INTRODUCTION

Thyroid carcinoma is the most common malignancy among the endocrine carcinomas, and its incidence has been increasing recently (1, 2). Gold standard of the treatment of thyroid carcinoma is surgery after cytologic confirmation, and the prognosis of the differentiated thyroid carcinoma is excellent. Currently, the estimation of prognosis and the treatment guidelines are based upon traditional TMN stages. However, a subset of thyroid carcinoma presents extremely unfavorable outcomes whereas some are indolent and grow slowly (3, 4), which cannot be fully explained by previous staging system. To discover additional prognostic markers and therapeutic targets, several molecules have been investigated so far. Among them, BRAF known to be associated with poor prognosis was most studied and several tyrosine kinase inhibitors targeting BRAF have been tried and some drugs showed promising results in the treatment of advanced thyroid carcinoma (5-7). However, the drugs are not beneficial to all of thyroid carcinoma because some cancers are not related to BRAF mutation and some acquires resistance to the drugs. In the way that novel target is needed to overcome the limitation of previous drugs, we noted Nodal which is a member of the TGF-β superfamily (Nodal, activins, bone morphogenetic proteins, myostatins, anti-Muellerian hormone)

Nodal, an embryonic morphogen, was found to be overexpressed in cancer tissues in studying embryonic signaling pathways in aggressive cancer (8). It is expressed around the transient embryonic structure, named the *Node* (9) which is the major source of progenitor cells for midline structures in vertebrates (10), and plays an integral role in processes of vertebrate development, endoderm/mesoderm specification, and establishing left-right axis asymmetry (11, 12). The role of Nodal in tumorigenicity was reported firstly in melanoma,

and its expression is known to be responsible for tumor cell plasticity and aggressiveness via organizing vascular-like networks, and Nodal is not expressed in normal skin (8). Inspired by the correlation between this re-activation of developmental signaling pathway in tumor cells and plasticity of aggressive tumors, further studies found that Nodal is also overexpressed in prostate and breast cancer while it is not expressed in normal adult tissues (13, 14). Accordingly, Nodal has drawn attention as a new therapeutic target due to its possible role in tumorigenicity and tumor progression. However, it has not been studied yet whether and how Nodal is expressed in thyroid tumors.

In this study, expression of Nodal in benign and malignant thyroid tumors was analyzed for the first time using immunohistochemistry in tissue microarray. With these results, we compared Nodal expression level according to the types of thyroid tumors, and evaluated correlation between the expression level and clinicopathological parameters in malignant tumors.

MATERIALS AND METHODS

Patient samples

Hematoxylin and eosin stained slides of PTC, follicular thyroid carcinoma (FTC), follicular adenoma (FA), adenomatous goiter (AG), and normal thyroid tissue from 315 patients were reviewed and formalin-fixed and paraffin-embedded block was selected. Slides and paraffin blocks were collected from the archives of the Department of Pathology, Seoul National University Boramae Hospital and the Department of Pathology, Seoul National University Hospital from January 1993 to December 2003. All samples were deprived of any patient identifiers and all experiments were conducted in accordance with the guidelines

proposed in The Declaration of Helsinki (http://www.wma.net) involving humans and approved by the Institutional Review Board of Seoul National University Hospital.

Construction of the tissue microarrays

Core tissue biopsies (2 mm in diameter) were taken from individual paraffin embedded thyroid tissues (donor blocks) and arranged in new recipient paraffin blocks (tissue array blocks) using a trephine apparatus (Super Biochips Laboratories, Seoul). Each tissue array block contained up to 60 cores, and total 6 tissue microarray blocks were made. Four micrometer-thick sections were cut from the completed array blocks and transferred to silanized glass slide.

Immunohistochemistry

Immunohistochemistry was performed using Ventana automated immunostainer (Benchmark XT, Ventana, Tuscon, AZ, USA). Primary antibodies used in this study were mouse monoclonal aniti-Nodal antibody (1:300 dilution; ab55676; Abcam, Cambridge, MA, USA), and rabbit polyclonal anti-Notch4 Antibody (1:100 dilution; sc-5594; Santacruz biotechnology, CA, USA). The intensity of positive tumor cells were graded on a scale of 0–3 (0, no staining; +1, weak stain; +2, moderate stain; +3, strong stain) by two experienced pathologist using consensus methods. Intensity score of 0 and +1 were categorized into low expression group while +2 and +3 into high expression group. Percentage of immunostained tumor cells was not considered because the cytoplasmic immunostaining were homogenous in all of the cases.

BRAF mutation analysis

The target locus was marked on hematoxylin and eosin (H&E) stained slides, and *BRAF* mutation analysis was conducted in paraffin-embedded sections as described

previously (15). Briefly, the BRAF exon 15 T1799A transversion (BRAF V600E) was amplified by polymerase chain reaction (PCR) from genomic DNA. After purification of the PCR products, direct DNA bidirectional sequencing was performed.

Statistical analysis

Results were analyzed with SPSS version 19 (SPSS, Inc. Chicago, IL, USA). Mean values with standard deviation were used for numeric data. Univariate analysis was performed using the Student′s *t*-, Chi-Square, or Fisher′s exact tests. For multivariable analysis, adjusted odds ratios (ORs) and 95% confidence intervals (CI) are reported. Differences were considered significant when $p < 0.05$.

RESULTS

Tissue microarray consists of tissues from PTC (n = 147), FTC (n = 58), FA (n = 57), AG (n = 54), and normal thyroid (n = 5). Clinicopathological characteristics of the tumors are summarized in Table 1. Nodal was immunostained in cytoplasm of the thyroid tumor cells sparing nucleus and normal thyroid follicular cells were not immunostained (Figure 1). Nodal was different according to the types of pathology (Table 2). The tumor proportion categorized into the high expression group was highest in the PTC (81.0%), and it was 48.3% in FTCs, 31.6% in FAs, and 35.2% in AGs ($p < 0.01$). Figure 2 demonstrates mean value of staining intensity and it was significantly higher in PTC than in FTC (2.26 vs. 1.74, $p < 0.01$). FTC showed higher value of intensity than FA (1.74 vs. 1.26, $p = 0.003$), and AG (1.74 vs. 1.13, $p = 0.02$).

Table 1 Composition of tissue microarray; Clinicopathological characteristics of the patients

	PTC (n = 147)	FTC (n = 58)
Gender (M : F)	24 : 120	8 : 47
Age (mean ± SD), years	44.7 ± 16.6	46.9 ± 15.5
Tumor size (mean ± SD), cm	2.5 ± 1.4	3.7 ± 2.0
Multiplicity	56 (38.1%)	8 (13.8%)
Extrathyroidal extension	110 (74.8%)	10 (17.2%)
Lymph node metastasis	74 (50.3%)	3 (5.2%)
Distant metastasis	2 (1.4%)	6 (10.3%)
AJCC TNM stage		
Lower stage (Ⅰ/Ⅱ)	83 (56.5%)	46 (79.3%)
Higher stage (Ⅲ/Ⅳ)	64 (43.5%)	12 (20.7%)
BRAF mutation	77/121 (63.6%)	0 (0%)
Recurrence	21 (14.3%)	1 (1.7%)

