WHSC1 Promotes Oncogenesis through Regulation of NIMA-Related Kinase-7 in Squamous Cell Carcinoma of the Head and Neck

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Abstract

Squamous cell carcinoma of the head and neck (SCCHN) is a relatively common malignancy with suboptimal long-term prognosis, thus new treatment strategies are urgently needed. Over the last decade, histone methyltransferases (HMT) have been recognized as promising targets for cancer therapy, but their mechanism of action in most solid tumors, including SCCHN, remains to be elucidated. This study investigated the role of Wolf homeodomain PHD-type zinc finger-1 (WHSC1), an NSD family HMT, in SCCHN. Immunohistochemical analysis of locoregionally advanced SCCHN, dysplastic, and normal epithelial tissue specimens revealed that WHSC1 expression and dimethylation of histone H3 lysine 36 (H3K36me2) were significantly higher in SCCHN tissues than in normal epithelium. Both WHSC1 expression and H3K36me2 levels were significantly correlated with histologic grade. WHSC1 knockdown in multiple SCCHN cell lines resulted in significant growth suppression, induction of apoptosis, and delay of the cell-cycle progression. Immunoblot and immunocytochemical analyses in SCCHN cells demonstrated that WHSC1 induced H3K36me2 and H3K36me3. Microarray expression profile analysis revealed NIMA-related kinase-7 (NEK7) to be a downstream target gene of WHSC1, and chromatin immunoprecipitation (ChIP) assays showed that NEK7 was directly regulated by WHSC1 through H3K36me2. Furthermore, similar to WHSC1, NEK7 knockdown significantly reduced cell-cycle progression, indicating that NEK7 is a key player in the molecular pathway regulated by WHSC1.

Implications: WHSC1 possesses oncogenic functions in SCCHN and represents a potential molecular target for the treatment of SCCHN.

Introduction

Over the last decade, epigenetic regulators have been implicated as key factors in many pathways relevant to cancer development and progression, such as cell-cycle regulation (1–3), invasiveness (4), signaling pathways (5), chemoresistance (6) and immune evasion (7). The three basic systems of epigenetic regulation are DNA methylation of gene regulatory regions, histone protein modifications, including acetylation, methylation, ubiquitination, phosphorylation and sumoylation, and noncoding RNAs. Histone methylation is dynamically regulated by two different types of enzymes called histone methyltransferases and histone demethylases. At present, approximately 50 different histone lysine methyltransferases (HKMT; ref. 8), 10 histone arginine methyltransferases (HRMT; ref. 8), and 30 histone demethylases (HDMT; ref. 9) have been identified, but their biologic functions are not fully characterized. However, because of their frequent overexpression and/or somatic mutations in a variety of cancer types, extensive efforts for the development of drugs targeting these enzymes have been initiated over the past several years (10, 11). In this regard, an important group of HKMTs are the nuclear receptor Suppressor of variegation 3–9 Su(var)3–9, Enhancer of zeste and Trithorax (SEIT)-domain-containing (NSD) family members of HKMTs (NSD-HKMTs) NSD1, NSD2/WHSC1/MMSET, and NSD3/WHSC1L1, which modulate the expression of genes through methylation of lysine 36 on histone H3 (12). These HKMTs share 70% to 75% homology in their amino acid sequences and contain 4 basic domains which are also conserved in other development-associated proteins: a Pro-Trp-Trp-Pro (PWWP) motif, which is a DNA methyl-lysine and methyl-arginine histone binder, a plant homeodomain PHD-type zinc finger (Cys3-His-Cys4) with methylation-binding affinity, a high-mobility-group (HMG) box which has DNA-binding capacity, and a SET domain that possesses the methyltransferase activity (13).

We previously reported that WHSC1 is overexpressed in various types of human cancer compared with nonneoplastic tissues. We also showed that knockdown of WHSC1 in bladder and lung cancer cell lines significantly suppressed the growth of cancer cells through cell-cycle arrest and that WHSC1 regulated the Wnt
pathway through interaction with β-catenin (14). Hudlebusch and colleagues also examined the expression of WHSC1 in 3,774 cancer tissues and 904 corresponding normal tissues and found significantly higher expression in a variety of cancers (15). Sec and colleagues described the haploinsufficiency of WHSC1 as a cause of Wolf-Hirschhorn syndrome, a growth developmental disorder, and reported the chromosomal translocation t(4;14)(p16.3; q32.3) of the WHSC1 gene and the immunoglobulin heavy-chain (IgH) promoter in multiple myeloma that led to significant overexpression of WHSC1 (16). Moreover, it has been shown that knockdown of WHSC1 in multiple myeloma cell lines remarkably suppresses growth and regulates apoptosis, cell cycle, invasion, and DNA repair pathways (17). Martinez-Garcia and colleagues showed that WHSC1-mediated oncogenesis in multiple myeloma is related to activation of gene expression of clusters of genes through increased dimethylation of lysine 36 on histone H3 (H3K36me2; ref. 18). Kuo and colleagues further reported that the genes involved in carcinogenesis of SCCHN. The Cancer Genome Atlas (TCGA) project recently reported that the NSD family of HKMTs is altered in 29% of patients with SCCHN in a mutually exclusive pattern, with 9% of patients having recurrent amplifications in WHSC1 (8p11.23) and 10% having recurrent mutations in NSD1, implying that alterations in these genes may function as driver events in the oncogenesis of SCCHN (23). Frequent and mutually exclusive genetic alterations in the NSD-HKMTs were also found in lung squamous cell carcinoma (28%) and breast invasive carcinoma (18%; ref. 23). On the basis of these data, we decided to further investigate WHSC1 as a potential novel therapeutic target for SCCHN. Cell culture

