의학석사 학위논문

다양한 갑상선암종에서 텔로머레이스 역전사효소 (TERT) 프로모터 돌연변이 예측인자로서의 TERT와 5-하이드록시메틸사이토신 면역조직화학의 평가

Evaluation of Telomerase Reverse Transcriptase (TERT) and 5-hydroxymethylcytosine Immunohistochemistry as Predictors of *TERT* Promoter Mutations in Various Thyroid Carcinomas

울 산 대 학 교 대 학 원

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이 논문을 의학석사학위 논문으로 제출함

2024년 2월

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2024년 2월

감사의 글

의학의 길을 걷기 시작한 순간부터 오늘에 이르기까지, 많은 분들의 따뜻한 격려와 지원이 있었기에 제가 여기까지 올 수 있었습니다. 이 짧은 글로 모든 분들에 대한 고마움을 담기에는 턱없이 부족하지만, 지난 2년여 동안 저와 함께 해주신 모든 이들에게 깊은 감사를 전합니다.

저의 지도교수님이신 송동은 교수님, 선배 의사로서 길을 안내해주시고, 부족한 점이 많은 저를 가르쳐 주신 깊은 은혜에 머리 숙여 감사드립니다. 묵묵히 저를 인내해주시고, 알뜰히 살펴주시며, 세심하게 배려해주셔서 정말 감사드립니다. 그리고 바쁘신 와중에도 논문 작업에 관심을 가져주시고 지도해주신 김원구 교수님과 성태연 교수님, 그리고 책에서는 배울 수 없는 귀중한 지식을 전해주신 모든 교수님들께도 깊은 감사의 인사를 드립니다.

제가 태어났을 때부터 항상 저를 믿어주시고 기도와 사랑으로 지원해주신 어머니와 보고 싶은 아버지, 그리고 사랑하는 나의 아내 다훤이에게는 많이 사랑하고 고맙다는 말을 전합니다. 또한, 가깝고 먼 곳에서 저의 길을 지켜봐주고 격려해준 가족들, 배움의 길을 함께했던 선후배들, 곁에서 저와 동고동락했던 친구들에게도 감사의 마음을 전합니다.

전공의 생활을 시작한 후 정말 많은 일들이 있었던 것 같습니다. 때로는 무겁고 쓸쓸한 마음에 근심이 가득했던 날들도 있었고, 더할 나위 없이 풍성한 나날들에 기쁨이 넘치기도 했습니다. 다사다난했던 이 시간들이 앞으로 펼쳐질 지난한 삶의 중요한 선택과 갈림길의 순간에 빛을 발휘할 귀중한 자양분이 되었으면 좋겠습니다.

저와 함께 했던 모든 분들이 있었기에 지금의 제가 있을 수 있었습니다. 앞으로도 여러분이 주신 도움과 응원에 힘입어 최선을 다하겠습니다. 좀 더 낮은 자세로 겸손하며 성실하게, 타인의 몸과 마음을 살필 줄 알며, 공감과 인정이 넘치는 사람이 되도록 노력하겠습니다. 다시 한 번 진심으로 감사드립니다.

2023년 12월

집으로 향하는 길 위에서

Abstract

Background Telomerase reverse transcriptase (*TERT*) promoter mutation is known to be associated with an aggressive clinical course in thyroid carcinomas. Therefore, the detection of *TERT* promoter mutation is important for proper patient management. 5-hydroxymethylcytosine (5hmC) is an epigenetic marker involved in the DNA demethylation pathway, and loss of 5hmC has been observed in various tumors. Loss of 5hmC has also been reported in thyroid carcinomas and is presented as a possible predictive biomarker for *TERT* promoter mutation.

Materials and Methods This study included 105 patients (44 patient with *TERT* mutant group and 61 patients in *TERT* wild group) with thyroid carcinoma and the presence of *TERT* promoter hotspot mutations was evaluated by Sanger sequencing. Immunohistochemistry (IHC) was conducted to evaluate TERT and 5hmC expression according to the *TERT* promoter mutation. H-scores were calculated for both of cancer lesion and its adjacent normal counterpart on the same slide in each case using an image analyzer.

Results The median H-scores of TERT IHC were significantly higher in the *TERT* mutant group than in the *TERT* wild group (47.15 vs 9.80, $p<0.001$). The sensitivity and specificity of TERT IHC for predicting *TERT* promoter mutations in thyroid carcinomas were 65.9% and 65.7%, respectively. Regardless of *TERT* promoter mutation status, the 5hmC H-scores were markedly lower in all subtypes of thyroid carcinomas compared to the normal counterparts. Significant differences in 5hmC H-scores were observed between N0 and N1a, and between N0 and N1b in total thyroid carcinoma, but not within the papillary thyroid carcinoma subgroup.

Conclusion TERT IHC showed higher expression levels in thyroid carcinomas with *TERT* promoter mutations than those without mutations, albeit with some difficulties in the proper interpretation. Additionally, the expression of 5hmC IHC was reduced in various thyroid carcinomas regardless of the status of *TERT* promoter mutations. Comprehensive futher studies are required to elucidate the predictive role of 5hmC IHC as a promising prognostic marker in various thyroid carcinomas.

Keywords Thyroid carcinoma, Immunohistochemistry, Image analysis, *TERT* promoter mutation, 5-hydroxymethylcytosine

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Introduction

Thyroid cancers are one of the most common endocrine malignancies and encompass a broad spectrum of biological behaviors ranging from indolent to highly aggressive forms. Accurate prognostic stratification at the time of diagnosis is crucial for optimal patient management and treatment for patients with thyroid cancer. The telomerase reverse transcriptase (*TERT*) promoter mutation has emerged as an important molecular marker, predicting the aggressive clinical course in thyroid carcinomas. The *TERT* promoter hotspot mutation (C228T and C250T) serves as a major indicator of unfavorable prognosis in differentiated thyroid carcinomas, and their frequency escalates from well-differentiated to poorly differentiated and to anaplastic thyroid carcinomas (ATC) [1, 2]. The presence of *TERT* promoter mutation is known to be associated with resistance to radioactive iodine treatment, distant metastasis, tumor recurrence, and the dedifferentiation process in thyroid cancers [1, 3-5].

