

H3K27me3 Protein Is a Promising Predictive Biomarker of Patients' Survival and Chemoradioresistance in Human Nasopharyngeal Carcinoma

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Trimethylation of lysine 27 on histone H3 (H3K27me3) is an epigenetic change which plays a critical role in tumor development and/or progression. However, the molecular status of H3K27me3 and its clinicopathologic/prognostic significance in nasopharyngeal carcinoma (NPC) have not been elucidated. In this study, the methods of Western blotting and immunohistochemistry (IHC) were utilized to examine the expression of H3K27me3 protein in NPC tissues and nonneoplastic nasopharyngeal epithelial tissues. Receiver operating characteristic (ROC) curve analysis was used to determine the cutpoint for H3K27me3 high expression. High expression of H3K27me3 could be observed in 127/209 (60.8%) of NPCs and in 8/50 (16.0%) normal nasopharyngeal epithelial tissues ($P < 0.001$). Further correlation analysis demonstrated that high expression of H3K27me3 was positively associated with tumor later T classification, tumor metastasis, advanced clinical stage and chemoradioresistance ($P < 0.05$). Moreover, high expression of H3K27me3 was closely associated with NPC patient shortened survival time as evidenced by univariate and multivariate analysis ($P < 0.05$). Consequently, a new clinicopathologic prognostic model with three poor prognostic factors (H3K27me3 expression, distant metastasis and treatment regimen) was constructed. The model could stratify risk significantly (low, intermediate and high) for overall survival and progression-free survival ($P < 0.0001$). These findings provide evidence that H3K27me3 expression, as examined by IHC, has the potential to be used as an immunomarker to predict NPC chemoradiotherapy response and patient prognosis. The combined clinicopathologic prognostic model may become a useful tool for identifying NPC patients with different clinical outcomes.

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INTRODUCTION

Nasopharyngeal carcinoma (NPC), an Epstein-Barr virus (EBV)-related head and neck cancer, is a leading lethal malignancy with the highest prevalence in Southeast Asia, especially in the Cantonese region of Southern China (1,2). Most NPC cells are poorly differentiated or undifferentiated with a great tendency to invade adjacent regions as well as to

metastasize to neck lymph nodes. Although early-stage NPC is highly resectable, local failure and distant metastasis are still the major issues for the poor outcome of patients with advanced stage NPC (3,4). Recently, platinum-based induction chemotherapy (IC), followed by chemoradiotherapy (CRT) or radiotherapy (RT), has been utilized to treat locally advanced NPC,

which shows benefits for organ preservation, loco-regional control and overall survival (5–7). Despite conventional TNM information having a strong prognostic significance in NPC (8), few predictive biomarkers of response to chemoradiotherapy exist in this cancer. Therefore, uncovering predictors of response to IC may improve our ability to anticipate tumor response to CRT or RT and thus, to identify patients who could benefit from a conservative treatment.

Histone modification and DNA methylation are epigenetic changes involved in silencing of various tumor suppressor genes, facilitating tumorigenesis and/or progression of different types of human cancer (9–11). One histone modification, methylation of lysine,

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has been found recently to relate to the transcriptional status of genes and chromatin structure (10). Trimethylation of lysine 27 on histone H3 (H3K27me3), a transcription-suppressive histone modification, is methylated by an enhancer of zeste homolog 2 (EZH2) (12). EZH2, the catalytic subunit of Polycomb repressive complex 2 (PRC2), contributes to the maintenance of cell identity, cell cycle regulation and tumorigenesis. Overexpression of the *EZH2* gene occurs in a variety of human malignancies, including breast, prostate, endometrial, gastric, colon, hepatocellular, bladder and oral cancers (13–20). Recently, several studies have reported that H3K27me3 plays a significant role in the development and/or progression of various human cancers, such as prostate, breast, ovarian, pancreatic and esophageal cancers and has a prognostic impact on patients' overall survival (21–24). In NPC, Lu et al (25) found that osteoprotegerin was ubiquitously deficient in NPC cells and that silencing this gene could increase H3K27 trimethylation. Up to date, however, the expression dynamics of H3K27me3 in NPCs and its clinicopathologic/prognostic significance have not been investigated.

In the present study, Western blotting and immunohistochemistry (IHC) were employed to examine the distribution and frequency of expression of H3K27me3 in a large cohort of NPC patients treated with RT or CRT. The purpose of our study was to determine if H3K27me3 expression could be used to assess CRT response and clinical outcome in NPC patients.

MATERIALS AND METHODS

Cell Lines and Cell Cultures

Eight NPC cell lines (CNE1, CNE2, C666, HONE1, HNE1, 5-8F, SUNE1 and 6-10B) and one immortalized normal nasopharyngeal cell line (NP69) were maintained in RPMI-1640 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C with 5% CO₂.