Squamous cell carcinoma cell lines UD-SCC2, SCC-23, SCC-25, SCC-35, UT-SCC-40, HN-SCC-135, HN-SCC-151, HN-SCC-166, PE/CA-PJ15, OECDM-1, BICR31, 93VU1477, FaDu, IQS-3, HN-5, and HN-6 were derived from patients with locoregionally advanced SCCHN and were kindly provided by Dr. Tanguy Seiwert (The University of Chicago, Chicago, IL). Detailed characteristics of each cell line are shown in Supplementary Table S1. UD-SCC-2, SCC-23, SCC-25, SCC-35, HN-SCC-135, HN-SCC-151, HN-SCC-166, and JSQ-3 were maintained in DMEM/F12 medium, 10% FBS, 1% penicillin/streptomycin, and 2 nmol/L L-glutamine. UT-SCC-40 cells were maintained in Eagle's medium, 10% FBS, 1% penicillin/streptomycin, and 2 nmol/L L-glutamine. PE/CA-PJ15 was maintained in IMDMEM, 10% FBS, 1% penicillin/streptomycin, and 2 nmol/L L-glutamine. HN-5, HN-6, BICR31, and 93VU1477 cells were maintained in DMEM with 10% FBS, 1% penicillin/streptomycin, and 2 nmol/L L-glutamine. OECM-1 and FaDu cells were maintained in RPMI medium, 10% FBS, 1% penicillin/streptomycin, and 2 nmol/L L-glutamine. KGM cells (normal human keratinocytes, Lonza, 00192627) were maintained in KGM-Gold keratinocyte growth medium supplemented by BPE, transferrin, insulin, hEGF, hydrocortisone, epinephrine, transferrin, and gentamicin/amphotericin B (KGM-Gold Bullet kit 00192060). Expression vector construction

An entire coding sequence of WHSC1 (GenBank Accession: BC166668) was amplified from human testis cDNAs using KOD-Plus Neo (TOYOBO, KOD-401) DNA polymerase and cloned into pcAGGSn3FC vector between NotI and XhoI restriction enzyme sites (pCAGGS-WT-WHSC1-FLAG). To prepare an enzyme-inactive WHSC1, the coding sequence of SET domain (pCAGGS-SET-FLAG).

Quantitative real-time PCR

Specific primers for human GAPDH (housekeeping gene), SDH (housekeeping gene), WHSC1, NEK7, MAPK8, and HIPK3 were designed (primer sequences in Supplementary Table S2). PCR reactions were performed using ViiA 7 real-time PCR system (Life Technologies) following the manufacturer's protocol.
Western blotting

Nuclear extracts were prepared using the Nuclear Extract Kit (Active Motif) to examine protein levels of WHSC1, and whole-cell extracts were used to examine protein levels of cytoplasmic NEK7 and ACTB. Primary antibodies used were anti-WHSC1 (Abcam, ab75359, dilution: 1:1,000), anti-NEK7 (Thermo Scientific, H,691.4, dilution: 1:1,000) and anti-ACTB (Sigma-Aldrich, A5441, dilution: 1:4,000). For detection of histone marks, nuclear extracts were prepared using the Nuclear Extract kit (Active Motif) and 1 μg of each extract was loaded for each experiment. Antibodies used were anti-H3K36me2 (Millipore, 07-369, dilution: 1:4,000), anti-H3K36me3 (Abcam, ab9050, dilution: 1:1,000), anti-FLAG antibody (Sigma-Aldrich, F7425, dilution: 1:4,000), anti-H3K36me2 (Millipore, 07-369, dilution: 1:1,000), and anti-H3 (Abcam, ab1791, dilution: 1:20,000). An anti-FLAG antibody (Sigma-Aldrich, F7425, dilution: 1:4,000) was used to assess efficiency of transfection of cell lines with FLAG-WHSC1-WT and FLAG-WHSC1-SET-deleted (ASSET).

