Chulalongkorn Medical Journal

Volume 68 | Issue 4 Article 5

October 2024

# Histone 3 and 4 acetylation patterns in nodular goiter and well-differentiated thyroid tumors compared with normal thyroid tissue

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## Recommended Citation

Keelawat, Somboon (2024) "Histone 3 and 4 acetylation patterns in nodular goiter and well-differentiated thyroid tumors compared with normal thyroid tissue," *Chulalongkorn Medical Journal*: Vol. 68: Iss. 4, Article 5.
DOI: https://doi.org/10.56808/2673-060X.5496
Available at: https://digital.car.chula.ac.th/clmjournal/vol68/iss4/5

# Histone 3 and 4 acetylation patterns in nodular goiter and well-differentiated thyroid tumors compared with normal thyroid tissue


## Abstract

**Purpose** To estimate histone modification (hypoacetylation or hyperacetylation) in thyroid lesions, using immunohistochemistry compared with their normal counterpart.

**Methods** FFPE sections of surgically removed PTC, FA, FTC, and nodular goiter samples were collected from the archives of the Department of Pathology, Faculty of Medicine, Chulalongkorn University from 2016-2018 and stained with anti-acetyl histone 3 antibody (H3K9/K14ac) and anti-acetyl histone 4 antibody (H4K5,8,12 and 16ac). The intensity and proportion of immunostaining of the lesions and their normal thyroid tissue counterparts were automatically scored by Aperio Image Scope software.

**Results** A total of 147 benign and malignant thyroid lesions cases, including 28 FA, 50 PTC, 19 FTC, and 50 nodular goiters, were studied. Deacetylation of both anti-acetyl histone 3 antibody (H3K9/K14ac) and anti-acetyl histone 4 antibody (H4K5,8,12 and 16ac) was detected in nodular goiter ($p=0.0016$ and $p=0.05$ in all cases).

**Conclusion** For the first time, this study demonstrates that nodular goiters have H3 and H4 deacetylation compared with their normal tissue counterpart. In contrast, these epigenetic events are not found in well-differentiated thyroid neoplasm (FA, FTC, and PTC).



## Keywords

Thyroid, histone acetylation, immunohistochemical study, epigenetics


## Cover Page Footnote

This study was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University (IRB No. 411/64). This article does not contain any studies with human participants or animals performed by any of the authors.

Chula Med J Vol. 68 No. 4 October - December 2024 ; 275 - 281 DOI : 10.56808/2673-060X.5496

Original article

# Histone 3 and 4 acetylation patterns in nodular goiters and well-differentiated thyroid tumors compared with normal thyroid tissue

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## Abstract

**Background:** Histone acetylation is a well-recognized epigenetic process involved in various cancer development pathways. However, thus far, its role in thyroid tumors and nodular goiter has been barely investigated.

**Objectives:** This study aimed to assess histone modifications (hypoacetylation or hyperacetylation) in various thyroid lesions, including papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), follicular adenoma (FA), and nodular goiter, through immunohistochemistry with anti-acetyl histone 3 (H3K9/K14ac) and 4 (H4K5, 8, 12, and 16ac) antibodies, compared with their normal counterparts to elucidate their role in the development of these lesions.

**Methods:** Formalin-fixed paraffin-embedded sections of surgically resected PTC, FA, FTC, and nodular goiter samples were collected from the archives of the Department of Pathology, Faculty of Medicine, Chulalongkorn University, from 2016 to 2018. These sections were then stained with anti-acetyl histone 3 (H3K9/K14ac) and 4 (H4K5, 8, 12, and 16ac) antibodies. Aperio ImageScope was used to automatically score the intensity and proportion of immunostaining of the lesions and their normal thyroid tissue counterparts.

