

Tristetraprolin Regulates Interleukin-6, Which Is Correlated With Tumor Progression in Patients With Head and Neck Squamous Cell Carcinoma

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BACKGROUND: Tumor-derived cytokines play a significant role in the progression of head and neck squamous cell carcinoma (HNSCC). Targeting proteins, such as tristetraprolin (TTP), that regulate multiple inflammatory cytokines may inhibit the progression of HNSCC. However, TTP's role in cancer is poorly understood. The goal of the current study was to determine whether TTP regulates inflammatory cytokines in patients with HNSCC. **METHODS:** TTP messenger RNA (mRNA) and protein expression were determined by quantitative real-time-polymerase chain reaction (Q-RT-PCR) and Western blot analysis, respectively. mRNA stability and cytokine secretion were evaluated by quantitative RT-PCR and enzyme-linked immunosorbent assay, respectively, after overexpression or knockdown of TTP in HNSCC. HNSCC tissue microarrays were immunostained for interleukin-6 (IL-6) and TTP. **RESULTS:** TTP expression in HNSCC cell lines was found to be inversely correlated with the secretion of IL-6, vascular endothelial growth factor (VEGF), and prostaglandin E2 (PGE₂). Knockdown of TTP increased mRNA stability and the secretion of cytokines. Conversely, overexpression of TTP in HNSCC cells led to decreased secretion of IL-6, VEGF, and PGE₂. Immunohistochemical staining of tissue microarrays for IL-6 demonstrated that staining intensity is prognostic for poor disease-specific survival ($P = .023$), tumor recurrence and development of second primary tumors ($P = .014$), and poor overall survival ($P = .019$). **CONCLUSIONS:** The results of the current study demonstrated that down-regulation of TTP in HNSCC enhances mRNA stability and promotes secretion of IL-6, VEGF, and PGE₂. Furthermore, high IL-6 secretion in HNSCC tissue is a biomarker for poor prognosis. In as much as enhanced cytokine secretion is associated with poor prognosis, TTP may be a therapeutic target to reduce multiple cytokines concurrently in patients with HNSCC. *Cancer* 2011;117:2677-89. © 2011 American Cancer Society.

KEYWORDS: cytokines, interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), prostaglandin E2 (PGE₂), messenger RNA (mRNA) stability.

Cytokines have a significant role in cancer, including roles in malignant transformation, tumor growth, survival, invasion, angiogenesis, and metastasis.¹⁻³ Several cytokines and proinflammatory factors, including interleukin-1 (IL-1), IL-4, IL-6, IL-8, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), prostaglandin E2 (PGE₂), and cyclooxygenase-2 (COX-2), are up-regulated in cancer.^{1,4-6} Some of these molecules mediate the role of inflammation in cancer. By promoting cell proliferation, invasion, and survival, cytokines facilitate tumor progression in multiple cancers, including colorectal, breast, head and neck, and non-small cell lung cancers.^{3,7,8} Because cytokines play a critical role in tumor progression, their expression is highly regulated at many levels: messenger RNA (mRNA) transcription, post-transcription, and the initiation of protein translation. Post-transcriptional regulation of cytokines occurs at different stages such as nuclear export, cytoplasmic localization, stability/degradation, and translation of RNA into protein.⁹ RNA-binding proteins (RNA-BPs) are trans-acting factors that regulate these events by binding *cis* elements on target mRNA. *Cis* elements such as adenine and uridine (AU)-rich elements (AREs) are located

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in the 5'- and 3'-untranslated region (UTR) of mRNA transcripts. Because cytokine transcripts contain AREs in their 3'UTR, proteins that promote degradation or stability of transcripts by binding to this region have therapeutic potential.

Expression of RNA-BPs that regulate cytokine mRNA stability may be altered in cancer.^{10,11} In breast cancer, overexpression of human-related antigen R (HuR), an RNA-BP that stabilizes mRNA, contributes to poor prognosis and the increased secretion of VEGF.¹² Conversely, in colon cancer, tristetraprolin (TTP), which decreases mRNA stability, is down-regulated, correlating with increased COX-2 expression.¹³ These changes in RNA-BPs underscore their significance in cancer progression.

HNSCC is the sixth most common cancer, annually affecting approximately 500,000 individuals worldwide.¹⁴ The 5-year survival rate is approximately 50%, primarily because of late detection. HNSCCs secrete multiple cytokines including VEGF, IL-6, and COX-2.¹⁵⁻¹⁸ Because COX-2 and IL-6 inhibitors decrease cell proliferation and invasion in HNSCC in vitro, cancer therapy targeted toward tumor-secreted inflammatory cytokines may inhibit tumor progression.^{3,17-19} Treating HNSCC with agents directed at a single cytokine is unrealistic because of the secretion of multiple cytokines with overlapping functions.²⁰⁻²² Given the importance of cytokines such as IL-6, PGE₂, tumor necrosis factor- α (TNF- α), VEGF, and epidermal growth factor in HNSCC progression,^{3,5,6} down-regulation of multiple cytokines simultaneously by targeting a common upstream protein may inhibit HNSCC progression. However, to our knowledge, the role of TTP in the regulation of multiple cytokines has not been investigated in HNSCC. Furthermore, the relation between TTP expression and cytokine secretion in HNSCC progression has not been established. In the current study, we investigated whether TTP regulates multiple inflammatory cytokines in HNSCC and the significance of the overexpression of one of these cytokines on tumor progression.

MATERIALS AND METHODS

Cell Culture

The human HNSCC cell lines UM-SCC-1, UM-SCC-5, UM-SCC-11A, UM-SCC-14A, UM-SCC-17B, UM-SCC-22B, UM-SCC-74A, and UM-SCC-81B and OSCC3 cells were cultured as described previously.^{23,24} UM-SCC-1, UM-SCC-5, UM-SCC-11A, UM-SCC-14A,

UM-SCC-17B, UM-SCC-22B, UM-SCC-74A, and UM-SCC-81B were validated by genotyping. OSCC3 cells were obtained from Dr. Peter Polverini at the University of Michigan and were genotyped in the University of Michigan DNA Sequencing Core. The human papillomavirus type 16 (HPV-16)-immortalized human oral keratinocyte cell line (HOK-16B, a generous gift from Dr. No-Hee Park, University of California at Los Angeles) was maintained in low-calcium (Ca²⁺) keratinocyte growth medium (Cascade Biologics, Portland, Ore).²⁵ Primary human oral keratinocytes (NHK) were cultured in oral keratinocyte growth medium (ScienCell, Carlsbad, Calif).

Western Blot Analysis

Whole-cell lysates were prepared by sonicating cell suspensions on ice, and Western blot analysis was performed as described.²⁴ Membranes were blocked and incubated in the primary antibody overnight at 4°C or for 1 hour at room temperature. Antibody concentrations were as follows: rabbit anti-TTP (Abcam, Cambridge, UK) at a dilution of 1:2000, actin (Cell Signaling Technology, Inc, Danvers, Mass) at a dilution of 1:3000, and horseradish peroxidase-conjugated donkey antirabbit immunoglobulin (Ig) G and goat antimouse secondary antibodies (dilutions of 1:2000 to 1:5000; Jackson ImmunoResearch Laboratories, Inc, West Grove, Pa). Immunoreactive proteins were observed using the SuperSignal West Pico Chemiluminescent Substrate system (Pierce, Rockford, Ill) and exposed to x-ray film.

Quantitative-Real-Time-Polymerase Chain Reaction

Total RNA was isolated from cells with QIAzol (Qiagen, Valencia, Calif). Complementary DNA (cDNA) was synthesized using SuperScript II (Invitrogen, Carlsbad, Calif). Quantitative real-time polymerase chain reaction (Q-RT-PCR) was performed with SYBR Green Master Mix on an Applied Biosystems ABI 7500 RealTime PCR System (Applied Biosystems, Foster City, Calif). Data were analyzed using the delta-delta cycle threshold method with normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and then expressed relative to NHK.

Adenoviral Infection and IL-1 β Induction

A DL3 replication-deficient serotype-5 adenovirus (Ad-5) containing cytomegalovirus (CMV)- β -galactosidase (gal) and human TTP were generated through the University of Michigan Vector Core as described.²⁶ The cell lines

UM-SCC-11A and UM-SCC-17B and OSCC3 cells (35×10^4) were transduced with 1000 multiplicity of infection (MOI) Ad-5- β -gal or Ad-5-TTP in serum-free media. Three hours after transduction, 10% fetal bovine serum (FBS) was added. Twenty-one hours later, HNSCC cell lines were serum starved for 4 hours and incubated with 1 ng/mL of IL-1 β or phosphate-buffered saline (PBS) in serum-free media. Conditioned media were collected after 24 hours.

