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Title: WHSC1 Promotes Oncogenesis through Regulation of NIMA-related-kinase-7 in Squamous Cell Carcinoma of the Head and Neck

Authors: Vassiliki Saloura¹, Hyun-Soo Cho¹, Kazuma Kyiotani¹, Houda Alachkar¹, Zhixiang Zuo¹, Makoto Nakakido¹, Tatsuhiro Tsunoda², Tanguy Seiwert¹, Mark Lingen¹, Jonathan Licht³, Yusuke Nakamura¹, and Ryuji Hamamoto¹

¹Section of Hematology and Oncology, University of Chicago, 5841 S. Maryland Ave, MC2115 Chicago, IL 60637 USA
²Laboratory for Medical Science Mathematics, RIKEN Center for Integrative Medical Sciences, 1-7-22 Suehirocho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan
³Section of Hematology and Oncology, Northwestern University, 251 East Huron Street Galter Suite 3-150, Chicago, IL 60611

Running Title: WHSC1 Upregulates NEK7 in Head and Neck Cancer

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Corresponding Author: Dr. Ryuji Hamamoto, PhD, 5841 S. Maryland Avenue, MC2115, University of Chicago, 60637. Phone: 773-702-0933, Fax: 773-702-9385, email address: rhamamoto@medicine.bsd.uchicago.edu

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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 (HMTs) 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-Hirschhorn syndrome candidate 1 (WHSC1), a NSD-family histone methyltransferase, in SCCHN. Immunohistochemical (IHC) 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 compared with normal epithelium. Both WHSC1 expression and H3K36me2 levels were significantly correlated with histological 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), chemo-resistance (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 non-coding 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 (HKMTs) (8), 10 histone arginine methyltransferases (HRMTs) (8) and 30 histone demethylases (HDMTs) (9) have been identified, but their biological functions are not fully characterized. However, due to 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 (SET)-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 70-75% homology in their amino-acid sequences and contain four 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 methyl-lysine 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 corresponding non-neoplastic tissues, and 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 et al. also examined the expression of WHSC1 in 3774 cancer tissues and 904 corresponding normal tissues, and found significantly higher expression in a variety of cancers (15). Stec et al. 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 (MM) 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 et al. showed that WHSC1-mediated oncogenesis in MM is related to activation of gene expression of clusters of genes through increased dimethylation of lysine 36 on histone H3 (H3K36me2) (18). Kuo et al. further reported that the main chromatin-modifying end-product of WHSC1 is H3K36me2 and showed that this was sufficient for activation of oncogenic programming favoring myelomagenesis (19). Furthermore, WHSC1 has been reported to facilitate p53-binding-protein 1 recruitment in the process of DNA damage repair, suggesting that overexpression of WHSC1 may potentially contribute to resistance of cancer cells to DNA-damaging chemotherapy agents (20).

Despite various advances in treatment, long-term survival for patients with squamous cell carcinoma of the head and neck (SCCHN) remains suboptimal, hence new
therapeutic options are urgently required. Epigenetic dysregulation has mainly been studied as a potential mechanism of progression from dysplasia to SCCHN through aberrant DNA promoter methylation of genes involved in carcinogenesis (21,22). Few studies though have explored the role of histone modifications in the pathogenesis of SCCHN. The Cancer Genome Atlas (TCGA) project recently reported that the NSD-family of HKMTs is altered in 29% of SCCHN patients in a mutually exclusive pattern, with 9% of patients having recurrent amplifications in WHSC1L1 (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%) (23). Based on these data, we decided to further investigate WHSC1 as a potential novel therapeutic target for SCCHN. In the present study, we demonstrate that WHSC1 is significantly overexpressed in SCCHN, its knockdown causes cell death through apoptosis and that it promotes cell cycle progression through activation of NEK7, indicating its potential role as an oncogenic force in SCCHN.

