STAT3/HOTAIR Signaling Axis Regulates HNSCC Growth in an EZH2-dependent Manner

Shanshan Sun1,2, Yansheng Wu1, Wenyu Guo1, Feng Yu3, Lingping Kong4, Yu Ren5, Yu Wang1, Xiaofeng Yao1, Chao Jing1, Chao Zhang6, Mingyang Liu2, Yuqing Zhang2, Minghui Zhao1, Zhaoqing Li1, Chuanqiang Wu1, Yu Qiao1, Jingxuan Yang2, Xudong Wang1, Lun Zhang1, Min Li1,2, and Xuan Zhou1

Abstract

Purpose: PI3K and STAT3 are frequently activated in cancer progression. However, little is known about the underlying mechanisms by which PI3K and STAT3 regulate head and neck squamous cell cancer (HNSCC) growth. The lncRNA HOX transcript antisense RNA (HOTAIR) was found to modulate the progression of HNSCC. In this study, we attempted to establish the correlation of PI3K/STAT3/HOTAIR signaling with the progression of HNSCC and its sensitivity toward platinum-based and targeted anti-EGFR combination therapy.

Experimental Design: We first analyzed the STAT3/HOTAIR and PI3K/AKT level in human HNSCC samples. We then activated or suppressed STAT3/HOTAIR and determined the effects on HNSCC cell proliferation in vitro and the growth of UM1 xenograft tumors, an orthotopic model of HNSCC. The sensitivity of HNSCC cells toward cisplatin and cetuximab was determined by in vitro assays.

Introduction

Human head and neck squamous cell carcinoma (HNSCC) is one of the most prevalent tumors, with approximately 550,000 new cases diagnosed yearly worldwide (1). This disease is characterized by high proliferation, regional lymph node metastasis, and poor prognosis (2). The standard treatment for HNSCC is a platinum-based (platinum, 5-FU, and cetuximab) regimen (3). The PI3K/AKT/mTOR pathway, downstream of EGFR signaling, is the most commonly activated pathway in HNSCC (4) implicated in HNSCC development, progression, and therapeutic resistance (5–7). A more recent report revealed that this pathway contributes to resistance to anti-EGFR therapy and chemotherapy (8).

Ablation of STAT3 has been well characterized in cancer cell proliferation, dedifferentiation, invasion, angiogenesis, and immune responses in various human cancers (9). Phosphorylated STAT3 dissociates from the activated tyrosine kinase receptors and forms a transcriptionally active STAT3–STAT3 dimer, which translocates into nuclei and regulates the transcription of downstream target genes by binding to specific DNA sequences in the promoter of target genes (10). Our previous studies have demonstrated the involvement of STAT3 in HNSCC carcinogenesis and the significance of STAT3 as a therapeutic target for patients with HNSCC (11–13).

There is considerable recent interest in the role of noncoding RNA (ncRNA), especially the long noncoding RNA (lncRNA), during cancer development. With more than 200 nucleotides, lncRNAs have been primarily implicated in transcriptional regulation by serving as scaffolds for assembling transcriptional regulators to modulate the activity of transcription factors (9). LncRNA HOX transcript antisense RNA (HOTAIR) is coded by the homeobox C gene (HOXC) locus and exerts diverse functions
in various malignancies (14, 15). In multiple human cancers, HOTAIR is aberrantly expressed, and it is a potential biomarker for assessing prognosis (16, 17). HOTAIR functions as an oncogene by recruiting EZH2 to catalyze H3K27 triple-methylation to suppress downstream tumor suppressor genes. In glioblastoma, HOTAIR regulates cell-cycle progression and invasion by activating the catenin signaling pathway (18). Moreover, HOTAIR promotes cancer cell invasion by repressing E-cadherin transcription and inducing epithelial–mesenchymal transition (EMT) in oral squamous cell carcinoma (19). Targeting HOTAIR and EZH2 caused mitochondria-related apoptosis and inhibited growth of HNSCC (20, 21). Importantly, HOTAIR expression level was associated with the sensitivity of lung adenocarcinoma cells to cisplatin (22).