siRNA transfection and cell growth assays

MISSION_sil RNA oligonucleotide duplexes were purchased from Sigma-Aldrich for targeting the human WHSC1 transcripts (SASI Has02_00309678 and SASI Has02_00309679). siNegative control (siNC) and siAS, which consists of 3 different oligonucleotide duplexes, were used as control siRNAs (Cosmo Bio). The siRNA sequences are described in Supplementary Table S3 and siAS (control) was obtained from QiAGEN (AllStars Negative Control siRNA, SI03650318). SCCHN cells were plated overnight in 24-well plates (2 × 10^4 to 4 × 10^5 cells per well) and were transfected with siRNA duplexes (50 nmol/L final concentration) using Lipofectamine RNAiMax (Life Technologies) for 144 hours (6 days) to 192 hours (8 days) with retransfection performed at day 5. The number of viable cells was measured using the Cell Counting Kit-8 (Dojindo) at days 6 to 8 (24, 25).

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed using ChIP Assay kit (Millipore, 17–29S) according to the manufacturer's protocol (26–29). Briefly, WHSC1 and fragmented chromatin complexes were immunoprecipitated with anti-WHSC1 (Abcam, ab75359, dilution: 1: 100) and anti-H3K36me2 (Millipore, 07-369, dilution: 1:100) antibodies 48 hours after transfection of UD-SCC-2 cells with siRNAs for WHSC1. DNA fragments were quantified by real-time PCR using NEK7-ChIP forward primer (5'-GGATGTTTACACCTTGTACA-GC-3') and NEK7-ChIP reverse primer (5'-GGTCCTCCGAGT-GCGGCCCTAGC-3'). We also conducted ChIP assays using primers for GAPDH (Takara Bio, #5311) and β-globin (Takara Bio, #5316) whose expressions were not changed after WHSC1 knockdown and confirmed that H3K36 dimethylation levels of these genes were not changed (Supplementary Fig. S1).

Cell-cycle analysis and apoptosis assays

The bromodeoxyuridine (BrdUrd) flow kit (BD Pharmingen) was used to determine the cell-cycle kinetics. The assay was performed according to the manufacturer's instructions (7, 30, 31). Briefly, cells were seeded overnight in 10-cm tissue culture dishes and treated with siRNAs (50 nmol/L) described as above using medium with 10% FBS without antibiotics for 72 hours, followed by addition of 10 μmol/L BrdUrd. Cells were harvested at 72 hours and fixed in a solution containing paraformaldehyde and saponin. Then samples were incubated with DNase for 1 hour at 37°C and fluorescein isothiocyanate (FITC)-conjugated anti-BrdUrd antibody (dilution: 1:50) was added for 20 minutes at RT. Total DNA was stained with 7-amino-actinomycin D (7-AAD), followed by flow cytometric analysis. Apoptosis was measured using the Annexin V Apoptosis Detection Kit (Biovision) according to the manufacturer's protocol.

Microarray hybridization and statistical analysis

UD-SCC2 cells were plated in 6-well dishes and treated with 2 different WHSC1-specific siRNAs (50 nmol/L) and 2 siRNA negative controls (siNegative Control, siAS) in triplicates for 48 and 72 hours. Purified total RNA isolated from these samples was labeled and hybridized onto Affymetrix GenChip U133 Plus 2.0 oligonucleotide arrays (Affymetrix) according to the manufacturer's instructions (32–34). Probe signal intensities were normalized by RMA and Quantile normalization methods (using R and Bioconductor). Next, signal intensity fluctuation due to interexperimental variation was estimated. Each experiment was replicated (n = 1 and 2), and the SD of log2(intensityEXP/intensityCONT) was calculated for each set of intensity ranges with the midpoints being at log2[(intensity + intensity2)/2] = 5, 7, 9, 11, 13, and 15. We modeled intensity variation using the formula SD(log2(intensityEXP/intensityCONT)]) = a{log2[(intensity + intensity2)/2]} + b and estimated parameters a and b using the method of least squares. Using these values, the SD of intensity fluctuation was calculated. The signal intensities of each probe were then compared between siWHSC1 (EXP) and controls (siNC or siAS) (CONT) and tested for up/downregulation by calculating the z-score: log2(intensityEXP/intensityCONT)]/[a{log2[(intensity + intensity2)/2]} + b]. Resultant P values for the replication sets were multiplied to calculate the final P value of each probe. These procedures were applied to each comparison: siNC versus siWHSC1, siAS versus siWHSC1, and siNC versus siAS, respectively. We determined up/downregulated gene sets as those that simultaneously satisfied the following criteria: (i) The Benjamini-Hochberg false discovery rate (FDR) ≤ 0.05 for siNC versus siWHSC1, (ii) FDR ≤ 0.05 for siAS versus siWHSC1 and the regulation direction is the same as (i) and (iii) siNC versus siAS has the direction opposite to (i) and (ii) or P > 0.05 for siNC versus siAS. Finally, we performed a pathway analysis using the hypergeometric distribution test, which calculates the probability of overlap between the up/downregulated gene set and each Gene Ontology (GO) category compared against another gene list that is randomly sampled. We applied the test to the identified up/downregulated genes to test whether or not they are significantly enriched (FDR ≤ 0.05) in each category of "Biological processes" (857 categories) as defined by the GO database.