Materials and Methods

Immunohistochemistry and head and neck cancer tissue microarrays

The expression pattern of WHSC1 in 149 SCCHN, 19 dysplastic and 18 normal epithelial tissue sections were examined by immunohistochemistry. SCCHN sections were derived from biopsies of patients with local or locoregionally advanced disease previous to treatment with either surgery with or without adjuvant chemoradiation, or definitive chemoradiation. Slides of paraffin-embedded squamous cell carcinoma tumor specimens, dysplastic and normal epithelial tissues were deparaffinized, rehydrated and sections were treated with antigen retrieval buffer (pH 6, DAKO, S2367) in a steamer for
20 min at 96°C. Anti-WHSC1 (Abcam, ab75359, dilution: 1:400) and anti-H3K36me2 (Cell Signaling Technology, #2901, dilution: 1:100) antibodies were applied on tissue sections for 1 h incubation at room temperature, followed by detection using the Bond Refine system (Leica). Following TBS wash, the antigen-antibody binding was detected with the Bond Refine polymer detection system (DS9800, Leica Biosystems) and the DAB+ chromogen (DAKO, K3468). Tissue sections were briefly immersed in hematoxylin for counterstaining and were covered with cover glass slides. An expert head and neck cancer pathologist performed semiquantitative analysis of WHSC1 and H3K36me2 staining using a four-grade scale defined as follows: negative, grade 0; mild, grade +1; moderate, grade +2; and strong staining intensity, grade +3. Use of tissues was approved by the University of Chicago Institutional Review Board (IRB 12-2125 and IRB 12-2117).

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, OECM-1, BICR31, 93VU147T, FaDu, JSQ-3, HN-5, HN-6 were derived from patients with locoregionally advanced SCCHN and were kindly provided by Dr. Tanguy Seiwert (The University of Chicago). 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% fetal bovine serum, 1% penicillin/streptomycin and 2 nM L-glutamine. UT-SCC-40 cells were maintained in Eagle’s medium, 10% fetal bovine serum, 1% penicillin/streptomycin and 2 nM L-glutamine. PE/CA-PJ15 was maintained in IDMEM, 10% fetal bovine serum, 1% penicillin/streptomycin and 2 nM L-glutamine. HN-5, HN-6, BICR31 and 93VU147T cells were maintained in DMEM medium with 10% fetal bovine serum, 1%
penicillin/streptomycin, and 2 nM L-glutamine. OECM-1 and FaDu cells were maintained in RPMI medium, 10% fetal bovine serum, 1% penicillin/streptomycin and 2 nM 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 was deleted from the entire coding sequence of WHSC1 (pCAGGS-WHSC1-ΔSET-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 manufacture’s protocol.

Western blot

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: 1:1000), anti-NEK7 (Thermo Scientific, H.691.4, dilution: 1:1000) and
anti-ACTB (Sigma-Aldrich, A5441, dilution: 1:4000). 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: 1:4000), anti-H3K36me3 (Abcam, ab9050, dilution: 1:1000), anti-phospho H3 serine 10 (Millipore, 06-570, dilution: 1:1000) and anti-H3 (Abcam, ab1791, dilution: 1:20000). An anti-FLAG antibody (Sigma-Aldrich, F7425, dilution: 1:4000) was used to assess efficacy of transfection of cell lines with FLAG-WHSC1-WT and FLAG -WHSC1-SET-deleted (ΔSET).

**siRNA transfection and cell growth assays**

MISSION _ siRNA oligonucleotide duplexes were purchased from Sigma–Aldrich for targeting the human WHSC1 transcripts (SASI Hs02_00309678 and SASI Hs02_00309679). siNegative control (siNC), which consists of three different oligonucleotide duplexes, were used as control siRNAs (Cosmo Bio, Tokyo, Japan). The siRNA sequences are described in Supplementary Table 3 and siAS (control) was obtained from QIAGEN (AllStars Negative Control siRNA, SI03650318). SCCHN cells were plated overnight in 24-well plates (2-4 x 10^4 cells/well) and were transfected with siRNA duplexes (50 nM final concentration) using Lipofectamine RNAimax (Life Technologies) for 144 h (6 days) – 192 h (8 days) with retransfection performed at day 5. The number of viable cells was measured using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) at days 6 – 8 (24,25).

**ChIP assays**

ChIP assays were performed using ChIP Assay kit (Millipore, 17-295) according to the manufacture’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 h 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'-GGATGTTTACACTCCTGACAGC-3') and NEK7-ChIP reverse primer 5'-GCGTCCGGAGTGCGAGCCTAGC-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 Figure S1).

