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Original Article

NPC2 expression in thyroid tumors and its possible diagnostic utility

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Abstract: Histopathologic diagnosis of thyroid lesions is sometimes difficult and may require the assistance of immunohistochemistry. Currently-used immunohistochemical biomarkers share the weakness of staining both papillary thyroid carcinoma and other non-papillary thyroid lesions. We examined NPC2 as an immunohistochemical marker in various thyroid lesions to determine the subcellular localization of the immunohistochemistry signal and evaluated the value of NPC2 as a diagnostic marker of papillary thyroid carcinoma. NPC2 immunostaining was performed on various thyroid tumors and tumor-like lesions. The immunostaining revealed significantly different patterns for papillary carcinomas and the other lesions. Papillary carcinomas exhibited moderate to strong granular cytoplasmic staining, often with basal membranous accentuation. In contrast, the other lesions showed mostly weak cytoplasmic staining, often with apical membranous accentuation. The subcellular localization of NPC2 provided insight into contrasting histopathologic morphology and reversed cellular polarity between the papillary patterns of papillary carcinomas and the follicular patterns of non-papillary carcinoma lesions. The diagnostic characteristics of NPC2 immunohistochemistry for non-follicular papillary carcinomas versus non-papillary carcinoma lesions were a sensitivity of 97.3%, specificity of 96.9%, positive predictive value of 94.7%, and negative predictive value of 98.4%. Significant differences were present between the two staining patterns in papillary carcinoma relative to mean age, nodal metastasis, and follicular and non-follicular variants (P = 0.02, P = 0.03, and P = 0.000, respectively). In conclusion, our evaluation of the subcellular localization of NPC2 using immunohistochemistry demonstrated possible value of NPC2 as a biomarker and provided insight into the morphologic characteristics of papillary carcinoma.

Keywords: NPC2, immunohistochemistry, papillary thyroid carcinoma, thyroid cancer, thyroid tumor

Introduction

The incidence of thyroid cancer is estimated as more than 50,000 cases a year in the United States and it is the fifth most prevalent cancer of females [1]. The incidence has steeply increased during recent decades [2]. In conjunction with the increased incidence of thyroid cancer is the need for more accurate pathologic diagnosis of thyroid lesions.

In thyroid tumors and tumor-like lesions, the pathologic diagnosis in many cases is straightforward; however, diagnostically difficult thyroid lesions are often encountered in which immunohistochemistry is applied to differentiate between benign and malignant lesions [3-5]. Differential diagnosis between papillary carcinoma and benign thyroid lesions often is difficult, but it is critical because treatment can be changed according to the pathologic diagnosis [3]. Immunohistochemical staining for galectin-3, HBME1, and CK19 is used to assist in the pathologic diagnosis of thyroid lesions, but the immunostaining may cause confusion and requires caution in its interpretation [6-8]. There is a need for new antibodies for immunohistochemistry in thyroid pathology in order to overcome the shortcomings of those currently available.

The *Niemann-Pick type C intracellular cholesterol transporter 2* (*NPC2*) gene encodes the NPC2 protein, which binds to and transports cholesterol between NPC1 and the lysosome [9]. High NPC2 protein expression has been

detected in various cancers of the breast, colon, thyroid, and lung [9, 10]. For instance, NPC2 expression in thyroid has been described, especially with high levels in papillary thyroid carcinoma (PTC) [10, 11]. However, there is currently no detailed information regarding immunohistochemistry of NPC2 in various thyroid lesions.

In the current study, we performed NPC2 immunohistochemistry of various thyroid lesions. The subcellular localization of the immunohistochemical signals was evaluated and the potential value of the immunostaining in the diagnosis of PTC was evaluated by comparing the staining of various thyroid tumors and tumor-like lesions.