PTC papillary thyroid carcinoma, *FTC* follicular thyroid carcinoma

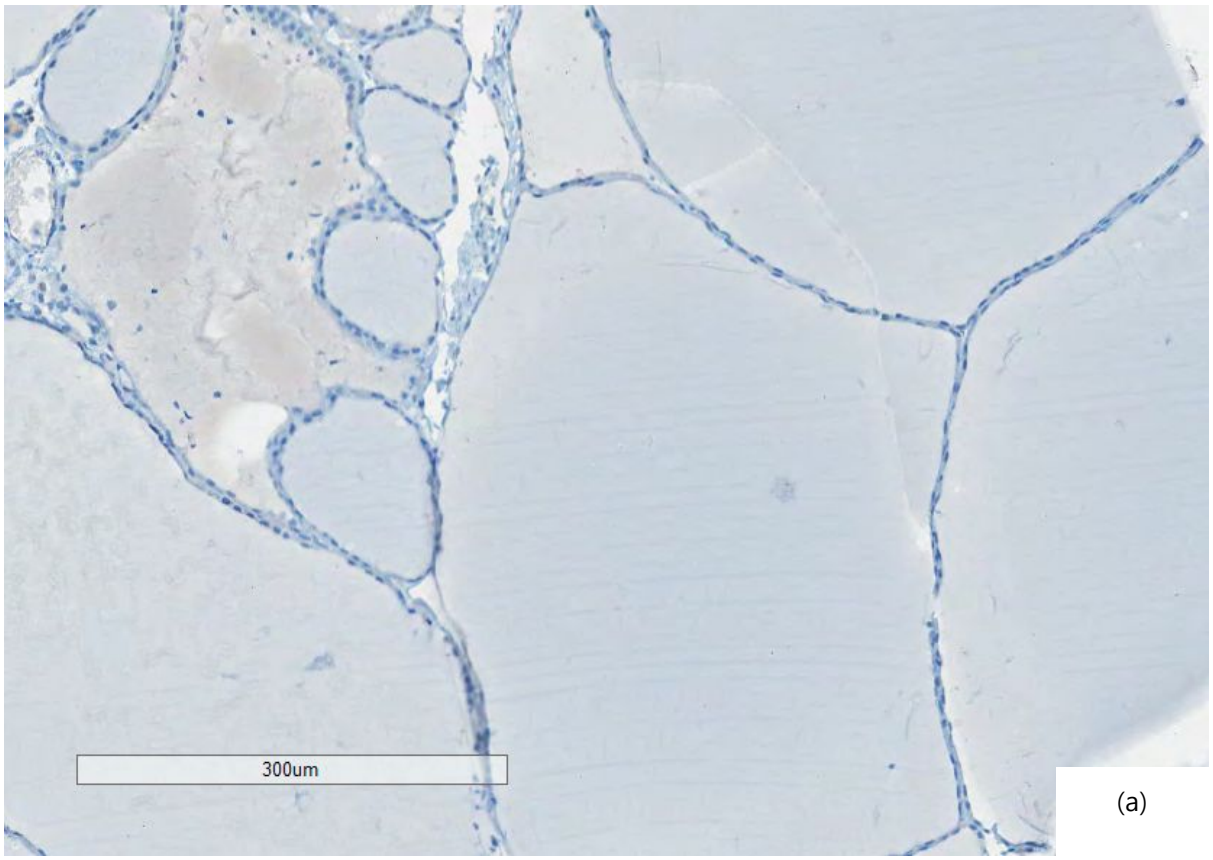
Table 2 Expression of Nodal in different types of thyroid tumors

	PTC	FTC	FA	AG	*p* value
0	0 (0%)	1 (1.7%)	8 (14.0%)	22 (40.7%)	<0.001
+1	28 (19.0%)	29 (50.0%)	31 (54.4%)	13 (24.1%)	
+2	53 (36.1%)	12 (20.7%)	13 (22.8%)	9 (16.7%)	
+3	66 (44.9%)	16 (27.6%)	5 (8.8%)	10 (18.5%)	
Total	147 (100%)	58 (100%)	57 (100%)	54 (100%)	

PTC papillary thyroid carcinoma, *FTC* follicular thyroid carcinoma, *FA* follicular adenoma, *AG* adenomatous goiter

Figure 1 Cytoplasmic Nodal expression in different thyroid carcinoma by IHC staining. (a) Negative Nodal staining in the normal thyroid follicular cells. (b) Weak staining in PTC. (c) Strong staining in PTC. (d) Weak staining in FTC. (e) Strong staining in FTC. (f) Weak staining in FA. (g) Strong staining in FA. (h) Weak staining in AG. (i) Strong staining in AG.

PTC papillary thyroid carcinoma, *FTC* follicular thyroid carcinoma, *FA* follicular adenoma, *AG* adenomatous goiter



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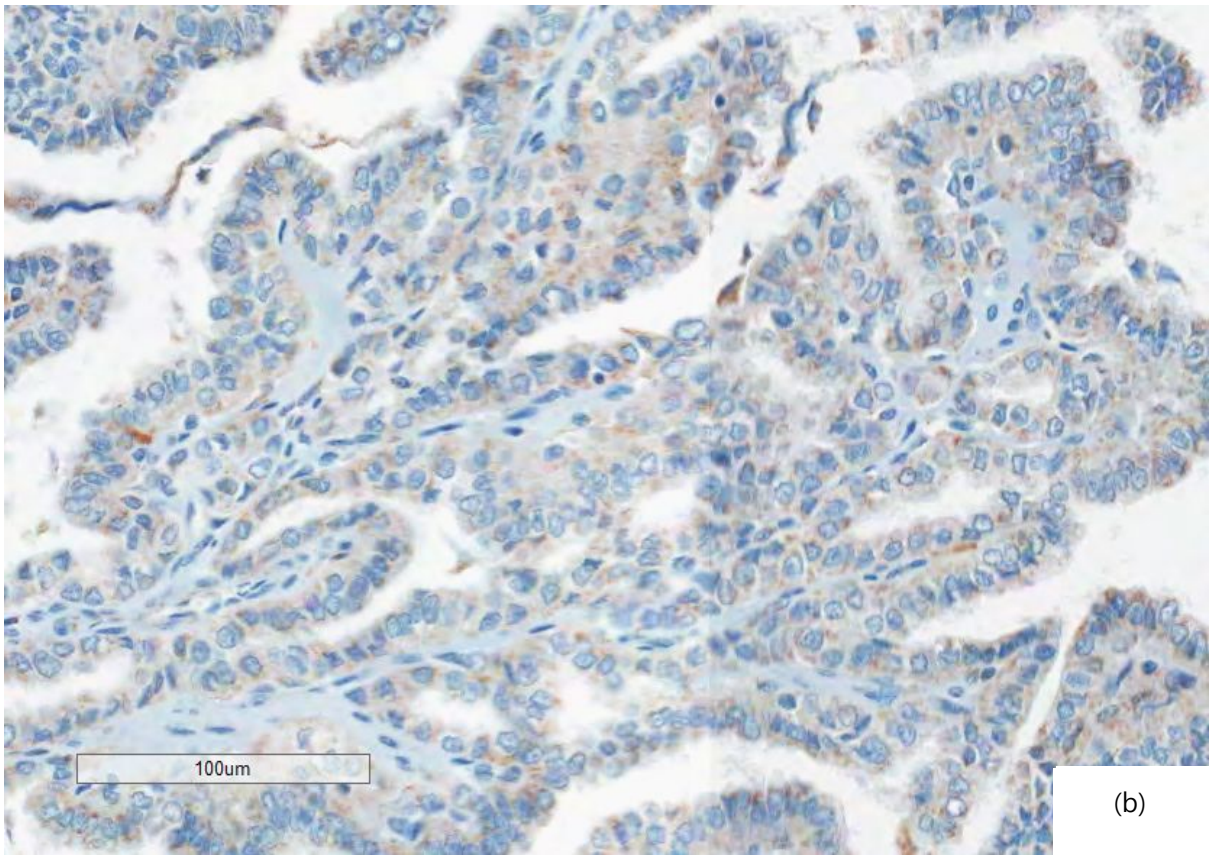