Cell cycle analysis and apoptosis assays

The 5-bromo-20-deoxyuridine (BrdU) flow kit (BD Pharmingen) was used to determine the cell cycle kinetics. The assay was performed according to the manufacturer's instructions (30-32). Briefly, cells were seeded overnight in 10cm tissue culture dishes and treated with siRNAs (50 nM) described as above using medium with 10% FBS without antibiotics for 72 h, followed by addition of 10 μM BrdU. Cells were harvested at 72 h and fixed in a solution containing paraformaldehyde and saponin. Then samples were incubated with DNAase for 1 h at 37°C and FITC-conjugated anti-BrdU antibody (dilution: 1:50) was added for 20 min at room temperature. 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 two different WHSC1-specific siRNAs (50nM) and two siRNA negative controls (siNegative control, siAS) in
triplicates for 48h and 72h. Purified total RNA isolated from these samples was labeled and hybridized onto Affymetrix GeneChip U133 Plus 2.0 oligonucleotide arrays (Affymetrix) according to the manufacturer’s instructions (33-35). Probe signal intensities were normalized by RMA and Quantile normalization methods (using R and Bioconductor). Next, signal intensity fluctuation due to inter-experimental variation was estimated. Each experiment was replicated (1 and 2), and the standard deviation (stddev) of log2(intensity2/intensity1) was calculated for each of a set of intensity ranges with the midpoints being at log2((intensity1 + intensity2)/2) = 5, 7, 9, 11, 13, and 15. We modeled intensity variation using the formula stddev(log2(intensity2/intensity1)) = a(log2((intensity1 + intensity2)/2)) + b and estimated parameters a and b using the method of least squares. Using these values, the standard deviation 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/down-regulation by calculating the z-score: log2(intensityEXP/intensityCONT)/(a(log2((intensityEXP + intensityCONT)/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 vs. siWHSC1, siAS vs. siWHSC1, and siNC vs. siAS, respectively. We determined up/down-regulated gene sets as those that simultaneously satisfied the following criteria: (1) The Benjamini-Hochberg false discovery rate (FDR) ≤ 0.05 for siNC vs. siWHSC1, (2) FDR ≤ 0.05 for siAS vs. siWHSC1 and the regulation direction is the same as (1), and (3) siNC vs. siAS has the direction opposite to (1) and (2) or P > 0.05 for siNC vs. siAS. Finally, we performed a pathway analysis using the hyper-geometric distribution test, which calculates the probability of overlap between the up/down-regulated gene set and each GO category compared against another gene list that is randomly sampled. We applied the test to the identified up/down-regulated 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 Gene Ontology 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 re-seeded in 2-chamber glass slides at a density of 2 x 10^4 cells/well (one well per experimental condition). After 24 h 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 min at 4°C to fix the cells. Subsequently cells were washed with PBS three times for 5 min each time at room temperature. 0.1% Triton X-100 was added for 3 min at room temperature to permeabilize the cells and samples were washed with PBS three times for 5 min each time. Then cells were blocked with 3% BSA for 1 h at room temperature and incubated with primary antibodies: mouse anti-WHSC1 (Abcam, ab75359, dilution: 1:500) and rabbit anti-H3K36me2 (Cell Signaling Technology, #2901, dilution: 1:500) in a 1 ml solution of 3% BSA at 4°C overnight. Next day, cells were washed 4 times with 1 ml of PBS and secondary antibodies were added (anti-mouse Alexa 594 -red- for FLAG, anti-rabbit Alexa 488 -green- for H3K36me2, dilution: 1:500) for 1 h at RT with gentle shaking. Following this, cells were washed 4 times with PBS and mounting medium with DAPI (VECTASHIELD®, Vector Laboratories) was added on each well. The wells were finally covered with a glass slide. Confocal microscopy (Leica 2D-Photon microscope) was used for the observation of stained cells. ImageJ software was used to analyze the images and quantify integrated intensities of the FLAG-WHSC1 and H3K36me2.
Statistical analysis

Spearman rank correlation coefficients were calculated to assess associations between WHSC1 and H3K36me2 expression levels (using the 4-point IHC scale). Expression levels were dichotomized as 0-1 vs. 2-3. IHC score, stage, T-stage, N-stage, grade, and smoking status were treated as an ordinal variable, age as a continuous variable, and gender was treated as a categorical variable. Association between IHC scores and each factor was evaluated using the Student’s t-test, Cochran-Armitage trend test, and Fisher’s exact test. Statistical analyses were carried out using the R statistic program (http://www.r-project.org/).