**Results:** A total of 97 malignant thyroid lesions, including (28 FA, 50 PTC, and 19 FTC samples), and 50 nodular goiters were evaluated. The deacetylation of both anti-acetyl histone 3 (H3K9/K14ac) and 4 (H4K5, 8, 12, and 16ac) antibodies was detected in nodular goiter ($P = 0.0016$ and $P < 0.0001$, respectively) in comparison with their normal counterparts. However, the difference in the acetylation status of FTC, PTC, and FA were not significant compared with that of their normal counterparts ($P > 0.05$ in all cases).

**Conclusion:** For the first time, this study demonstrates the H3 and H4 deacetylation of nodular goiters compared with their normal tissue counterparts. In contrast, these epigenetic events are not found in well-differentiated thyroid neoplasms (FA, FTC, and PTC).

**Keywords**: Epigenetics, histone acetylation, immunohistochemical study, thyroid.

Cancer requires genetic and epigenetic events for its development.[1, 2] The field of human genetics has significantly progressed since the discovery of the deoxyribonucleic acid (DNA) in the 1960s, which has reformed the sphere of medical oncology. More recently, several epigenetic mechanisms of oncogenesis have been discovered. These molecular events play vital roles in cancer development.[3] They are defined as heritable changes in gene expression (phenotype) that do not lead into the corresponding change in DNA sequence (genotype). Epigenetic processes ensure the effective packaging of the genetic material to fit within the mammalian nucleus. In eukaryotic cells, DNA is packed as chromatin, with nucleosomes as functional units. Each nucleosome is composed of an octamer of four core histones (H3, H4, H2A, and H2B), which are wrapped by 147 base pairs of DNAs.[1, 4] Lysine residues in these histone tails have a positive charge that interacts with the negatively charged phosphate backbone of DNAs.[5] The N-tail of histones inside the nucleosome octamer

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Received: May 10, 2024
Revised: June 20, 2024
Accepted: July 26, 2024

is the well-known target for specific chromatin epigenetic posttranslational modifications (PTMs). PTMs affect nucleic acids and proteins, which are crucial for chromatin structure regulation without changing the DNA sequence. These chromatin modifications influence the DNA transcriptional machinery, which affects gene expression. PTMs are involved in oncogenesis because they can silence tumor-suppressor genes and enhance the expression of oncogenesis. Chromatin stability and integrity are determined by the balance between acetylation and deacetylation.[6]

So far, several studies have examined epigenetic changes in various malignancies in the literature, for instance, an investigation of acetylation levels in prostate, breast, and colorectal carcinomas.[7, 8] However, only a few studies on the acetylation status of thyroid neoplasms, which is the most common endocrine malignancy, ranked fourth by prevalence and seventh by incidence in Thailand.[9] Therefore, this study aimed to estimate the level of histone acetylation in well-differentiated thyroid neoplasms and nodular goiters compared with their normal thyroid tissue counterparts, using anti-acetyl histone 3 and 4 (H3 and H4, respectively) immunohistochemistry (IHC).

## Materials and methods

### *Samples*

Cases of papillary thyroid carcinoma (PTC) (n = 50), follicular thyroid carcinoma (FTC) (n = 19), follicular adenoma (FA) (n = 28), and nodular goiter (n = 50) from 2016 to 2018 were retrieved from the archives of Department of Pathology, Faculty of Medicine, Chulalongkorn University. Cases were selected based on the availability of the materials, including tumor tissues and normal thyroid samples, and were re-evaluated for consistency of diagnoses and block selection by a pathologist (Keelawat S). The diagnoses were made according to the fourth edition of the World Health Organization classification of tumors of endocrine organs (PMID 30537125).

This study was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University (IRB no. 411/64).

### *IHC study*

Formalin-fixed paraffin-embedded sections of all specimens, including lesions and normal thyroid tissues of the same patients, were retrieved from the archive. Specifically, 3 μm-thick sections of tissues were placed on positively charged slides (SuperFrost Plus, Menzer-Glaser, and Freiburg, Germany) and deparaffinized with xylene and alcohol. For the IHC staining method, the manufacturer's recommended protocol of the automated Ventana BenchMark XT (Ventana Medical Systems, USA) was followed. After antigen retrieval, the slides were incubated with anti-acetyl H3 and H4 rabbit polyclonal antibody (Catalog no. 06-599, Lot no. 3022883 and Catalog no. 06-866, Lot no. 2459612, respectively, Millipore, Germany) at a dilution of 1:200 and 1:300, respectively, for 1 h at 37°C. Thereafter, the UltraView DAB detection kit (Ventana Medical Systems, USA) was used for secondary antigen and chromogen.