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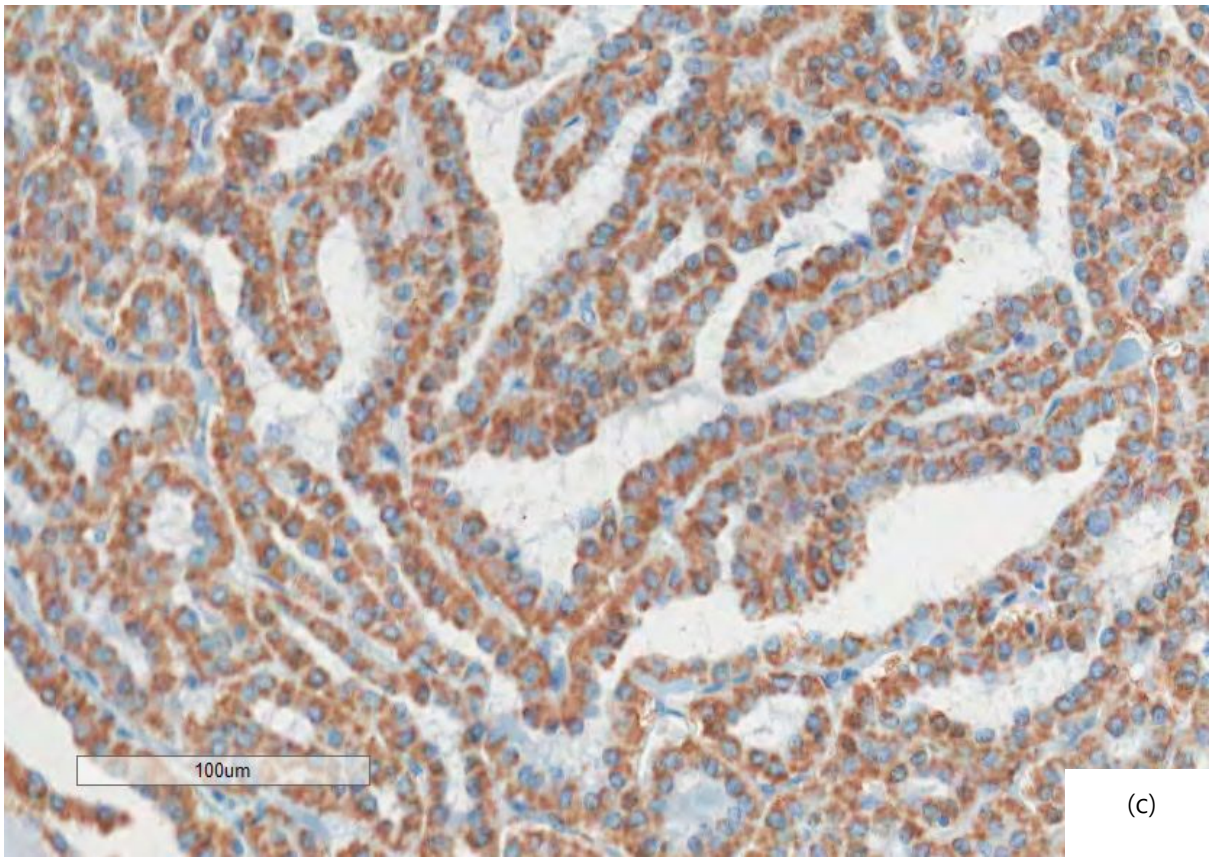

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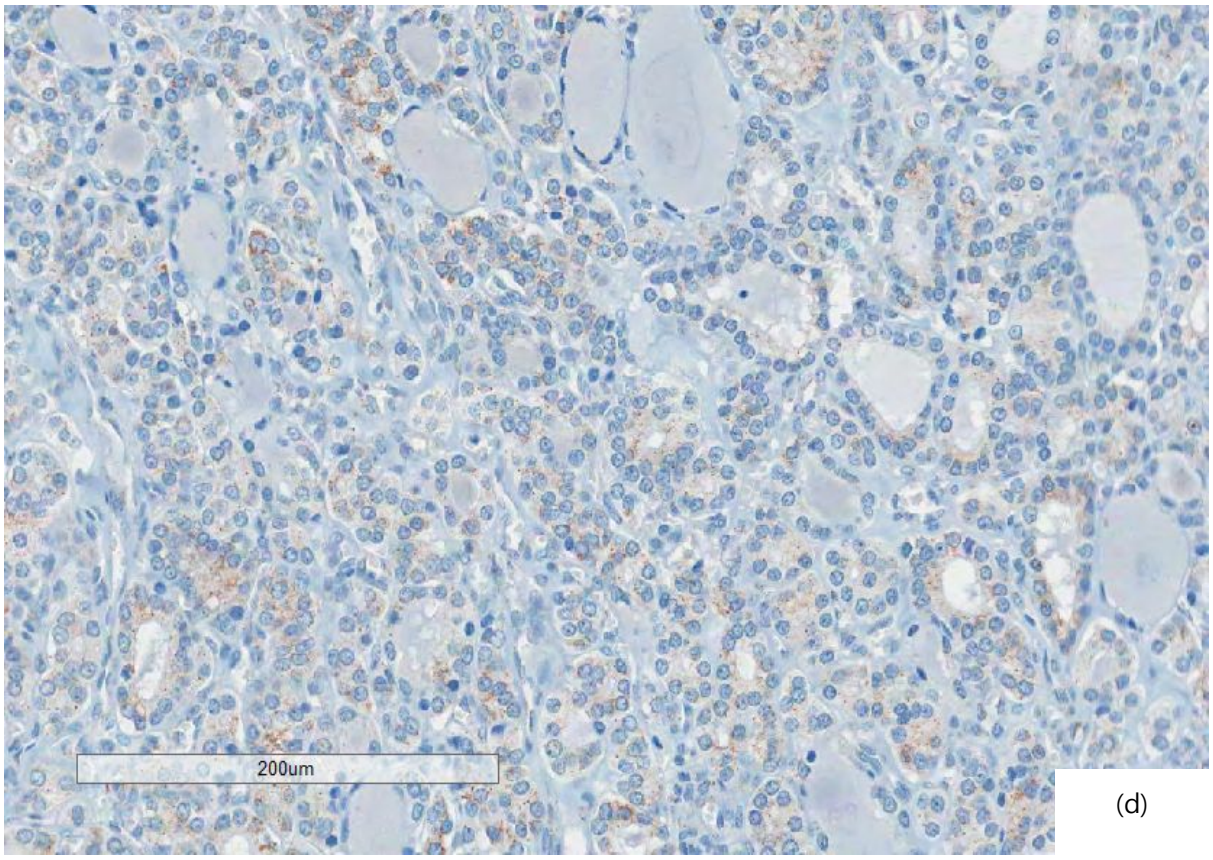