Activation of STAT3 reportedly depends on the phosphorylation of EZH2 at Ser21, suggesting a potential interaction of EZH2 and STAT3 in cancers. We hypothesized that STAT3 regulates EZH2 and HOTAIR to impact the growth of HNSCC. The purpose of this study was to demonstrate the connection between STAT3 regulation of EZH2 and HOTAIR in HNSCC. An estimated 30% to 40% of HNSCC cases have mutations in the PIK3CA pathway, including mutations in PIK3CA, the catalytic subunit of PI3K, which is related to decreased survival and late-stage disease (4, 23). Our results suggest that HOTAIR and EZH2 are downstream effectors of STAT3 signaling in HNSCC with PI3K activation. These findings provide a rationale for targeting STAT3/EZH2/HOTAIR signaling to treat patients with HNSCC.

Materials and Methods

HNSCC samples and pathologic characterization

A total of 28 HNSCC tumor samples were collected at the Department of Maxillofacial and Otorhinolaryngology Oncology, Tianjin Medical University Cancer Institute & Hospital (Tianjin, China) from January to December, 2015. IHC staining was used to examine STAT3, pSTAT3-705, PI3K, pPI3K, and pAkt-473 levels in all tissue samples. For each HNSCC specimen, IHC-positive cells with respect to subcellular localization, intensity, and distribution were quantified using a visual grading system based on the extent of staining (percentage of positive cancer cells graded on a scale from 0 to 3: 0, ≤10%; 1, 11%–30%; 2, 31%–60%; or 3, >60%) and the intensity of staining (graded on a scale of 0–3; 0, none; 1, weak staining; 2, moderate staining; or 3, strong staining). The combination of extent (E) and intensity (I) of staining was obtained by the product of E × I, called EI, varying from 0 to 9 for each spot. HOTAIR expression was determined by FISH assay. All slides were blindly and independently evaluated by two pathologists. In cases of discrepancy, the pathologists reviewed the slides together to achieve a consensus. All samples were collected with informed consent according to the Human Tissue Sample Usage Guidelines of the Tianjin Medical University Medical Ethics Committee.

Analysis of TCGA database of HNSCC RNA-seq datasets

We investigated the pairwise coexpression between STAT3, PIK3R1, AKT1 and its downstream targets NFKB1, MTOR, BAD, and MDM2 based on the RNA sequencing (RNA-seq) datasets for HNSCC from The Cancer Genome Atlas (TCGA) database. Transcriptome read counts of total 508 samples were available for this analysis. We transformed read counts of each gene to RPKM values and calculated Pearson correlation coefficients to demonstrate gene coexpression. Data manipulation, statistical analysis, and visualization were accomplished using R 3.0.2.

Cell culture and reagents

Human HNSCC cell lines SCC25, CaLe27, and UM-SCC1 (UM1) were gifts from Prof. Jinsong Hou (Guanghua School of Stomatolgy, Hospital of Stomatolgy, Sun Yat-sen University, China). The SCC15 cell line was purchased from ATCC. The Tsca, Trab1113, and Hep-2 cell lines were purchased from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, P. R. China). The Tb3.1 cell line was a gift from the Ninth People's Hospital, Shanghai Jiaotong University (Shanghai, China). All experimental cell lines were maintained in DMEM/F12 1:1, DMEM, minimum essential medium, or RPMI1640 (HyClone) media, supplemented with 10% FBS ( Gibco) and 5% CO2 at 37°C in an incubator.

WP1066 (Selleck Chemicals) and DDP (Sigma) were dissolved in DMSO (Solarbio) for use and storage. IL-6 (Sigma) was dissolved in 0.1% BSA-DMEM for use and storage. Cetuximab (Merck Drugs & Biotechnology) solution was prepared according to the manufacturer's protocol. Final working concentrations of WP1066, cetuximab, and DDP were 6 μmol/L, 10–20 µg/ml, and 0.5 μg/ml, respectively.

