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Overexpression of Histone H3 Lysine 27 Trimethylation Is Associated with Aggressiveness and Dedifferentiation of Thyroid Cancer

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Abstract

A variety of epigenetic dysregulations are observed in thyroid malignancies. EZH2, the catalytic subunit of polycomb repressive complex 2, is upregulated in advanced thyroid cancers. EZH2 can catalyze trimethylation of histone H3 at lysine 27 (H3K27me3) and contribute to transcriptional silencing of target genes. Here, we investigated the immunohistochemical expression of H3K27me3 in neoplastic and normal thyroid tissues. Normal thyroid epithelial cells typically exhibited nuclear staining of moderate intensity. A similar expression pattern was observed in nodular goiters and follicular adenomas. By contrast, strong H3K27me3 expression was evident in 80% (8/10) lymphocytic thyroiditis, 63% (80/127) papillary thyroid cancer, 41% (7/17) follicular thyroid cancer, and 73% (8/11) poorly differentiated and anaplastic thyroid cancer. In differentiated thyroid cancer, strong H3K27me3 expression was associated with extrathyroidal extension ($p < 0.001$), lymphovascular invasion ($p = 0.029$), lymph node metastasis ($p = 0.006$), and higher risk of recurrence ($p = 0.003$). Our results indicate that H3K27me3 overexpression may be implicated in aggressiveness and dedifferentiation of thyroid cancer. In addition to prognostication, the predictive value of H3K27me3 expression deserves further investigation given the recent development of epigenetic targeting agents.

Keywords H3K27me3 · EZH2 · Thyroid cancer · Immunohistochemistry

Introduction

Genetic alterations in cancer include driver mutations, gene fusions, and copy number alterations. In contrast, epigenetic modifications that do not change the DNA sequence can also regulate transcriptional activation or repression. A number of epigenetic dysregulations have been reported in thyroid cancer [1]. Among epigenetic regulators, enhancer of zeste homolog 2 (EZH2) is the catalytic subunit of polycomb repressive complex 2 (PRC2), which methylates lysine 27 of histone H3 to promote transcriptional silencing [2]. Higher EZH2 expression was associated with recurrence in papillary thyroid cancer [3]. Moreover, strong nuclear EZH2 staining is characteristic of undifferentiated thyroid cancer [4]. As such, EZH2 is an attractive target for the treatment of thyroid cancer, and some EZH2 inhibitors have shown encouraging results in clinical trials [5].

The canonical function of EZH2 is to catalyze trimethylation of histone H3 at lysine 27 (H3K27me3), leading to the silencing of its target genes. Nonetheless, the expressions of EZH2 and H3K27me3 are not always concordant [6]. Although EZH2 overexpression portends a poor prognosis in most cancer types, low expression of H3K27me3 was found to correlate with shorter overall survival time in breast, ovarian, and pancreatic cancers [7]. Recently, loss of H3K27me3 is considered as being highly specific for malignant peripheral nerve sheath tumor [8]. It remains unknown whether benign and malignant thyroid tumors have aberrant H3K27me3 expression. In this study, we further explored the prognostic implication for H3K27me3 expression in thyroid cancer.

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Materials and Methods

Under institutional review board approval, the paraffin-embedded archival pathologic specimens from 144 patients with differentiated thyroid cancer were randomly collected from the archives of the Department of Pathology, MacKay Memorial Hospital. Additional formalin-fixed, paraffin-embedded blocks investigated in this study included 10 nodular goiters, 10 follicular adenomas, 10 samples of lymphocytic thyroiditis, 7 poorly differentiated thyroid cancers, and 4 anaplastic thyroid cancers. All of the tissues were histologically reviewed, and the diagnosis was made based on the most current World Health Organization criteria, which adopted the Turin consensus criteria for the histopathological diagnosis of poorly differentiated carcinoma [9]. Minimal extrathyroidal extension was considered as a prognostic factor according to the seventh edition of the American Joint Committee on Cancer (AJCC) staging system [10].