Patients and Tissue Specimens

In this study, 209 specimens of NPC were collected in Sun Yat-Sen University Cancer Center and in Guangdong Provincial People's Hospital, Guangzhou, China, between January 1991 and August 2000. The cases selected were based on the following criteria: pathologically confirmed as nonkeratinizing carcinoma of nasopharynx (World Health Organization types of II or III) with available biopsy specimens for tissues microarray (TMA) construction; no previous malignant disease or a second primary tumor; without radiotherapy, chemotherapy and surgery treatment history; Karnofsky ≥ 70 ; received RT, IC/RT or IC/CRT regimen and follow-up regularly. Patients with unavailable biopsy tissue for constructing TMA were excluded from our study to provide adequate samples for pathological diagnosis. On signing informed consent, we registered 209 primary NPCs from the Department of Pathology of our institutes, and 50 samples of normal nasopharyngeal mucosa were used for controls. In addition, for Western blotting assay, 22 pairs of fresh NPC tissues and adjacent nonneoplastic nasopharyngeal mucosa specimens were collected in 2008 and 2011 in our institute. All these NPC samples selected contained at least 70% carcinoma tissues identified in the whole tissue sample with the help of frozen section examination. The routine staging workup comprised of a detailed physical examination, fiber optic nasopharyngoscopy, magnetic resonance imaging (MRI) of the entire neck, chest X-ray, abdominal sonography, a complete blood count and a biochemical profile. The clinical stage was defined according to the 1992 NPC staging system of China (26). The Institute Research Medical Ethics Committee of Sun Yat-Sen University granted approval for this study.

Treatment

In our RT group, the radiotherapy was administered as two Gy daily fractions, 5 d per week, for a total intended dose of 66–78 Gy. The neck received 50 to 70 Gy,

depending on the lymph node invasiveness; 50 Gy for lymph node-negative-invaded necks and 60 to 70 Gy for lymph node-positive-invaded necks. In the IC/RT group, patient received two cycles of floxuridine + cisplatin (floxuridine 750 mg/m², d 1–5; cisplatin 35–40 mg/m², d 1–3) chemotherapy and underwent radiotherapy thereafter at 1 wk intervals. In IC/CRT group, 1 wk after completion of two cycles of floxuridine + cisplatin (floxuridine 750 mg/m², d 1–5; cisplatin 35–40 mg/m², d 1–3), patients received radiotherapy and concurrent cisplatin (35 mg/m² weekly) chemotherapy.

Evaluation and Follow-Up

The response of RT or CRT was assessed clinically for primary lesion based on fiber optic nasopharyngoscopy and MRI 1 month after treatment according to the following criteria: complete response (CR) was defined as the complete resolution of all assessable lesions; partial response (PR) was defined as a reduction by 50% or more of the sum of the lesions and no progression of assessable lesions; no change (NC) was indicated by a reduction <50% or increase <25% in tumor size. All these conditions had to last for at least 4 wks with no appearance of new lesions. Progressive disease (PD) was defined as an increase of $\geq 25\%$ in tumor size or the appearance of new lesions. In our study, CR, PR, NC and PD were achieved in 164, 24, 16 and 5 patients at the evaluation time, respectively.

The patients were followed every 3 months for the first year and then every 6 months for the next 2 years and finally annually, thereafter. The diagnostic examinations consisted of fiber optic nasopharyngoscopy, MRI, CT, chest X-ray, abdominal ultrasonography and bone scan when necessary to detect recurrence and/or metastasis. The total follow-up period was defined as the time from diagnosis to the date of death or the last date censused if patients were still alive. Disease progression was defined as cases in which the tumor evaluated as PD after primary treatment or recurrence

after CR (local progression) and/or cases in which new distant metastasis occurred (distant progression).

Western Blotting Analysis

Equal amounts of whole cell and tissue lysates were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred on a polyvinylidene difluoride (PVDF) membrane (Pall Corp., Port Washington, NY, USA). The tissues were then incubated with primary rabbit monoclonal antibodies against H3K27me3 (Cell Signaling Technology, Beverly, MA, USA, 1:1000 dilution). The immunoreactive signals were detected with enhanced chemiluminescence kit (Amersham Biosciences, Uppsala, Sweden). The procedures followed were conducted in accordance with the manufacturers' instructions.

Tissue Microarray (TMA)

TMA was constructed using the method described previously (27). In brief, the paraffin-embedded tissue blocks and the corresponding histological H&E-stained slides were overlaid for tissue TMA sampling. Duplicate of 0.6 mm diameter cylinders were punched from representative tumor areas of individual donor tissue block, and re-embedded into a recipient paraffin block at a defined position, using a tissue arraying instrument (Beecher Instruments, Silver Spring, MD, USA).

Immunohistochemistry (IHC)

The TMA slides were deparaffinized in xylene, rehydrated through graded alcohol, immersed in 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity and antigen retrieved by pressure cooking for 3 min in citrate buffer (pH = 6). For blocking nonspecific binding, the slides were preincubated with 10% normal goat serum at room temperature for 20 min. Subsequently, the slides were incubated with rabbit monoclonal antibody anti-H3K27me3 (Cell Signaling Technology, 1:100 dilution), overnight at 4°C in a moist chamber. The slides were sequentially incu-

Table 1. Correlation between the expression of H3K27me3 and clinicopathologic features in nasopharyngeal carcinomas.