### *Image acquisition*

Whole-slide images of both hematoxylin and eosin slides and anti-histone antibody IHC slides were acquired by scanning conventional glass slides using Aperio CS2 whole-slide scanner (Leica Biosystems, Germany) with a 40 × lens and one focus layer without Z-stacking (i.e., several focus planes). The default autofocus mode was used; however, in a few cases, scanning required manual focus to optimize image sharpness. Image files (.svs format) were stored on a Windows-based computer running the Aperio Scan Scope software.

### *Automated digital image analysis*

All slides stained with the two antibodies were digitalized by Aperio CS2 slide scanner (Aperio Technologies, USA). Image analysis was performed using Aperio ImageScope v.12.2.2.5015 (Aperio Technologies). Tumor lesions and normal tissues from the same patients were compared. The H-score quantifies biomarker expression from whole-slide scanned IHC images. Initially, in the H-score algorithm, individual cells and their subcellular compartments such as nucleus, cytoplasm, and cell membrane are first identified, and based on the relative expression of the biomarker, the cells are classified as either positive or negative. Positive cells are further classified into high (3+), medium (2+), or low (1+) intensity. This captures both the intensity and proportion of the biomarker of interest and comprises values between 0 and 300, subsequently offering a dynamic range to quantify biomarker abundance.[10] To minimize selection bias, five fields of 0.5 × 0.5 cm area were selected for the examination and arbitrarily selected at the superior, inferior, left, right, and center aspects. At least 5,000 nuclei were scored for each case **(Figure 1).**

The immunostaining intensity was automatically scored using commercially available nuclear v.9 algorithm (Aperio Technologies). The grading scale was quantitatively defined as follows: 0 (negative), 1+ (weak nuclear staining), 2+ (moderate), and 3+ (strong). The number of nuclei in each category was determined simultaneously **(Figures 2)**. Both the intensity and proportion scores were then averaged to provide an IHC score that represents the degree of histone acetylation for each case according to the following formula:

H-Score = (% of 3+ nuclei × 3) + (% of 2+ nuclei × 2) + (% of 1+ nuclei × 1)

### *Statistical analysis*

Descriptive statistics was performed in Microsoft Office Excel 2016 (Microsoft, CA). Further statistical analysis was performed with GraphPad Prism 9. Data were expressed as means ± standard deviations (SD), and Student's paired *t*-test was used to compare variables between the thyroid lesions and their normal counterparts. Pearson (parametric) correlation was used to determine the relationship between the variables. A $P < 0.05$ was considered significant in all tests.