For patients with differentiated thyroid cancer, total thyroidectomy with prophylactic central neck dissection or therapeutic dissection was performed [11]. The risk of recurrence was assessed using the 2015 American Thyroid Association management guidelines for adult patients with differentiated thyroid cancer [12]. The presence of *BRAF* mutation (c.1799T>A; p.V600E) was determined by Sanger sequencing as we previously reported [13].

The immunohistochemical analysis for H3K27me3 was performed as previously described [14]. In brief, the deparaffinized tissue sections were boiled for 20 min in citrate buffer for antigen retrieval. Following inactivation of endogenous peroxidase activity and blocking non-specific antibody binding, the sections were incubated overnight with primary antibody. The monoclonal rabbit antibody targeting H3K27me3 (clone RM175) was obtained from RevMab Biosciences, San Francisco, CA, USA. The clone had no cross-reactivity with H3K27me1, H3K27me2, or other methylations in histone H3. Subsequently, the sections were incubated with the secondary antibody conjugated with horseradish peroxidase for 30 min. The immunoreaction was then visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB) brown chromogen, and the slides were counterstained with hematoxylin. Paraffin sections of malignant melanoma were used as positive controls [15]. Sections incubated with isotype rabbit IgG instead of the primary antibody were used as negative controls, which showed no specific immunostaining.

Images were acquired with automated multichannel microscopy using the TissueFAXS platform (TissueGnostics, Vienna, Austria) equipped with an 8-bit Baumer Optronic HXG40c digital camera. The intensity and distribution of H3K27me3 immunostaining were evaluated by two independent investigators. Staining intensity was scored as 0 (no staining), 1+ (weak staining), 2+ (moderate staining), and 3+ (strong staining). *H* scores were calculated by multiplying the intensity and the percentage of positively stained cells (0 to 100%) [16]. Given that the normal and benign thyroid tissues typically had a moderate and variable staining pattern, neoplastic tissues that exhibited homogenously strong immunostaining were considered as overexpression.

The optical density of immunostaining was further quantified using the ImageJ plugin, ImmunoRatio [17]. Specifically, the percentage of brown peroxidase positivity was calculated from color deconvolution of each image. Quantification of immunostaining was compared with manual immunohistochemical scoring results.

All of the analyses were performed using Statistical Product and Service Solutions (SPSS) Statistics version 25 (IBM, Armonk, NY, USA). The chi-square test, Fisher's exact test, and the Cochran-Armitage test for trend were used to analyze categorical variables as appropriate [18]. The correlation between manual immunohistochemical scores and quantified optical density was analyzed by Spearman's rank correlation. All tests were two-sided, and p value of less than 0.05 was considered statistically significant.

Results

The expression of H3K27me3 was evaluated by immunohistochemical staining in benign and malignant thyroid lesions. We first examined matched noncancerous tissues of 144 differentiated thyroid cancers. Normal thyroid follicular epithelial cells typically exhibited moderate-intensity nuclear H3K27me3 expression (Fig. 1). Positively stained cells had a heterogeneous distribution. Nonetheless, the percentage of positive cells was virtually always > 50%.

A moderate and variable nuclear staining pattern was seen in nodular goiters and follicular adenomas similar to that of normal thyroid tissues (Fig. 2). Strong H3K27me3 expression was noted in 8 out of 10 samples of lymphocytic thyroiditis. The remaining two samples had moderate-intensity H3K27me3 immunostaining. We further analyzed 144 cases of differentiated thyroid cancer, which consisted of 127 papillary and 17 follicular thyroid cancers. The moderate and strong expression was observed in 37 (29%) and 80 (63%) papillary thyroid cancers, respectively (Fig. 3). Ten (8%) papillary cancers exhibited absent or decreased expression of H3K27me3. As for follicular thyroid cancer, the moderate and strong expression was observed in 6 (35%) and 7 (41%) cases, respectively. Four (24%) had weak immunostaining.