Enzyme-Linked Immunosorbent Assay for IL-6, VEGF, and PGE₂.

Conditioned medium from NHK, HOK-16B, OSCC3, and HNSCC cell lines (UM-SCC-1, UM-SCC-11A, UM-SCC-14A, UM-SCC-17B, UM-SCC-22B, UM-SCC-74A, and UM-SCC-81B) was processed as described.²⁷ The total cell number was measured with a trypan blue enumeration assay. IL-6 and VEGF secreted into the medium were measured by a noncompetitive enzyme-linked immunosorbent assay (ELISA) whereas PGE₂ was measured using a competitive ELISA (R&D Systems, Minneapolis, Minn).

Transient TTP Knockdown

The UM-SCC-1, UM-SCC-22B, and UM-SCC-81B cell lines were transfected with ON-TARGET *plus* TTP (*Zfp36*) SMART Pool (Dharmacon Inc, Lafayette, Colo) small interfering RNA (siRNA). The UM-SCC-1 cell line was transfected with Nucleofectin (Amaxa Biosystems, Gaithersburg, Md) as described.^{23,28} The UM-SCC-22B and UM-SCC-81B cell lines (25×10^4 in 6 well dishes) were transfected with 1 μ g of siRNA using Lipofectomine RNAimax (Invitrogen), as described.²⁹ Four hours after transfection, 10% FBS was added. Conditioned media and whole-cell lysates were collected as described earlier.

Stable TTP Knockdown and mRNA Decay

For stable knockdown of TTP, the UM-SCC-1 and UM-SCC-22B cell lines were infected with short hairpin RNA (shTTP and sh vesicular stomatitis virus glycoprotein [VSVG] control) in lentiviral particles in a VSVG backbone (University of Michigan Vector Core). Stable cell lines were selected in 10 μ g/mL of puromycin (Sigma Chemical Company, St. Louis, Mo). For mRNA decay experiments, 35×10^4 cells were plated in 60-mm dishes. The next day, the cells were serum starved for 4 hours and treated with 10 nM of actinomycin D for 0, 1, 2, and 3 hours (Sigma Chemical Company). After incubation, cells were washed with PBS and RNA was harvested.

Invasion Assay

In vitro cell invasion was determined according to the manufacturer's instructions (BD Biosciences, Franklin Lakes, NJ), as described previously.^{23,28} Cells were washed and plated on Matrigel-coated inserts at 2.5 to 3.5×10^4 cells in Dulbecco modified Eagle medium (DMEM) in triplicate. For control experiments, cells were plated in DMEM on identical inserts that were not coated with Matrigel. The lower chamber contained DMEM with 10% FBS as a chemoattractant. After incubation for 24 hours, cells that had migrated to the lower surface of the membrane were fixed and stained with 10% methanol and crystal violet. To determine the percentage invasion, the average number of cells per field on the invasion inserts was divided by the average number of cells per field on the control inserts and multiplied by 100.

Migration Assay

Cells were plated at 95% density in 24-well plates in triplicate. Twenty-four hours after plating, cells were serum starved for 6 hours, then treated with 25 mM of mitomycin C (Sigma Chemical Company) for 2 hours in serum-free conditions. Media was removed and a scratch was made with a 200- μ L pipette tip. Immediately after the scratch, complete media with 25 mmol of mitomycin C was added and 3 fields in each replicate were photographed at 0 hours and at 24 hours. The area between the migration fronts was determined using ImagePro Plus 5.1 (Media Cybernetics Inc, Bethesda, Md) and the percentage migration at 24 hours was calculated.

University of Michigan Oral Cavity/Oropharyngeal Cancer Organ Preservation Trial

This randomized clinical trial of patients with stage III/IV HNSCC (TNM staging system) compared patients treated concurrently with chemotherapy and radiotherapy versus those treated with surgery and radiotherapy after induction chemotherapy.³⁰ A tissue microarray (TMA) constructed from pretreatment specimens from this clinical trial was used for immunohistochemical studies after Institutional Review Board approval.

Immunohistochemistry

Immunodetection in tissue sections was performed as described²⁴ with affinity-purified anti-IL-6 (R&D Systems; 25 μ g/mL), affinity-purified TTP antibody (Abcam; 3 μ g/mL), or the corresponding IgG controls at the same concentrations.

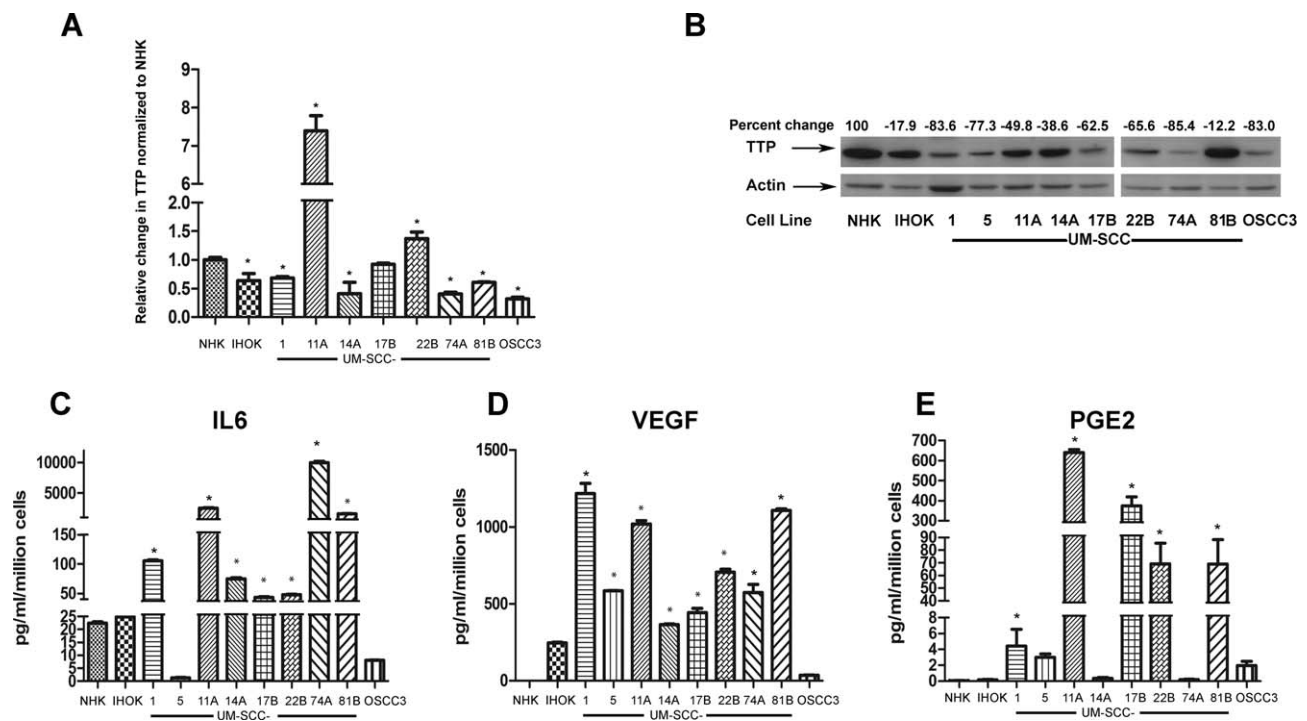


Figure 1. Tetrastrolin (TTP) is down-regulated in head and neck squamous cell carcinoma (HNSCC) cell lines. (A) Total RNA was isolated from normal human keratinocytes (NHK), HOK-16B immortalized keratinocytes (IHOK), and HNSCC cell lines. Complementary DNAs (cDNAs) were prepared and quantitative real-time-polymerase chain reaction was performed using SYBR Green Master Mix (Applied Biosystems, Foster City, Calif). (B) Whole-cell lysates from NHK, IHOK, and HNSCC cell lines were electrophoresed and immunoblotted with TTP antibody. Actin was used as a loading control. TTP expression was quantified by densitometry, normalized to actin and then to NHK, and expressed as the percentage change. (C, D, and E) Conditioned media from NHK, IHOK, and HNSCC cell lines was collected at 24 hours (*indicates $P < .001$ compared with NHK). Enzyme-linked immunoadsorbent assay experiments were performed in triplicate with similar results.