To detect the presence of *TERT* promoter mutations, molecular methods including Sanger sequencing and next-generation sequencing (NGS) have been usually employed in the clinical setting. However, these techniques can be time-consuming, technically demanding, and not always cost-effective process for routine screening. Immunohistochemistry (IHC) can stand out as a potential alternative method because of its well-established protocols, affordability, and simplicity [6]. While TERT IHC might not be suitable for predicting the presence of *TERT* promoter mutations in follicular thyroid carcinoma (FTC) [7], its efficacy in other types of thyroid carcinomas remains less elucidated.

Beyond the genetic mutations, epigenetic modifications have emerged as important prognostic determinants in thyroid tumors. DNA methylation, a crucial epigenetic mechanism, plays an important role in regulating gene expression. Abnormalities of DNA methylation, such as hypermethylation of tumor suppressors and hypomethylation of oncogenes, have been related to possible tumorigenesis in thyroid cancer [8]. 5-hydroxymethylcytosine (5hmC) is an intermediate in the DNA demethylation pathway, and recent studies suggested a marked decrease in 5hmC levels in various cancers [9]. A recent study reported the relevance of 5hmC loss to *TERT* promoter mutations in papillary thyroid carcinoma (PTC), proposing that 5hmC IHC might offer an alternative method to predict the presence of *TERT* promoter mutation [10]. Furthermore, the potential role of 5hmC as a valuable biomarker for predicting the presence of lymph node (LN) metastasis in PTCs has also been suggested [11].

In this study, we investigated the immunohistochemical expression of TERT and 5hmC according to the status of *TERT* promoter mutations in various thyroid carcinomas. This study aimed to elucidate the diagnostic utility of these markers in

predicting the presence of *TERT* promoter mutations and the aggressiveness of various thyroid carcinomas.

Materials and Methods

1. Study cohort

This cohort study included 105 patients who were diagnosed with follicular cell-derived thyroid carcinomas after surgery at Asan Medical Center from October 2020 to September 2022. *TERT* promoter hotspot mutations were evaluated for all the patients in this cohort. Clinicopathologic data of the patients were retrieved from electron medical records. *TERT* promoter hotspot mutations (C250T, C228T) were detected by Sanger sequencing, which was performed using formalin-fixed paraffin-embedded (FFPE) tissue from surgical specimens following the manufacturer's instruction (3500 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA). The pathological subtypes of the patients in this study were classified into four groups. These groups included PTC (n=71), follicular variant of papillary thyroid carcinoma (FVPTC, n=16), FTC (n=14), and high-grade thyroid carcinoma (HGTC, n=4). The category of HGTC included poorly differentiated thyroid carcinoma (PDTC), differentiated high-grade thyroid carcinoma (DHGTC), and ATC. Additionally, we also included 6 patients with benign control groups such as follicular adenoma (n=2) and thyroid follicular nodular disease (n=4). Pathological diagnoses were reviewed by two pathologists for all cases, and pathologic staging was rendered according to the 8th edition of American Joint Committee on Cancer staging system. Informed consents were obtained from all patients, and this study was approved by the Institutional Review Board of Asan Medical Center (2020-1219).

2. Immunohistochemistry

To evaluate the expression of TERT and 5hmC, we conducted IHC using FFPE tissue samples obtained from surgical specimens. Whenever possible, the same block used in the Sanger sequencing was utilized. We used a monoclonal anti-TERT antibody (ab32320, Abcam, Cambridge, UK) and a polyclonal anti-5hmC antibody (39769, Active Motif, Tokyo, Japan) at an optimal dilution (1:100 and 1:11000, respectively) which were determined through a serial dilution. The tissue samples were sectioned into 4 μm-thick sections and stained using the OptiView DAB IHC Detection Kit on the BenchMark XT automatic immunostaining device (Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer's instructions. Normal spermatocytes from testicular tissue were used as a positive control for TERT, and normal thyroid follicular cells were used as a positive control for 5hmC. Positive staining for TERT IHC was defined as a cytoplasmic, perinuclear, or nuclear patterns, while nuclear positive staining was interpreted for 5hmC IHC.

3. Image analysis

The slides were digitally scanned using a Pannoramic 250 Flash II (3DHISTECH, Budapest, Hungary) at 20X magnification, with a resolution within the range of 0.22-0.23 μm/pixel. To interpret the results of IHC, we employed Qupath (v0.4.3) as an image analysis software. The stain vectors for TERT and 5hmC IHC stainings were preprocessed separately. Representative areas of approximately $2mm^2$ were manually annotated in both the lesion and normal counterpart on the same slide. When the tumor had a diagnostic high-grade portion, the annotation included the high-grade area. The remaining non-neoplastic follicles were preferentially selected as normal counterpart, if this was not possible, the adjacent soft tissue was selected.

The H-score was automatically calculated using the 'positive cell detection' command with three-tiered intensity threshold parameters, separately for both the lesion and normal counterpart. In this command, the intensity threshold for 1+, 2+, and 3+ were set at 0.1, 0.2, and 0.3, respectively, while the remaining options were maintained at their default settings. The H-score was calculated using the following formula: 1 × (% of cells with 1+ staining) + 2 × (% of cells with 2+ staining) + 3 × (% of cells with 3+ staining). This formula yielded a continuous scale ranging from 0 to 300. The H-score 'Cell: DAB OD mean' was adapted to analyze TERT expression, and 'Nucleus: DAB OD mean' was used for 5hmC expression.

4. Statistical analysis

Statistical analysis and data visualization were performed using R version 4.3.1 and RStudio (v2023.06.01). To compare the differences between *TERT* mutant and *TERT* wild groups, the chi-square test, Cochran-Armitage test, and Wilcoxon rank-sum test were properly employed. The diagnostic efficacy of TERT IHC was assessed using a Receiver Operating Characteristic (ROC) curve to determine its sensitivity and specificity. The Kruskal-Wallis test was utilized to analyze the differences among PTC, FVPTC, FTC, and HGTC subgroups, and the differences in the 5hmC H-score among T and N categories. In cases where significant differences were detected by the Kruskal-Wallis test, post hoc tests were subsequently conducted to discern pairwise subgroup differences. The HGTC group in the *TERT* wild group was excluded from the post hoc analysis because it had only one case. All p-values were two-sided, and $p < 0.05$ was considered statistically significant.