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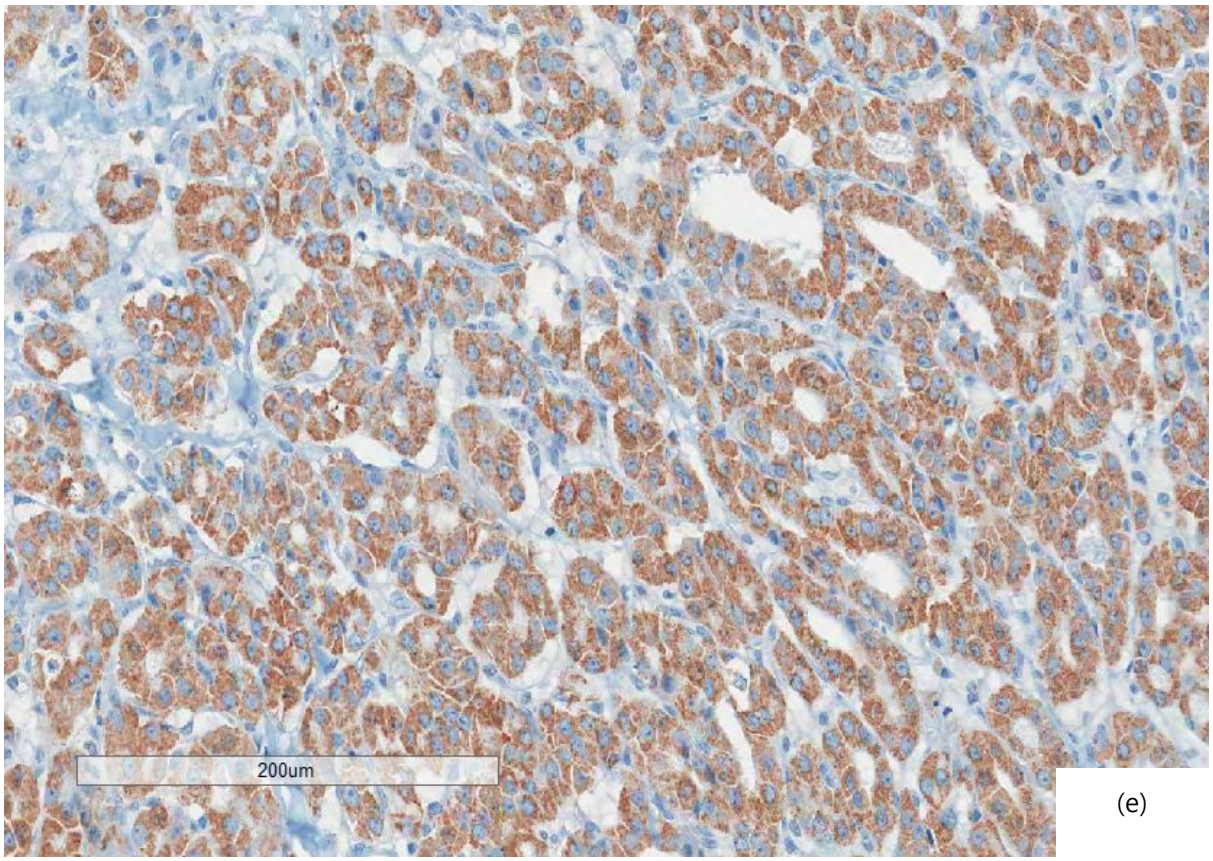

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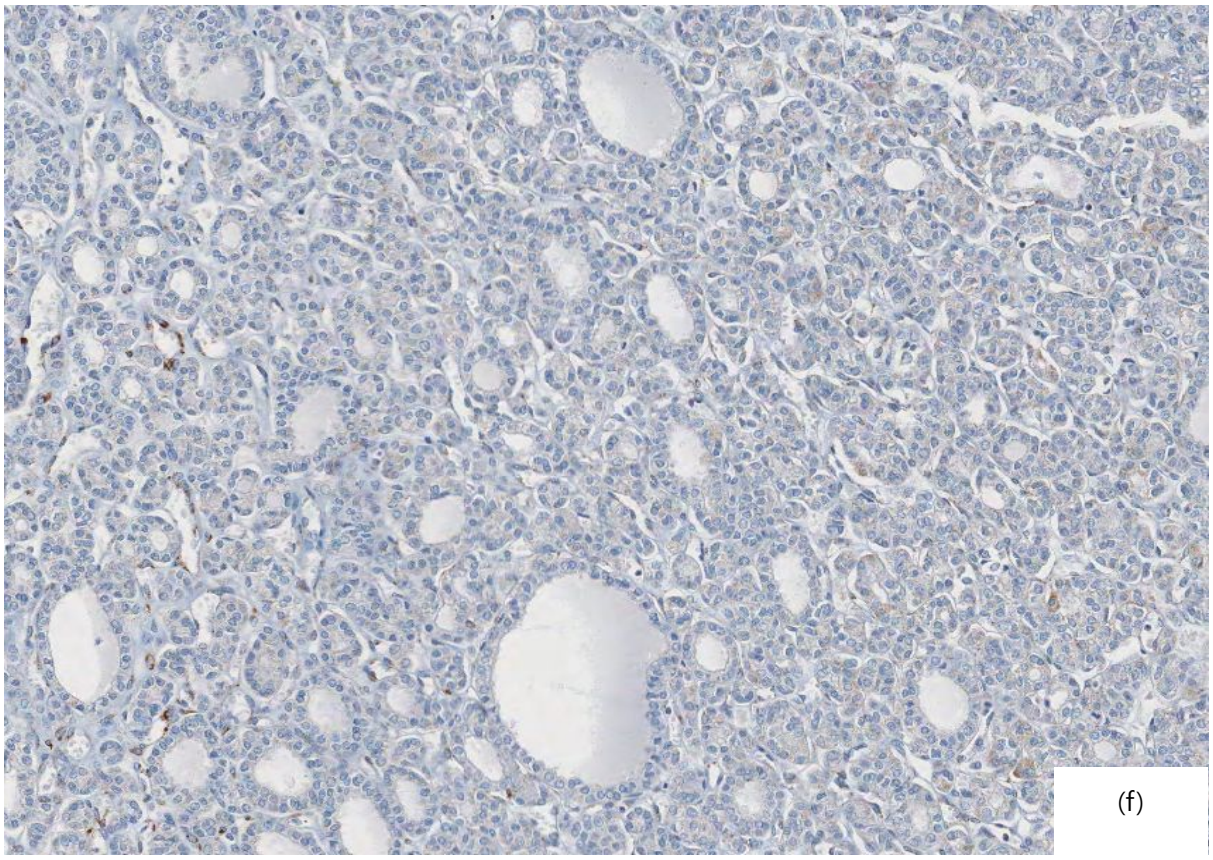

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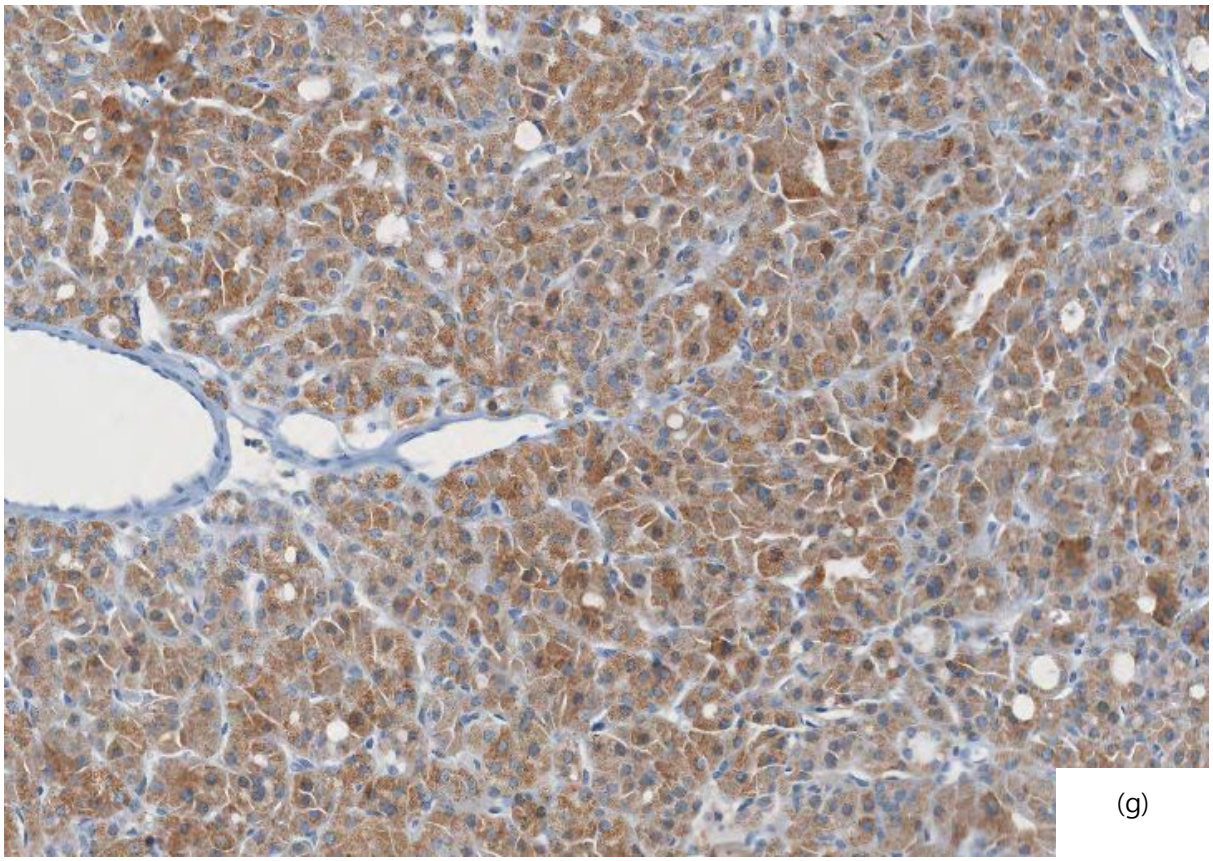