Next, we evaluated whether the dedifferentiation process influences the H3K27me3 expression. Notably, strong H3K27me3 staining was observed in 5 out of 7 poorly differentiated thyroid cancers and 3 of 4 anaplastic cancers (Fig. 4). The remaining 2 poorly differentiated cancers and one anaplastic cancer had moderate staining intensity. The staining

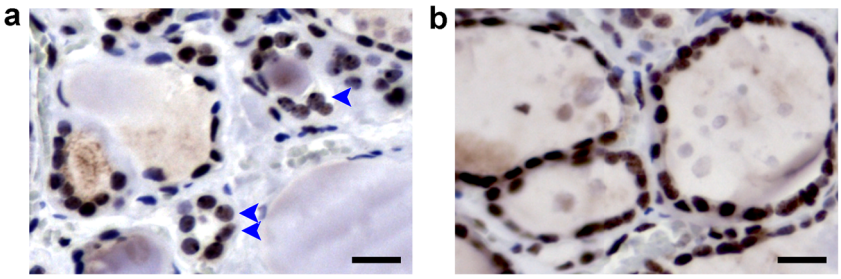

Fig. 1 H3K27me3 immunostaining of normal thyroid epithelial cells in a female (**a**) and a male (**b**) patient. Eccentric intranuclear dots (arrowheads) reflect the inactivated X chromosome. Scale bars, 20 μm

pattern was quite homogeneous in poorly differentiated cancers but slightly heterogeneous in anaplastic cancers.

Our manual scoring of immunostaining intensity was compared with ImmunoRatio automated calculation of the percentage of the positive nuclear area (Fig. 5). A good correlation between manual scoring and automated quantification was noted (Spearman's rho = 0.736, $p < 0.001$).

According to the predefined criteria, some 87 (60%) of 144 differentiated thyroid cancers exhibited strong H3K27me3 expression and were classified as H3K27me3 overexpression. The clinicopathological analysis showed that H3K27me3 overexpression was associated with extrathyroidal extension, lymphovascular invasion, lymph node metastasis, and higher risk of recurrence in differentiated thyroid cancer (Table 1). Differentiated thyroid cancer with H3K27me3 overexpression tended to have a higher prevalence of concomitant lymphocytic thyroiditis, but the difference did not reach statistical significance. Furthermore, papillary thyroid cancer with H3K27me3 overexpression had a marginally higher frequency of harboring *BRAF* mutation (75% vs 60%, $p = 0.083$).

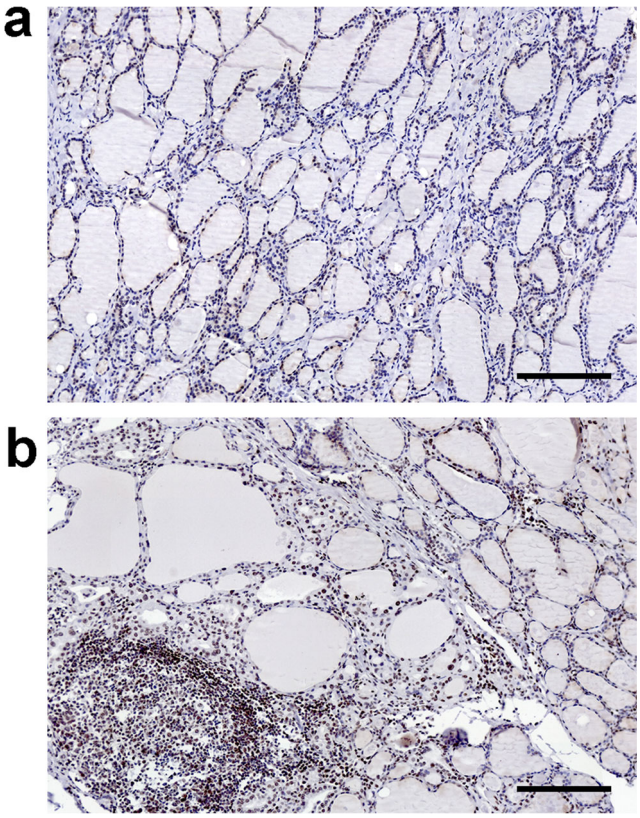

Fig. 2 Representative photomicrographs of H3K27me3-immunostained slides from nodular goiter (**a**) and lymphocytic thyroiditis (**b**). Scale bars, 200 μm