Results

1. Baseline clinicopathologic characteristics of patients

In this cohort study, 44 patients with *TERT* promoter mutations (*TERT* mutant group) and 61 patients without *TERT* promoter mutations (*TERT* wild group) were included (Table 1). Most of the *TERT* promoter mutations were C228T (86%) mutation, and six cases had C250T mutation, of which five were PTCs. The mean ages in the *TERT* mutant group and the *TERT* wild group were 61.3 years and 48.9 years, respectively ($p < 0.001$). The mean tumor size was larger in the *TERT* mutant group than in the *TERT* wild group (3.46cm vs 2.61cm, $p=0.108$). For aggressive subtypes of carcinomas, all eight cases of the tall cell subtype of PTC were included within the *TERT* mutant group. The *TERT* mutant group also included one angioinvasive (AI) and one widely invasive (WI) FTC, as well as one AI-FVPTC. In contrast, the *TERT* wild group included one WI-FTC and one AI-FVPTC. In the case of HGTC, the *TERT* mutant group included two ATCs and one PDTC, while the *TERT* wild group had just one PDTC (Table 2).

Table 1. Epidemiological and Clinical Data in the Cohort

		Total	***TERT* wild**	***TERT* mutant**	*p*-value
Total number		105	61	44	
Age, y	Mean	54.1 ± 15.4	48.9 ± 15.1	61.3 ± 12.9	**<0.001**
Gender, n	Female	70 (66.7)	45 (73.8)	25 (56.8)	0.108
	Male	35 (33.3)	16 (26.2)	19 (43.2)	
Size, n	Mean, cm	2.96 ± 2.09	2.61 ± 1.67	3.46 ± 2.50	0.125
	≤ 2cm	43 (41.0)	28 (45.9)	15 (34.1)	
	2-4cm	37 (35.2)	21 (34.4)	16 (36.4)	
	> 4cm	25 (23.8)	12 (19.7)	13 (29.5)	
T category, n	T1-2	49 (46.7)	33 (54.1)	16 (36.4)	
	T3	45 (42.8)	23 (37.7)	22 (50.0)	0.092
	T4	11 (10.5)	5 (8.2)	6 (13.6)	
N category, n	N0	46 (43.8)	32 (52.5)	14 (31.8)	
	N1a	21 (20.0)	10 (16.4)	11 (25.0)	0.064
	N1b	38 (36.2)	19 (31.1)	19 (43.2)	
M category, n	M0	98 (93.3)	58 (95.1)	40 (90.9)	0.398
	M1	7 (6.7)	3 (4.9)	4 (9.1)	

n, number. Numbers in parentheses represent percentages within each category.

Table 2 . Epidemiological and Clinical Data in Various Thyroid Carcinomas

		PTC		FVPTC		FTC		HGTC	
TERT		wild	mutant	wild	mutant	wild	mutant	wild	mutant
Number of cases		40	31	12	4	8	6	1	3
Age, y	Mean	46±14	59±13	49±14	65±11	48±14	64±11	78	71±5
Gender, n	Female	32 (80)	20 (65)	8 (67)	2 (50)	5 (63)	3 (50)	0 (0)	0 (0)
	Male	8 (20)	11 (35)	4 (33)	2 (50)	3 (37)	3 (50)	1 (100)	3 (100)
Size, n	≤ 2cm	24 (60)	15 (48)	3 (25)	0 (0)	1 (12)	1 (17)	0 (0)	0 (0)
	2-4cm	13 (33)	10 (33)	6 (50)	3 (75)	2 (25)	1 (17)	0 (0)	1 (33)
	> 4cm	3 (7)	6 (19)	3 (25)	1 (25)	5 (63)	4 (66)	1 (100)	2 (67)
T category, n	T1-2	22 (55)	12 (39)	8 (67)	2 (50)	3 (37)	0 (0)	0 (0)	0 (0)
	T3	13 (32)	14 (45)	4 (33)	1 (25)	5 (63)	2 (33)	1 (100)	3 (100)
	T4	5 (13)	5 (16)	0 (0)	1 (25)	0 (0)	4 (67)	0 (0)	0 (0)
N category, n	N0	12 (30)	3 (10)	11 (92)	3 (75)	8 (100)	6 (100)	1 (100)	2 (67)
	N1a	9 (23)	10 (32)	1 (8)	1 (25)	0 (0)	0 (0)	0 (0)	0 (0)
	N1b	19 (47)	18 (58)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (33)
M category, n	M0	39 (97)	31 (100)	11 (92)	3 (75)	7 (88)	4 (67)	1 (100)	2 (67)
	M1	1 (3)	0 (0)	1 (8)	1 (25)	1 (12)	2 (33)	0 (0)	1 (33)

n, number. Numbers in parentheses represent percentages within each category.

2. TERT IHC expression

The TERT positive immunostaining predominantly showed a cytoplasmic pattern, and occasionally, perinuclear or nuclear patterns (Figure 1). The carcinoma group showed a lower H-score compared to normal counterpart (Figure 2A and 2B). The median H-score for benign control group was 68.30, representing the highest score among all groups (Table 3). In comparisons according to the status of *TERT* promoter mutations, the *TERT* mutant groups showed higher H-scores than the *TERT* wild groups (Figure 2C and 2D). The median H-score in the total *TERT* mutant groups was 47.15, but it was 9.80 in the *TERT* wild group ($p < 0.001$). In both PTC and FVPTC, the *TERT* mutant groups had significantly higher median H-scores than the *TERT* wild groups ($p < 0.001$ and $p=0.013$, respectively).