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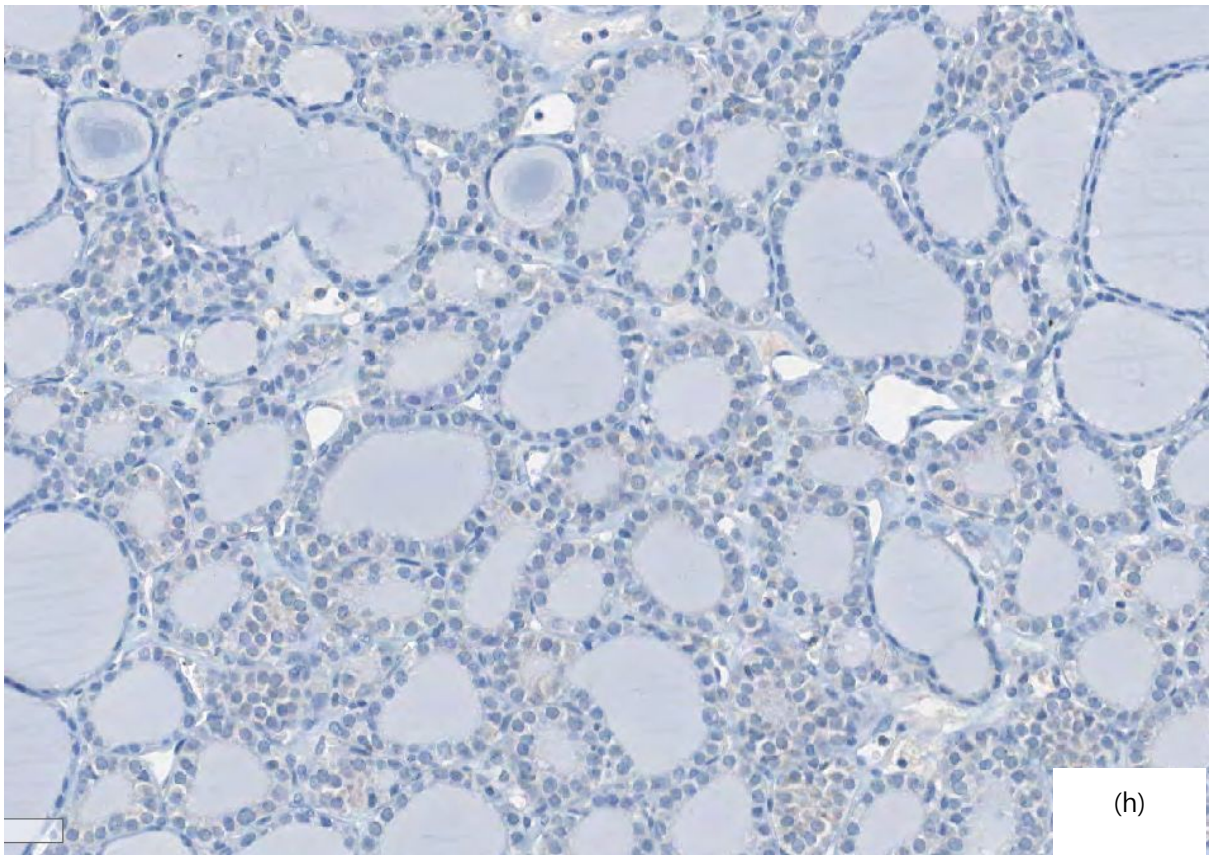

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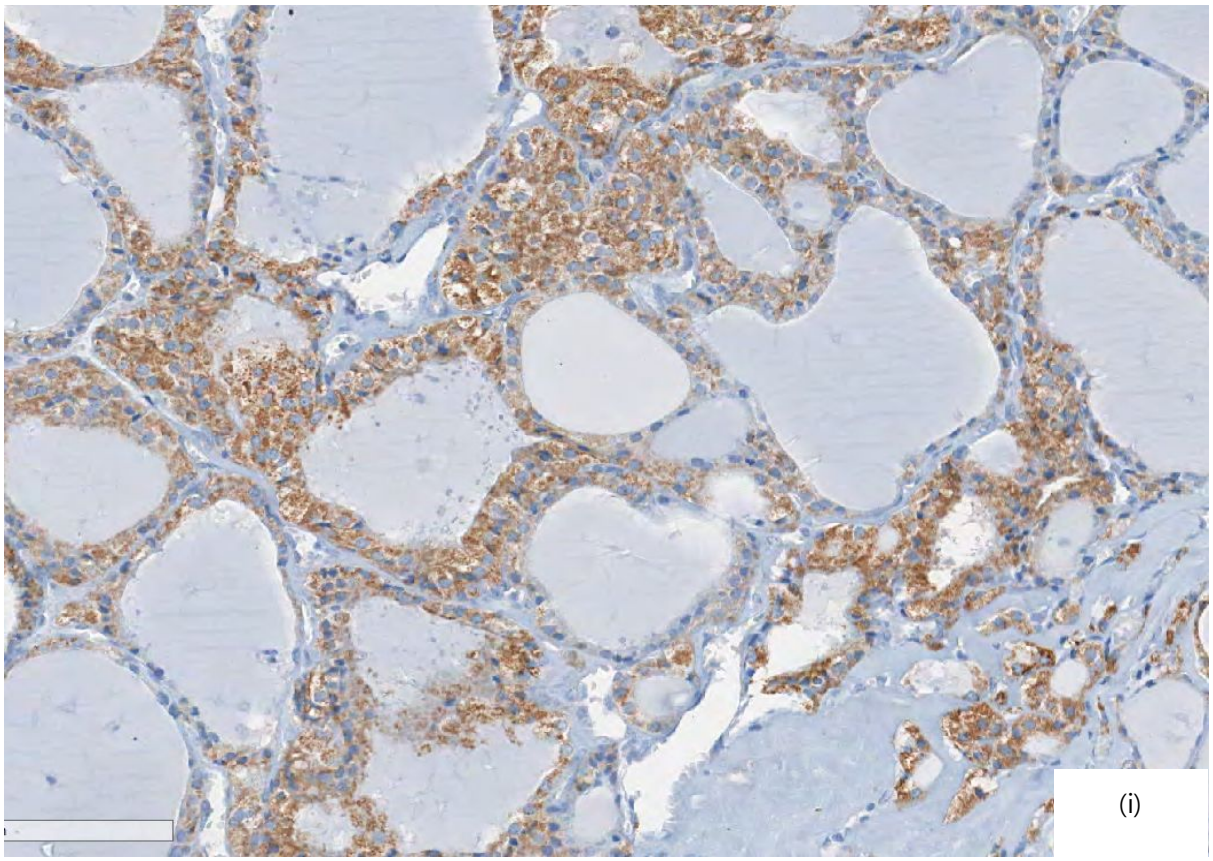
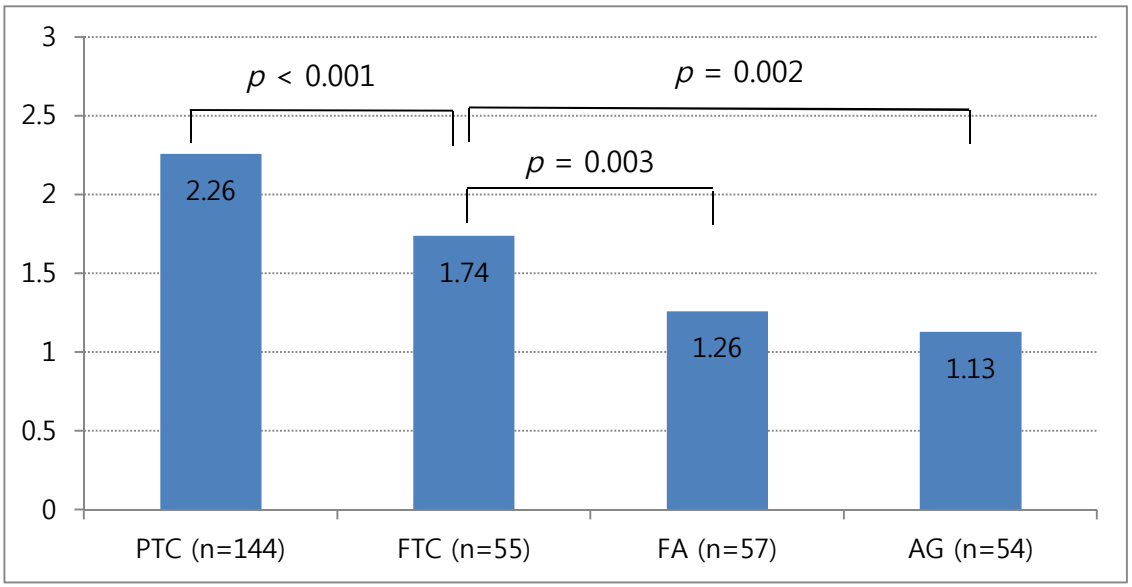