Discussion

Polycomb group proteins possess histone-modifying activities that result in transcriptional repression. Such epigenetic mechanism plays a fundamental role in regulating cellular differentiation and development. In cancer cells, histone modifications also contribute to tumor cell heterogeneity [19]. It is worth noting that mutations in genes encoding SWI/SNF chromatin remodeling complex or histone methyltransferases are enriched in advanced thyroid cancers, indicating that epigenetic alterations are involved in the progression and dedifferentiation of thyroid cancer [20]. Previous studies have shown that EZH2 is overexpressed in poorly differentiated and anaplastic thyroid cancer and predicts aggressive behavior of these cancers [4, 21]. An in silico analysis of papillary thyroid cancer demonstrated a loss of thyroid hormone responsiveness and dysregulation of retinoic acid metabolism, highlighting the putative activation of EZH2 [22]. Interestingly, we found that papillary thyroid cancer harboring telomerase reverse transcriptase (TERT) promoter mutations had higher EZH2 expression levels [23].

Accumulating evidence suggests that the oncogenic function of EZH2 related to PRC2 functioning, namely, repression of tumor suppressor genes through H3K27me3 [24]. Nonetheless, H3K27me3 expression is not solely determined by the activity of PRC2. H3K27me3 mark can be removed by the KDM6A (UTX) and KMD6B (JMJD3) demethylases [25]. Our previous transcriptome analysis showed that the expression of KDM6A and KMD6B was slightly decreased in papillary thyroid cancer without significant difference [26]. In the present study, for the first time we demonstrated that the H3K27me3 expression was upregulated in malignant thyroid tumors, particularly in those with less differentiated phenotype.

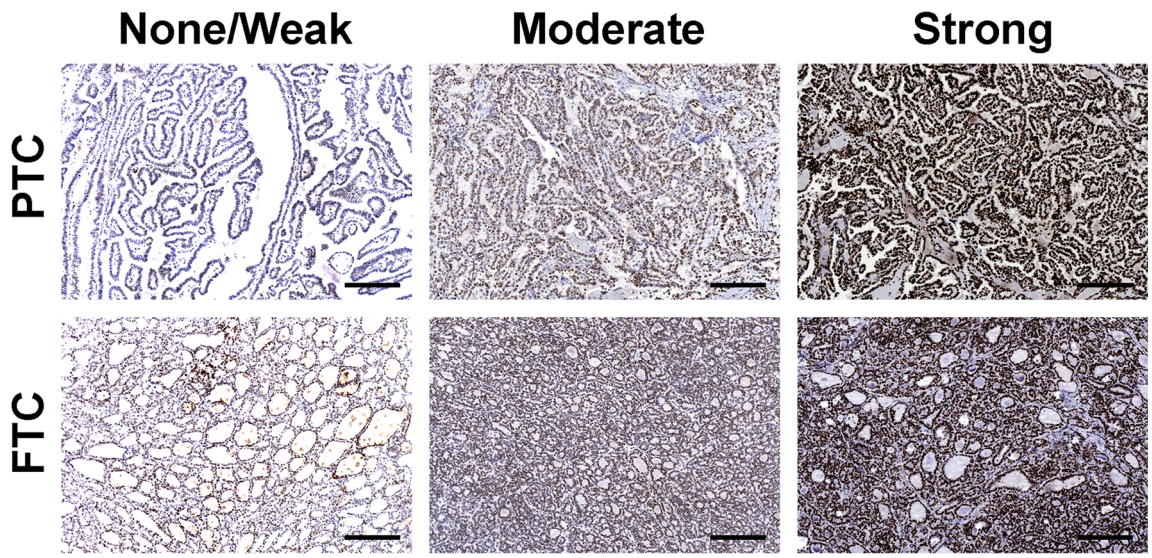

Fig. 3 Representative images of H3K27me3 expression in papillary (PTC) and follicular (FTC) thyroid cancer showing none/weak, moderate, and strong immunohistochemical staining. Scale bars, 200 μm