Statistical Analysis

Statistical analysis of in vitro assays was performed using a Student *t* test or 1-way analysis of variance (ANOVA). A *P* value $< .05$ was considered to be statistically significant. For analysis of TMA data, interpretation and scoring were performed by a board certified pathologist, as described.^{23,28} The covariates of interest were T classification and N classification, which were analyzed as ordinal data. The outcomes of interest were overall survival, disease-specific survival (DSS), time to indication of surgery at the primary tumor site, and time to disease recurrence or the development of second primary tumors. The Spearman correlation coefficient was used to evaluate univariate associations between markers and numerical and ordinal variables of interest. The Cox proportional hazards model was used to relate time-to-event outcomes to marker levels and other numerical and ordinal covariates. Statistical analyses of TMA data were performed using SAS statistical software (version 9.0; SAS Institute Inc, Cary, NC). A

2-tailed *P* value $\leq .05$ was considered to be statistically significant.

RESULTS

TTP Is Down-Regulated in HNSCC

TTP expression was evaluated by quantitative real-time-PCR and normalized to GAPDH. The TTP transcript was lower in 6 HNSCC cell lines compared with NHK (Fig. 1A) ($P < .05$, Student *t* test). The UM-SCC-11A and UM-SCC-22B cell lines had significantly more TTP mRNA than NHK ($P < .05$, Student *t* test). Whole-cell lysates from 9 HNSCC cell lines and from 2 nonmalignant cell lines NHK and immortalized keratinocytes (HOK-16B, designated as IHOK) were immunoblotted with anti-TTP antibody. As shown in Figure 1B (immunoblot and densitometric analyses), TTP expression was higher in NHK and IHOK compared with HNSCC cell lines when normalized to actin as a loading control.

OSCC3 cells expressed the least amount of TTP compared with NHK (83% reduction), whereas the UM-SCC-81B cell line demonstrated the least reduction in TTP expression compared with NHK. There was an overall decrease in TTP expression of 62% (standard deviation [SD], 24.6%) in HNSCC cell lines compared with NHK.

IL-6, VEGF, and PGE₂ were quantified by ELISA in conditioned media from normal and IHOK and HNSCC cells to correlate TTP expression with cytokine secretion. In 7 of the 11 HNSCC cell lines, IL-6 secretion was found to be significantly up-regulated (*indicates $P < .001$, 1-way ANOVA for all cell lines noted) in HNSCC cell lines compared with NHK. VEGF and PGE₂, the other 2 cytokines evaluated, demonstrated a similar trend. The increase in VEGF secretion was significant in 8 of 9 HNSCC cell lines (*indicates $P < .001$, 1-way ANOVA). The increase in PGE₂ secretion was significant in 5 of 9 HNSCC cell lines ($P < .001$, 1-way ANOVA). Of the cell lines evaluated, NHK secreted the least VEGF and PGE₂ (Figs. 1D and 1E, respectively). IHOK demonstrated increased VEGF and PGE₂ secretion compared with normal cells, but less than that observed in the HNSCC cell lines. The UM-SCC-81B cell line secreted high levels of all 3 cytokines evaluated, despite high TTP expression in these cell lines (Fig. 1B). However, in the majority of cell lines (>65%), TTP expression was found to be inversely correlated with cytokine secretion.

Overexpression of TTP Reduces Cytokine Secretion in HNSCC

Three HNSCC cell lines with approximately 50% reduced endogenous TTP expression compared with NHK (Fig. 1B) were used for overexpression studies. To verify TTP overexpression, whole-cell lysates were generated from HNSCC cell lines transduced with Ad-5 that contained either TTP or β -gal (control) and were immunoblotted for TTP. As shown in Figure 2A, TTP was overexpressed in the UM-SCC-11A cell line UM-SCC-17B cell line, and OSCC3 cells transduced with Ad-5-TTP compared with cells transduced with control vector.

IL-1 β is secreted by inflammatory cells adjacent to a tumor and activates the p38 mitogen-activated protein kinase (MAPK) pathway within tumor cells.³¹ Mitogen-activated protein kinase-activated protein kinase 2 (MK2), the downstream target of p38 MAPK, inactivates TTP by phosphorylation.^{32,33} Therefore, we investigated the effect of TTP on cytokine secretion in the presence and absence of IL-1 β . In mock-transduced cells, IL-1 β increased cytokine secretion in all 3 cell lines, which is

consistent with inactivation of residual TTP. However, overexpression of TTP led to a significant reduction in IL-6, VEGF, and PGE₂ secretion in all 3 cell lines (Figs. 2B, 2C, and 2D) (*indicates $P < .001$) when compared with cells transfected with control vector, even in the presence of IL-1 β .

In a complementary approach, TTP expression was down-regulated by RNA interference (RNAi) in 3 HNSCC cell lines representing a spectrum of TTP expression. Knockdown of TTP, verified by immunoblot analysis, led to increased secretion of IL-6, VEGF, and PGE₂ (Fig. 3). In the UM-SCC-1 cell line, small interfering TTP (siTTP) mediated a significant increase in all 3 cytokines evaluated (Fig. 3A) ($P < .05$). IL-6 secretion was increased more than PGE₂ secretion in the UM-SCC-22B cell line ($P < .05$). VEGF secretion demonstrated a similar trend (Fig. 3B). In the UM-SCC-81B cell line, which strongly expresses TTP (Fig. 1A), siTTP also induced a significant increase in the secretion of IL-6, VEGF, and PGE₂ (Fig. 3C) ($P < .05$). Collectively, overexpression and knockdown data suggest that TTP inversely regulates IL-6, VEGF, and PGE₂ secretion in HNSCC.

TTP Regulates Cytokine mRNA Stability in HNSCC

To determine whether TTP targets cytokine mRNA for degradation in HNSCC, cells were treated with actinomycin D, which inhibits DNA polymerase. Stable cell lines with shTTP or shVSVG in the UM-SCC-1 and UM-SCC-22B cell lines were generated. Knockdown of TTP, confirmed by immunoblot analysis (Fig. 4A), increased cytokine mRNA stability. For the UM-SCC-1 cell line, mRNA transcripts of IL-6 and COX-2 in shTTP-transduced cells remained between 90% and 100% stable over a 3-hour period. In contrast, in cells transduced with shVSVG, mRNA transcripts degraded to nearly 50% at 3 hours (Fig. 4B) ($P < .05$). Similarly, in the UM-SCC-22B cell line, IL-6 and COX-2 mRNAs were more stable in cells transduced with shTTP (approximately 100% after 3 hours) compared with control (shVSVG) cells, in which IL-6 and COX-2 mRNA were degraded in 3 hours (Fig. 4C) ($P < .05$).

Loss of TTP Enhances Invasion and Migration

In HNSCC, invasion and migration facilitate tumor progression. To determine the functional significance of loss of TTP, invasion and migration assays were performed