Materials and methods

Clinical specimens

A total of 249 formalin-fixed paraffin-embedded specimens of thyroid tumors and tumor-like lesions obtained during thyroidectomies performed between 2002 and 2012 at Korea University Medical Center Anam Hospital were randomly selected from pathology archives. The specimens included 73 non-follicular variant PTCs, 47 follicular variant PTCs (FVPTCs), 24 follicular carcinomas, 24 follicular adenomas, 21 nodular goiters, 20 medullary carcinomas, 15 oncocytic adenomas, 9 anaplastic carcinomas, 8 lymphocytic thyroiditis, 5 nodular goiters with oncocytic change, 2 hyalinizing trabecular tumors, and 1 poorly differentiated carcinoma. The study was approved by the Korea Medical Center Anam Hospital Institutional Review Board (ED12099). The institutional review board also approved a waiver of patient informed consent for the study.

Immunohistochemistry

The paraffin-embedded specimens were sectioned 4-μm thick, deparaffinized in xylene, and dehydrated in graded alcohols. Antigen retrieval was performed in a microwave oven for 15 min in 10 mM citrate buffer pH 6.0. Endogenous peroxidase was blocked with 3% H_2O_2-methanol solution and the slides then incubated in 10% normal goat serum for 30 min to prevent non-specific staining. The slides were then moved to a Dako Autostainer (Dako, Glostrup, Denmark) and incubated with NPC2 primary antibody (1:500; Atlas Antibody, Stockholm, Sweden) for 1 h at room temperature. The slides were incubated with Dako Envision Kit reagents for 30 min. For visualization, 0.02% 3,3'-diaminobenzidine tetrahydrochloride in 0.05 M Tris buffer with 0.01% hydrogen peroxide was added as chromogen. The slides were counterstained with hematoxylin and coverslipped. Sections of normal epididymis specimens were used as positive controls.

Interpretation of Immunohistochemistry

Subcellular localization of NPC2 based on immunohistochemical staining was evaluated and recorded by two pathologists independently and consensus was reached through discussion [12]. Granular cytoplasmic staining was present in many of the thyroid tumors with apical or basal membranous accentuation occurring concurrently with the cytoplasmic staining in some. Nuclear staining was not present. Staining intensity and the percentage of positively stained cells were graded. Staining intensity was recorded as negative, weak, moderate, or strong. The proportion of positive-staining cells was categorized as diffuse or focal staining.

Statistical analysis

Statistical analyses were performed using SPSS 26.0 software (IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp.). Independent T-test was used to compare age differences among the expression groups. Chi-square and Fisher's exact tests were performed to compare NPC2 expression between disease entities. *P*-values less than 0.05 were considered significant.

Results

Thyroid tumors and tumor-like lesions immunostained for NPC2 exhibited granular cytoplasmic staining with or without membranous accentuation (**Figure 1**). The intensity of the cytoplasmic staining varied. Membranous accentuation, where present, was either apical or basal. A noticeable difference in the NPC2 expression pattern was noted between PTC specimens and non-PTC lesions.

Most non-follicular variant PTC specimens showed diffuse, moderate to strong granular cyto-