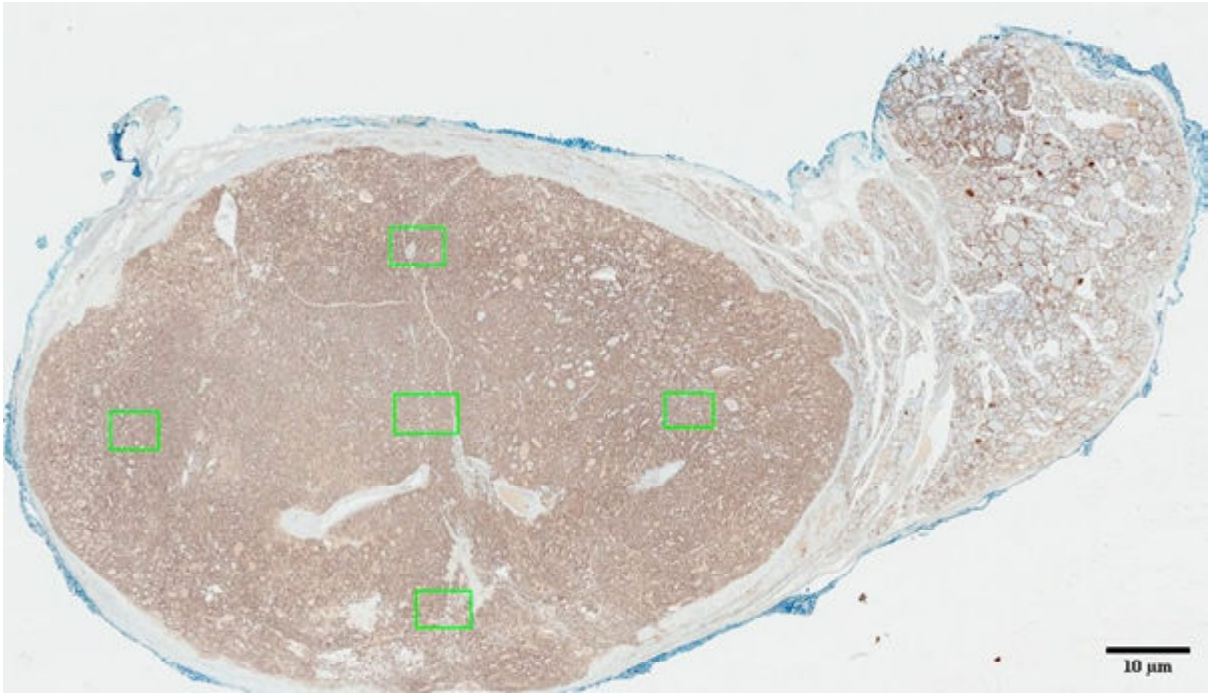



**Figure 1**. Example of image analysis with five fields of 0.5 x 0.5 cm area arbitrarily selected as superior, inferior, right, left, and center aspect.

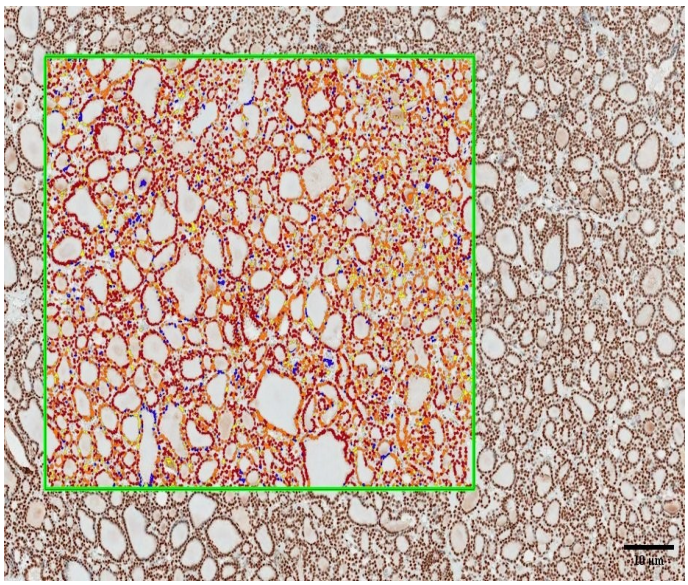
## Results

A total of 147 thyroid lesions were analyzed in the study, including 97 malignant tumors consisting of 50 PTCs, 19 FTCs, 28 FAs, and 50 benign lesions of nodular goiters. The clinical and demographic data of the patients are provided in **Table 1**.

For patients with nodular goiter, the anti-acetyl histone antibody score (H-score) of their normal thyroid tissue counterparts had significantly higher acetylation levels of 98.9 ± 77.3 and 169.1 ± 63.0 (mean ± SD) for both anti-acetyl H3 (H3K9/K14ac) and H4 (H4K5, 8, 12, and 16ac) antibodies, respectively. Compared with nodular goiters, the average scores were 80.4 ± 73.5 and 134.5 ± 75.5, respectively, revealing significant differences ($P$ = 0.0016 and $P < 0.0001$, respectively).