	All cases	H3K27me3 protein		P value ^a
		Low expression	High expression	
Sex				0.560
Female	59	25 (42.4%)	34 (57.6%)	
Male	150	57 (38.0%)	93 (62.0%)	
Age at diagnosis (years)				0.583
≤45	89	33 (37.1%)	56 (62.9%)	
>45	120	49 (40.8%)	71 (59.2%)	
Histological classification (WHO) ^b				0.363
Type II	54	24 (44.4%)	30 (55.6%)	
Type III	155	58 (37.4%)	97 (62.6%)	
T classification				0.000
1	26	19 (73.1%)	7 (26.9%)	
2	68	37 (54.4%)	31 (45.6%)	
3	69	17 (24.6%)	52 (75.4%)	
N classification				0.024
0	40	23 (57.5%)	17 (42.5%)	
1	96	34 (35.4%)	62 (64.6%)	
2	51	15 (29.4%)	36 (70.6%)	
Distant metastasis				0.021
0	158	69 (43.7%)	89 (56.3%)	
1	51	13 (25.5%)	38 (74.5%)	
Clinical stage				0.000
I	10	8 (80.0%)	2 (20.0%)	
II	55	37 (67.3%)	18 (32.7%)	
III	83	23 (27.7%)	60 (72.3%)	
RT response				0.505
CR	31	23 (74.2%)	8 (25.8%)	
IC/RT response				0.016
CR	67	29 (43.3%)	38 (56.7%)	
Non-CR ^c	21	3 (14.3%)	18 (85.7%)	
IC/CRT response				0.006
CR	66	32 (48.5%)	34 (51.5%)	
Non-CR ^c	13	1 (7.7%)	12 (92.3%)	

^aChi-square test.

^bWorld Health Organization.

^cIncluding PR, NC and PD.

bated with a secondary antibody (Envision, Dako, Denmark) for 1 h at room temperature, and stained with DAB (3,3-diaminobenzidine). Finally, the sections were counterstained with Mayer's hematoxylin, dehydrated and mounted. A negative control was obtained by replacing the primary antibody with a normal rabbit IgG. Known immunostaining positive slides were used as positive controls.

IHC Evaluation

Nuclear immunoreactivity for H3K27me3 protein was scored using a

semiquantitative method by evaluating the number of positive tumor cells over the total number of tumor cells. Scores were assigned by using 5% increments (0%, 5%, 10% . . . 100%). Expression for H3K27me3 was assessed by three independent pathologists (M-Y Cai, HL Rao and D Xie) who were blinded to clinicopathologic data. Their conclusions were in complete agreement in approximately 85.1% of the cases, which identified this scoring method was highly reproducible. If two or all of them were consistent with the results they reported, the value was selected. In case completely different re-

sults occurred, all three worked together to confirm the score.

Selection of Cutoff Score

Receiver operating characteristic (ROC) curve analysis was performed to determine cutoff score for tumor "high expression" by using the 0,1-criterion (28). At the H3K27me3 score, the sensitivity and specificity for each outcome under study was plotted, thus generating various ROC curves. The score was selected as the cutoff value which was closest to the point with both maximum sensitivity and specificity. Tumors designated as low expression of H3K27me3 were those with the scores below or equal to the cutoff value, while tumors of high expression were those with scores above the value. To use ROC curve analysis, the clinicopathologic characteristics were dichotomized: T classification (T1–T2 versus T3–T4), N classification (N0–N1 versus N2–N3), distant metastasis (M0 versus M1), clinical stage (I–II versus III–IV), cancer progression (Yes versus No) and survival status (death due to NPC versus censored).

Statistical Analysis

Statistical analysis was performed by using the SPSS statistical software package (standard version 13.0; SPSS, Chicago, IL, USA). ROC curve analysis was applied to determine the cutoff score for high expression of H3K27me3. The correlation between H3K27me3 expression and clinicopathologic features of NPC patients was analyzed by χ^2 test. For univariate survival analysis, survival curves were obtained with the Kaplan-Meier method. The Cox proportional hazards regression model was used to identify the independent prognostic factors. Differences were considered significant if the *P* value from a two-tailed test was less than 0.05.