Immunocytochemistry

FaDu cells (SCCHN cell line with low expression of WHSC1) were plated in two 10-cm dishes, and next day, they were transfected with FLAG-WHSC1-WT using Fugene HD (Roche Applied Science, 8 μg plasmid, 24 μL Fugene HD, 450 μL OPTIMEM) in 10 mL of cell culture medium with 10% FBS. After 2 days of transfection, cells were trypsinized and resuspended in 2-chamber glass slides at a density of 2 × 10^4 cells per well (one well per experimental condition). After 24 hours of incubation, medium was removed and cells were washed 2 times with 1 mL of PBS. Following suctioning of PBS, 1 mL of 4% paraformaldehyde was added to each well for 30 minutes at 4°C to fix the cells. Subsequently, cells were washed with PBS.
33% of SCCHN sections were positive for WHSC1 expression levels (\( \text{H3K36me2} \)) in TMAs of patients with locoregionally advanced SCCHN (\( n = 96 \)). Normal squamous epithelium sections (\( n = 16 \)) were used as baseline reference. Dysplastic squamous epithelial sections were also stained (\( n = 13 \)). Slides were counterstained with hematoxylin and eosin.

Figure 1.
WHSC1 is strongly overexpressed in locoregionally advanced SCCHN. A, IHC staining for WHSC1 (Abcam, ab75359) in TMAs (tissue microarrays) of patients with locoregionally advanced SCCHN (\( n = 149 \)). Normal squamous epithelium sections (\( n = 18 \)) were used as baseline reference. Dysplastic squamous epithelial sections were also stained (\( n = 19 \)). Slides were counterstained with hematoxylin and eosin. WHSC1 is mildly expressed in the basal and parabasal layers of normal squamous epithelium with nuclear localization. Lymph node sections were used as a positive control for WHSC1 staining. B, WHSC1 protein expression levels are significantly higher in SCCHN than in normal epithelium and dysplastic squamous epithelium (\( \text{P} < 0.0001; \text{Mann–Whitney U test} \)). C, IHC staining for H3K36me2 (Cell Signaling Technology, #2901) in TMAs of patients with locoregionally advanced SCCHN (\( n = 96 \)). Normal squamous epithelium sections (\( n = 16 \)) were used as baseline reference. Dysplastic squamous epithelial sections were also stained (\( n = 13 \)). Slides were counterstained with hematoxylin and eosin. H3K36me2 is mildly expressed in the basal and parabasal layers of normal squamous epithelium with nuclear localization. Lymph node sections were used as a positive control for H3K36me2 staining. D, H3K36me2 protein expression levels are significantly higher in SCCHN and dysplastic squamous epithelium than in normal squamous epithelium (\( \text{P} = 0.0003 \) and \( \text{P} = 0.0485; \text{Mann–Whitney U test} \)). E, high WHSC1 protein levels (+2, +3) correlate with poor grade in locoregionally advanced SCCHN. About 87% of the poorly differentiated SCCHN samples had high WHSC1 expression levels (\( \text{P} = 0.0032; \text{Cochrane–Armitage test} \)). F, high H3K36me2 levels (+2, +3) correlate with poor grade in locoregionally advanced SCCHN. Almost 100% of the poorly differentiated SCCHN samples had high H3K36me2 expression levels (\( \text{P} = 0.0033; \text{Cochrane–Armitage test} \)).
as a continuous variable, and gender was treated as a categorical variable. Association between IHC scores and each factor was evaluated using the Student t test, Cochran–Armitage trend test, and Fisher exact test. Statistical analyses were carried out using the R statistic program (http://www.r-project.org/).

Results

Correlation of WHSC1 expression and dimethylated H3K36 in advanced SCCHN

We first examined the expression levels of WHSC1 and dimethylation status of lysine 36 on histone H3 (H3K36me2) in SCCHN by IHC analysis. We used tissue microarrays derived from 149 patients with locoregionally advanced SCCHN for WHSC1 and of 96 patients for H3K36me2. We also obtained clinical information for 92 of 149 cases examined for WHSC1 and 54 of 96 cases for H3K36me2. Nineteen dysplastic epithelium and 18 normal squamous epithelium samples were also examined for WHSC1 and H3K36me2. Figure 1A shows representative results of IHC applying a 4-grade scoring system (IHC score 0, +1, +2, +3). In the cases with strong staining (+3), WHSC1 protein was observed in both the cytoplasm and the nucleus, whereas in cases with mild staining (+1) WHSC1 was observed mainly in the nucleus. IHC analysis revealed moderate or strong (+2, +3) staining for WHSC1 in 73% of SCCHN samples and absent or mild (0, +1) staining in the remaining 27% of cases, whereas 95% of the normal epithelium samples showed mild (+1) staining. WHSC1 expression was significantly higher in SCCHN than in dysplastic and normal epithelium tissues (P < 0.0001; Mann–Whitney U test). Significant differences in WHSC1 expression among normal, dysplastic, and SCCHN cases were also confirmed by the Kruskal–Wallis test (P < 0.0001; Fig. 1B).