RNA extraction and qRT-PCR assays

Total RNA was extracted using TRIzol reagents (LifeTechnologies) following the manufacturer's protocol, and was reverse transcribed into cDNA with the GoScript Reverse Transcription System kit (Promega). qRT-PCR was performed with a GoTaq qPCR Master Mix Kit (Promega) according to the manufacturer's instructions. The primer of HOTAIR was “GGTAGAAAAAGCAACCACGAAGC” (forward) and “AGGTGGAGGAGTGGGTGTCGCTGTT” (reverse). The primer of GAPDH was “CCCGGAAACTCTGGCGCTGATG” (forward) and “AGTGGAGGAGTGGCGTCCTGTT” (reverse). The qRT-PCR procedure was performed under the following conditions: 15 minutes at 95°C, followed by 40 cycles of 5 seconds at 95°C and 35 seconds at 60°C. GAPDH was used as a loading control.

Protein extraction and Western blot analysis

Cancer cells in culture were washed twice with ice-cold PBS and treated with RIPA lysis buffer (Solarbio) to extract total cell lysates. Extracted proteins were transferred to polyvinylidene difluoride
membranes (Millipore) and probed with the primary antibodies against STAT3, pSTAT3-T705/S727, pEZH2-S21, pEZH2-T487 (Abcam), panAKT, pAKT-473, p16, p21, cleaved caspase-3, p-p16,100, cyclin-D1 (Cell Signaling Technology); EZH2, Bcl-2, Bax, and GAPDH (Zhongshan Biotechnology). GAPDH was used as a loading control in total cell extracts.

**Knockdown of HOTAIR with siRNA and overexpression of HOTAIR and STAT3 via plasmid transient transfection**

The siRNAs for HOTAIR knockdown were purchased from GenePharma Company and were transfected into cells with Lipofectamine 3000 (Life Technologies), following the recommended protocol. Two siRNA molecules (siHOTAIR1: 5’-CAG-CCCAAUUUAAGAAUUATT-3’; siHOTAIR2: 5’-GGGACGACAGAGAAGUAAIT-3’) were used. For STAT3 or HOTAIR overexpression, we transiently transfected 2.5 μg of each plasmid into HNSCC cells. The STAT3-expressing plasmid was kindly provided by the Key Laboratory of Cancer Prevention and Therapy of Tianjin, Tianjin Cancer Institute (Tianjin, China). The LZRS-HOTAIR plasmid was purchased from the Addgene website (plasmid #26110).

**IL6 and WP1066 treatment**

Recombinant IL6 cytokine was purchased from Sigma-Aldrich. For IL6 stimulation, HNSCC cells were starved overnight and were then treated with IL6 (20 ng/mL) for 48 hours (24, 25). The STAT3 small-molecule inhibitor, WP1066, was purchased from Selleck Chemicals. WP1066 was first introduced in 2007 as a JAK inhibitor in acute myelogenous leukemia and glioma; it is currently used in clinical trials for treatment of acute myelogenous leukemia and glioma; it is currently considered as a STAT3 phosphorylation (Tyr705 residue) blocker (26, 27). WP1066 has shown robust antitumor effects by inhibiting tumor cell proliferation, migration, and invasion (28, 29). The treatment dosage and time have been described in our previous research (11, 12). Cetuximab (10 μg/mL) was used to treat cells for 24 hours, as described previously (30).

**RNA FISH assay**

FISH assay was carried out according to our previous study (31). RNA in situ hybridization was performed using a Linc-pint probe designed by Servicebio Company. Slides were incubated with FITC-labeled target probes in dark overnight, and counterstained with 4’,6-diamidino-2-phenylindole (DAPI; Life Technologies). All images were obtained with a DP-71 fluorescence microscope (Olympus).

**Immunoprecipitation assay**

Cal27 cells were lysed in co-immunoprecipitation (co-IP) buffer. Total lysate of each sample was precleared by incubation with 20 μL of protein G-Sepharose for 1 hour at 4°C. The precleared supernatants were subjected to overnight IP using STAT3 antibody (Abcam) or control IgG antibody at 4°C, followed by addition of 40 μL of protein G-Sepharose for 1 hour at 4°C. The immunoprecipitates were washed three times with co-IP lysis buffer, and were denatured with SDS loading buffer. Western blot analysis was used to determine the pEZH2-S21 level.