RESULTS

Patients' Characteristics

The clinicopathologic characteristics of the NPC patients are summarized in

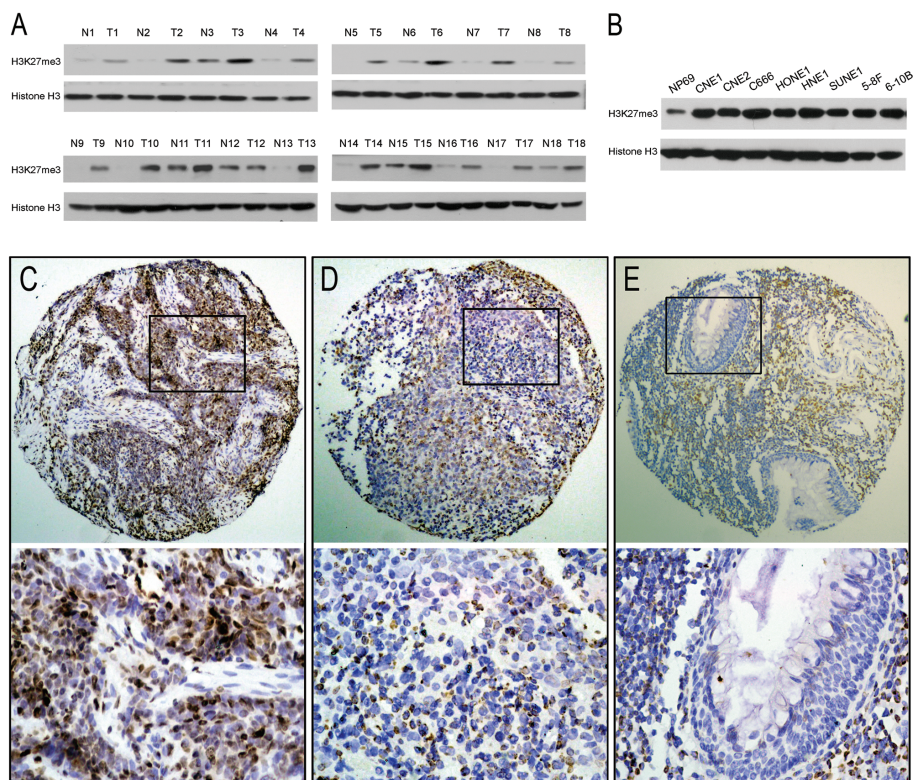


Figure 1. The expression of H3K27me3 in NPC cell lines and tissues. (A) Upregulated expression of H3K27me3 was observed in 17/18 of NPC tissues compared with nonneoplastic nasopharyngeal mucosal tissues. T, NPC tissue; N, nonneoplastic nasopharyngeal mucosal tissue. (B) The eight NPC cell lines (that is, CNE1, CNE2, C666, HONE1, HNE1, 5–8F, SUNE1 and 6–10B) exhibited relatively higher levels of H3K27me3 expression than that in the immortalized normal human nasopharyngeal epithelial cell line (NP69). (C) High expression of H3K27me3 was shown in a NPC case (case 36), in which more than 75% of carcinoma cells revealed positive staining of H3K27me3 protein in nuclei (100). (D) Another NPC case (case 27) demonstrated low expression of H3K27me3, in which less than 30% of carcinoma cells showed positive staining of H3K27me3 protein in nuclei (100). (E) Normal nasopharyngeal mucosal tissue showed nearly negative expression of H3K27me3 protein, though immunoreactivity also could be seen in some stromal lymphocytes (100). The lower panels indicated the higher magnification (400 \times) from the area of the box in the upper panels.

Table 1. This NPC cohort consisted of 150 (71.8%) men and 59 (28.2%) women, with median age of 47 years. Average follow-up period was 72.9 months (median, 73.0 months; range, 3.0 to 233.0 months). In our study, a total of 42 NPC patients were treated by radiotherapy only, whereas 88 patients were treated with induction chemotherapy plus radiotherapy. A total of 79 patients received induction chemotherapy prior to concurrent chemoradiotherapy. At evaluation time, CR, PR, NC and PD were achieved in 164, 24, 16 and 5 patients, respectively.

Expression Patterns of H3K27me3 in Nasopharyngeal Cells and Tissues by Western Blotting

Western blotting analysis demonstrated that, in primary NPCs, 17/22 (77.3%) of cases had upregulated H3K27me3 expression, as compared with that in adjacent nonneoplastic nasopharyngeal tissues. The 17 NPC cases with upregulated expression of H3K27me3 are shown in Figure 1A. In nasopharyngeal cell lines, high levels of H3K27me3 expression were examined in all eight NPC cell lines (that is, CNE1, CNE2, C666, HONE1, HNE1,

5–8F, SUNE1 and 6–10B), while the immortalized normal human nasopharyngeal epithelial cell line NP69 showed a relatively lower level of endogenous H3K27me3 (Figure 1B).

IHC Analysis of H3K27me3 Expression in Nasopharyngeal Tissues

For H3K27me3 IHC staining, nuclear immunoreactivity was observed primarily in the NPC cells, though occasionally immunoreactivity also could be seen in the stromal lymphocytes (Figures 1C–E). Immunoreactivity of H3K27me3 protein in NPC ranged from 0% to 100% (see Figures 1C, D). For the 19 noninformative TMA samples (samples with too few tumor cells, <300 cells per case, and lost samples), IHC staining was performed by using whole tissue slides.