(i)

Figure 2 Mean value of intensity of Nodal expression



PTC papillary thyroid carcinoma, *FTC* follicular thyroid carcinoma, *FA* follicular adenoma, *AG* adenomatous goiter

Table 3 shows the difference of clinicopathological parameters according to Nodal expression level in PTC. Nodal was expressed at high level in 119 (81.0%) tumors. There was no significant difference between the low and high expression group in terms of gender, tumor size, multifocality, extrathyroidal extension, lymph node metastasis, distant metastasis, TNM stage, and recurrence. High Nodal expression was associated with older age (36.2 ± 18.1 vs. 46.7 ± 15.7, $p < 0.002$) and the presence of BRAF mutation (33.3% vs. 70.0%, $p = 0.001$). BRAF mutation analysis was performed in 121 (82.3%) patients, and there was no significant difference between the BRAF-tested group and –not tested group in terms of gender, age, size, and stage. In multivariable analysis (Table 4), the presence of BRAF mutation was an independent predictive factor with high Nodal expression (OR 4.562, 95 % CI 1.534 – 13.570). The association between the age and Nodal expression was shown in the

PTC without BRAF mutation whereas it was not in the PTC with BRAF mutation (Table 5). Table 6 demonstrates BRAF mutation status is not different according to the age (p = 0.873). In FTC, Nodal was highly expressed in 28 (48.3%) tumors. The mean age of the high expression group was significantly higher than in the low expression group (46.0 ± 13.8 vs 56.6 ± 13.7, p = 0.006), (Table 7). There was no difference according to the Nodal expression level in terms of gender, tumor size, multifocality, extrathyroidal extension, lymph node metastasis, distant metastasis, TNM stage, and recurrence.

Table 3 Expression of Nodal in papillary thyroid carcinoma according to clinicopathological parameters

	Nodal expression		p value
	Low	High	
Number of tumors	28 (19.0%)	119 (81.0%)	
Gender (M : F)	5 : 23	20 : 99	1.000
Age (mean ± SD), years	36.2 ± 18.1	46.7 ± 15.7	0.002
< 45	20 (71.4%)	60 (50.4%)	0.045
≥ 45	8 (28.6%)	59 (49.6%)	
Tumor size (mean ± SD), cm	2.7 ± 1.5	2.5 ± 1.4	0.502
≤2cm	11 (39.3%)	58 (48.7%)	0.367
>2cm	17 (60.7%)	61 (51.3%)	

Table 3 Continued

	Nodal expression		*p* value
	Low	High	
Multifocality	12 (42.9%)	44 (37.0%)	0.564
Extrathyroidal extension	18 (64.3%)	92 (77.3%)	0.153
Lymph node metastasis	16 (57.1%)	58 (48.7%)	0.424
Distant metastasis	1 (3.6%)	1 (0.8%)	0.346
AJCC TNM stage			
Lower stage (Ⅰ/Ⅱ)	20 (71.4%)	63 (52.9%)	0.076
Higher stage (Ⅲ/Ⅳ)	8 (28.6%)	56 (47.1%)	
BRAF status			
Wild type	14 (66.7%)	30 (30.0%)	0.001
Mutation	7 (33.3%)	70 (70.0%)	
Recurrence	7 (25.0%)	14 (11.8%)	0.128

Table 4 Multivariable analysis of clinicopathological risk factors associated with high Nodal expression in papillary thyroid carcinoma

Covariates	Adjusted odds ratio	95 % confidence interval	*p* value
Male sex	1.230	0.268–5.640	0.790
Age ≥ 45	1.532	0.503–4.671	0.453
Size >2cm	0.342	0.107–1.088	0.069
Multifocality	0.595	0.189–1.870	0.374
Extrathyroidal extension	2.190	0.647–7.418	0.208
Lymph node metastasis	0.787	0.241–2.569	0.691
Distant metastasis	0.380	0.014–10.074	0.563
BRAF mutation	4.562	1.534–13.570	0.006
Recurrence	0.689	0.177–2.692	0.593

Table 5 Nodal expression level in PTC according to the BRAF mutation status

	Nodal expression		*p* value
	Low	High	
Age (mean ± SD), years			
BRAF status; wild type	35.7 ± 18.4	47.2 ± 15.6	0.038
BRAF status; mutation	42.1 ± 21.4	56.9 ± 15.9	0.566

Table 6 Correlation between the age and the BRAF status

	BRAF wild type	BRAF mutation	*p* value
Age $<$ 45	27 (61.4%)	38 (49.4%)	0.202
Age $\geq$ 45	17 (38.6%)	39 (50.6%)	

Table 7 Expression of Nodal in follicular thyroid carcinoma according to clinicopathological parameters

	Nodal expression		*p* value
	Low	High	
Number of tumors	30 (51.7%)	28 (48.3%)	
Gender (M : F)	5 : 25	3 : 25	0.707
Age (mean ± SD), years	41.3 ± 15.5	52.8 ± 13.4	0.004
< 45	19 (63.3%)	10 (35.7%)	0.036
≥ 45	11 (36.7%)	18 (64.3%)	
Tumor size (mean ± SD), cm	3.7 ± 2.0	3.7 ± 1.9	0.920
Multifocality	4 (13.3%)	4 (14.3%)	1.000
Extrathyroidal extension	6 (20.0%)	4 (14.3%)	0.732
Lymph node metastasis	1 (3.3%)	2 (7.1%)	0.605
Distant metastasis	2 (6.7%)	4 (14.3%)	0.415
AJCC TNM stage			
Lower stage (Ⅰ/Ⅱ)	25 (83.3%)	21 (75.0%)	0.434
Higher stage (Ⅲ/Ⅳ)	5 (16.7%)	7 (25.0%)	
Recurrence	0 (0%)	1 (3.6%)	0.483

DISCUSSIONS

Nodal signaling pathway in melanoma was described by Strizzi et al (16). Nodal signal is generated when Nodal binds to a heteromeric complex composed of the EGF-like glycoprotein co-receptor Cripto-1, and types I and II activin-like kinase receptors (ALK4/7 and ActRIIB, respectively). Consequently, this leads to activation and nuclear translocation of the Smad 2/3/4 complex where it regulates expression of genes, such as those involved in stem cell maintenance and cellular plasticity, in addition to inducing its own expression (16, 17). In immunohistochemical study on melanocytic tumors, the low expression of Nodal in dysplastic nevi, and its increasing expression with the progression of malignant lesions were reported (18). In prostate tumors, Lawrence et al. (13) showed that Nodal was overexpressed in cancer tissues and Nodal enhances the growth of prostate cancer cells.