**Colony formation assay**

Five-hundred or 1,000 Cal27 or UM1 cells were seeded into 6-well plates and were treated with WP1066, cisplatin, cetuximab, or a combination at designated concentrations. After 14 days, cells were fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich) and stained with crystal violet (Zhongshan Biotechnology). The colonies were further visualized under an inverted microscope and then photographed. The relative survival rate (%) was calculated using the following formula: relative survival rate (%) = (number of colonies counted in experimental plate per 100 mm²/number of colonies counted in control plate per 100 mm²) × 100%. Experiments were repeated in triplicate.

**Bromodeoxyuridine assay**

WP1066-treated cells were incubated with bromodeoxyuridine (BrdUrd) solution (Solarbio) for 1 hour at 37°C. The cells were then fixed in 4% PFA and permeabilized with 0.3% Triton X-100 (Solarbio) for 10 minutes. After blocking with 10% BSA in PBS for 1 hour, cells were incubated with primary antibody against BrdUrd (Santa Cruz Biotechnology) overnight at 4°C. Cells were then incubated with the FITC-labeled secondary antibody (Zhongshan Biotechnology) for 1 hour at room temperature. Nuclei were stained with DAPI (Life Technologies), and images were captured with a DP-71 fluorescence microscope (Olympus).

**Flow cytometry assay**

Treated cells were digested and prepared as a single-cell suspension, and then fixed in 70% ethanol. Following the Cell Cycle Kit (Keygen Biotech) instructions provided by the manufacturer, the cell-cycle distribution was measured using a flow cytometer (Thermo Fisher Scientific).

**Cell viability and proliferation assays**

Cells were seeded into 96-well plates at a density of 4,000 cells per well. After drug treatment, a total volume of 20 μL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT, Solarbio; 5 mg/mL) was added for 4 hours at 37°C in a dark room. After removal of the medium and MTT, 200 μL DMSO was added into each well, and the optical density (OD) was measured with a spectrophotometer at 490 nm. The blank control group was only treated with medium. OD value of cells was drawn with time as the x-axis and absorbance as the y-axis. The inhibition rate was defined as the absorbance of OD value as the following formula: proliferation inhibition rate = (1 – OD of experiment group/OD of control group) × 100% (32).

**Immunofluorescence staining**

WP1066-treated UM1 cells were incubated with STAT3, pEZH2-S21, and pEZH2-T487 primary antibodies overnight at 4°C, followed by incubation with FITC or TRITC-labeled secondary antibody (Zhongshan Biotechnology) for 1 hour at room temperature. Nuclei were counterstained with DAPI reagent (Life Technologies). All images were captured with a DP-71 fluorescence microscope (Olympus).

**Luciferase reporter assay**

The upstream 1,000-bp promoter region of HOTAIR was PCR-amplified and cloned into a luciferase reporter vector (pEZX-PG04 vector, GeneCopoeia). Mutated binding sites of STAT3 on this region’s (A=T, C=C) sequences were also cloned into the same luciferase reporter vector. Cal27 cells were cotransfected with STAT3 plasmid and pEZX-PG04 (WT/MT) plasmid. After 24 hours of transient transfection, cells were treated with IL6 or WP1066 for 48 hours. After treatment, cells were collected, and the luciferase reporter assay was performed according to the
STAT3 (Tyr705; Supplementary Fig. S1C). We found significantly higher expression of total STAT3 and PI3K in human P1C and D, stage HNSCC tumors (tumor diameter greater than 2 cm; Fig. 1A). In all analyzed HNSCC tumor samples, total STAT3 expression level was positively correlated with PI3K (Fig. 1B; r = 0.60; MTOC, r = 0.54; and MDM2, r = 0.49). Furthermore, we found a negative correlation between STAT3 and BAD (r = −0.35), a proapoptotic gene negatively regulated by AKT (Supplementary Fig. S2).