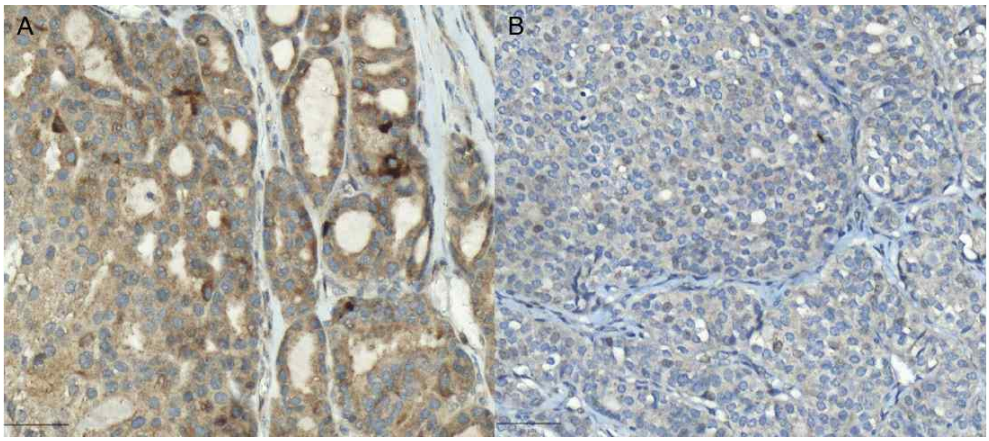

Figure 1. TERT immunohistochemical staining. (A) Poorly differentiated thyroid carcinoma with *TERT* promoter mutation shows mostly cytoplasmic staining. (B) Poorly differentiated thyroid carcinoma without *TERT* promoter mutation expresses faint cytoplasmic and nuclear staining. (*Scale bar in the bottom left: 50um*)

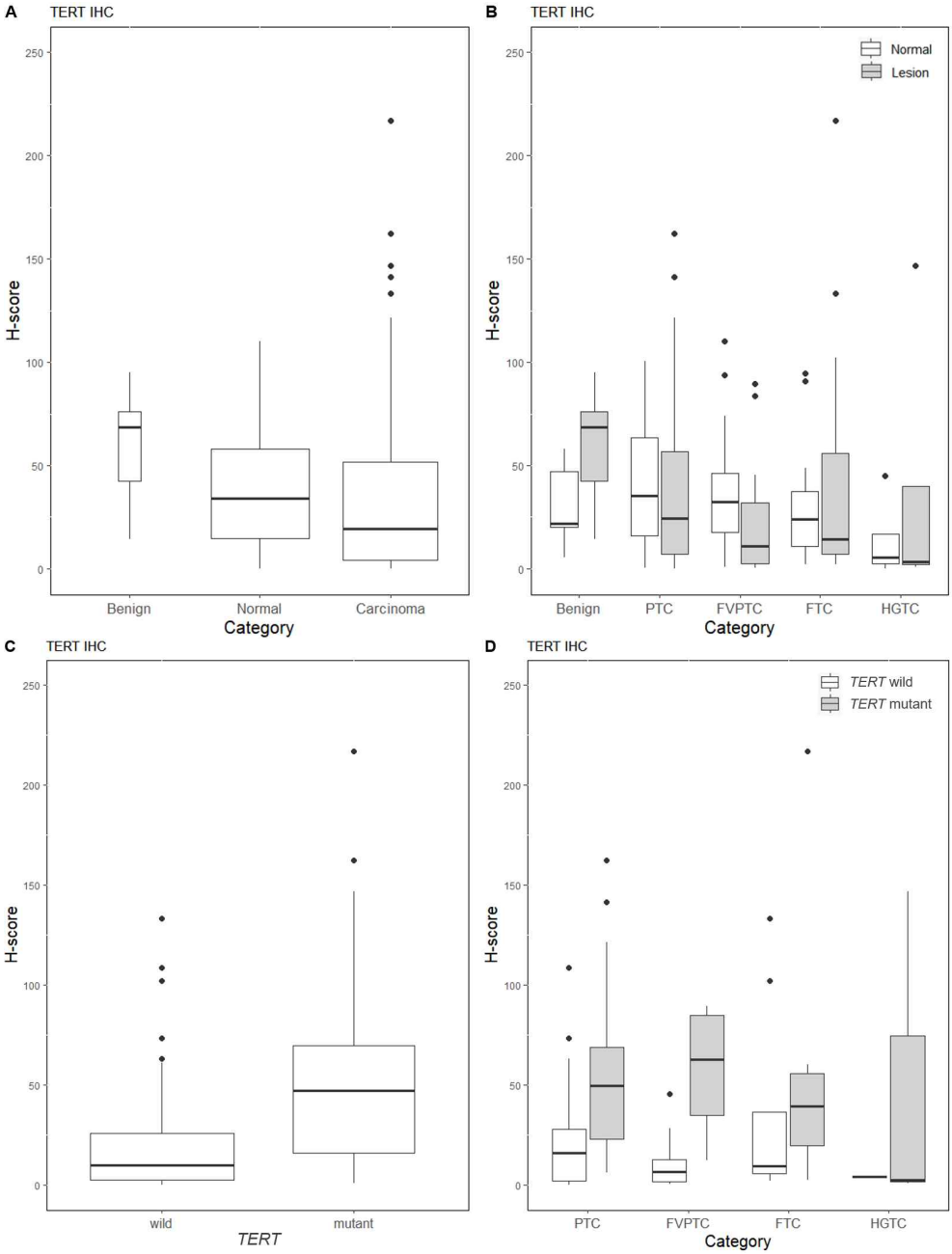

Figure 2. Box plots of TERT immunohistochemistry H-scores in each group. (A) The normal group comprises all the corresponding normal counterparts for carcinoma cases. The carcinoma group shows the lowest median H-score. (B) All carcinoma subgroups have lower median H-scores than their respective normal counterparts. (C) The total *TERT* mutant group shows higher H-score than the *TERT* wild group, and (D) this trend is consistent within each subgroup.

