHN.

MMPs have a critical role in remodeling the extracellular matrix. While remodeling is important for wound healing, excessive MMP production is associated with invasion (35). Invasion is an essential event in tumor progression. We previously showed that MMP9/2 promote invasion and are prognostic of poor outcomes in SCCHN (14). Although we and others showed that loss of TTP promotes invasion in cancer (11, 36) 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 SCCHN, which was significantly suppressed when IL-6, MMP2 or MMP9 was downregulated with siRNA. Concurrent knockdown of IL6 and MMP9 lead to such severe apoptosis that the remaining cells were insufficient to perform invasion assays (not shown). These findings are consistent with an overlap in function between multiple pro-invasive mediators. In support of the possibility that loss of TTP promotes invasion via multiple proteins, loss of TTP enhances MMP1, urokinase plasminogen activator (uPA) and IL-8 in other cancers (36-37), 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 (38-39), this mechanism has not been investigated in cancer, which is important given that each cell type has a unique signaling repertoire. Our data from two different SCCHN cell lines using biochemical and siRNA approaches, demonstrate that p38 phosphorylates and inactivates TTP, and increases secretion of pro-inflammatory mediators.

Rap1, a critical signaling mediator in SCCHN, facilitates adhesion, proliferation, survival, migration and invasion in SCCHN (17, 40). Two isoforms exist in SCCHN, rap1A and rap1B. Although active, GTP-bound rap1B has a critical role in ERK/MAPK activation in SCCHN (40) 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 SCCHN, this does not eliminate the possibility that other secreted proteins activate p38 via rap1B in SCCHN.

Together the novel OCE and CAM models of invasion of human SCCHN 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, i.e. above the BM (41). Destruction of the BM and invasion of tumor cells into the underlying connective tissue are required for progression of a precancerous lesion to SCCHN. Thus, the BM is the first structural barrier to invasion (13), but current models for human SCCHN do not recapitulate this structure. Two dimensional (2D) invasion assays lack the BM and the complexity of the connective tissue. Alternative 3D assays that investigate invasion on a collagen matrix lack BM and connective tissue. The novel in vitro OCE 3D invasion model described here, recapitulates invasion through a BM 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 BM and the cellular and structural complexity of the connective tissue itself (13), we developed an in vivo model for invasion of human SCCHN using the chick embryo CAM. Mouse models of human SCCHN are inadequate for invasion because tumor cells are injected directly into the connective tissue thereby bypassing the BM of the surface epithelium. The CAM has been used for breast, brain and ovarian cancers (19, 22, 42) but not SCCHN. The CAM consists of surface chorionic epithelium separated from the underlying connective tissue by an intact epithelial-derived BM that contains type IV collagen (13, 19). Tissues underlying the CAM contain types I and III collagen and blood vessels (43). Human SCCHN cells are seeded on top of the chorionic epithelium. In order to invade the underlying connective tissue, the SCCHN cells must destroy the BM thereby recapitulating the process that occurs in human SCCHN. Invasion is easily visualized and quantified. Thus, the CAM model of invasion simulates the BM, the complexity of the connective tissue and microscopic features that simulate invasion of human SCCHN. Studies in the OCE and CAM models highlight the role of TTP-mediated invasion in SCCHN.

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 (44). Overexpression of TTP decreases tumor growth and VEGF secretion in colon cancer (45) and decreases invasion in breast cancer (36). Moreover when treating patients with deficient TTP, a synonymous polymorphism in TTP genes predicted failure to respond to Herceptin/Trastuzumab in HER2-positive-breast cancer patients (46).
Taken together, our findings provide insight into a master regulatory mechanism that promotes invasion in SCCHN. To establish this mechanism we developed in vitro and in vivo models for SCCHN that recapitulate the complexity of invasion in human oral mucosa. By elucidating TTP-mediated invasion, we uncovered a possible explanation for failure of SCCHN to respond favorably to inhibitors targeting a single pro-inflammatory mediator. The studies presented here suggest that targeting the p38-TTP cascade is an attractive treatment strategy in SCCHN.

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FIGURE LEGENDS

**Figure 1.** IL-6 and MMP9 are predictive of poor outcome in SCCHN. Immunohistochemistry was performed on tissue sections of a human SCCHN tissue microarray. Interactions with IL-6 and MMP9 were determined with a COX interaction model. Patient groups are indicated by color lines. Low MMP9 and low IL-6 staining intensity (Red, n=14); Low MMP9 and high IL-6 (Yellow, n=8); High MMP9 and low IL-6 (Blue, n=6); High MMP9 and high IL-6 (Green, n=7). Subjects who did not experience the events were censored ($p=0.020$).