In this study, we chose Cal27 and UM1 cell lines for further exploration. In SCC15 DDP-resistant (cisplatin-R) cells, we found that pSTAT3-705 and pEZH2-S21 were triggered by IL6 (20 ng/mL) induction, while these proteins were downregulated by WP1066 (6 μmol/L) treatment (Supplementary Fig. S1B). However, Thr-487, a phosphorylation site that was necessary for EZH2 degradation (34), was inhibited by IL6 and was activated by WP1066 treatment (Supplementary Fig. S1B).

The IL6/STAT3 axis is closely correlated with lncRNA HOTAIR in HNSCC

EZH2 protein expression has been shown to be induced by IL6 in growth factor–dependent cell lines (35). To investigate the relationship between IL6 and EZH2, we treated HNSCC cells with IL6 (0, 5, 10, 20, 50 ng/mL) for different time points (0, 30 minutes, 3, 6, and 12 hours). Western blot analysis revealed that pSTAT3 and EZH2 were activated in time- and dose-dependent manners (Fig. 2A). Twenty-four hours after IL6 (20 ng/mL) treatment, HOTAIR expression level increased by about twofold in Cal27 and UM1 cells (Fig. 2B). In SCC15-cisplatin-R cells, IL6 treatment or transfection of STAT3 expression vector triggered HOTAIR expression, while WP1066 (6 μmol/L) treatment significantly suppressed HOTAIR expression (Fig. 2C).

Targeting STAT3/EZH2 signaling significantly impacts cell-cycle progression and proliferation of HNSCC cells

We previously showed that reduction of STAT3 by WP1066 could induce cell-cycle blockage (11). We sought to examine the effects of STAT3/HOTAIR signaling on cell cycle and proliferation of HNSCC cells, by employing FCM, colony formation, and BrdUrd assays. FCM analysis showed that the number of UM1 and Cal27 cells in S-phase was increased by STAT3 or HOTAIR overexpression, accompanied by a reduction in cells in G1 and G2 phases (Fig. 3A; P < 0.05). Colony formation was remarkably inhibited by WP1066 treatment in Cal27 and UM1 cells (Fig. 3B; P < 0.05); BrdUrd assay (Fig. 3C) revealed that WP1066 treatment significantly suppressed S phase in both Tca8113 and UM1 cells (P < 0.05).

STAT3 enhances HOTAIR transcription by interacting with pEZH2-Serine21, but not pEZH2-T487

To further understand how HOTAIR transcription was enhanced by STAT3, we employed IF assay to determine the in situ expression of STAT3 and pEZH2 in UM1 cells. As shown in Fig. 4A, WP1066-treated UM1 cells showed decreased nuclear STAT3 and pEZH2-S21 signals, while nuclear pEZH2-T487 expression was increased. The predicted STAT3-binding site on HOTAIR promoter region is shown in Supplementary Fig. S3.

IL6 treatment increased the fluorescence ratio in Cal27 cells transfected with the wild-type HOTAIR promoter, but not the mutant HOTAIR promoter. WP1066 treatment also decreased the fluorescence ratio in Cal27 cells with the wild-type HOTAIR promoter, but not the activity of mutant HOTAIR promoter (Fig. 4B).
To further investigate the interaction between STAT3 and pEZH2, a FLAG-tagged STAT3 plasmid was transiently transfected into Cal27 cells. FLAG antibody was used for IP assay, which showed a direct binding of STAT3 with pEZH2-S21, but not with pEZH2-T487 (Fig. 4C). Consistently, activation or inhibition of STAT3/HOTAIR signaling with IL6 or WP1066 modulated EZH2 phosphorylation sites and the level of pAKT-473 and P-gp100 in Cal27 and UM1 cells (Fig. 4D). Overexpression of STAT3 or HOTAIR in SCC25 cells led to upregulated pAkt-473 and P-gp100 expression (Fig. 4E), which were closely correlated with cell proliferation and multidrug resistance (36).