Table 3. TERT H-scores in Each Group

	H-score (median)				
	Benign	**PTC**	**FVPTC**	**FTC**	**HGTC**
Normal	21.62	35.24	32.34	24.05	5.33
Lesion	68.30	24.54	10.81	14.33	3.33
p-value	0.093	0.069	**0.029**	0.982	0.99
	Total	**PTC**	**FVPTC**	**FTC**	**HGTC**
***TERT* wild**	9.80	16.01	6.47	9.39	4.25
***TERT* mutant**	47.15	49.76	62.94	39.29	2.41
p-value	**<0.001**	**<0.001**	**0.013**	0.345	0.99

In the ROC curve analysis of TERT IHC, the optimal cutoff value for predicting *TERT* promoter mutation in various thyroid carcinomas was determined to be 37.145 (Figure 3). At this threshold, the sensitivity and specificity of TERT IHC were 65.9% and 65.7%, respectively. The Area Under the Curve (AUC) was 0.651 (0.559-0.743, 95% confidence interval).

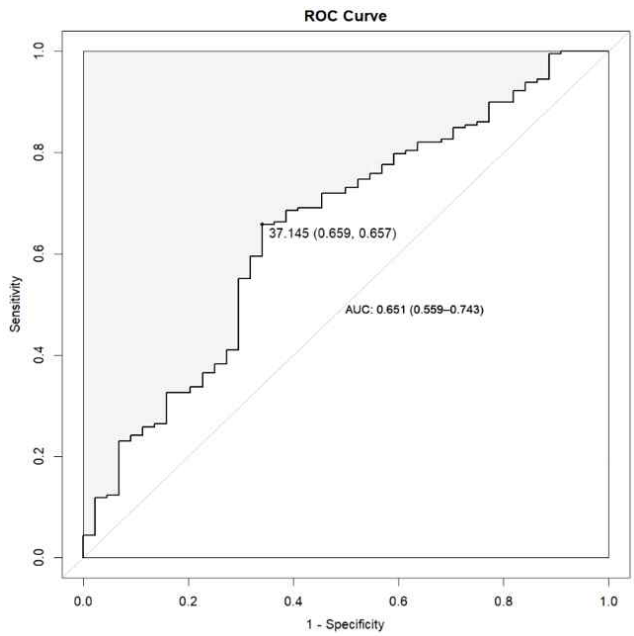

Figure 3. ROC curve of TERT immunohistochemistry. The optimal cutoff value is 37.145. The sensitivity and specificity are 65.9% and 65.7%, respectively.

3. 5hmC IHC expression

The 5hmC positive immunostaining showed strong nuclear expression in both normal counterpart and benign control group (Figure 4). The expression was markedly reduced in carcinoma compared with the normal counterpart (median H-score, 38.41 vs 171.06, $p < 0.001$) (Figure 5A). In all carcinoma subgroups, the median H-scores were significantly lower than their normal counterparts (Figure 5B and Table 4). There were no significant differences in H-scores between the *TERT* mutant and wild groups (Figure 5C, 5D, and Table 4). Among the carcinoma subgroups regardless of the status of *TERT* promoter muations, only a slight difference between PTC and FVPTC was identified (p=0.051).

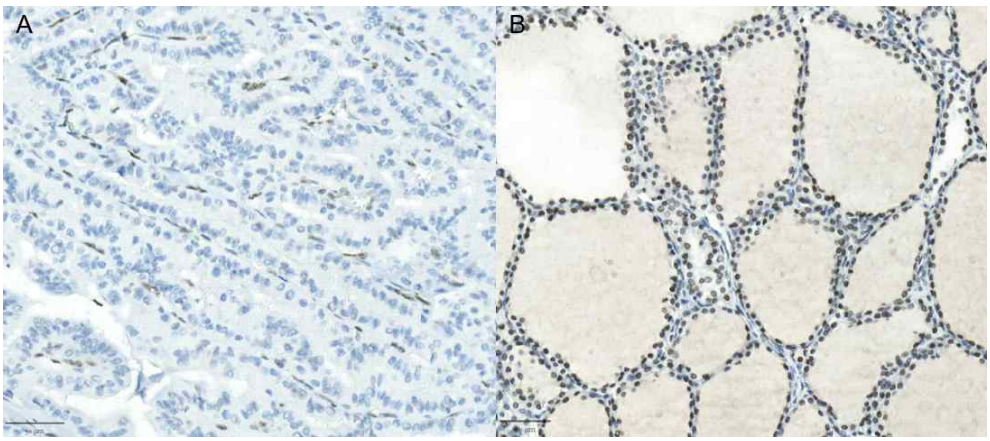

Figure 4. 5hmC immunohistochemical staining. (A) Tall cell subtype of papillary thyroid carcinoma with *TERT* promoter mutation shows a marked loss of expression compared with endothelial cell. (B) Follicular adenoma without *TERT* promoter mutation retains nuclear expression. (*Scale bar in the bottom left: 50um*)