Results

Correlation of WHSC1 expression and di-methylated H3K36 in advanced SCCHN

We first examined the expression levels of WHSC1 and di-methylation status of lysine 36 on histone H3 (H3K36me2) in SCCHN by immunohistochemical (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. 19 dysplastic epithelium and 18 normal squamous epithelium samples were also examined for WHSC1 and H3K36me2. Figure 1A shows the representative results of IHC applying a four-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, while 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, while 95% of the normal epithelium samples showed mild (+1) staining. WHSC1 expression was significantly higher in SCCHN compared to 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 (40/96) and moderate in 38% of the cases (37/96). Approximately 80% of 13 dysplastic tissues stained moderately (10/13), but one case was judged to be score +3. In the normal controls, nearly 30% was moderately or strongly stained (5/16) and the remaining 70% was mildly stained (11/16). H3K36me2 levels were significantly higher in SCCHN and dysplastic tissues compared to 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’s rank correlation coefficient, $\rho = 0.545$, $P < 0.0001$).

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 clinicopathological parameters were not associated with WHSC1 and dimethylated H3K36me2, we found significant correlation of both WHSC1 and H3K36me2 with poorly differentiated histological 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**

In order to clarify the physiological 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 compared to 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 two-independent specific siRNAs targeting WHSC1 (siWHSC1 #1 and #2). As shown in Figure 2B, WHSC1 expression was knocked down at both mRNA and protein levels. Subsequently, we transfected these siRNAs into one HPV-positive (UD-SCC-2) and three HPV-negative cell lines (UM-SCC-35, HN-SCC-151 and PE/CA-PJ15) endogenously overexpressing WHSC1 and examined their effect on cell growth using the Cell Counting Kit-8 (Dojindo). WHSC1 knockdown resulted in significant growth suppression of all four 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 siWHSC1-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-di-methylated H3K36 and anti-tri-methylated H3K36 antibodies. A significant decrease in di- and tri-methylated H3K36 was observed in the cells treated with siWHSC1 compared to 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-ΔSET (enzyme-inactive type of WHSC1) for 2 days (14), and western blots were performed to assess changes in di- and tri-methylated H3K36. Both di- and tri-methylated H3K36 levels in FaDu cells transfected with FLAG-WHSC1-WT were higher than those in FLAG-WHSC1-ΔSET 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 Image J software and found statistically higher staining of H3K36me2 in FLAG-WHSC1-WT cells compared to the untrasfected cells ($P < 0.0001$, Mann-Whitney $U$-test) (Fig. 3D). Interestingly, we also observed that the mean H3K36me2 fluorescence levels of FLAG-WHSC1-ΔSET-transfected cells were significantly lower compared to 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 biological function of WHSC1 and elevated H3K36 methylation in SCCHN cells. We transfected UD-SCC2 cells with two control siRNAs and two independent WHSC1 siRNAs and total RNA was extracted to conduct microarray expression profile analysis (Affymetrix platform) at 48 h and 72 h 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 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 (36,37). 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 (38). 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 SCCHN cells (39). 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 (37,40), 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 level (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 chromatin immunoprecipitation (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 di-methylation 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 either siNC, siWHSC1 or siNEK7, and we synchronized the cell cycle at the G0/G1 phase with aphidicolin 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 (40,41), and our results appear to be consistent with these findings. In addition, we prepared cell lysates 24 h after the release of cell cycle and conducted western blot for phosphorylated H3 Serine 10, a maker of mitosis. Consistently, phosphorylated H3S10 was decreased in cells treated with either siWHSC1 or siNEK7 compared with controls (Fig. 5C), indicating that mitotic cells were diminished after treatment with either siWHSC1 or siNEK7. Since 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

Histone methyltransferases are a group of histone modifiers that are emerging as attractive candidates for drug development (42-45). An inhibitor of EZH2, a histone methyltransferase, has already been introduced in phase I trials with the goal to target patients with refractory DLBC and follicular lymphoma with the Y641 and A677 EZH2 activating mutations (46,47). In this study, we showed that the histone methyltransferase WHSC1 is significantly overexpressed in 73% of locoregionally advanced SCCHN tissues and provide evidence that supports the significant pathophysiological 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 de-differentiation reprogramming of epithelial cells. This is in accordance with the observation that WHSC1 is highly expressed during embryonic development (16,48), though its physiological 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 prior to the initiation of differentiation towards 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 NIMA-related-kinase-7 (NEK7) and that NEK7 is a direct downstream target gene of WHSC1 in SCCHN cells. NEK7 belongs to the NIMA-related (NEK) family of protein kinases which have a prominent role in cell cycle control and mitotic spindle formation (49-51). A number of studies on the NEK family of kinases have shown their potential role in oncogenesis (37,52-56), while a recent study reported that high levels of NEK7 were associated with poor survival in patients with gallbladder carcinoma (52). Furthermore, the TCGA database revealed that amplification of NEK7 frequently occurred in various types of cancer (Supplementary Fig. S5), suggesting 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-dependent 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 squamous cell carcinoma of the head and neck. 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