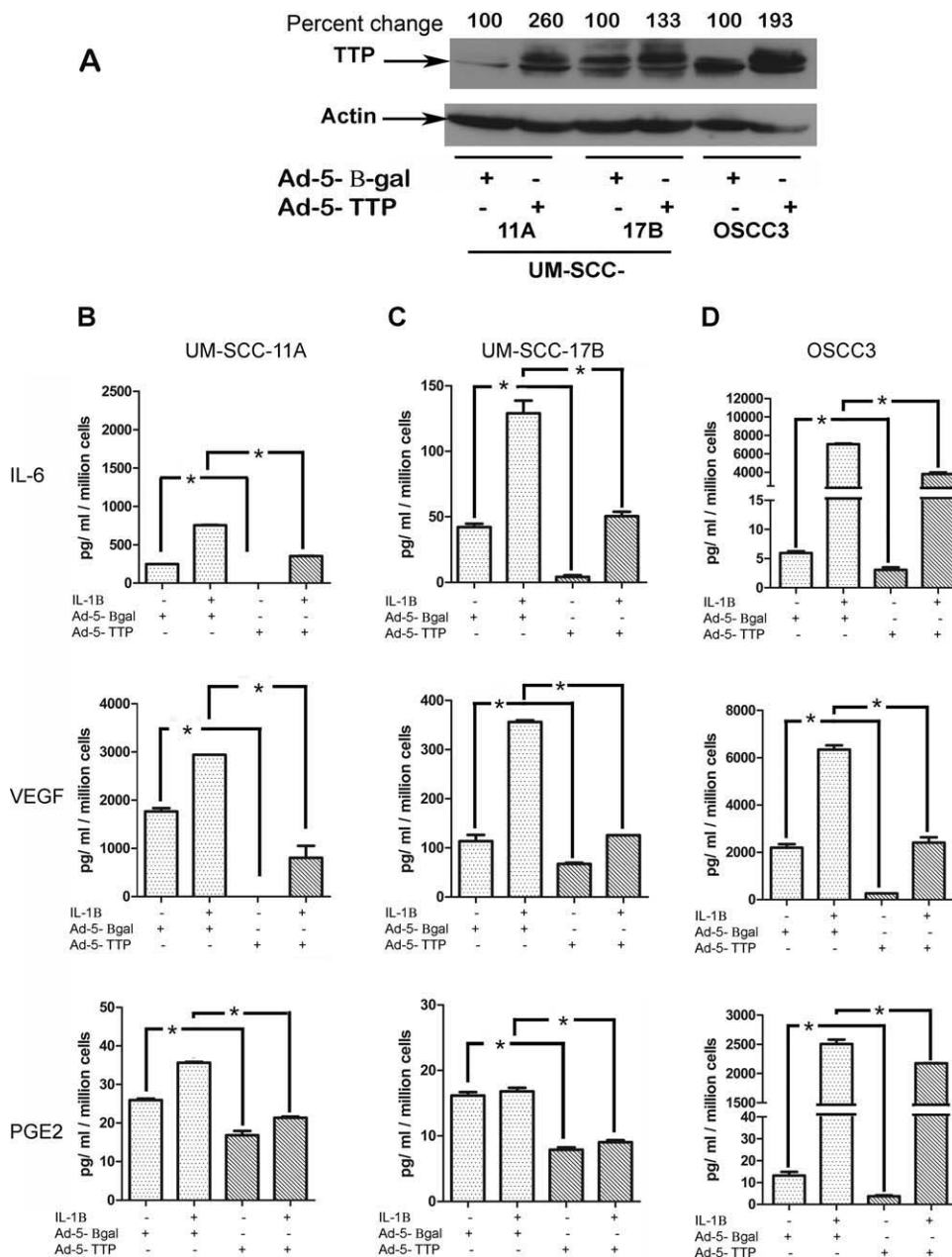


Figure 2. Overexpression of tristetraprolin (TTP) in head and neck squamous cell carcinoma (HNSCC) cell lines decreases cytokine secretion. (A) The UM-SCC-11A cell line, (B) UM-SCC-17B cell line, and (C) OSCC3 cells were transduced with serotype-5 adenovirus containing β -galactosidase (β -gal) (control) or TTP. Cell lysates and conditioned media were harvested. Cell lysates were electrophoresed and immunoblotted with TTP antibody. Conditioned media was used to quantify interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), and prostaglandin E2 (PGE₂) secretion by enzyme-linked immunoadsorbent assay. All experiments were performed in triplicate (*indicates $P < .001$). Standard error bars are present in all graphs. + indicates positive; -, negative.

with the UM-SCC-1 cell line after shRNA-mediated knockdown of TTP (Fig. 4A). Loss of TTP in the UM-SCC-1 cell line increased invasion nearly 300% compared with control cells transduced with empty vector (Fig. 5A). Migration assays demonstrated that suppression of

TTP increases cell migration. In the UM-SCC-1 cell line, the percentage area migrated, normalized to time 0, was 75% in shTTP cells whereas control cells migrated 15% (Fig. 5B). Similar results for invasion and migration were observed in the UM-SCC-22B cell line (data not shown).

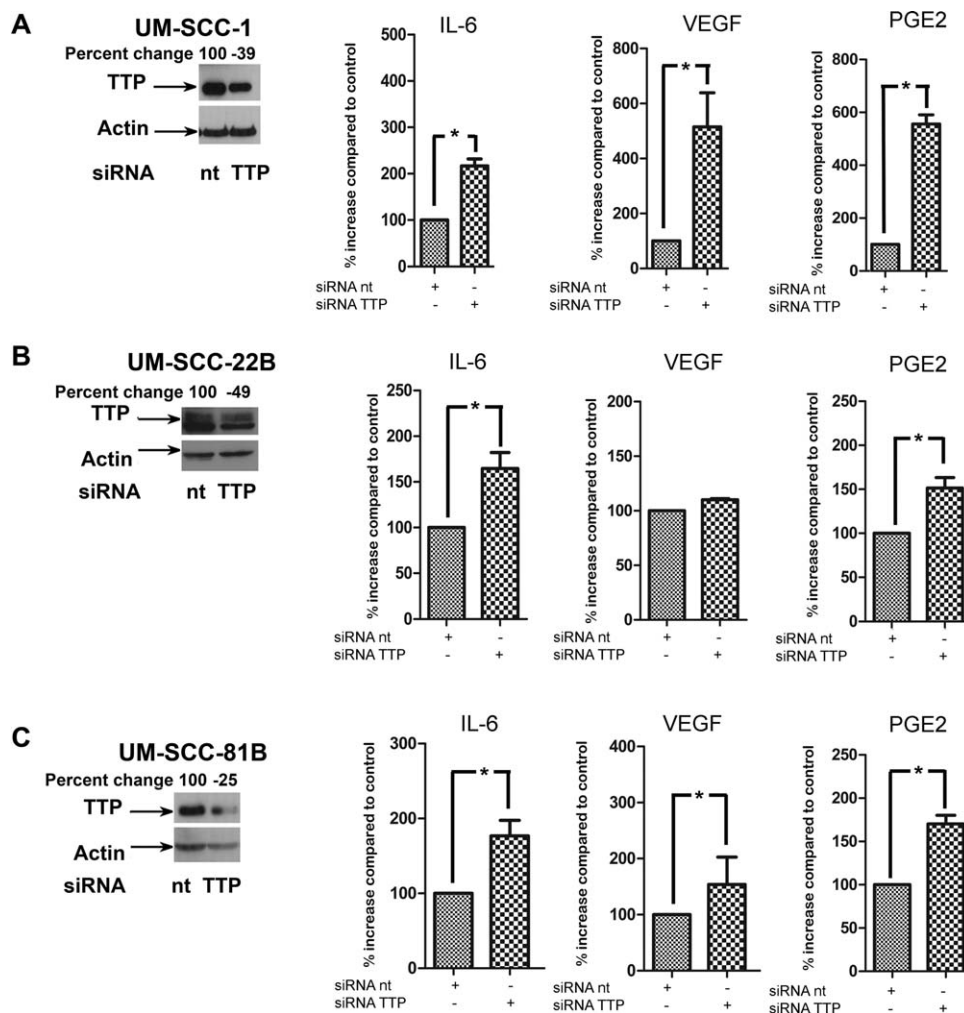


Figure 3. Tristetraprolin (TTP) knockdown induces cytokine secretion in head and neck squamous cell carcinoma cells. (A) The UM-SCC-1, (B) UM-SCC-22B, and (C) UM-SCC-81B cell lines were transfected with small interfering RNA (siRNA) against TTP or nontarget (nt) siRNA. Whole-cell lysates were electrophoresed and immunoblotted with TTP antibody. Actin was used as loading control. Conditioned media was assayed in triplicate for interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), and prostaglandin E₂ (PGE₂) by enzyme-linked immunosorbent assay. All experiments were performed in triplicate. Data are representative of the mean and standard deviation of 3 replicates within an experiment (*indicates $P < .05$). + indicates positive; -, negative.