The prognostic implications of H3K27me3 expression might be cell type- and cancer type-dependent. As aforementioned, low expression of H3K27me3 was linked to shorter overall survival in breast, ovarian, and pancreatic cancers [7]. On the contrary, high expression of H3K27me3 was associated with shorter cancer-specific survival in hepatocellular, bladder, and oral squamous cell carcinoma [27–29]. Furthermore, worse progression-free survival and chemoradiotherapy effectiveness were observed in patients with esophageal squamous cell carcinoma displaying high H3K27me3 expression [30]. In this study, we observed a higher frequency of extrathyroidal extension, lymphovascular invasion, and lymph node metastasis in differentiated thyroid cancer with H3K27me3 overexpression. Our findings are consistent with those of Kampilafkos and colleagues who demonstrated that the H3K27me3 level was higher at the invasion front of melanoma [15]. These observations suggest that H3K27me3 may play a role in the invasive capacity of cancer cells.

Consistent with a previous report [31], we observed the presence of Barr body-pattern eccentric intranuclear dot which reflects the inactivated X chromosome in many female tissues. In male tissues, diffuse nuclear staining was observed. The findings confirmed that H3K27me3 immunostaining is helpful for sex determination. It is intriguing to note that H3K27me3 expression was upregulated in most cases of lymphocytic thyroiditis. Concomitant lymphocytic thyroiditis was also slightly more frequent in differentiated thyroid cancer with H3K27me3 overexpression. In melanoma, higher H3K27me3 level was associated with the presence of lymphocytic infiltration [15]. NF-κB has been shown to regulate the

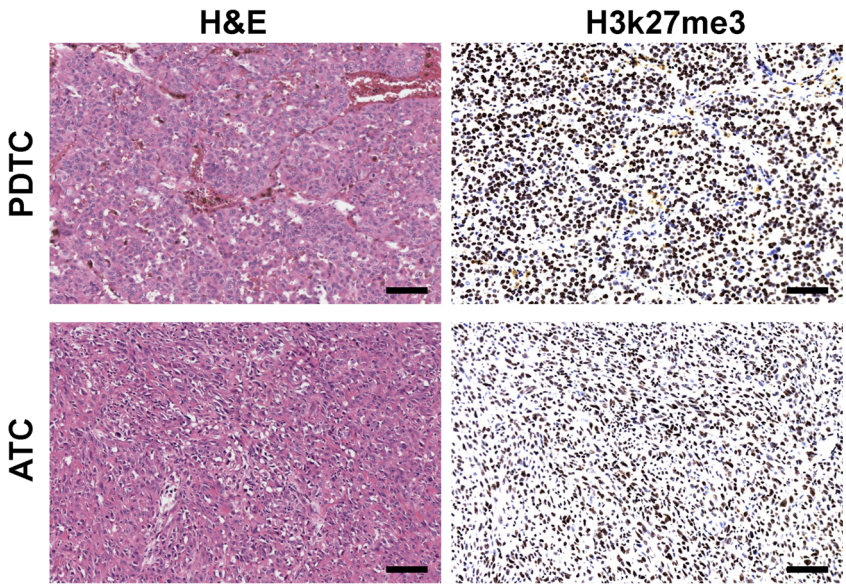

Fig. 4 Representative microphotographs of hematoxylin and eosin (H&E) stain and H3K27me3 immunostaining of poorly differentiated thyroid cancer (PDTC) and anaplastic thyroid cancer (ATC). Scale bars, 100 μm