Y. Nakamura is a stock holder and a scientific advisor of Oncotherapy Science and also has research grants from Oncotherapy Science. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: V. Saloura, Y. Nakamura, R. Hamamoto

Development of methodology: V. Saloura, Y. Nakamura, R. Hamamoto

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): V. Saloura, H-S. Cho, H. Alachkar, M. Nakakido, T. Seiwert, M. Lingen, R. Hamamoto

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V. Saloura, K. Kiyotani, H. Alachkar, Z. Zuo, M. Nakakido, T. Tsunoda, R. Hamamoto

Writing, review, and/or revision of the manuscript: V. Saloura, J. Licht, Y. Nakamura, R. Hamamoto

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Seiwert

Study supervision: Y. Nakamura, R. Hamamoto

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Figure Legends:

Figure 1. WHSC1 is strongly overexpressed in locoregionally advanced SCCHN. A, immunohistochemical 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 a nuclear localization. This expression increases significantly in SCCHN with a mixed cytoplasmic and nuclear expression: 27%, 40% and 33% of SCCHN sections were +1, +2 and +3 respectively. Lymph node sections were used as a positive control for WHSC1 staining. B, WHSC1 protein expression levels are significantly higher in SCCHN compared to normal epithelium and dysplastic squamous epithelium (P < 0.0001, *Mann-Whitney U-test). WHSC1 protein expression levels also increase significantly among normal, dysplastic squamous epithelium and SCCHN (P < 0.0001, **Kruskal-Wallis test). C, immunohistochemical staining for H3K36me2 (Cell Signaling Technology, #2901) in TMAs (tissue microarrays) 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 compared to normal squamous epithelium (P = 0.0003 and P = 0.0485, *Mann-Whitney U-test). H3K36me2 expression also increase significantly among normal, dysplastic squamous epithelium and SCCHN (P = 0.0007, **Kruskal-Wallis test). E, high WHSC1 protein levels (+2, +3) correlate with poor grade
in locoregionally advanced SCCHN. 87% of the poorly differentiated SCCHN samples had high WHSC1 expression levels ($P = 0.0032$, Cochrane-Armitage test). F, high H3K36me2 levels (+2, +3) correlate with poor grade in locoregionally advanced SCCHN. 100% of the poorly differentiated SCCHN samples had high H3K36me2 expression levels ($P = 0.003$, Cochrane-Armitage test).

**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 to a normal control keratinocyte cell line (KGM). mRNA levels were normalized by GAPDH. B, knockdown of WHSC1 mRNA and protein levels using two 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 four SCCHN cell lines with endogenous overexpression of WHSC1. siRNA-mediated WHSC1 knockdown caused significant growth suppression in one HPV-positive (UD-SCC-2) and three HPV-negative (SCC-35, HN-SCC-151, CE/PA-PJ15) SCCHN cell lines after 6-8 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 quadruples and statistical comparisons performed by the Student’s $t$-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 to siNC-treated cells. Three independent experiments were performed and
the average of ratios of relative increase in apoptosis \( r = \frac{\text{percentage of early + late apoptotic SCC2 cells treated with siWHSC1}}{\text{percentage of early + late apoptotic SCC2 cells treated with siNC}} \) was calculated and statistical comparisons were performed by the Student's \( t \)-test (*\( P < 0.05 \)).

**Figure 3.** WHSC1 induces global histone di- and tri-methylation 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, ab9050) 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 150kD WHSC1) and pCAGGS-WHSC1-ΔSET-FLAG (150kD WHSC1 with deleted SET-domain) plasmids and western blotting was performed for H3K36me2, H3K36me3 and WHSC1. H3K36me2 and H3K36me3 levels were higher in the WT-WHSC1-FLAG compared to the WHSC1-ΔSET-FLAG-transfected FaDu cells. Histone H3 was used as a loading control. C, FaDu cells were transfected with FLAG-WHSC1-WT and immunocytochemistry was performed with anti-FLAG (Sigma-Aldrich, M2, dilution: 1:500, red) and anti-H3K36me2 (Cell Signaling Technology, #2901, dilution: 1:500, green) antibodies. Nuclei were stained with DAPI. Transfected FaDu cells exhibited higher WHSC1 and H3K36me2 integrated densities. D, FaDu cells transfected with FLAG-WHSC1-WT cells showed significantly higher levels of H3K36me2 expression compared to untransfected cells (\( P = 0.0082 \), Mann-Whitney \( U \)-test).