**Figure 2:** 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 percent 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 percent of shC. (D) Left Panel: 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 panel: Quantification of total number of invasive islands. Right panel: Quantification of total number of invaded cells, normalized to total cell number. Data are representative of 2 independent experiments with two replicates in each experiment. (E) Left panel: UM-SCC-1-shC and -shTTP CAM sections were
stained with hematoxylin and eosin. Images were taken under bright-field and fluorescence (GFP) at 20X and merged to visualize cells invading past the basement membrane (BM - white arrows). Right panel: Quantification of invasive islands. Results are representative of 3 independent experiments. (F) CAM sections were immunostained for collagen IV to identify the BM (yellow arrows). Images were taken at 20X under fluorescence and merged (green-tumor cells, red-Col IV, blue-DAPI). White arrows show invading tumor cells (also Fig S3). (*p<0.01)

**Figure 3: Downregulation of TTP promotes invasion via IL-6, MMP9 and MMP2.** (A) UM-SCC-1 cells were transiently transfected with NT or siTTP. Gelatin zymography (left panel) was used to quantify MMP9/2 in conditioned medium and expressed as percent of corresponding control (middle panel). TTP knockdown was verified (right panel). Data are representative of 3 independent experiments. (B) Whole-cell lysates from UM-SCC-1-shTTP cells transiently transfected with siIL-6, siMMP9, siMMP2 or NT were immunoblotted with IL-6, MMP9, or MMP2 and actin antibodies. Signal intensity (DU) was quantified, normalized to actin, and expressed as percent of control. (C) Invasion of UM-SCC-1-shTTP cells transiently transfected with siIL-6, siMMP9, siMMP2 or NT was quantified and expressed as percent of NT. Data are representative of two independent experiments with three replicates in each experiment. (*p<0.01)

**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 Q-RT-PCR from UM-SCC-1 cells transfected with NT and siTTP. Data are representative of two independent experiments with three replicates in each experiment. Right Panel: Whole-cell
lysates were immunoblotted with TTP and actin. Signal intensity (DU) was quantified, normalized to actin, and expressed as percent of NT. (B) Left three panels: UM-SCC-1shC or shTTP cells were treated with actinomycin D followed by RNA isolation and quantification via Q-RT-PCR. Right panel: 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 three panels: UM-SCC-1 cells transfected with NT or siTTP were co-transfected 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 three independent experiments with three replicates in each experiment. Right panel: Whole cell lysates were immunoblotted with TTP and actin antibodies. Signal intensity (DU) was quantified, normalized to actin, and expressed as percent of NT. (*p<0.01)

**Figure 5: Rap1B induces p38-mediated phosphorylation of TTP, which promotes secretion of pro-inflammatory mediators.** (A) Whole cell lysates from UM-SCC-1 cells treated with IL-1β were immunoblotted with phospho-p38 (pp38), p38, rap1B and actin. Active GTP bound rap1B (rap1B_{GTP}) was retrieved by a ralGDS pull-down assay. p38 was used for normalization of pp38 and rap1B was used for normalization of rap1B_{GTP}. Signal intensity (DU) was quantified, normalized to its corresponding loading control and expressed as percent of time zero. Data are representative of three independent experiments. (B) Cells were transfected with two siRNAs targeting rap1B (si6 and si7) or NT and stimulated with IL-1β or PBS (control) for 10 minutes. Whole cell lysates were immunoblotted with pp38, p38 and rap1B. Signal intensity (DU) was quantified, normalized to p38 and expressed as percent of stimulated NT. Experiments were performed in triplicate. (C) Whole cell lysates from SCCHN and normal keratinocytes (HOK)
were immunoblotted for pp38, p38 and actin. Signal intensity (DU) was quantified, normalized to p38 then actin and expressed as percent of HOK. (D) TTP was immunoprecipitated from UM-SCC-1, -11A and -81B and were immunoblotted with phosphoserine or TTP. Signal intensity (DU) was quantified, normalized to input and expressed as percent of UM-SCC-1. (E) UM-SCC-1 cells were serum starved and treated with 10μM of SB203580. Clarified cell lysates were immunoprecipitated with TTP antibody, and blotted with anti-TTP and anti-phosphoserine antibodies. (F) Cells transfected with sip38 or NT were incubated with PBS (control) or IL-1β for 10 minutes. Top two panels: Immunoprecipitation was performed as above. Signal intensity (DU) was quantified, normalized to input and expressed as percent of unstimulated NT. Lower three panels: Whole cell lysates from NT and sip38 were also immunoblotted with pp38, p38 and actin. Signal intensity (DU) was quantified, normalized to p38 then actin and expressed as percent of unstimulated NT. Three independent experiments were performed. (G) Cells were transfected with sip38 or NT and cell lysates were immunoblotted with p38 and actin antibodies. Signal intensity (DU) was quantified, normalized and expressed as percent of NT. IL-6 secretion was determined with two replicates in each of three independent experiments. (*p<0.01)

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.
Figure 1
**Figure 2**

**A**

Percent Invasion

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**B**

Migration

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**D**

Quantification of invasive islands

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**H&E**

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**F**

COL IV

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Clinical Cancer Research

Inactivation or loss of TTP promotes invasion in head and neck cancer via transcript stabilization and secretion of MMP9, MMP2 and IL-6

Elizabeth A. Van Tubergen, Rajat Banerjee, Min Liu, et al.

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