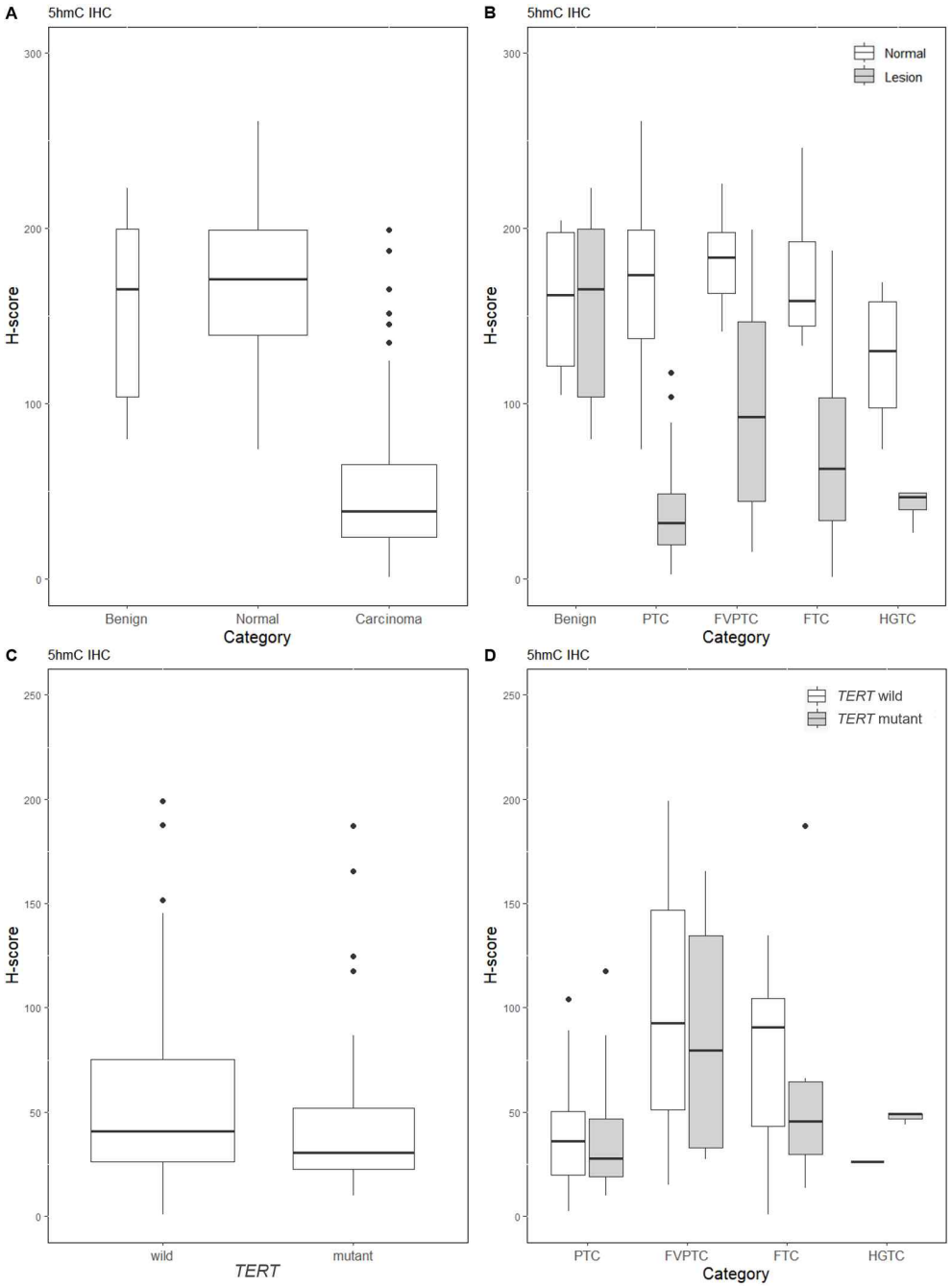

Figure 5. Box plots of 5hmC immunohistochemistry H-scores in each group. (A) The carcinoma group shows the lowest H-score. (B) All carcinoma subgroups have lower median H-scores compared with their normal counterparts. (C) The *TERT* mutant and wild groups show similar H-scores. (D) Regardless of the status of TERT promoter mutations, the PTC subgroup shows lower median H-score than FVPTC or FTC.

Table 4. 5hmC H-scores in Various Thyroid Carcinomas

	H-score (median)				
	Benign	**PTC**	**FVPTC**	**FTC**	**HGTC**
Normal	162.00	173.61	183.30	158.60	130.06
Lesion	165.46	31.87	92.58	62.74	46.60
p-value	0.99	**<0.001**	**<0.001**	**<0.001**	**<0.001**
	Total	**PTC**	**FVPTC**	**FTC**	**HGTC**
***TERT* wild**	40.73	36.02	92.58	90.71	26.26
***TERT* mutant**	30.34	27.76	79.50	45.51	49.10
p-value	0.164	0.457	0.684	0.414	0.5

We evaluated the difference of 5hmC IHC expression across T and N categories in 105 patients with various thyroid carcinomas (Figure 5). There were significant differences in 5hmC H-score between N0 and N1a (p=0.009), and between N0 and N1b (p=0.006). (Figure 6B). However, no significant differences were observed among N categories within PTC subgroup (p=0.09) (Figure 6D), nor among any of T categories (Figure 6A and 6C).