Selection of Cutoff Score for High Expression of H3K27me3

The ROC curves for each clinicopathologic feature (Figure 2) show the arrow on the curve closest to the point (that is, 0.0, 1.0), which maximizes both sensitivity and specificity for the outcome (28). Tumors with scores above the obtained cutoff value were considered as highly-expressed H3K27me3, leading to the greatest number of tumors correctly classified as having or not having the clinical outcome. The corresponding area under the curve (AUC) and cutoff scores were collected and shown in Figure 2 and Table 2, respectively. In our current study, ROC curve analysis for clinical stage had the shortest distance from the curve to the point (0.0, 1.0), and we selected the cutoff value determined by clinical stage. Thus, the cutoff score for high expression of H3K27me3 was defined when more than 55% carcinoma cells had positive staining of H3K27me3.

Association of H3K27me3 Expression with NPC Patients' Clinicopathologic Features

In our study, high expression of H3K27me3 was examined in 127/209 (60.8%) of NPCs and in 8/50 (16.0%) normal nasopharyngeal mucosal tissues ($P <$

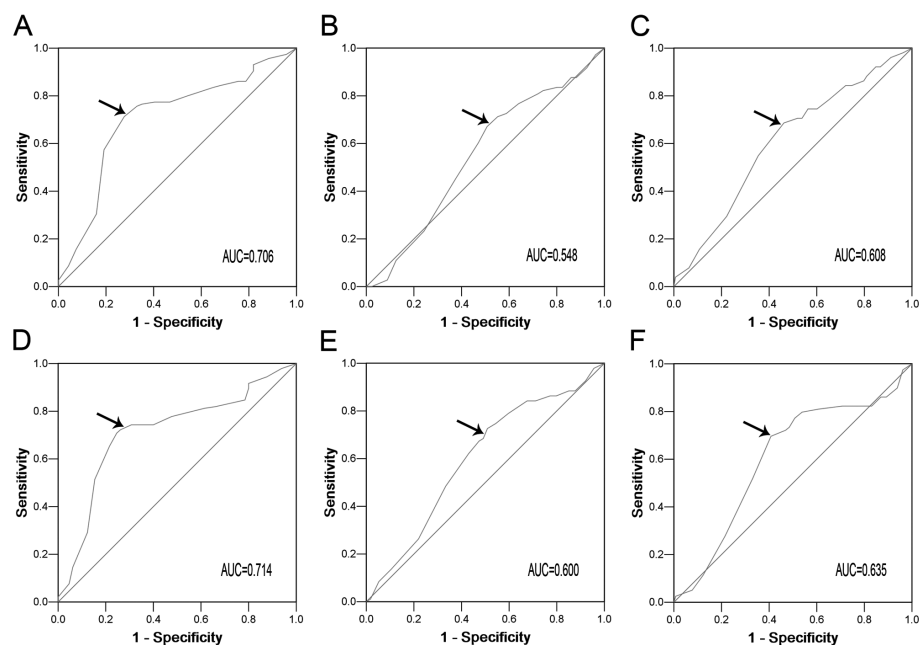


Figure 2. Receiver operating characteristic curve analysis was employed to determine the cutoff score for the high expression of H3K27me3. The sensitivity and specificity for each outcome were plotted: (A) T classification ($P < 0.0001$); (B) N classification ($P = 0.249$); (C) Distant metastasis ($P = 0.021$); (D) Clinical stage ($P < 0.0001$); (E) Cancer progression ($P = 0.013$); (F) Survival status ($P = 0.011$).

0.001, Fisher exact test). The rates of high expression of H3K27me3 in NPCs with respect to several standard clinicopathologic features were detailed in Table 1. The results demonstrated that high expression of H3K27me3 was positively correlated with tumor T classification, N classification, distant metastasis and advanced clinical stage ($P < 0.05$, Table 1). There was no significant association between H3K27me3 expression and other clinicopathologic features, such as patient gender, age and tumor histological classification ($P > 0.05$, Table 1).

Predictive Significance of H3K27me3 Expression in Treatment Response of NPC Patients

Primary CR was achieved in 78.5% (164/209) of the NPC patients. Moreover, H3K27me3 expression also was the factor that showed a negative correlation with treatment response in the IC/RT and IC/CRT groups, in which high expression of H3K27me3 was observed more frequently in the non-CR subset

than in the CR subset ($P < 0.05$, Table 1). Nevertheless, there was no significant correlation between H3K27me3 expression and treatment response in the RT group ($P > 0.05$, Table 1).

To investigate the predictive value of H3K27me3 expression in NPC therapeutic response, ROC curve analysis was performed in our study. For ROC curve analysis, the treatment response was dichotomized: CR versus PR + NC + PD. The result showed a promising predic-

Table 2. The corresponding cutoff score of H3K27me3 expression for each clinicopathological feature according to ROC curve analysis.