Figure 1.
Correlation of STAT3/HOTAIR and PI3K expression in HNSCC tissues and cells. A, STAT3 and PI3K expression in six HNSCC cell lines, as examined by Western blot analysis. B, Pearson correlation analysis between STAT3 and PI3K in HNSCC tissue samples (n = 28; P < 0.05, r = 0.62). C, HOTAIR expression in T > 2 cm group of HNSCC is higher than in T ≤ 2 cm group (P < 0.05). D, HOTAIR expression in HNSCC with different T stages (P < 0.05), as assayed with FISH. E, Representative IHC images of STAT3 and PI3K in human specimens (scale bar = 100 μm).
Blocking the STAT3/HOTAIR axis potentiates the efficacy of cisplatin and cetuximab and their antiproliferative effect in HNSCC cells

To evaluate the effect of STAT3/HOTAIR signaling inactivation combined with chemotherapy or anti-EGFR therapy, we treated HNSCC cells with WP1066 or HOTAIR siRNA in combination with cisplatin or cetuximab. In cetuximab-treated UM1 cells, protein level of P-gp100, EGFR, STAT3, pAKT-473, cyclin-D1, and Bcl-2 were significantly downregulated (Fig. 5A). In UM1 or Cal27 cells treated with a combination of cetuximab and WP1066 or HOTAIR siRNA, the protein levels of P-gp100, pAKT-473, cleaved caspase-3, Bcl-2, and cyclin-D1 were inhibited, while the expression of Bax, p16, and p21 were increased (Fig. 5B). Cell viability assays demonstrated that WP1066 sensitized UM1 and SCC25 cells to cetuximab and DDP (Fig. 6A), together with a decreased colony formation (Fig. 6B). Furthermore, Fig. 6C and D suggested that cetuximab in combination with WP1066 or HOTAIR knockdown showed better inhibition than cetuximab alone in UM1 cells ($P < 0.05$). Similarly, WP1066 and HOTAIR knockdown sensitized UM1 cells to DDP treatment, as shown in colony formation assays (Fig. 6E and F; $P < 0.05$).

HOTAIR overexpression promotes HNSCC tumor growth in vivo

To further illustrate the biological effect of HOTAIR interference in HNSCC in vivo, we established an orthotopic tumor model by injecting the HOTAIR-overexpressing UM1 cells into the oral cavity floor. HOTAIR overexpression resulted in a significant increase of bioluminescence from the oral tumors, tumor weight, and volume compared with the Lenti-NC groups (Fig. 7A–D, $P < 0.05$). We next performed IHC staining to evaluate the pathologic changes in these UM1 orthotopic tumors. In HOTAIR-overexpressing UM1 cells, the expression of EZH2, pEZH2-S21, STAT3, P-gp100, Bcl-2, and cyclin-D1 were decreased, compared with the
control animals. Levels of pEZH2-T487, cleaved caspase-3, and p21 in HOTAIR-overexpressing UM1-derived tumors were lower than levels in control animals (Fig. 7E).

**Discussion**

Combination strategies, such as targeted therapy (cetuximab-based treatment) and chemotherapy, have provided new options for patients with HNSCC. However, an essential topic in HNSCC treatment is how to increase the response to chemotherapy or targeted therapy (37). Abnormal activation of STAT3 signaling pathway is widely recognized as one major molecular event that is associated with cancer cell proliferation and chemoresistance, including HNSCC cells (38). In this study, we demonstrated that inhibition of STAT3 globally suppressed HNSCC proliferation and enhanced the sensitivity of HNSCC cells toward chemotherapy and targeted anti-EGFR therapy via modulating HOTAIR and EZH2.