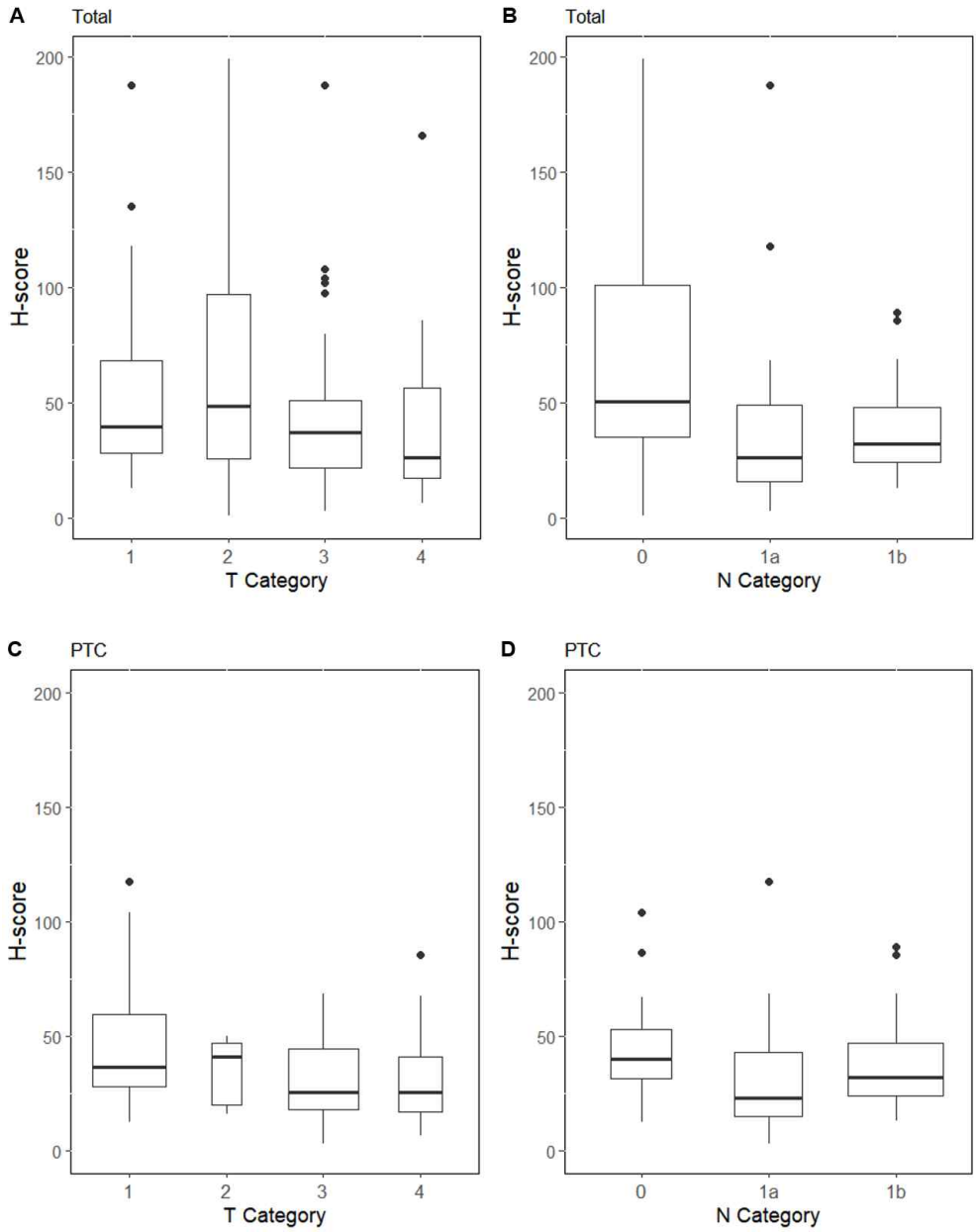

Figure 6. Box plots of 5hmC immunohistochemistry H-scores across T and N categories. (A) H-scores in each T categories, and (B) N categories for total thyroid carcinomas. N0 shows a slightly higher median H-score than N1a or N1b. (C) H-scores in each T categories and (D) N categories for the PTC subgroup. All median H-scores are below 50, showing a narrow interquartile range relative to total thyroid carcinomas.

Discussion

Human TERT is a catalytic protein subunit of the telomerase complex responsible for synthesizing telomeres, and its production and activity are typically suppressed in most normal cells except for cells like germline or progenitor cells [12, 13]. However, the genetic and epigenetic changes in the *TERT* gene, such as promoter mutations, can lead to increased *TERT* expression and telomerase activity, potentially inducing cellular immortality [14-16]. In normal thyroid follicles, *TERT* expression is generally reduced, but partial TERT immunoreactivity was reported in some thyroid cancers [7, 13, 17, 18]. TERT can also have various intracellular localizations, such as nucleus, cytoplasm, and mitochondria, corresponding to its non-canonical functions [19, 20]. In addition, different *TERT* promoter mutations can coexist within segments of a single tumor and cause the heterogeneous distribution pattern of positive TERT immunostaining [21]. In our study, TERT IHC staining displayed heterogeneity in many cases with variations in both the intensity and the distribution of positive expression even within the same individual tumor, and this heterogeneity made the interpretation of TERT IHC more challenging. Although the *TERT* mutant group revealed higher median H-scores than the *TERT* wild group with statistical significance, the magnitude of the expression difference was not remarkable. Furthermore, considerable positive expression was observed even in the *TERT* wild group, and both benign lesions and normal counterparts also showed higher median H-scores.

TERT IHC has been explored in various tumor types, and its utility has been evaluated. In infiltrating gliomas, the sensitivity and specificity of TERT IHC were around 60% [22], similar to that of our present study. Paulsson et al. demonstrated no correlation between TERT IHC and *TERT* mRNA expression or the risk of recurrence in thyroid tumors [7]. Although it is well-established that the presence of *TERT* promoter mutations correlates with a poor prognosis in thyroid cancers, the efficacy of TERT IHC as a predictor of *TERT* promoter mutations or as indicator of poor prognosis remains unclear.

5hmC is an intermediate produced during the demethylation process when 5-methylcytosine is oxidized by the Ten-eleven translocation (*TET*) family of proteins [23]. Reduced TET activity diminishes demethylation, leading to a decrease in the level of 5hmC. It is well-known that hypermethylation of CpG islands occurs in various cancers, and abnormalities in *TET* proteins have been suggested as one of the mechanisms contributing to this aberrant DNA hypermethylation [9, 24-26]. Besides, reduced 5hmC levels have been observed in multiple cancers, including thyroid cancers [11, 27-29]. Our study also confirmed a significant reduction of 5hmC expression in various thyroid carcinomas compared with their normal counterparts or benign thyroid follicular lesions. However, the extent of reduced

5hmC expression did not show significant differences among various subgroups of thyroid carcinomas. Considering that PTC, FVPTC, and FTC subgroups have their distinct DNA methylation profiles, and the types of driver mutations like *BRAF* and *RAS* are potentially linked to unique methylation profiles [30], it can be anticipated that the levels of demethylation and the consequent 5hmC reductions might vary among thyroid cancers. Nevertheless, the relationship between the status of DNA methylation and 5hmC loss in thyroid cancers remains to be elucidated.

A previous study by Oishi et al. demonstrated a lower 5hmC level in PTCs with *TERT* promoter mutation compared with the wild type [10]. In our cohort, we found no correlation between reduced 5hmC IHC expression and the presence of *TERT* promoter mutation across PTC, FVPTC, FTC, and HGTC subgroups. Another study using two different antibodies for 5hmC IHC in FTCs also reported that 5hmC IHC has a low sensitivity as a predictor for the presence of *TERT* promoter mutations [31]. Although we did not measure 5hmC quantitatively and IHC results might not fully reflect the extent of 5hmC reduction, the role of 5hmC IHC in predicting of the presence of *TERT* promoter mutations is considered uncertain.