H3K36me2 staining was observed in all of 16 normal, 13 dysplastic, and 96 SCCHN samples (Fig. 1C), but their intensities were significantly different. H3K36me2 levels were strong in approximately 40% of the SCCHN cases (40 of 96) and moderate in 38% of the cases (37 of 96). Approximately 80% of 13 dysplastic tissues stained moderately (10 of 13), and one case was scored as +3. In the normal controls, nearly 30% was moderately or strongly stained (5 of 16) and the remaining 70% was mildly stained (11 of 16). H3K36me2 levels were significantly higher in SCCHN and dysplastic tissues than in normal controls (normal-dysplastic epithelium: P = 0.0485, normal-SCCHN: P = 0.0003, Mann–Whitney U test). The Kruskal–Wallis test confirmed significant differences in H3K36me2 levels among normal epithelium, dysplastic epithelium, and SCCHN (P = 0.0007; Fig. 1D). In addition, we examined the correlation between WHSC1 expression levels and dimethylated H3K36me2 in 123 samples and found a statistically significant positive correlation (Spearman rank correlation coefficient, ρ = 0.545, P < 0.0001).

We then investigated the correlation of the WHSC1 expression and dimethylated H3K36me2 with various clinical parameters, including grade, tumor size (T), nodal stage (N), TNM stage, smoking status, HPV status, age, and gender, in 92 (for WHSC1) and 53 (for H3K36me2) patients for whom clinical information was available. Results are summarized in Table 1. Although most of the clinicopathologic parameters were not associated with WHSC1 and dimethylated H3K36me2, we found significant

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Table 1. Clinicopathologic correlations of dichotomized WHSC1 and H3K36me2 expression by IHC in locoregionally advanced SCCHN
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**Figure 2.**
WHSC1 is overexpressed and its knockdown causes significant growth suppression and apoptosis in SCCHN cell lines. A, quantitative real-time PCR of WHSC1 in 15 SCCHN cell lines compared with a normal control keratinocyte cell line (KGM). mRNA levels were normalized by GAPDH. B, knockdown of WHSC1 mRNA and protein levels using 2 different WHSC1-specific siRNAs (siWHSC1 #1 and siWHSC1 #2). UD-SCC-2 cells were treated with control siRNA (siNC), siWHSC1 #1, and siWHSC1 #2 for 3 days. mRNA and regular protein extraction were performed. WHSC1 (150 kD) was blotted with anti-WHSC1 (HPA015801, Sigma-Aldrich). ACTB was used as a loading control. C, cell cytotoxicity assays by Cell Counting Kit-8 (Dojindo) in 4 SCCHN cell lines with endogenous overexpression of WHSC1. siRNA-mediated WHSC1 knockdown caused significant growth suppression in one HPV-positive (UD-SCC-2) and 3 HPV-negative (SCC-35, HN-SCC-151, CE/PA-PJ15) SCCHN cell lines after 6 to 8 days of treatment (*, *P < 0.05). siWHSC1 treatment of a cell line with low expression of WHSC1 (FaDu) did not cause growth suppression. Each condition was plated in quadruplicates, and statistical comparisons were performed by the Student t test. D, assessment of apoptosis in UD-SCC-2 cells using the Annexin V assay. UD-SCC-2 cells were plated in 10-cm dishes and treated with siNC and siWHSC1 for 5 days. Cells were collected and processed using an annexin V assay (Biodivision). Annexin V-positive cells increased by an average of approximately 2 times in the siWHSC1-treated cells compared with siNC-treated cells. Three independent experiments were performed and the average of ratios of relative increase in apoptosis (r = [percentage of early + late apoptotic SCC2 cells treated with siWHSC1]/[percentage of early + late apoptotic SCC2 cells treated with siNC]) was calculated and statistical comparisons were performed by the Student t test (*, *P < 0.05).

correlation of both WHSC1 and H3K36me2 with poorly differentiated histologic grade (WHSC1: *P = 0.0032, H3K36me2: *P = 0.003, Cochrane–Armitage test; Fig. 1E and F). In this cohort of patients, overall and recurrence-free survival analyses were not feasible due to lack of power.