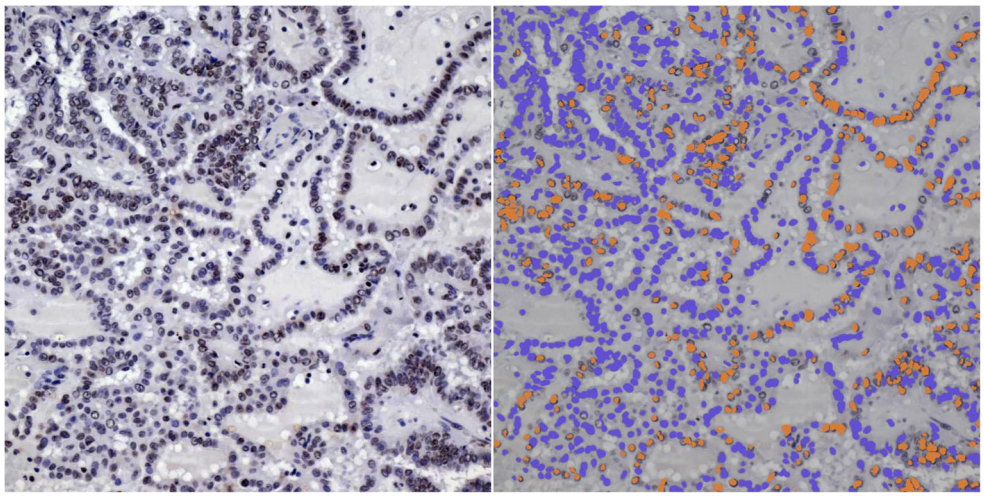

Fig. 5 Representative images of ImmunoRatio automated calculation of the percentage of the positive nuclear area in one case of papillary thyroid cancer with moderate H3K27me3 expression

EZH2 expression [32]. Whether H3K27me3 modulation serves as a link between inflammation and cancer deserves further investigation.

Table 1 Clinicopathological features and H3K27me3 expression of 144 cases of differentiated thyroid cancer

	H3K27me3 overexpression (−) ($n = 57$)	H3K27me3 overexpression (+) ($n = 87$)	p value
Histology			0.084
Papillary	47 (82%)	80 (92%)	
Follicular	10 (18%)	7 (8%)	
Sex			0.809
Male	8 (14%)	11 (13%)	
Female	49 (86%)	76 (87%)	
Age			0.521
< 55 years	44 (77%)	63 (72%)	
≥ 55 years	13 (23%)	24 (28%)	
Tumor size			0.795
0–2 cm	24 (42%)	33 (38%)	
2–4 cm	27 (47%)	47 (54%)	
> 4 cm	6 (11%)	7 (8%)	
Lymphocytic thyroiditis			0.050
Absent	56 (98%)	77 (89%)	
Present	1 (2%)	10 (11%)	
Extrathyroidal extension			< 0.001
None	43 (75%)	38 (44%)	
Minimal	12 (21%)	36 (41%)	
Advanced	2 (4%)	13 (15%)	
Lymphovascular invasion			0.029
Absent	49 (86%)	61 (70%)	
Present	8 (14%)	26 (30%)	
Lymph node metastasis			0.006
N0	39 (68%)	39 (45%)	
N1a	13 (23%)	33 (38%)	
N1b	5 (9%)	15 (17%)	
Risk of recurrence			0.003
Low risk	26 (46%)	21 (24%)	
Intermediate risk	27 (47%)	49 (56%)	
High risk	4 (7%)	17 (20%)	

Activated mitogen-activated protein kinase signaling by *BRAF* mutation can promote the expression of the PRC2 components and H3K27me3 [33]. In the present study, we found that H3K27me3-overexpressing papillary thyroid cancer tends to have a higher frequency of *BRAF* mutation although the difference did not reach statistical significance. It may partly explain that papillary thyroid cancers harboring *BRAF* mutation are less sensitive to radioactive iodine therapy, and BRAF inhibition can potentiate redifferentiation of iodine-refractory *BRAF*-mutant papillary thyroid cancer [34]. In this regard, it will be interesting to examine whether H3K27me3 overexpression negatively correlates with the expression of the sodium-iodide symporter. Targeting PRC2 may be a potential approach to restore radioiodine avidity, particularly in *BRAF*-mutant cancers.

In summary, our analysis of H3K27me3 expression in a panel of neoplastic and normal thyroid tissues demonstrated that H3K27me3 overexpression is associated with aggressiveness and dedifferentiation of thyroid cancer. Histone modifications are subject to partial or complete reversal of the alterations. Currently, a variety of PRC2 inhibitors are under development for cancer treatment. The expression level of H3K27me3 in thyroid cancer may provide valuable prognostic and predictive information to guide tailored treatment strategies.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval The study was approved by the Institutional Review Board of MacKay Memorial Hospital. All procedures performed in studies involving human participants were in accordance with the ethical standards of MacKay Memorial Hospital and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. For this type of study, formal consent is not required.

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