A few reports suggested that tumors with decreased 5hmC expression might be associated with worse clinical outcomes. Malignant melanoma and malignant gliomas like glioblastoma with reduced 5hmC expression were related to the shorter survival [32, 33]. The relationship between 5hmC loss and the prognosis remains unclear in thyroid cancers. While Tong et al. reported that a loss of 5hmC might indicate increased propensity for LN metastasis in PTCs, another study comparing clinicopathological factors in PTCs and 5hmC IHC found no association with T or N categories [10, 11]. Our data suggested that a significantly lower 5hmC expression was associated with cervical LN metastasis in thyroid carcinomas. However, there was no significant reduction of 5hmC IHC expression in advanced T or N categories of the PTC subgroup. Although ATC, well-known for its poorest prognosis, exhibited a more significant 5hmC reduction compared to differentiated thyroid carcinomas in other studies [10, 34], the implications of 5hmC reduction in the prognosis of thyroid carcinomas remain uncertain. Further comprehensive studies with larger cohorts for each subgroup of thyroid carcinomas are needed.

In our study, we used QuPath to evaluate the results of IHC staining. Qupath is an open-source software designed for analyzing whole slide images in digital pathology. Compared with manual interpretation, it can yield more objective results with greater reproducibility [35]. Moreover, when immunostainings like PD-L1 are expressed in both neoplastic cell and non-neoplastic cells and exhibits heterogeneous distribution, Qupath provides tools to classify different type of cells and offers information for each compartment [36]. Although we attempted to apply these functions in our study, we found that the process was time-consuming and showed

incomplete classification primarily resulted from the diverse tumor cell morphologies and stromal cell distributions observed in each case. Thus, stromal cells were not completely excluded from our results, and this might influence the H-score outcomes, even though the stromal cells only constituted a minor fraction of the entire tumor. Meanwhile, our study employed surgical specimens of thyroid carcinomas and selected a part of the tumor for the image analysis. Image analyzers might also be used for smaller samples like a core needle biopsy of the thyroid, but they should be cautiously interpreted, considering possible heterogeneity of IHC expression and confounding pre-analytic factors [37].

Interestingly, the PTC subgroup showed a lower 5hmC median H-score in comparison to other subgroups. This might be attributed to the characteristic cytopathological features of PTC, such as nuclear cytoplasmic pseudoinclusions and nuclear clearing, which could potentially influence the intensity measurement of positive nuclear staining for the 5hmC IHC [38]. The significant variations of 5hmC levels across diverse types of thyroid carcinomas need to be elucidated in more extensive studies using reproducible, quantitative measurement methods.

Conclusion

We demonstrated that TERT IHC showed higher expression levels in thyroid carcinomas with *TERT* promoter mutations than those without mutations, although some difficulties in the proper interpretation remain. Additionally, our study revealed a reduction of 5hmC expression in various thyroid carcinomas. The presence of *TERT* promoter mutation did not correlate with reduced 5hmC expression in thyroid carcinomas, and the potential role of 5hmC IHC as a promising prognostic marker in thyroid carcinomas needs to be elucidated in comprehensive further studies.

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국문요약

배경 : 텔로머레이스 역전사효소 (TERT) 프로모터 돌연변이는 갑상선암종의 나쁜 예후와 관련되어 있다. 따라서 TERT 프로모터 돌연변이의 유무를 파악하는 것은 환자의 적절한 치료와 관리를 위해 중요하다. 5-하이드록시메틸사이토신 (5hmC) 은 DNA 비메틸화 경로에 관여하는 유전자 조절 인자이며, 다양한 종양에서 5hmC의 감소가 관찰되었다. 갑상선암종에서도 5hmC의 감소가 보고되었으며, 5hmC가 TERT 프로모터 돌연변이를 예측할 수 있는 바이오마커로서 제시되었다.

방법 : 총 105명의 갑상선암종 환자(44명의 TERT 돌연변이 그룹, 61명의 TERT 야생형 그룹)가 포함되었으며, TERT 프로모터 호발 부위 돌연변이에 대한 정보는 Sanger 염기서열 분석을 통해 평가되었다. TERT와 5hmC의 발현을 평가하기 위해 면역조직화학 염색을 수행하였고, 디지털 이미지 분석을 사용하여 각 종양에서 병변과 그 주변의 정상 부분 모두에 대한 H-점수를 계산하였다.

결과 : TERT 면역염색의 H-점수 중앙값은 TERT 돌연변이 그룹에서 TERT 야생형 그룹보다 유의하게 높았다 (47.15 vs 9.80, $p < 0.001$). 갑상선암종에서 TERT 프로모터 돌연변이에 대한 TERT 면역염색의 민감도와 특이도는 각각 65.9%와 65.7%였다. TERT 프로모터 돌변연이의 유무와 관계없이 모든 갑상선암종 하위 그룹에서 5hmC 면역염색의 H-점수는 정상 대조군에 비해 현저하게 낮았다. 전체 갑상선암종에서는 N0와 N1a 그룹, 그리고 N0과 N1b 그룹 사이의 5hmC H-점수에 유의한 차이가 관찰되었지만, 갑상선유두암 그룹 내에서는 유의한 차이를 보이지 않았다.

결론: TERT 면역조직화학 염색에서 TERT 프로모터 돌연변이가 있는 갑상선암종이 돌연변이가 없는 그룹에 비해 더 높은 발현 수준을 나타냈지만 이에는 적절한 해석의 어려움이 동반된다. 또한, 다양한 갑상선암종에서 5hmC 면역조직화학 염색의 발현은 감소했다. TERT 프로모터 돌연변이의 유무는 갑상선암종에서 5hmC 발현과 상관관계는 없었지만, 갑상선암종의 예후를 예측하는 유망한 마커로서의 5hmC 면역조직화학 염색의 역할은 향후 큰 규모의 연구를 통해 규명되어야 할 것으로 생각된다.

중심단어 : 갑상선암종, 면역조직화학, 이미지 분석, TERT 프로모터 돌연변이, 5-하이드록시메틸사이토신