**Overexpression of WHSC1 in SCCHN cell lines and its critical role in cell proliferation and survival**

To clarify the physiologic function of WHSC1 in SCCHN, we examined the expression profile and the effect of WHSC1 knockdown on the proliferation of SCCHN cells. Quantitative real-time PCR demonstrated that all SCCHN cell lines examined showed higher levels of WHSC1 than in a control human normal keratinocyte cell line (KGM; Fig. 2A). We next transfected UD-SCC-2 cells, an HPV-positive SCCHN cell line highly expressing WHSC1, with control siRNA (siNC) and 2-independent specific siRNAs targeting WHSC1 (siWHSC1 #1 and #2). As shown in Fig. 2B, WHSC1 expression was knocked down at both mRNA and protein levels. Subsequently, we transfected these siRNAs into 1 HPV-positive (UD-SCC-2) and 3 HPV-negative cell lines (UM-SCC-35, HN-SCC-151, and PE/CA-PJ15) endogenously overexpressing WHSC1 and examined their effect on
WHSC1 Upregulates NEK7 in Head and Neck Cancer

Figure 3.
WHSC1 induces global histone di- and trimethylation of H3K36 in SCCHN cells. A, WHSC1 knockdown causes global decrease in H3K36me2 and H3K36me3 in PE/CA-PJ15 cells. After treatment of UD-SCC-2 cells with siWHSC1 for 5 days, Western blotting was performed for H3K36me2 (Millipore, 07-369), H3K36me3 (Abcam, ab8050), and WHSC1 (Abcam, ab75359) in nuclear extracts of UD-SCC-2 cells. Histone H3 (Abcam, ab1791) was used as a loading control. B, FaDu cells with endogenously low levels of WHSC1 were transfected with pCAGGS-WT-WHSC1-FLAG (wild-type 150-kD WHSC1) and pCAGGS-WHSC1-SET-FLAG (150-kD WHSC1 with deleted SET domain) plasmids and Western blotting was performed for H3K36me2, H3K36me3, and WHSC1. Histone H3, H3K36me2 and H3K36me3 levels were higher in the WT-WHSC1-FLAG than in the WHSC1-SET-FLAG-transfected FaDu cells. Histone H3 was used as a loading control. C, FaDu cells were transfected with FLAG-WHSC1-WT and FLAG-WHSC1-ΔSET (enzyme-inactive type of WHSC1) for 2 days and Western blotting was performed for H3K36me2, H3K36me3, and WHSC1. Histone H3, H3K36me2 and H3K36me3 levels were higher in the WT-WHSC1-FLAG than in the WHSC1-SET-FLAG-transfected FaDu cells. Histone H3 was used as a loading control. D, FaDu cells transfected with FLAG-WHSC1-WT cells showed significantly higher levels of H3K36me2 expression than untransfected cells (P = 0.0082; Mann-Whitney U test).

Cell growth using the Cell Counting Kit-8 (Dojindo). WHSC1 knockdown resulted in significant growth suppression of all 4 cell lines (Fig. 2C), suggesting that WHSC1 possesses a significant role in the proliferation of SCCHN cells. Treatment of FaDu cells with low expression of WHSC1 with WHSC1-specific siRNAs did not result in growth suppression (Fig. 2C and Supplementary Fig. S2), indicating that the observed effect in SCCHN cells was unlikely to be due to an off-target effect. To examine whether WHSC1 knockdown induced death through apoptosis, we performed Annexin V assays in UD-SCC-2 cells. WHSC1 knockdown resulted in doubling of the Annexin V-positive cells from 25.4% in the siRNA control–treated (siNC) group to 51.3% in the WHSC1–treated group (Fig. 2D). These data indicate that knockdown of WHSC1 induces growth suppression and apoptosis of SCCHN cells.

Induction of global histone methylation changes of H3K36 by WHSC1 in SCCHN cells

To evaluate whether overexpression of WHSC1 induces global histone methylation changes in SCCHN cells, we used a knockdown system of PE/CA-PJ15 cells transfected with a WHSC1-specific siRNA (siWHSC1 #1). Following 5 days of culture, nuclear extracts were prepared and immunoblotted with anti-dimethylated H3K36 and anti-trimethylated H3K36 antibodies. A significant decrease in di- and trimethylated H3K36 was observed in the cells treated with siWHSC1 compared with those treated with control siRNA (Fig. 3A). To evaluate the effect of WHSC1 in the aforementioned histone marks in a gain-of-function system, FaDu cells with very low expression of WHSC1 (Supplementary Fig. S2) were transfected with FLAG-WHSC1-WT and FLAG-WHSC1-SET (enzyme-inactive type of WHSC1) for 2 days (14), and Western blot analyses were performed to assess changes in di- and trimethylated H3K36. Both di- and trimethylated H3K36 levels in FaDu cells transfected with FLAG-WHSC1-WT were higher than those in FLAG-WHSC1-SET–transfected FaDu cells (Fig. 3B).