FA showed a lower level of anti-acetyl H3 and H4 antibodies (124.0 ± 74.4 and 157.9 ± 74.4, respectively) than the adjacent normal thyroid (130 ± 70 and 166.2 ± 53.0, respectively). However, the differences were not significant ($P$ = 0.33 and $P$ = 0.533, respectively).

**A**



**B**

| Attribute | Value |
|---|---|
| Percent Positive Nuclei | 80.0554 |
| Intensity Score | 2 |
| (3+) Percent Nuclei | 14.0654 |
| (2+) Percent Nuclei | 44.1867 |
| (1+) Percent Nuclei | 21.8033 |
| (0+) Percent Nuclei | 19.9446 |
| Average Positive Intensity | 177.069 |
| Average Negative Intensity | 229.009 |
| (3+) Nuclei | 1067 |
| (2+) Nuclei | 3352 |
| (1+) Nuclei | 1654 |
| (0+) Nuclei | 1513 |
| Total Nuclei | 7586 |
| Average Nuclear RGB Intensity | 152.161 |
| Average Nuclear Size (Pixels) | 644.777 |
| Average Nuclear Size (um^2) | 41.1737 |
| Area of Analysis (Pixels) | 22602460. |
| Area of Analysis (mm^2) | 1.4433318429333999 |
| *** Algorithm Inputs *** | *** Algorithm Inputs *** |
| Algorithm | Nuclear v9 |
| Version | 9.1 |

**Figures 2.** Example of nuclear staining for H4K12ac antibody **(A)** with image analysis; **(B)** and result of analysis for the selected field with grading of nuclear intensity as negative (blue), weakly positive (1+, yellow), moderately positive (2+, orange), and strongly positive (3+, red).

**Table 1.** A clinical and demographic data of the patients enrolled in this study (n = 147).

| Variables | N (%) |
|---|---|
| **Age (years)** | 48.4 (± 15.2) |
| < 20 | 4 (2.7%) |
| 21 - 40 | 40 (27.2% |
| 41 - 60 | 69 (46.9%) |
| > 60 | 34 (23.1%) |
| **Gender** | |
| Male | 26 (17.7%) |
| Female | 121 (82.3%) |
| **Laterality** | |
| Right | 56 (47.1%) |
| Left | 44 (29.9%) |
| Bilateral/ total | 47 (32.0%) |
| **Thyroid function** | |
| Hyperthyroidism | 22 (15.0%) |
| Hypothyroidism | 11 (7.5%) |
| Euthyroidism | 85 (57.8%) |
| Not available | 29 (19.7%) |

In PTC, the acetylation level of anti-acetyl H3 antibodies was slightly lower (118.2 ± 74.0) than its adjacent normal thyroid tissue (120.5 ± 104.1). Nonetheless, the differences were not significant ($P$ = 0.86). The average level of anti-acetyl H4 antibody was higher in PTC (156.1 ± 66.2) than its normal counterpart (144.5 ± 113.2). Again, they were not significantly different ($P$ = 0.46).

For FTC, the acetylation level was slightly higher for both anti-acetyl H3 and H4 antibodies at 106.9 ± 78.2 and 142.3 ± 92.9, respectively, than that of its normal counterparts (101.8 ± 63.6 and 119.4 ± 73.3, respectively). However, the differences were not and nearly significant ($P$ = 0.82 and $P$ = 0.05, respectively). The comparison of the H-scores between the normal thyroid tissue and thyroid lesions (goiter, PTC, FTC, and FA) is illustrated in **Table 2** and **Figure 3.**