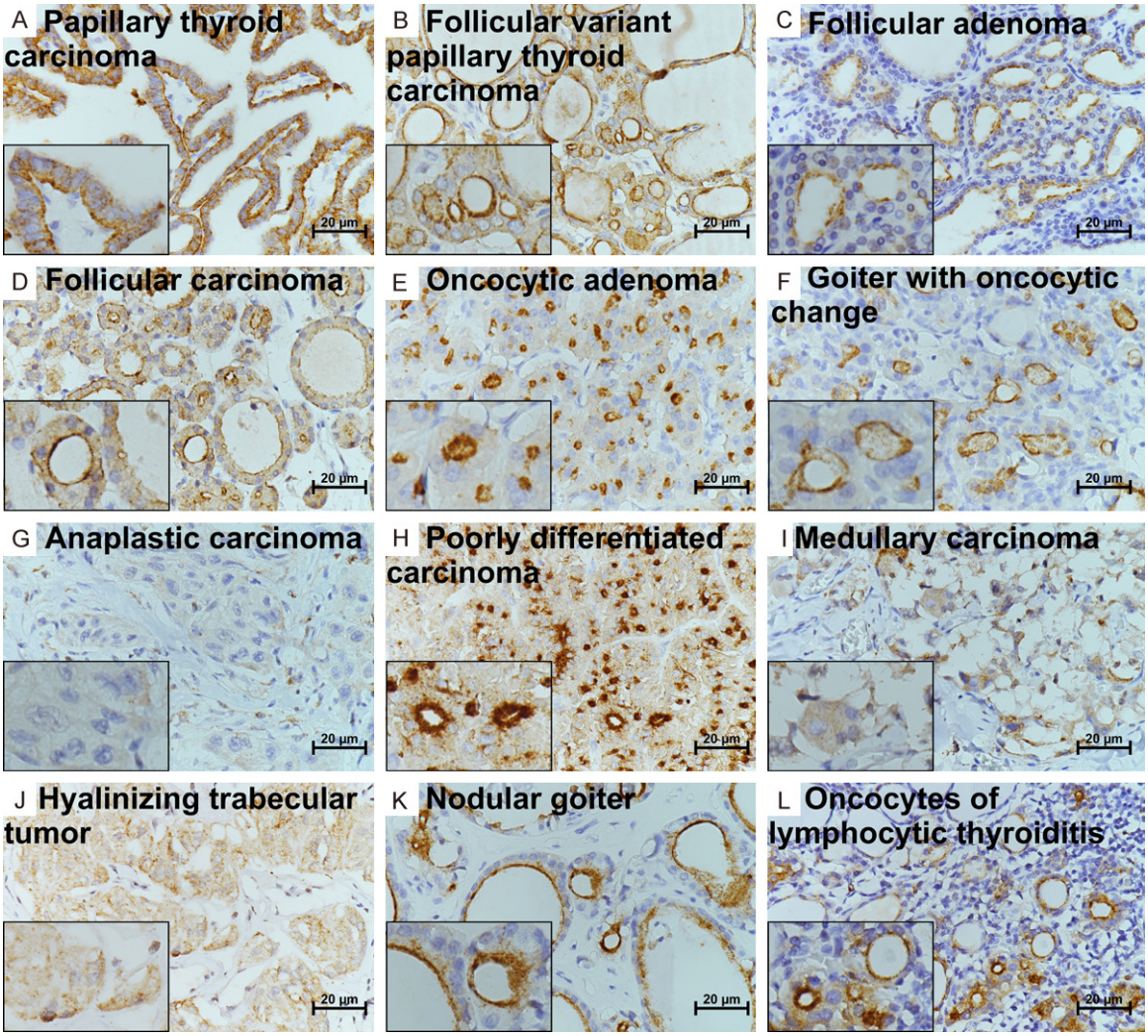

Figure 1. NPC2 immunohistochemistry of thyroid tumors and tumor-like lesions. A. Papillary thyroid carcinoma, non-follicular variant. B. Follicular variant papillary thyroid carcinoma. C. Follicular adenoma. D. Follicular carcinoma. E. Oncocytic adenoma. F. Nodular goiter with oncocytic change. G. Anaplastic carcinoma. H. Poorly differentiated carcinoma. I. Medullary carcinoma. J. Hyalinizing trabecular tumor. K. Nodular goiter. L. Oncocytes of lymphocytic thyroiditis (×400).

plasmic staining with or without basal membranous accentuation (**Figure 1A**). FVPTCs showed varied staining patterns. Many showed diffuse, moderate to strong granular cytoplasmic staining with apical membranous accentuation (**Figure 1B**). Most follicular adenomas showed diffuse, weak granular cytoplasmic staining with apical membranous accentuation (**Figure 1C**). Most follicular carcinomas showed diffuse, weak to moderate granular cytoplasmic staining with apical membranous accentuation (**Figure 1D**). Most oncocytic adenomas showed diffuse, weak to moderate granular cytoplasmic staining with apical membranous accentuation (**Figure 1E**). Most nodular goiters with oncocytic change showed diffuse, weak to moderate granular cytoplasmic staining with apical membranous accentuation, especially in oncocytes (**Figure 1F**). Most anaplastic carcinomas showed weak cytoplasmic staining (**Figure 1G**). The only poorly differentiated carcinoma showed diffuse, weak granular cytoplasmic staining with strong apical membranous accentuation (**Figure 1H**). Most medullary carcinomas showed weak cytoplasmic staining (**Figure 1I**). Hyalinizing trabecular tumors showed diffuse, moderate granular cytoplasmic staining with one of the two showing basal membranous accentuation (**Figure 1J**). Most nodular goiters showed focal, weak granular cytoplasmic stain-