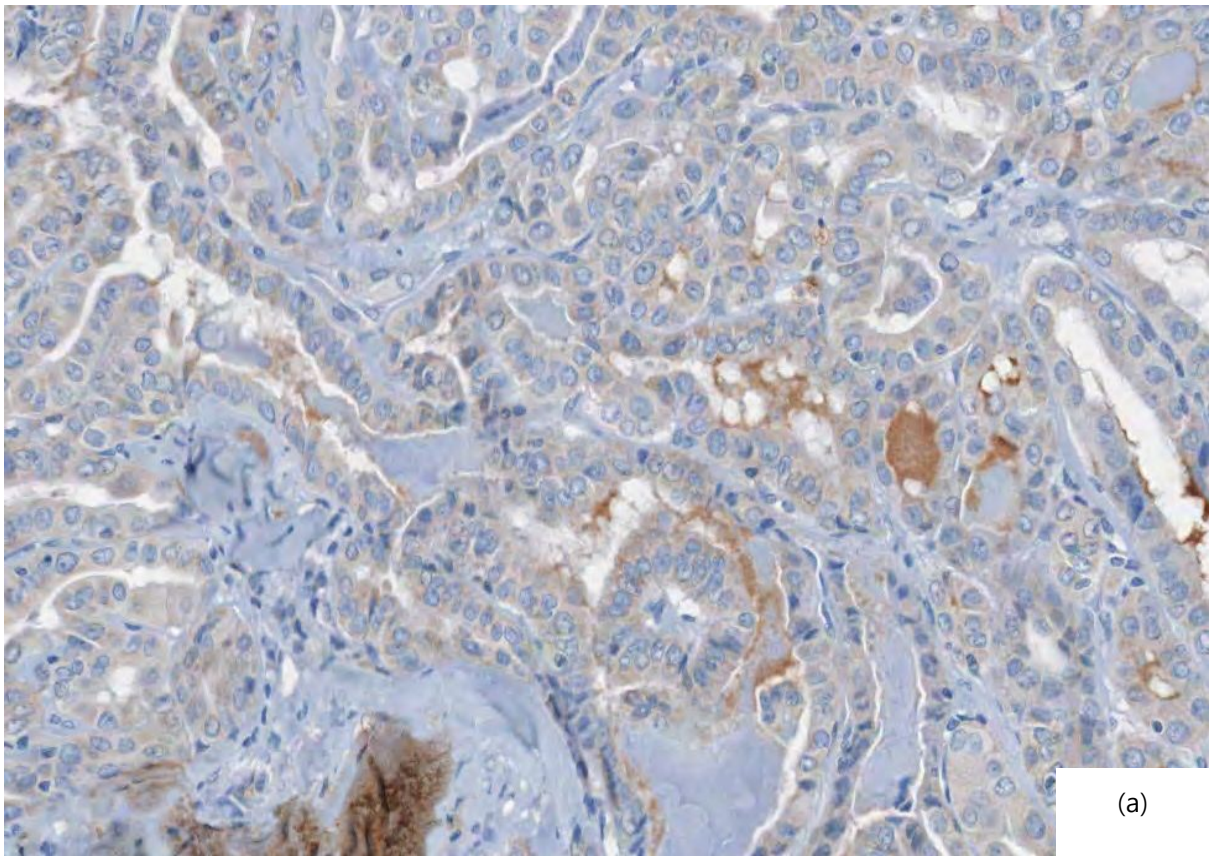
In accordance with the previous studies, Nodal expression was significantly higher in PTC and FTC than in benign thyroid tumors such as AG and FA. Overexpression of Nodal was more prominent in PTC than in FTC (2.26 vs. 1.74, $p < 0.01$), and high Nodal staining was associated with poor prognostic factors such as older age (≥ 45), higher TNM stage, and the presence of BRAF mutation in PTC. And there was no correlation between older age and the BRAF mutation status in PTC, suggesting that older age was not a confounder in the correlation of BRAF mutation with the high Nodal expression. In fact, the presence of BRAF mutation was an independent predictive factor with high Nodal expression in multivariable analysis. BRAF mutation is associated with TGF-β expression and TGF-β is associated with the occurrence of extrathyroidal extension and lymph node metastasis (19-21). BRAF mutation activates TGF-β/Smad signaling and consequently induces the repression of sodium iodide symporter (NIS) (19), and Nodal also activates Smad2/3/4 complex to promote tumor progression and vasculogenic mimicry like TGF-β (16). It is interesting that BRAF mutation

and high Nodal expression were associated in this study and both BRAF mutation and Nodal activate Smad2/3/4 complex which leads to tumor aggressiveness.

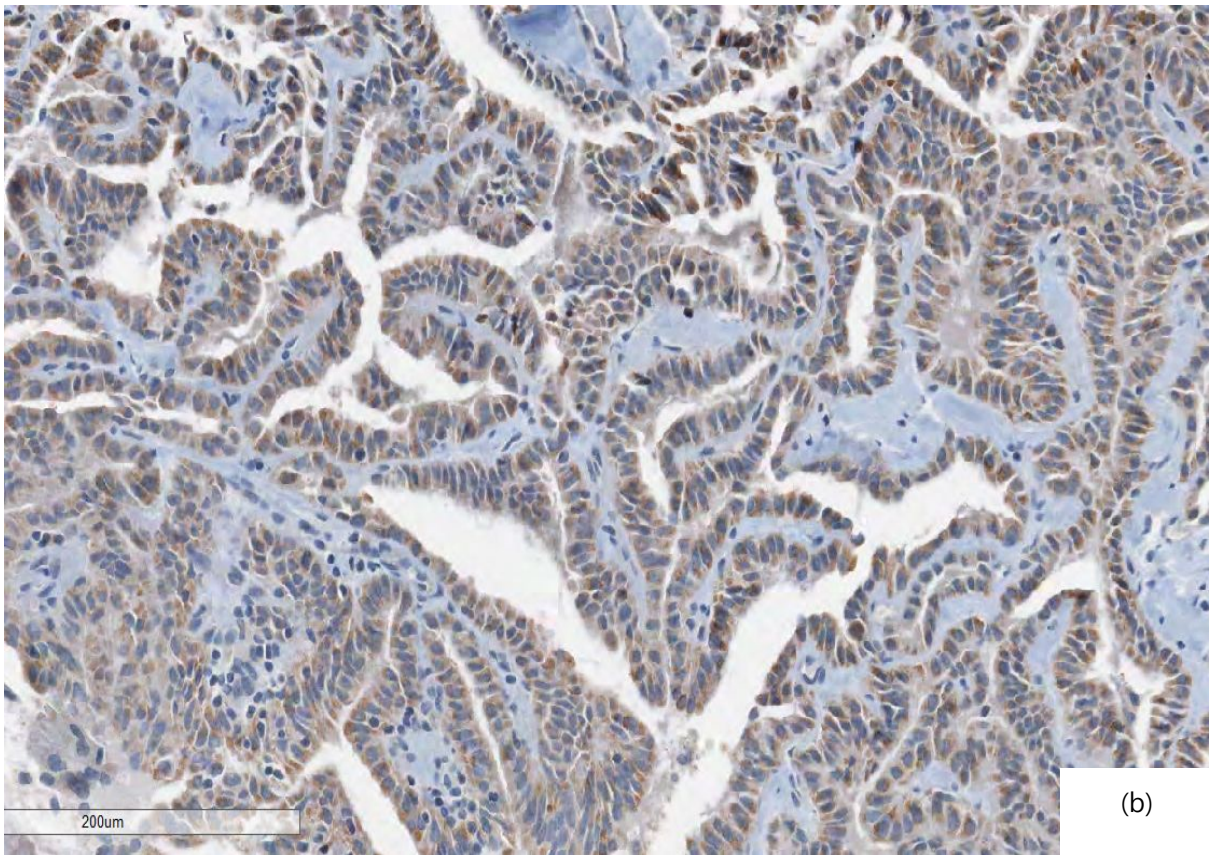
In terms of age, it is noteworthy that the mean age was significantly higher in the high Nodal expression group than in the low expression group when PTC has wild type BRAF status ($p = 0.038$). In addition, there was an association between the older age and the high Nodal expression in FTC which is never related to BRAF mutation ($p = 0.032$). Taken together, the association between the old age and the high Nodal expression is more prominent when there is no BRAF mutation. We can postulate that both of the presence of the BRAF mutation and aging activate or enhance Nodal re-expression in thyroid tumors and aging becomes a dominant regulator of Nodal expression when there is no BRAF mutation. In fact, we found that the benign tumors such as FA and AG showed low but substantial grade of Nodal expression compared to negative expression in the normal thyroid tissues.