Feature	Cutoff score (%)	<i>P</i> value
T classification	60	0.000
N classification	65	0.249
Distant metastasis	55	0.021
Clinical stage	55	0.000
Cancer progression	65	0.013
Survival status	55	0.011

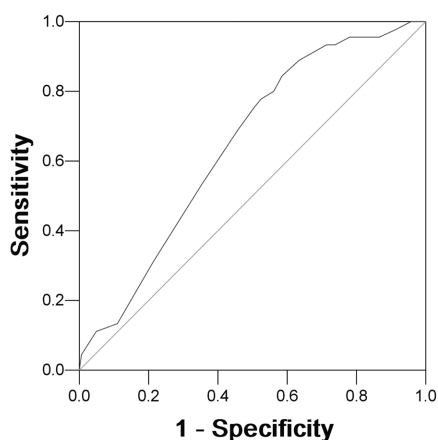


Figure 3. The predictive value of H3K27me3 expression regarding NPC patients' treatment response. Receiver operating characteristic curve analysis for H3K27me3 expression was performed to assess NPC treatment response (AUC = 0.644, $P = 0.003$).

ive value of H3K27me3 regarding to NPC treatment response (AUC = 0.644, $P = 0.003$, Figure 3).

Association between Clinicopathologic Features, H3K27me3 Expression, and NPC Patient Survival: Univariate Survival Analysis

To confirm the representativeness of the NPC cohort in this study, we first tested well established prognostic factors of patient survival. Kaplan–Meier analysis evaluated a significant impact of well-known clinicopathologic prognostic parameters, such as T classification ($P = 0.012$), N classification ($P < 0.0001$), distant metastasis ($P < 0.0001$) and clinical stage ($P < 0.0001$) on patients' survival (Table 3). Assessment of NPC patient survival also revealed that high expression of H3K27me3 was correlated significantly with poor overall survival ($P < 0.0001$, Table 2 and Figure 4A) and progression-free survival ($P = 0.001$, Figure 4B).

Independent Prognostic Factors of Nasopharyngeal Carcinoma: Multivariate Survival Analysis

Since variables observed to have prognostic influence by univariate analysis

Table 3. Univariate and multivariate analysis of different prognostic variables in 209 patients with nasopharyngeal carcinoma.

Variable	All cases	Univariate analysis ^a		Multivariate analysis ^b	
		Mean survival (months)	<i>P</i> value	Hazards ratio (95% CI)	<i>P</i> value
Sex			0.545		
Female	59	128.9			
Male	150	158.5			
Age at surgery (years)			0.237		
≤45	89	119.4			
>45	120	162.9			
Histological classification (WHO)			0.059		
Type II	54	180.5			
Type III	155	120.3			
T classification			0.012	0.829 (0.444–1.549)	0.557
T1–T2	94	158.5			
T3–T4	115	142.5			
N classification			0.000	1.368 (0.815–2.297)	0.235
N0–N1	136	172.2			
N2–N3	73	79.1			
Distant metastasis			0.000	2.000 (1.217–3.289)	0.006
0	158	171.2			
1	51	69.3			
Clinical stage			0.000	1.836 (0.739–4.563)	0.191
I–II	65	175.7			
III–IV	144	140.0			
Treatment regimen			0.001	0.692 (0.493–0.972)	0.034
RT ^d	42	112.9			
IC ^e /RT	88	132.3			
IC/CRT ^f	79	185.3			
H3K27me3 expression			0.000	1.830 (1.040–3.220)	0.036
Low	82	172.7			
High	127	135.3			

CI, confidence interval; WHO, World Health Organization.

^aLog-rank test.

^bCox regression model.

may covariate, the expression of H3K27me3 as well as other clinicopathologic features that were significant in univariate analysis (T classification, N classification, distant metastasis, clinical stage and treatment regimen) were examined in multivariate analysis (Table 3). We found that high expression of H3K27me3 was evaluated as an independent risk factor for adverse overall patient survival (hazards ratio: 1.830; 95% confidence interval: 1.040–3.220; $P = 0.036$). Of the other variables, distant metastasis and treatment regimen also were found to be independent prognostic predictors for overall survival (Table 3).

New Prognostic Model with H3K27me3 Expression, Distant Metastasis and Treatment Regimen

According to the results of our univariate and multivariate analyses, we proposed a new clinicopathologic prognostic model with three prognostic factors, that is, H3K27me3 expression, distant metastasis and treatment regimen. We designated a high-risk group as the presence of all three factors, an intermediate-risk group as the presence of two factors (regardless of their identity), and a low-risk group as the presence of one factor or none. Our results revealed that the model could significantly stratify the risk