Increased IL-6 Secretion Is Correlated With Human HNSCC Progression

Immunohistochemical studies were performed on normal epithelial and HNSCC tumor tissue and a TMA of pretreatment tissue specimens from HNSCC. Normal epithelial tissue was found to demonstrate strong TTP staining in the basal one-third of the epithelium (Fig. 6A Left, indicated by arrows), whereas the invasive epithelial islands in the HNSCC tissue did not demonstrate strong TTP staining (Fig. 6A Right, inset). IgG controls were found to be negative (data not shown). Figure 6B (Top and Bottom) demonstrates high IL-6 and low TTP stain-

ing within the same HNSCC core. IgG controls were appropriately negative (data not shown). Low TTP and high IL-6 intensity were found to be correlated with poor DSS, ($P = .043$) (Fig. 6C). In addition, TMA findings revealed that high IL-6 intensity is prognostic for bad patient outcomes, specifically poor DSS ($P = .023$) (Fig. 6D), tumor recurrence or the development of a second primary tumor ($P = .014$) (Fig. 6E), and poor overall survival ($P = .019$) (data not shown). Thus, these findings based on a small sample size indicate that IL-6 and TTP expression are inversely correlated and that IL-6 is associated with poor patient outcome.

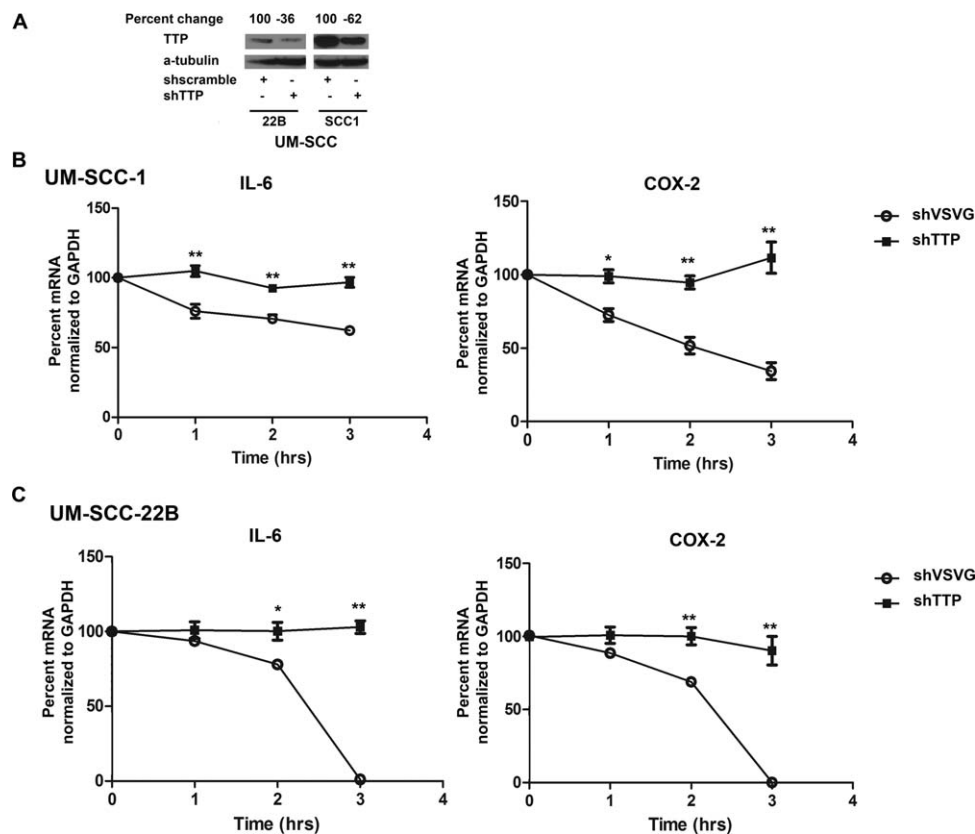


Figure 4. Tristetraprolin (TTP) knockdown increases cytokine messenger RNA (mRNA) half-life. (A) Whole-cell lysates from the UM-SCC-1 and UM-SCC-22B cell lines that stably expressed short hairpin vesicular stomatitis virus glycoprotein (shVSVG) or short hairpin TTP (shTTP) were immunoblotted. (B and C) RNA was purified, complementary DNAs (cDNAs) were prepared, and quantitative real-time-polymerase chain reaction was performed using SYBR Green Master Mix (Applied Biosystems, Foster City, Calif). Cycle threshold values for interleukin-6 (IL-6) and cyclooxygenase-2 (COX-2) were normalized to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and expressed as a percentage of time 0. Data are representative of the mean and standard deviation of 3 individual experiments (*indicates $P < .05$; **, $P < .001$).

DISCUSSION

Changes in oncogenic mRNA stability can alter the production of inflammatory cytokines. For example, up-regulation of HuR, an RNA-BP that stabilizes mRNA, enhances COX-2 and VEGF production.^{34,35} This increase in cytokine production is detrimental because tumor-derived cytokines facilitate oncogenic phenotypes such as invasion, proliferation, and survival, thereby promoting tumor growth.^{1,3,7,13,36} Consistent with this theory, increased COX-2 production in colon cancer increases tumor invasion.⁸ The results of the current study demonstrate that down-regulation of TTP in HNSCC stabilizes transcripts and promotes the secretion of multiple cytokines, including IL-6, VEGF, and COX-2, and increases invasion and migration. Furthermore, IL-6 and TTP expression are inversely correlated in human HNSCC tissue. High IL-6 expression is predictive of poor DSS, tumor recurrence, and the development of sec-

ond primary tumors. On the basis of the findings in this study performed in a small group of patients, patients with HNSCC tumors with high IL-6 staining intensity appear to have a much poorer prognosis than those patients with tumors expressing IL-6 at low levels.

HNSCC is a disease with a poor prognosis, with 5-year survival rates of approximately 50%.³⁷ HNSCCs secrete multiple growth, inflammatory, and angiogenic factors, including VEGF, IL-6, IL-8, and GM-CSF, which promote tumor progression.^{3,4,5} IL-6 promotes tumor growth in mice by promoting the survival and proliferation of HNSCC cells.² Other cytokines, such as VEGF, promote angiogenesis and metastasis.^{37,38}

The critical role of cytokines in the progression of many cancers makes them an attractive treatment target.^{3,4,36,39} In a murine model, IL-6 monoclonal antibodies decreased the size of prostate cancer xenografts significantly.⁴⁰ However, targeting individual cytokines is

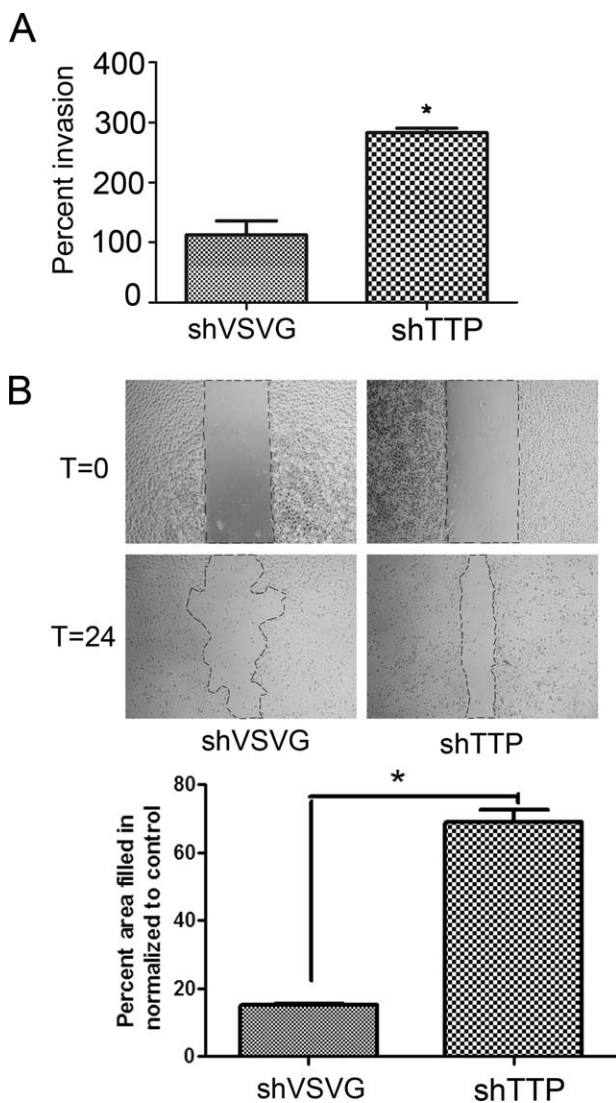


Figure 5. Tristetraprolin (TTP) knockdown increases head and neck squamous cell carcinoma invasion and migration. The UM-SCC-1 cell line was stably transduced with short hairpin TTP (shTTP) or short hairpin vesicular stomatitis virus glycoprotein (shVSVG). (A) Invasion is shown. Transduced cells were plated in serum-free media on Matrigel-coated inserts or control inserts. The percentage invasion was calculated. (B) Migration is shown. The UM-SCC-1 cell line was treated with 25 nmol of mitomycin C (Sigma Chemical Company, St. Louis, Mo). Media was removed and a scratch was made. Immediately after the scratch, complete media Dulbecco modified Eagle medium with 25 nmol of mitomycin C was added and the cells were photographed at 0 hours and at 24 hours. Data are representative of 2 independent experiments with 3 replicates within an experiment (*indicates $P < .02$).

unlikely to be of therapeutic benefit in humans. For example, results from a clinical trial in patients with multiple myeloma with antibodies against IL-6 indicated that there was no improvement in clinical outcome for these patients.³⁹ This suggests that cytokines secreted can com-

pensate for one another and therefore this limits the effect of an inhibitor specific for each cytokine. Targeting a common regulatory mechanism for multiple cytokines such as IL-6, VEGF, and PGE₂ in HNSCC may decrease tumor progression and improve response to treatment.