To further examine the relationship between WHSC1 overexpression and H3K36me2 levels in SCCHN cells, we performed immunocytochemistry (ICC) using anti-FLAG and anti-H3K36me2 antibodies in FaDu cells transfected with the FLAG-WHSC1-WT (Fig. 3C). We quantified the fluorescence intensity by Fiji Imagej software and found statistically higher
staining of H3K36me2 in FLAG-WHSC1-WT cells than in the untransfected cells (P < 0.0001; Mann–Whitney U test; Fig. 3D). Interestingly, we also observed that the mean H3K36me2 fluorescence levels of FLAG-WHSC1-ASET–transfected cells were significantly lower than that of untransfected cells (P = 0.0367; Mann–Whitney U test), indicating a possible dominant-negative effect of FLAG-WHSC1-deltaSET in H3K36me2. These results indicate that overexpression of WHSC1 enhances methylation of histone H3 lysine 36 in SCCHN cells.

**Direct regulation NEK7 transcription by WHSC1**

Next, we attempted to identify the genes directly regulated by WHSC1 to further clarify the biologic function of WHSC1 and elevated H3K36 methylation in SCCHN cells. We transfected UD-SCC2 cells with 2 control siRNAs and 2 independent WHSC1 siRNAs and total RNA was extracted to conduct microarray expression profile analysis (Affymetrix platform) at 48 and 72 hours after transfection. We used these time points to avoid confounding of our analysis by death-associated pathways, given that cell death with WHSC1 knockdown was noted only after at least 4 days of siRNA treatment. Hypergeometric distribution analysis revealed significant downregulation of 26 genes by more than 50% reduction (Supplementary Fig. S3 and Supplementary Table S4). Among these genes, NIMA-related kinase-7 (NEK7), homeodomain-interacting protein kinase-3 (HIPK3), and mitogen-activated protein kinase 8 (MAPK8) were previously reported to be involved in cell-cycle regulation and mitosis, apoptosis, and chemoradioresistance. NEK7 is a serine/threonine kinase which is required for cell-cycle progression through mitotic spindle formation and cytokinesis.
WHSC1 delays cell-cycle progression through transcriptional regulation of NEK7. A, histograms of cell-cycle analysis of UD-SCC-2 cells synchronized with aphidicholin (5 μg/mL). UD-SCC-2 cells were plated in 10-cm dishes and treated with siNC, siWHSC1, and siNEK7 for 3 days. On the second day of transfection, cells were exposed to aphidicholin for 48 hours. Following that, cells were released and cell-cycle analysis was performed at 0 hour (A) and 36 hours (B). B, 4-M phase cells were decreased from 44.1% in the siNC group to 19.4% and 30.5% in the siWHSC1 group and siNEK7 group, respectively. Three independent experiments were performed and demonstrated delay in cell-cycle progression. B, numerical analysis of the flow cytometry result (A), classifying cells by cell-cycle status. C, Western blotting for phospho-H3-Ser10 (Millipore, 06–381) in regular extracts from UD-SCC-2 cells treated with siNC, siWHSC1, and siNEK7 at 24 hours after release from aphidicholin synchronization. Phospho-H3-Ser10 decreased in both siWHSC1 and siNEK7 groups compared with controls.

(35, 36). HIPK3 is a serine/threonine kinase which was shown to negatively regulate apoptosis by phosphorylating FADD and inhibiting the interaction between FADD and caspase-8, thus inducing resistance to Fas-R-mediated apoptosis (37). MAPK8 is a serine/threonine kinase involved in epithelial transformation, migration, differentiation, and transcriptional regulation and has also been implicated in induction of radioresistance in SCC1 cells (38). These potential downstream genes were validated with quantitative real-time PCR in UD-SCC-2 cells after knockdown of WHSC1 (Fig. 4A and Supplementary Fig. S4). Among these genes, NEK7 was previously reported to regulate proliferation of cancer cells (36, 39), which was a phenotype observed with WHSC1 knockdown. In addition, NEK7 was confirmed to be significantly decreased after knockdown of WHSC1 at the protein level. Therefore, we focused on NEK7 for further analysis.

Decrease of NEK7 expression after WHSC1 knockdown was also observed in the HPV-negative PE/CA-PJ15 cell at the transcriptional and protein levels (Fig. 4B and C). Concordantly, transfection of FaDu cells with the FLAG-WHSC1-WT plasmid significantly enhanced the expression levels of NEK7 (Fig. 4D).

To assess whether WHSC1 directly regulates the transcription of NEK7, we conducted ChIP assays in UD-SCC-2 cells transfected with either siNC or siWHSC1 using ChIP-grade antibodies for WHSC1 and H3K36me2 (Fig. 4E). Quantitative PCR for NEK7 showed a 54% reduction in the gene levels between the siNC-treated and siWHSC1-treated UD-SCC-2 cells, with a concordant 47% decrease in the levels of H3K36me2. This result supports that NEK7 is transcriptionally regulated by WHSC1 through dimethylation of histone H3 lysine 36.