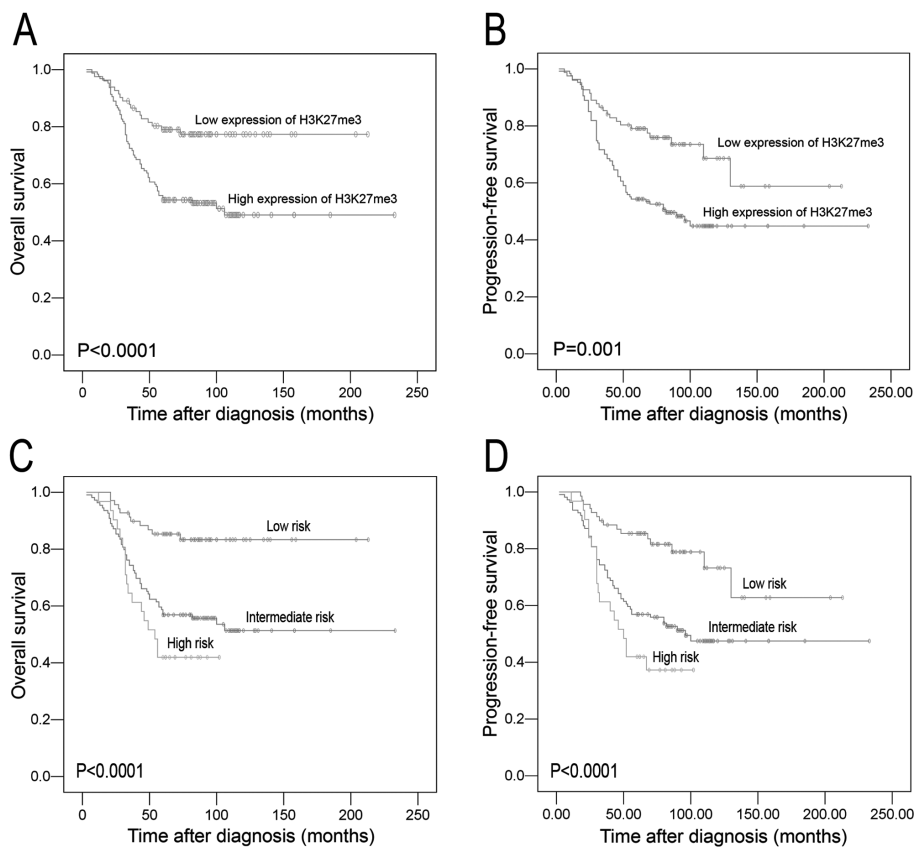


Figure 4. The association of H3K27me3 expression with NPC patients' survival. (A) Kaplan-Meier survival analysis of H3K27me3 expression for overall survival and (B) progression-free survival (log-rank test). (C) Comparison of overall survival and (D) progression-free survival according to a new combined clinicopathologic prognostic model (including H3K27me3 expression, distant metastasis and treatment regimen).

(low, intermediate and high) for either overall survival (Figure 4C, $P < 0.0001$) or progression-free survival (Figure 4D, $P < 0.0001$).

Correlation between the Expression of H3K27me3 and EZH2 in NPC Tissues

Our recent study revealed that high expression of EZH2 was observed in 131/209 (62.7%) of NPC tissues in the same cohort (29). In this study, we further evaluated the potential correlation between expression of H3K27me3 and EZH2 in our NPC cohort. The results showed a positive correlation between expression of H3K27me3 and EZH2 in NPCs (Figures 5A, B). For the 118 NPC cases with high expression of H3K27me3, an average of 64.7% of the carcinoma cells stained positive with

EZH2 protein; the percentage was significantly higher than that (49.0%) in the remaining 91 NPCs with low expression of H3K27me3 ($P < 0.001$, independent sample t test; Figure 5C).

DISCUSSION

Histone modification is an epigenetic change that plays a crucial role in tumorigenesis (9). One such modification, the trimethylation of H3K27 is mediated by proteins in the Polycomb group (PcG) family of genes which was originally identified as genes suppressing the development of extra sex combs in *Drosophila* (30). It has been suggested that maintenance of the H3K27me3 epigenetic mark during cell division is pivotal for normal embryogenesis and cell identity (31). Moreover, H3K27

trimethylation has been shown to be correlated with the development and/or progression of different human cancers (10). Up to this date, however, the expression dynamics of H3K27me3 in NPCs and its potential impact on NPC tumorigenesis and/or prognosis have not been elucidated.

In the present study, the expression levels of H3K27me3 protein was detected initially in a series of carcinomatous and nonneoplastic human nasopharyngeal tissues and cells. Our results, using Western blot analysis, clearly demonstrated that all NPC cells examined had high levels of endogenous H3K27me3, while the immortalized normal nasopharyngeal epithelial cell line NP69 showed a relatively lower level of H3K27me3. In nasopharyngeal tissues, we found that the majority of NPCs had a higher expression of H3K27me3 than that in adjacent nonneoplastic nasopharyngeal tissues. Next, the expression dynamics of H3K27me3 was examined by IHC in a large cohort of NPC tissues using a NPC-TMA with complete follow-up data. The results demonstrated that the frequency of high expression of H3K27me3 in NPC tissues was significantly larger than that in nonneoplastic nasopharyngeal epithelial tissues. These findings suggest that upregulated expression of H3K27me3 may provide a selective advantage in NPC tumorigenic processes. In our study, further correlation analysis revealed that high expression of H3K27me3 in NPCs was associated closely with tumor T classification, N classification, distant metastasis and clinical stage. These findings were similar to that in others studies (23,24,32), in which H3K27me3 protein frequently was overexpressed in esophageal and hepatocellular carcinomas and it was positively correlated with tumor aggressiveness and/or advanced clinical stage. Taken together, these data suggest that upregulation of H3K27me3 may facilitate the invasive/metastatic phenotypes of different human cancers, including NPC.