Cytokine mRNA expression is tightly regulated in resting cells through continuously active mRNA decay mechanisms. Induction of mRNA decay via inflammatory signals facilitates rapid changes in the cellular production of cytokines through alterations in binding of RNA-BP.⁴¹ RNA-BPs regulate multiple cytokines by inducing decay or inhibiting translation. RNA-BPs may bind to multiple AREs, possibly contributing to competition for binding. At least 20 different proteins that can bind to ARE segments have been identified to date, including TTP, HuR, butyrate response factor (BRF)-1 and BRF-2, ARE/poly(U)-binding/degradation factor-1 (AUF-1), T cell intracellular antigen-1 (TIA-1) and T-cell-restricted intracellular antigen-related protein (TIAR). However, only a subset of RNA-BPs has been shown to influence the stability or translational efficiency of target mRNAs.

One of the most well-studied RNA-BPs is TTP, which targets mRNA for rapid degradation by binding to the AREs in the 3'UTR.⁴² Studies with knockout mice suggest that TTP is crucial in regulating TNF- α and IL-6.⁴³ Because TTP regulates mRNAs of multiple inflammatory cytokines by increasing turnover and inducing decay, loss of TTP may lead to the increased production of multiple cytokines, which may be associated with tumor progression. In some cancers, such as colon cancer, loss of TTP contributes to tumor growth by increasing COX-2, VEGF, or matrix metalloproteinase-1 (MMP-1) production.^{12,13,44,45} However, these studies did not evaluate how TTP overexpression influences the production of multiple cytokines. In the current study, we demonstrated that knockdown of TTP in HNSCC stabilizes the transcripts of multiple cytokines and enhances invasion and migration.

Because of the host-tumor microenvironment, inflammatory mediators, such as IL-1 β , produced by inflammatory cells that are adjacent to the tumor may activate the p38 MAPK pathway within the tumor cells.³¹ Therefore, in the current study, we investigated the effect of TTP on cytokine secretion in the presence or absence of IL-1 β . However, TTP overexpression was found to decrease the secretion of cytokines regardless of stimulation with IL-1 β . Although the absolute value of the reduction (ie, pg/mL/million cells) was greater with the addition of IL-1 β , the fold decrease in cytokine secretion

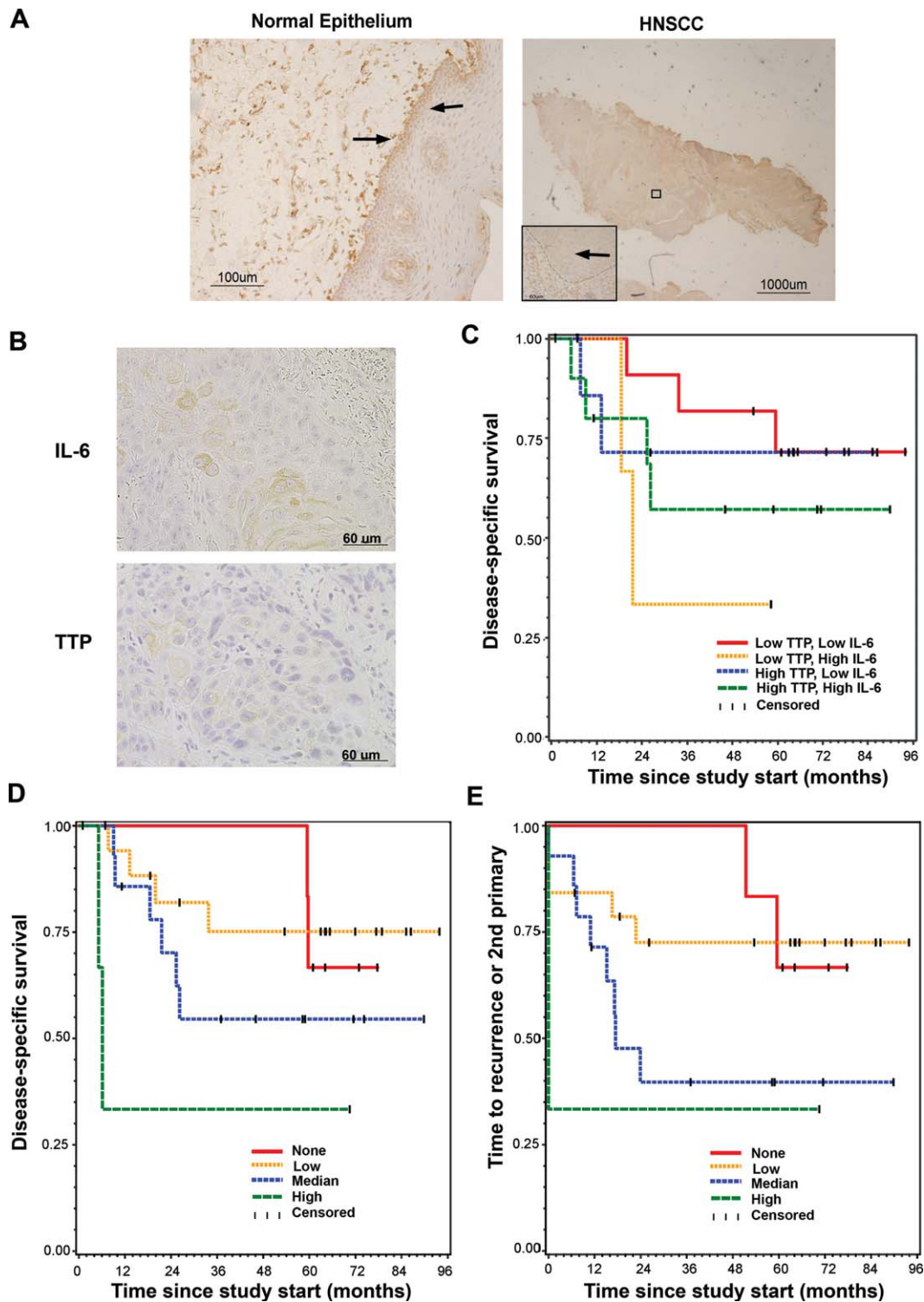


Figure 6. Interleukin-6 (IL-6) intensity is prognostic for disease-specific survival and tumor recurrence in patients with head and neck squamous cell carcinoma (HNSCC). (A) Immunohistochemistry was performed on tissue sections of normal human epithelium (*Left*; bar = 100 µm) and human HNSCC (*Right*; bar = 1000 µm) with tristetraprolin (TTP) antibody. Arrows indicate staining in the basal one-third of normal epithelium. *Inset*: Higher magnification of the outlined box demonstrating invasive epithelium is shown (bar = 60 µm). Arrow indicates invasive epithelium. (B) Immunohistochemistry was performed on tissue sections of a human HNSCC tissue microarray with the IL-6 and TTP antibodies. The slides were counterstained with hematoxylin. The top and bottom panels represent high IL-6 and low TTP staining, respectively, in the same HNSCC tissue section (bars = 100 µm). (C) Low TTP and high IL-6 are correlated with poor disease-specific survival. Patient groups are indicated by colored lines. Red line indicates low TTP and low IL-6 (n = 9 patients); yellow line, low TTP and high IL-6 (n = 3 patients); blue line, high TTP and low IL-6 (n = 11 patients); green line, high TTP and high IL-6 (n = 10 patients). Events (indicated by a drop in the graph lines) were deaths from HNSCC. Subjects who did not experience the events, such as patients who died of unrelated causes, were censored. (D and E) High IL-6 intensity is prognostic for (D) poor disease-specific survival and (E) the development of tumor recurrence and second primary tumors. Patient groups are indicated by colored lines. Red line indicates no IL-6 (n = 6 patients); yellow line, low IL-6 (n = 19 patients); blue line, medium IL-6 (n = 14 patients); green line, high IL-6 (n = 3 patients). IL-6 intensity is also prognostic for poor overall survival (data not shown).

after TTP overexpression was greater in unstimulated compared with IL-1 β -treated cells, which is consistent with inactivation of part of the overexpressed TTP by IL-1 β .