**Table 2.** Comparison of H-scores between normal thyroid tissue and the lesions (Goiter, PTC, FTC and FA).

| **Goiter (n = 50)** | **Normal mean (SD)** | **Goiter mean (SD)** | ***P*-value Paired *t*-test** |
|---|---|---|---|
| H3ac | 98.9 (± 77.3) | 80.4 (± 73.5) | 0.0016 |
| H4ac | 169.1 (± 63.0) | 134.5 (± 75.5) | < 0.0001 |
| **PTC (n = 50)** | **Normal mean (SD)** | **PTC mean (SD)** | ***P*-value Paired *t*-test** |
| H3ac | 120.5 (± 104.1) | 118.2 (± 74.0) | 0.86 |
| H4ac | 144.5 (± 113.2) | 156.1 (± 66.2) | 0.46 |
| **FTC (n = 19)** | **Normal mean (SD)** | **FTC mean (SD)** | ***P*-value Paired *t*-test** |
| H3ac | 101.8 (± 63.6) | 106.9 (± 78.2) | 0.82 |
| H4ac | 119.4 (73.3) | 142.3 (± 92.9) | 0.05 |
| **Follicular adenoma (n = 28)** | **Normal mean (SD)** | **Follicular adenoma mean (SD)** | ***P*-value Paired *t*-test** |
| H3ac | 130.3 (± 70.0) | 124.0 (± 74.4) | 0.33 |
| H4ac | 166.2 (± 53.0) | 157.9 (± 74.4) | 0.53 |

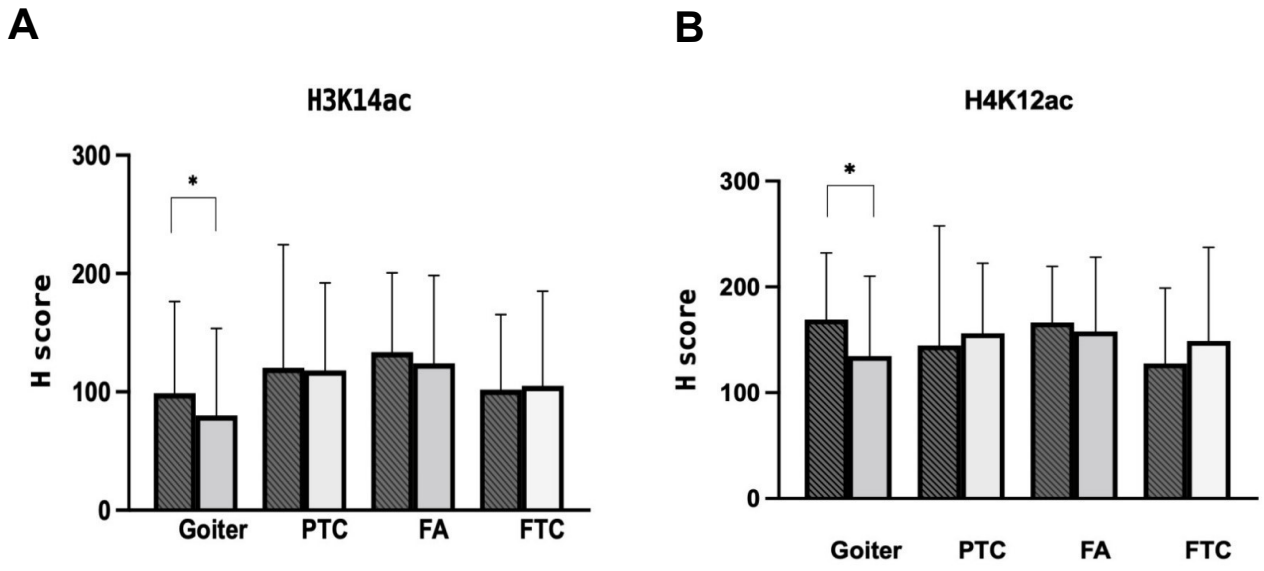



**Figure 3**. Bar Plot of H3K14ac **(A)** and H4K12ac; **(B)** in lesions (blank column) and normal counterpart (pattern). Columns represent means and standard deviation (SD). Statistically significant differences between the scores are depicted by solid-line brackets with *$P$ < 0.05.