**Figure 4.** NEK7 is a direct downstream target and is transcriptionally regulated by WHSC1 through H3K36 di-methylation in HPV-positive and HPV-negative SCCHN cell
lines. A, quantitative real-time PCR of NEK7 after siRNA-mediated knockdown of WHSC1 in HPV-positive UD-SCC-2 cells with two different siRNAs at 48 h and 72 h. NEK7 was significantly downregulated at 48 h and 72 h in the siWHSC1-treated cells compared to controls. Conditions were plated in triplicates. Statistical comparisons between siWHSC1 and siNC groups were performed using the Student’s t-test (*P < 0.05). B, quantitative real-time PCR of NEK7 after siRNA-mediated knockdown of WHSC1 in HPV-negative PE/CA-PJ15 cells with two different siRNAs at 72 h. NEK7 is significantly downregulated at 72 h in the siWHSC1-treated cells compared to controls. Conditions were plated in triplicates. Statistical comparisons between siWHSC1 and siNC groups were performed using the Student’s t-test (*P < 0.05). C, confirmation of NEK7 decrease at the protein level after WHSC1 knockdown in PE/CA-PJ15 cells. Regular and nuclear protein extracts were blotted for NEK7 (Thermo-Scientific, H.691.4) and WHSC1 (Abcam, ab75359) respectively. ACTB was used as a loading control. D, quantitative real-time PCR for NEK7 after transfection of FaDu cells with pFLAG-WHSC1-WT. NEK7 mRNA transcript levels increased by 35% compared to control FaDu cells transfected with mock-plasmid (FLAG-Mock). Conditions were performed in triplicates and statistical comparisons between the mock- and WT-WHSC1-FLAG-transfected cells were performed using the Student’s t-test (*P < 0.05). E, chromatin immunoprecipitation assay (ChIP) was performed in UD-SCC-2 cells treated with siWHSC1 for 3 days. Top, schematic diagram of the NEK7 promoter region. PCR amplified fragments are positioned by nucleotide number relatives to TSS (arrows). Bottom, real-time PCR analysis using primer pairs as described under “Materials and Methods”. Immunoprecipitation was performed with ChIP grade anti-WHSC1 (Abcam, ab75359) and anti-H3K36me2 (Millipore, 07-369) antibodies. Amplicons were significantly decreased in both anti-WHSC1 IP group and anti-H3K36me2 IP group. Y-axis shows a percentage of the input chromatin. Results are the mean ±SD of three
independent experiments, and $P$-values were calculated using Student’s $t$-test ($*P < 0.05$).

**Figure 5.** 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 10cm dishes and treated with siNC, siWHSC1 and siNEK7 for three days. On the second day of transfection, cells were exposed to aphidicholin for 48 h. Following that, cells were released and cell cycle analysis was performed at 0 h (A) and 36 h (B). G2/M phase cells were decreased from 44.1% in the siNC group to 19.4% in the siWHSC1 group and 30.5% in the siNEK7 group, while G0/G1 phase cells increased from 26.3% to 49.6% in the siWHSC1 group and 36.6% in the siNEK7 group. 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, 06-570) in regular extracts from UD-SCC-2 cells treated with siNC, siWHSC1 and siNEK7 at 24 h after release from aphidicholin synchronization. Phospho-H3-Ser10 decreased in both siWHSC1 and siNEK7 groups compared to controls.
Table 1. Clinicopathological correlations of dichotomized WHSC1 and H3K36me2 expression by IHC in locoregionally advanced SCCHN.

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Figure 1. Saloura et al.
Figure 2. Saloura et al.
Figure 3. Saloura et al.
**Figure 4. Saloura et al.**

(A) Relative mRNA expression levels of WHSC1 and NEK7 under different conditions.

(B) Similar to (A) but with additional conditions.

(C) Western blot analysis showing relative protein levels.

(D) Relative NEK7 expression levels under different treatments.

(E) NEK7 transcriptional regulation region with ChIP-qPCR results showing percentage input at TSS and ChIP-flr.
Figure 5. Saloura et al.
WHSC1 Promotes Oncogenesis through Regulation of NIMA-related-kinase-7 in Squamous Cell Carcinoma of the Head and Neck

Vassiliki Saloura, Hyun-Soo Cho, Kazuma Kyiotani, et al.

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