In this study, the expression of H3K27me3 was correlated positively

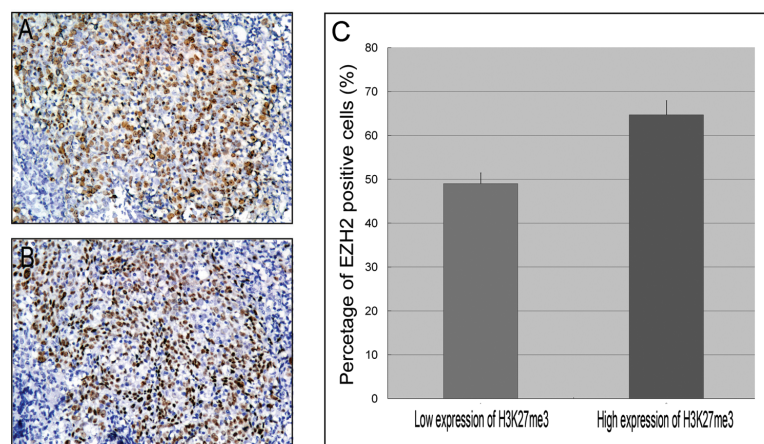


Figure 5. Correlation between the expression of H3K27me3 and EZH2 in NPC tissues. (A) High expression for EZH2 was observed in an NPC (case 63), in which more than 70% of tumor cells showed nuclear positive staining of EZH2 protein (200x). (B) Overexpression of H3K27me3 was examined in the same NPC case 63 (200x). (C) In 118 NPC cases with high expression of H3K27me3, an average of 64.7% of the NPC cells stained positive with EZH2 (right column), a percentage of cancer cells that was significantly larger than that (49.0%) in 91 NPCs with low expression of H3K27me3 (left column) ($P < 0.001$, independent sample t test).

with EZH2 levels in our NPC cohort. Interestingly, we further found that H3K27me3 immunoreactivity had a significant association with NPC patients' chemoradioresistance, but did not correlate with patients' radioresistance. A previous report documented that in ovarian cancer cells, overexpression of a dominant-negative point mutant of H3K27me3 (H3-K27R) derepressed epigenetically silenced tumor suppressor genes and reversed the platinum-resistant phenotype of this tumor (33). Similarly, other groups reported that EZH2 was upregulated in cisplatin-resistant ovarian cancer cells, and knock-down of EZH2 by RNA interference resulted in a decreased level of H3K27me3 and resensitized drug-resistant cancer cells to cisplatin (34). The mechanism(s) of resistance reversal on loss of H3K27 methylation is likely due to changed gene expression and "loosened" chromatin which in turn affect platinum adduct formation by reducing steric hindrance of DNA target sequences (33). We know that cisplatin-based combination chemoradiotherapy is the current standard first-line therapy, and that tumor re-

sponse to IC predicts response to CRT and patient survival in locally advanced NPC. Our findings suggest that increased expression of H3K27me3 might be an important factor related to cancer chemotherapy resistance and, therefore, may contribute to a decreased treatment response to CRT treatment in NPC patients. These data, collectively, might provide an explanation as to why a significant correlation between high expression of H3K27me3 and therapeutic resistance was observed in CRT-treated NPC patients, but not in RT-treated patients. Clearly, further work is needed to clarify the mechanisms of H3K27me3 in regulating NPC response to CRT in detail.

With regard to the prognostic impact of H3K27me3 on different types of human cancer, Wei et al (22) reported that loss expression of H3K27me3 was linked to poor prognosis in patients with breast, ovarian and pancreatic cancers. In esophageal and hepatocellular carcinomas, however, we and other groups found that high expression of H3K27me3 was positively associated with tumor high invasiveness and/or poor patient survival (23,24,32). Similarly, in the pres-

ent study, of a large cohort of NPCs, we did observe that high expression of H3K27me3 was a strong and independent predictor of shortened cancer-specific survival as evidenced by univariate and multivariate analysis. Considering that the mechanism(s) by which EZH2-mediated H3K27 methylation leads to gene silencing may vary among gene targets and among organisms (35), it is not very difficult for us to understand that the functions of H3K27me3 and its underlying mechanism(s) to impact cancer progression may be tumor-type specific. Furthermore, in this study, we propose, for the first time, a new prognostic model in NPC patients with H3K27me3 expression, distant metastasis and treatment regimen. Our data provided evidence that this kind of prognostic model can reflect the aggressive nature of NPC effectively and it may serve as a useful prognostic index for NPC.

In summary, our report describes the expression pattern of H3K27me3 in human NPCs and the examination of H3K27me3 expression by IHC could be used as an effective additional tool in identifying those NPC patients at increased risk of tumor invasiveness and/or chemoradioresistance. H3K27me3 also may serve as a potential novel immunomarker to predict the prognosis of NPC patients. Furthermore, our data, as provided in this report, suggest that H3K27me3 could be an encouraging molecular target for the development of novel combinatorial therapeutic strategies aimed at overcoming chemotherapy resistance to NPC.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the re-

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