## Discussion

Although histones have long been considered simple packaging units for DNAs, recent studies have revealed that histones and nucleosome architecture modifications actively influence transcriptional regulation and, thus, may actively participate in many pathways involved in the cell cycle. [11]

Among the epigenetic changes known thus far, histone acetylation is a fundamental process that strongly affects gene expression regulation. Any changes or disruption of this phenomenon has been linked to carcinogenesis. [12] In fact, acetylated histone is associated with active gene transcription and deacetylated histones to gene silencing. [13, 14] Many human malignant tissues show variable degrees of expression of histone deacetylases and histone acetyltransferases. Various processes, including cell cycle progression, chromosome dynamics, DNA recombination, DNA repair, and apoptosis, are influenced by histone acetylation. Thus, the control of aberrant activity and/or expression of these proteins has been favorable in the treatment of diseases such as cancer.

Moreover, the potential to manipulate histone acetylation in vivo by different anticancer drugs brings in medical interest for its studies. [15]

In this study, we tested whether the levels of acetylated histones are modified in thyroid lesions compared with their adjacent normal counterparts. Compared with normal tissue, levels of acetylated H3 at residues K9/K14 and H4 at residue K12 were lower in nodular goiter samples and marginally higher in PTC and FTC samples. These data propose that acetylated levels of H3 at residue K9/14 reduce in thyroid goiter. However, we have not correlated our findings with other studies because no studies have compared the level of histone acetylation in goiter to that of normal thyroid tissue.

Many studies have shown a lower level of H3K18 in various cancers, e.g., prostate cancers. Seligson B, *et al*. demonstrated that lower levels of acetylated H3K18 is associated with cancer recurrence. [16] The same group reported that reduced levels of acetylated H3K18 are also associated with poorer clinical outcomes in patients with lung and kidney cancer. [17] A large cohort study in breast cancer by the Nottingham group similarly reported that low levels of acetylated H3K18 are associated with shorter disease-free survival and breast cancer-specific survival. [18]

Thus far, only one study has analyzed these alterations in thyroid neoplasms, which demonstrated that H3 at K9-K14 residue was increased in both PTC and FTC. [7] However, our findings are not in concordance with these results. We postulate that this discordance is attributed to the difference in antibody clones and evaluation methods employed in the two studies. Unlike the previous study [7], we used normal thyroid tissue from the same patient for comparison. We also minimized possible sources of error by performing image analysis using unbiased field selection with > 5,000 analyzed nuclei in each sample with the standardized and widely used Aperio platform. Our study had a larger sample size, which is another research advantage. [7]

Despite the lack of positive findings for neoplastic lesions in this study, the results still contribute to the scientific literature because, so far, very few studies have explored this issue. Although the statistical analyses did not yield significant results, a slightly different trend was observed between benign lesions (FA and goiter) and malignancies (PTC and FTC), i.e., the former tends to show decreased acetylation, whereas the latter has a tendency toward hyperacetylation **(Figure 3).** These findings provide grounds for future diagnostic use, particularly in distinguishing between benign lesions (e.g., adenomatous goiter and follicular adenoma) and malignancy, particularly follicular carcinoma. Thus, to clarify this issue, further larger-scale studies are required.

## Conclusion

To the best of the authors' knowledge, this study is the first to demonstrate that nodular goiters have H3 and H4 deacetylation compared with their normal tissue counterparts. However, these epigenetic events do not occur in well-differentiated thyroid neoplasms, such as FA, FTC, and PTC.

### Acknowledgements

This research was supported by the Ratchadapisek-somphot Endowment Fund, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (Grant No. RA61/020, SK)

### Conflict of interest statement

Each author has completed an ICMJE disclosure form. None of the authors declare any potential or actual relationship, activity, or interest related to the content of this article.

### Data sharing statement

All data generated or analyzed during the present study are included in this published article. Further details are available for noncommercial purposes from the corresponding author on reasonable request.

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