WP1066, a potent STAT3 inhibitor, was derived from a non-ATP–competitive JAK2 inhibitor, AG490 (39). Compared with AG490, WP1066 has a favorable, lower IC50 in blocking STAT3 activation (26). In recent studies, WP1066 has been
demonstrated to affect multiple cancer types in vivo and in vitro (26, 40, 41). It suppresses the JAKs/STAT3 signaling pathway by inhibiting STAT3 activation and downregulating the expressions of JAKs downstream target genes starting from STAT3 (42). Importantly, WP1066 inhibits STAT3 phosphorylation via inhibiting the phosphorylation of JAK2 and inducing rapid and specific JAK2 degradation (43). Its remarkable anticancer effect in regulating proliferation, apoptosis, motility, and other biological functions in cancers results from blocking STAT3 phosphorylation, a finding that has been validated in the literature (44).

Although the explicit mechanisms explaining cooverexpression of STAT3, PI3K, and HOTAIR in HNSCC remain unclear, emerging evidence indicates that STAT3 and HOTAIR contribute to the proliferation and cell-cycle progression in many cancers (45, 46). Perhaps the interaction of STAT3 with HOTAIR promoter region increases HOTAIR level, as blocking STAT3 reduces HOTAIR expression (47). We blocked STAT3 activation with small-molecule STAT3 inhibitor WP1066, which effectively abrogated IL6-induced STAT3 expression and activation at the Tyr705 and Ser727 residues, the primary phosphorylation sites for STAT3 (48, 49). WP1066 treatment inhibits the growth of the cisplatin-resistant HNSCC cell
line SCC15, as well as UM1 and Cal27 cells. Importantly, STAT3 acts as a transcription factor to trigger HOTAIR in HNSCC cells.

In this study, activated STAT3 induces HOTAIR transcription via EZH2 phosphorylation. We elucidated that the regulatory mechanism of transcription factor STAT3 on IncRNA HOTAIR is by targeting the phosphorylation of EZH2 at Ser-21 but rather than Tyr487, as demonstrated by IP assay in Cal27 cells. Luciferase reporter analysis revealed that activated STAT3 signaling by IL6 or blocked STAT3 signaling with WP1066 could influence the interaction between STAT3 and HOTAIR promoter, as the mutant promoter did not respond. These findings suggest that HOTAIR/EZH2 functions as important downstream STAT3 effectors to mediate the biological function of the STAT3 signaling cascade.

Furthermore, our findings support that EZH2 is involved in STAT3-mediated cancer progression. First, different phosphorylation sites of EZH2 function differently in tumor cells (50). For instance, S21 phosphorylation of EZH2 was reported to be activated by JNK/STAT3/Akt signaling, leading to oncogenesis (51, 52). In contrast to pEZH2-S21, phosphorylation of Thr-345 and Thr-487 promotes EZH2 ubiquitination and subsequent degradation by the proteasome (34, 53). Thus, pEZH2-T487 disrupts EZH2 binding with other PRC2 components, such as SUZ12 and EED, and thereby inhibits EZH2-dependent epigenetic silencing, impairing the invasion ability of tumor cells (54).

We found that WP1066 treatment inhibits pEZH-S21 expression and triggers pEZH-T487 in both UM1 and Cal27 cells. Our results add STAT3 to a growing list of upstream pathways of EZH2 expression in a panel of lung adenocarcinoma, colorectal, and pancreatic cancer KRAS-mutant cell lines (52). Inhibiting the PI3K/AKT signaling pathway attenuated EZH2 axis on HNSCC cell proliferation, cell-cycle progression, and sensitivity to cisplatin and cetuximab. We therefore hypothesize that PI3K/AKT signaling may participate upstream of the referred STAT3-EZH2 axis and affect the overall growth regulation of HNSCC. In future studies, we will test how PI3K/AKT signaling is involved in the STAT3/HOTAIR/EZH2 axis in human HNSCC and explore the potential value of this axis as a therapeutic window.