Notch signaling is necessary for Nodal expression at the developmental stage of embryo (22). Notch signaling involves the cleavage of the Notch intracellular domain, and it translocate to the nucleus where it regulates the expression of Nodal (16). In melanoma, regulation of Nodal by Notch4 is established in vitro study (17). In that study, Notch4 expression correlated with Nodal expression in multiple aggressive melanoma cell lines, and Notch4 neutralizing antibody reduced Nodal transcription and cellular proliferation. But it was unknown whether Notch regulates Nodal in other cancers. We also performed immunohistochemical staining of Notch4 in the same tissue microarray (Figure 3). There was a strong correlation between the Nodal and the Notch4 expression suggesting regulation of Nodal by Notch signaling pathway in PTC (Table 8).

Figure 3 Cytoplasmic Notch4 expression in PTC by IHC staining. (a) Weak staining in PTC. (b) Strong staining in PTC.



(a)



(b)

Table 8 Correlation between the Nodal and Notch4 expression in PTC and FTC

			Notch4 expression		*p* value
			Low	High	
PTC	Nodal expression	Low	27 (96.4%)	1 (3.6%)	0.010
		High	88 (73.9%)	31 (26.1%)	
FTC	Nodal expression	Low	29 (96.7%)	1 (3.3%)	0.097
		High	23 (82.1%)	5 (17.9%)	

Nodal expression in thyroid carcinoma was investigated for the first time in the present study. Therefore, its clinical significance is not evaluable at the present time. However, previous *in vitro* studies had showed the possibility that Nodal might be a novel candidate for a targeted therapy. In melanoma cell lines, down-regulation of Nodal reduced plasticity and aggressiveness and the treatment of the cells with a general inhibitor of TGF-b/Activin/Nol signaling resulted in a reduction in tumor cell invasion (8). In the breast cancer cell lines, an anti-Nodal antibody reduced Nodal expression levels (23). The other molecules which are involved in Nodal signaling pathway such as Smad2/3/4 complex, and crypto might be potential targets of specific mitogen-activated protein kinase pathway inhibitors.

This study has several limitations. Firstly, we could not conclude in which mechanism BRAF and Nodal is associated because of the nature of observational study. Secondly, we could not show Nodal expression in a defined molecular setting such as BRAF transfected versus vector only control cell lines. Lastly, we had relatively small number of FTCs to evaluate clinical significance. FTC has totally different molecular profile than PTC, and RAS mutation is predominant while BRAF mutation is not present. Investigation of the correlation between Nodal expression and RAS mutation may offer better understanding in the nature of Nodal in thyroid carcinoma.

CONCLUSIONS

In summary, we showed for the first time that Nodal is expressed in the differentiated thyroid carcinoma as well as benign thyroid tumors, and the expression level was higher in the thyroid carcinoma than in the benign tumors. We also found that Nodal expression was associated with poor prognostic factors such as BRAF mutation in PTC and older age in FTC.

These findings suggest a role of Nodal as a prognostic marker or a potential therapeutic target in thyroid carcinoma. Further investigations to demonstrate precise signaling pathway and the clinical significance are needed.

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국문 초록

서론: 태아형성원 (embryonic morphogen)인 Nodal 은 transforming growth factor (TGF)-β superfamily 의 한 종류로써, 악성흑색종, 유방암, 전립선암에서 암 발생에 관여한다고 알려져 있으며, TGF-β 는 갑상선유두암에서 BRAF 돌연변이 및 나쁜 예후인자와 연관이 있다. 그러나 현재까지 갑상선암에서의 Nodal 의 역할에 대해서는 연구된 바가 없다. 따라서 본 연구에서는 조직미세배열 (tissue microarray, TMA)에서 Nodal 면역화학염색을 함으로써 갑상선암에서의 Nodal 발현 양상에 대해 알아보고자 하였다.

방법: 1993 년부터 2003 년 사이에 모아진 갑상선 종양으로 TMA 를 구축하였고 유두암 144 개, 여포암 57 개, 샘종성갑상샘종 57 개, 여포선종 54 개, 정상 갑상선 조직 5 개로 구성되었다. Nodal 염색은 두 명의 병리의에 의해 0 에서 3 까지의 단위로 판독되었고 0 과 1 을 저발현군, 2 와 3 을 고발현군으루 구분하여 두 군을 비교하였다.

결과: 유두암, 여포암, 샘종성갑상샘종, 여포선종의 염색스코어는 각각 2.26, 1.74, 1.23, 1.13 이었고 정상 갑상선 조직에서는 발현되지 않았다. 유두암은 여포암보다 높은 Nodal 발현을 보였고 ($p < 0.01$), 여포암은 샘종성갑상샘종과 여포선종보다

높은 Nodal 발현을 보였다. (p = 0.003, p = 0.002). 여포암에서 Nodal 고발현군은 많은 나이, 높은 병기, BRAF 돌연변이의 존재와 유의하게 연관이 있었으며 다변량분석에서는 BRAF 돌연변이가 높은 Nodal 발현의 독립적인 연관인자였다. (OR 4.366, 95 % CI 1.450 – 13.147). 여포암에서는 Nodal 고발현군은 많은 나이와 연관이 있었다.

결론: 결론적으로 유두암과 여포암은 양상 갑상선종양에 비해 높은 Nodal 발현을 보였으며, 높은 Nodal의 발현은 유두암에서는 BRAF 돌연변이, 여포암에서는 많은 나이와 관련되어 있었다. Nodal은 갑상선분화암의 잠재적인 예후 인자 또는 치료 목표로서의 가치가 있을 것으로 전망되며 향후 Nodal 신호전달체계에 대한 추가적인 연구가 필요할 것이다.

주요어 : Nodal, 갑상선암, 갑상선 유두암, BRAF 돌연변이, 조직미세배열

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