TTP expression was found to be reduced in all HNSCC cell lines compared with NHK. We observed that there was a 62% (SD, 24.6%) reduction in TTP expression in HNSCC compared with NHK. However, reduced TTP expression was not found to correlate 100% with increased cytokine secretion in HNSCC cells, which may be because of phosphorylation-mediated inactivation of TTP. MK2, the downstream target of p38 MAPK, phosphorylates TTP at serine sites 52 and 178 and renders the protein inactive by inducing binding of TTP to 14-3-3 protein.^{32,33} We observed that TTP expression was inversely correlated with cytokine secretion except for the UM-SCC-81B cell line, which was found to strongly express TTP but secreted high levels of cytokines. This may be the result of TTP phosphorylation because active p38 MAPK, which inactivates TTP by phosphorylation, is up-regulated in HNSCC. We were unable to immunoprecipitate TTP using available antibodies, but p38 MAPK is active in this cell line. Collectively, these data suggest that loss or inactivation of TTP contributes to the production of multiple cytokines in HNSCC. Because p38 MAPK can be constitutively active in HNSCC,¹⁵ the results of the current study suggest that inhibition of p38 MAPK in HNSCC would decrease cytokine secretion by preventing inactivation of TTP.

We also observed that TTP knockdown increased the secretion of multiple cytokines, but the effects on individual cytokines varied. This suggests that other pathways or multiple RNA-BPs are involved in the regulation of cytokines and the final effect on secretion may be determined by a balance among these factors. For example, TTP overexpression in mouse embryonic fibroblasts isolated from TTP knockout mice decreased VEGF but increased proliferation, whereas TTP overexpression in HeLa cells decreased cellular proliferation,¹² suggesting that other proteins may influence proliferation in different cell types. These studies underscore the importance of delineating how cytokines are regulated in the context of a particular cell or tissue.

IL-6 is a pleiotropic cytokine that functions during inflammation, immunity, bone metabolism, neural development, reproduction, and hematopoiesis.⁴⁶ Although epithelial malignancies may have elevated IL-6, to the best of our knowledge, the presence of tumor-derived IL-6 in HNSCC has not been investigated in the context of

tumor progression.⁴⁷ The identification of biomarkers that are prognostic of poor survival will allow for the selection of tumors for more aggressive treatment. The current study findings, derived from a small group of patients, suggest that HNSCCs with low TTP and high IL-6 expression have poorer DSS than HNSCCs with high TTP and low IL-6 expression. Similarly, in breast cancer, down-regulation of TTP is a negative prognostic indicator associated with increased tumor grade and mortality.¹² However, TTP expression may not be the actual prognostic indicator in HNSCC. Our findings with IL-6, independent of TTP expression, support this theory. HNSCCs with high IL-6 expression have poor outcomes, including poor DSS, tumor recurrence, and the development of second primary tumors. Consistent with these observations, high serum IL-6 in HNSCC patients is correlated with poor overall survival and an increase in IL-6 during treatment is associated with tumor recurrence.^{37,48} Furthermore, high serum IL-6 is associated with resistance to radiotherapy.⁴⁹

The results of the current study demonstrate that loss of TTP in HNSCC increases invasion and cell migration. Similarly, in breast cancer, invasion is decreased when TTP expression is re-established.⁴⁵ The increased invasion and migration may be because of increased cytokine production or TTP may directly regulate proteins responsible for migration in HNSCC.⁴² TTP stabilizes multiple transcripts that affect invasion and tumor progression, including VEGF, COX-2, urokinase plasminogen activator, MMP-1, and IL-8.^{13,44,45,50}

Given the importance of cytokines in invasion and proliferation, both of which contribute to HNSCC progression, disruption of cytokine secretion is an attractive treatment strategy. However, targeting cancers with a cocktail of inhibitors is impractical and, as mentioned earlier, targeting individual cytokines, such as monoclonal antibodies against IL-6 in patients with multiple myeloma, is ineffective in improving patient survival.³⁶ Therefore, up-regulation of a protein, such as TTP, that inhibits multiple cytokines is an attractive treatment strategy, particularly if it can be combined with p38 inhibitors, because p38, which inactivates TTP, is constitutively active in HNSCC.¹⁵ However, because of the importance of cytokines in the immune response, the potential for systemic immunosuppression as a side effect of p38 inhibitors would be a challenge, particularly because it may promote tumor progression.^{51,52} This may be overcome by strategies that specifically target the treatment to tumor cells. Future studies will explore the mechanisms by which

TTP-mediated cytokine secretion regulates tumor progression and the feasibility of targeting this protein to control tumor growth.