To examine whether WHSC1-mediated transcriptional regulation of NEK7 resulted in a phenotypic effect on cell-cycle progression, flow cytometry for cell-cycle analysis was performed in UD-SCC-2 cells after knockdown of WHSC1 or NEK7. UD-SCC-2 cells were transfected with siNC, siWHSC1, or siNEK7, and we synchronized the cell cycle at the G0–G1 phase with aphidicholin treatment. After release of the cell cycle in growth arrested cells, cell-cycle analysis using flow cytometry showed that the percentage of cells in the G2–M phase was decreased from 44.1% in the siNC-treated cells to 19.4% and 30.5% in the siWHSC1- and siNEK7-treated cells respectively. At the same time, the percentage of cells in the G0–G1 phase was increased from 26.3% in the siNC-treated cells to 49.6% and 38.6% in the siWHSC1- and siNEK7-treated cells, respectively (Fig. 5A and B). Previous reports showed that NEK kinases, including NEK7, play important roles in the cell-cycle checkpoint regulation at G1–S, intra-S, and G2–M phases in addition to their established functions during mitosis (39, 40), and our results appear to be consistent with these findings. In addition, we prepared cell lysates 24 hours after the release of cell cycle and conducted Western blotting for phosphorylated H3 Serine 10 (Millipore, 06–381) in regular extracts from UD-SCC-2 cells treated with siNC, siWHSC1, or siNEK7 compared with controls (Fig. 5C), indicating that mitotic cells were diminished after treatment with either siWHSC1 or siNEK7. Because WHSC1 knockdown produced a similar...
phenotypic effect with NEK7 knockdown, that is, a delay in the cell-cycle progression, NEK7 is likely to be one of the key factors in the molecular pathway regulated by WHSC1 in SCCHN cells. Taken together, these results suggest that WHSC1 plays a critical role in the cell-cycle progression of SCCHN cells through direct activation of NEK7 expression.

**Discussion**

HMTs are a group of histone modifiers that are emerging as attractive candidates for drug development (41–44). An inhibitor of EZH2, an HMT, has already been introduced in phase I trials with the goal to target patients with refractory diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma with the Y641 and A677 EZH2 activating mutations (45, 46). In this study, we showed that the HMT WHSC1 is significantly overexpressed in 73% of locoregionally advanced SCCHN tissues and provide evidence that supports the significant pathophysiologic role of WHSC1 in SCCHN. WHSC1 expression significantly increased with transition from normal to dysplastic epithelium and squamous cell carcinoma, indicating a vital role of WHSC1 in the initial stages of head and neck carcinogenesis. High WHSC1 expression and H3K36me2 levels were associated with poor differentiation, which suggests that WHSC1 may drive a dedifferentiation reprogramming of epithelial cells. This is in accordance with the observation that WHSC1 is highly expressed during embryonic development (16, 47), although its physiologic function in this setting has not been elucidated yet. It is possible that WHSC1 may allow for the maintenance of stemness and cellular plasticity which is normally required during embryonic development before the initiation of differentiation toward a specific cell fate.

Our results show that WHSC1 is important for cell proliferation and its knockdown induces delay in the cell-cycle progression in SCCHN cell lines. We identified that this effect is mediated by NEK7 and that NEK7 is a direct downstream target gene of WHSC1 in SCCHN cells. NEK7 belongs to the NEK family of protein kinases which have a prominent role in cell-cycle control and in SCCHN cells. NEK7 belongs to the NEK family of protein kinases which have a prominent role in cell-cycle control and in SCCHN cells. NEK7 and that activation of NEK7 may be important in oncogenesis. Taken together, the WHSC1–NEK7 pathway is likely to play a critical role in the oncogenesis of SCCHN. Further functional analysis is warranted to explore the importance of this pathway as a target of SCCHN therapy, as well as other aspects of the WHSC1-dependence network, such as its potential effect on apoptosis and chemoradioresistance pathways through regulation HIPK3 and MAPK8.

In conclusion, this study underlines the possible oncogenic activity of WHSC1 in SCCHN. As the methylation networks of WHSC1 in cancer are still largely unknown, research in this field will advance our knowledge and potentially accelerate the development of truly novel therapeutics for SCCHN. Development of specific inhibitors targeting WHSC1 may be a promising approach to improve the treatment outcomes for patients with SCCHN.

**Disclosure of Potential Conflicts of Interest**

J. Licht reports receiving a commercial research grant from Epizyme and is a consultant/advisory board member for AbbVie, GlaxoSmithKline, and Celgene. Y. Nakamura reports receiving a commercial research grant from and is a consultant/advisory board member for Oncotherapy Science. No potential conflicts of interest were disclosed by the other authors.

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**References**


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