In summary, As+ treatment triggers reactive oxygen species (ROS) and induces EZH2 S21 phosphorylation through JNK and STAT3-dependent Akt activation in human bronchial epithelial cells (52). Inhibiting the PI3K/AKT signaling pathway attenuated EZH2 expression in a panel of lung adenocarcinoma, colorectal, and pancreatic cancer KRAS-mutant cell lines (52). Our findings illustrate the antitumor effect of targeting the STAT3/HOTAIR/EZH2 axis on HNSCC cell proliferation, cell-cycle progression, and sensitivity to cisplatin and cetuximab. We therefore hypothesize that PI3K/AKT signaling may participate upstream of the referred STAT3-EZH2 axis and affect the overall growth regulation of HNSCC. In future studies, we will test how PI3K/AKT signaling is involved in the STAT3/HOTAIR/EZH2 axis in human HNSCC and explore the potential value of this axis as a therapeutic window.

In summary, we revealed that IncRNA HOTAIR transcription was upregulated by STAT3 signaling through pEZH2-S21, but not pEZH2-T487, in HNSCC cells (Supplementary Fig. S1D). Blockade of this pathway is associated with PI3K/AKT signaling inhibition, leading to reduced resistance to cisplatin and cetuximab and proliferation of HNSCC cells. Combined treatment with WP1066 and cetuximab or cisplatin induced synergistic antiproliferative and proapoptotic effects on the cetuximab/cisplatin sensitivity of HNSCC cells. Knockdown of HOTAIR with siRNA combined with cisplatin or cetuximab enhanced the suppressive effects. Therefore, our study highlighted new insights about how STAT3/EZH2 Regulates HNSCC Growth
the crosstalk of the STAT3/HOTAIR/EZH2 and PI3K/AKT pathways might impact cisplatin and cetuximab sensitivity in HNSCC.

Conclusion
We demonstrate that activated STAT3 binds to the promoter of the HOTAIR encoding gene to increase HOTAIR transcription, thereby enhancing EZH2-mediated epigenetic silencing in HNSCC. We also illustrate that EZH2-dependent crosstalk with STAT3 signaling has important clinical implications for PI3K-activated HNSCC treatment. These data suggest that inhibition of STAT3 signaling can inhibit HNSCC cell proliferation and sensitize these cells to chemotherapy or targeted therapy by regulating multiple downstream factors. EZH2-dependent regulatory mechanism of STAT3 signaling might be a promising therapeutic target for HNSCC treatment.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: Y. Zhang, M. Li, X. Zhou
Development of methodology: C. Zhang, C. Wu, J. Yang

Figure 7.
HOTAIR overexpression promotes HNSCC growth and inhibits apoptosis in an orthotopic model in vivo. A, Tumor volume of NC or HOTAIR-overexpressed UMI-derived orthotopic tumors as determined by a bioluminescence imaging system. B-D, Tumor volume and weight of NC or HOTAIR-overexpressed UMI-derived orthotopic tumors. E, Representative images of IHC staining of EZH2, pEZH2-S21, pEZH2-T487, P-gp100, Bax, Bcl-2, cleaved caspase-3, and p21 in tissue from mice with NC or HOTAIR-overexpressed UMI-derived orthotopic tumors (scale bar = 100 μm).

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Sun, Y. Wu, W. Guo, Y. Wang, X. Yao, C. Jing, Z. Li, C. Wu, X. Wang, L. Zhang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Sun, Y. Wu, Y. Wang, Y. Zhang, M. Zhao, Z. Li, X. Wang, L. Zhang, M. Li
Acknowledgments

This work was supported by grants from the National Science Foundation of China (NSFC81572492 and 81702529), the National Clinical Research Center for Cancer (NCCRCC), and Special Program of Talents Development for Excellent Youth Scholars in Tianjin.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 16, 2016; revised January 27, 2017; accepted March 6, 2018; published first March 14, 2018.

References


STAT3/HOTAIR Signaling Axis Regulates HNSCC Growth in an EZH2-dependent Manner

Shanshan Sun, Yansheng Wu, Wenyu Guo, et al.

Clin Cancer Res 2018;24:2665-2677. Published OnlineFirst March 14, 2018.

Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-16-2248

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2018/03/14/1078-0432.CCR-16-2248.DC1

Cited articles
This article cites 59 articles, 11 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/24/11/2665.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/24/11/2665.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://clincancerres.aacrjournals.org/content/24/11/2665.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.