CONFLICT OF INTEREST DISCLOSURES

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REFERENCES

- Aggarwal BB, Gehlot P. *Inflammation and cancer: how friendly is the relationship for cancer patients? Curr Opin Pharmacol.* 2009;9:351-369.
- Dong G, Loukinova E, Smith CW, Chen Z, Van Waes C. Genes differentially expressed with malignant transformation and metastatic tumor progression of murine squamous cell carcinoma. *J Cell Biochem Suppl.* 1997;28-29:90-100.
- Kanazawa T, Nishino H, Hasegawa M, et al. Interleukin-6 directly influences proliferation and invasion potential of head and neck cancer cells. *Eur Arch Otorhinolaryngol.* 2007;264:815-821.
- Chen Z, Malhotra PS, Thomas GR, et al. Expression of proinflammatory and proangiogenic cytokines in patients with head and neck cancer. *Clin Cancer Res.* 1999;5:1369-1379.
- Pries R, Nitsch S, Wollenberg B. Role of cytokines in head and neck squamous cell carcinoma. *Expert Rev Anticancer Ther.* 2006;6:1195-1203.
- Pries R, Wollenberg B. Cytokines in head and neck cancer. *Cytokine Growth Factor Rev.* 2006;17:141-146.
- Singh B, Berry JA, Shohar A, Ramakrishnan V, Lucci A. COX-2 overexpression increases motility and invasion of breast cancer cells. *Int J Oncol.* 2005;26:1393-1399.
- Tsujii M, Kawano S, DuBois RN. Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. *Proc Natl Acad Sci U S A.* 1997;94:3336-3340.
- Anderson P. Post-transcriptional control of cytokine production. *Nat Immunol.* 2008;9:353-359.
- Audic Y, Hartley RS. Post-transcriptional regulation in cancer. *Biol Cell.* 2004;96:479-498.
- Carrick DM, Blackshear PJ. Comparative expression of tristetraprolin (TTP) family member transcripts in normal human tissues and cancer cell lines. *Arch Biochem Biophys.* 2007;462:278-285.
- Brennan SE, Kuwano Y, Alkharouf N, Blackshear PJ, Gorospe M, Wilson GM. The mRNA-destabilizing protein tristetraprolin is suppressed in many cancers, altering tumorigenic phenotypes and patient prognosis. *Cancer Res.* 2009;69:5168-5176.
- Young LE, Sanduja S, Bemis-Standoli K, Pena EA, Price RL, Dixon DA. The mRNA binding proteins HuR and tristetraprolin regulate cyclooxygenase 2 expression during colon carcinogenesis. *Gastroenterology.* 2009;136:1669-1679.
- Parkin DM, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: Globocan 2000. *Int J Cancer.* 2001;94:153-156.
- Riebe C, Pries R, Kemkers A, Wollenberg B. Increased cytokine secretion in head and neck cancer upon p38 mitogen-activated protein kinase activation. *Int J Mol Med.* 2007;20:883-887.
- Nystrom ML, McCulloch D, Weinreb PH, et al. Cyclooxygenase-2 inhibition suppresses alphavbeta6 integrin-dependent oral squamous carcinoma invasion. *Cancer Res.* 2006;66:10833-10842.
- Peters WH, Lacko M, Te Morsche RH, Voogd AC, Oude Ophuis MB, Manni JJ. COX-2 polymorphisms and the risk for head and neck cancer in white patients. *Head Neck.* 2009;31:938-943.
- Sappayatosok K, Maneerat Y, Swadison S, et al. Expression of pro-inflammatory protein, iNOS, VEGF and COX-2 in oral squamous cell carcinoma (OSCC), relationship with angiogenesis and their clinico-pathological correlation. *Med Oral Patol Oral Cir Bucal.* 2009;14:E319-E324.
- Kurihara Y, Hatori M, Ando Y, et al. Inhibition of cyclooxygenase-2 suppresses the invasiveness of oral squamous cell carcinoma cell lines via down-regulation of matrix metalloproteinase-2 production and activation. *Clin Exp Metastasis.* 2009;26:425-432.
- Egloff AM, Grandis JR. Targeting epidermal growth factor receptor and SRC pathways in head and neck cancer. *Semin Oncol.* 2008;35:286-297.
- Paul WE. Pleiotropy and redundancy: T cell-derived lymphokines in the immune response. *Cell.* 1989;57:521-524.
- Sok JC, Coppelli FM, Thomas SM, et al. Mutant epidermal growth factor receptor (EGFRvIII) contributes to head and neck cancer growth and resistance to EGFR targeting. *Clin Cancer Res.* 2006;12:5064-5073.
- Mitra RS, Goto M, Lee JS, et al. Rap1GAP promotes invasion via induction of matrix metalloproteinase 9 secretion, which is associated with poor survival in low N-stage squamous cell carcinoma. *Cancer Res.* 2008;68:3959-3969.
- Mitra RS, Zhang Z, Henson BS, Kurnit DM, Carey TE, D'Silva NJ. Rap1A and rap1B ras-family proteins are prominently expressed in the nucleus of squamous carcinomas: nuclear translocation of GTP-bound active form. *Oncogene.* 2003;22:6243-6256.
- Park NH, Min BM, Li SL, Huang MZ, Cherick HM, Doniger J. Immortalization of normal human oral keratinocytes with type 16 human papillomavirus. *Carcinogenesis.* 1991;12:1627-1631.
- Patil CS, Liu M, Zhao W, et al. Targeting mRNA stability arrests inflammatory bone loss. *Mol Ther.* 2008;16:1657-1664.
- Henson BS, Neubig RR, Jang I, et al. Galanin receptor 1 has anti-proliferative effects in oral squamous cell carcinoma. *J Biol Chem.* 2005;280:22564-22571.
- Goto M, Mitra RS, Liu M, et al. Rap1 stabilizes beta-catenin and enhances beta-catenin-dependent transcription and invasion in squamous cell carcinoma of the head and neck. *Clin Cancer Res.* 2010;16:65-76.
- Gillenwater AM, Zhong M, Lotan R. Histone deacetylase inhibitor suberoylanilide hydroxamic acid induces apoptosis through both mitochondrial and Fas (Cd95) signaling in head and neck squamous carcinoma cells. *Mol Cancer Ther.* 2007;6:2967-2975.
- Wolf GT, Urba S, Hazuka M. Induction chemotherapy for organ preservation in advanced squamous cell carcinoma of

- the oral cavity and oropharynx. *Recent Results Cancer Res.* 1994;134:133-143.
31. Ng DC, Long CS, Bogoyevitch MA. A role for the extracellular signal-regulated kinase and p38 mitogen-activated protein kinases in interleukin-1 beta-stimulated delayed signal transducer and activator of transcription 3 activation, atrial natriuretic factor expression, and cardiac myocyte morphology. *J Biol Chem.* 2001;276:29490-29498.
 32. Heidenreich O, Neining A, Schratz G, et al. MAPKAP kinase 2 phosphorylates serum response factor in vitro and in vivo. *J Biol Chem.* 1999;274:14434-14443.
 33. Winzen R, Kracht M, Ritter B, et al. The p38 MAP kinase pathway signals for cytokine-induced mRNA stabilization via MAP kinase-activated protein kinase 2 and an AU-rich region-targeted mechanism. *EMBO J.* 1999;18:4969-4980.
 34. Brosens LA, Keller JJ, Pohjola L, et al. Increased expression of cytoplasmic HuR in familial adenomatous polyposis. *Cancer Biol Ther.* 2008;7:424-427.
 35. Levy AP, Levy NS, Goldberg MA. Post-transcriptional regulation of vascular endothelial growth factor by hypoxia. *J Biol Chem.* 1996;271:2746-2753.
 36. Hideshima T, Akiyama M, Hayashi T, et al. Targeting p38 MAPK inhibits multiple myeloma cell growth in the bone marrow milieu. *Blood.* 2003;101:703-705.
 37. Druzgal CH, Chen Z, Yeh NT, et al. A pilot study of longitudinal serum cytokine and angiogenesis factor levels as markers of therapeutic response and survival in patients with head and neck squamous cell carcinoma. *Head Neck.* 2005;27:771-784.
 38. Grunstein J, Roberts WG, Mathieu-Costello O, Hanahan D, Johnson RS. Tumor-derived expression of vascular endothelial growth factor is a critical factor in tumor expansion and vascular function. *Cancer Res.* 1999;59:1592-1598.
 39. Bataille R, Barlogie B, Lu ZY, et al. Biologic effects of anti-interleukin-6 murine monoclonal antibody in advanced multiple myeloma. *Blood.* 1995;86:685-691.
 40. Smith PC, Keller ET. Anti-interleukin-6 monoclonal antibody induces regression of human prostate cancer xenografts in nude mice. *Prostate.* 2001;48:47-53.
 41. Anderson P. Intrinsic mRNA stability helps compose the inflammatory symphony. *Nat Immunol.* 2009;10:233-234.
 42. Stoecklin G, Stoeckle P, Lu M, Muehleemann O, Moroni C. Cellular mutants define a common mRNA degradation pathway targeting cytokine AU-rich elements. *RNA.* 2001;7:1578-1588.
 43. Taylor GA, Carballo E, Lee DM, et al. A pathogenetic role for TNF alpha in the syndrome of cachexia, arthritis, and autoimmunity resulting from tristetraprolin (TTP) deficiency. *Immunity.* 1996;4:445-454.
 44. Lee HH, Son YJ, Lee WH, et al. Tristetraprolin regulates expression of VEGF and tumorigenesis in human colon cancer. *Int J Cancer.* 2010;126:1817-1827.
 45. Al-Souhibani N, Al-Ahmadi W, Hesketh JE, Blackshear PJ, Khabar KS. The RNA-binding zinc-finger protein tristetraprolin regulates AU-rich mRNAs involved in breast cancer-related processes. *Oncogene.* 2010;29:4205-4215.
 46. Keller ET, Wanagat J, Ershler WB. Molecular and cellular biology of interleukin-6 and its receptor. *Front Biosci.* 1996;1:d340-d357.
 47. Kishimoto T. Interleukin-6: from basic science to medicine—40 years in immunology. *Annu Rev Immunol.* 2005;23:1-21.
 48. Duffy SA, Taylor JM, Terrell JE, et al. Interleukin-6 predicts recurrence and survival among head and neck cancer patients. *Cancer.* 2008;113:750-757.
 49. De Schutter H, Landuyt W, Verbeken E, Goethals L, Hermans R, Nuyts S. The prognostic value of the hypoxia markers CA IX and GLUT 1 and the cytokines VEGF and IL 6 in head and neck squamous cell carcinoma treated by radiotherapy +/- chemotherapy. *BMC Cancer.* 2005;5:42.
 50. Suswam E, Li Y, Zhang X, et al. Tristetraprolin down-regulates interleukin-8 and vascular endothelial growth factor in malignant glioma cells. *Cancer Res.* 2008;68:674-682.
 51. Basu A, Datta D, Zurakowski D, Pal S. Altered VEGF mRNA stability following treatments with immunosuppressive agents: implications for cancer development. *J Biol Chem.* 2010;285:25196-25202.
 52. Fantini MC, Becker C, Kiesslich R, Neurath MF. Drug insight: novel small molecules and drugs for immunosuppression. *Nat Clin Pract Gastroenterol Hepatol.* 2006;3:633-644.