Table 1. NPC2 staining pattern in thyroid tumors and tumor-like lesions

	PTC Staining Pattern (n)	Non-PTC Staining Pattern (n)	*p* value
Non-follicular PTC	71	2	0.000*
Non-PTC	4	125	

PTC indicates papillary thyroid carcinoma. *Fisher's exact test.

Table 2. Test characteristics of NPC2 immunohistochemistry in thyroid regarding non-follicular PTC vs. non-PTC

Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Diagnostic accuracy (%)
97.3	96.9	94.7	98.4	97.0

PTC indicates papillary thyroid carcinoma; PPV, positive predictive value; NPV, negative predictive value.

ing with apical membranous accentuation (**Figure 1K**). Finally, oncocytic foci of lymphocytic thyroiditis showed focal, weak to moderate granular cytoplasmic staining with apical membranous accentuation (**Figure 1L**).

The staining patterns were divided into two groups, those with the PTC pattern and those with the non-PTC pattern. The PTC staining pattern included diffuse, moderate to strong cytoplasmic staining with or without basal membranous accentuation. In contrast, the non-PTC staining pattern included the remaining staining results, which exhibited weak cytoplasmic staining and moderate or strong cytoplasmic staining with apical membranous accentuation.

PTC versus the other thyroid lesions

Most of non-follicular variant PTCs showed the PTC staining pattern, while most of the other tumors showed the non-PTC staining pattern. The difference between the two patterns was statistically significant (P = 0.000) (**Table 1**). Basal membranous accentuation was noted in 19 of the 73 PTCs (26%). The two PTCs with the non-PTC staining pattern showed strong cytoplasmic staining with apical membranous accentuation. Four non-PTC tumors exhibited the PTC staining pattern. These included a follicular carcinoma with strong cytoplasmic staining, another follicular carcinoma with moderate cytoplasmic staining and basal membranous staining, a hyalinizing trabecular tumor with moderate cytoplasmic staining and basal membranous accentuation, and a medullary carcinoma with moderate cytoplasmic staining. The diagnostic characteristics of NPC2 immunohistochemistry for non-follicular PTCs versus non-PTC lesions were a sensitivity of 97.3%, specificity of 96.9%, positive predictive value of 94.7%, negative predictive value of 98.4%, and diagnostic accuracy of 97.0% (**Table 2**).

Follicular and non-follicular PTCs

Mean age and lymph node metastasis of the PTCs exhibiting the PTC staining pattern compared to those exhibiting the non-PTC staining pattern were significantly different (P = 0.02 and P = 0.03, respectively). However, there were no significant differences for sex, tumor size, or tumor multiplicity. (P = 0.29, P = 0.39, and P = 0.53, respectively)

The pattern distribution between FVPTCs and non-follicular variant PTCs was significantly different (P = 0.000). There was no significant difference between FVPTCs with nodal metastasis and FVPTCs without nodal metastasis (P = 0.63) (**Table 3**).

Discussion

We evaluated NPC2 immunohistochemistry in various thyroid tumors and tumor-like lesions with the results showing distinctions between PTC and non-PTC lesions. Although many of the thyroid lesions demonstrated positive staining, we noticed differences in the staining intensity and subcellular localization of the immunohistochemistry signal. Staining intensity was mostly strong in PTCs and weak to moderate in non-PTC lesions. The currently used immunohistochemistry markers for thyroid lesions include galectin-3, HBME1, and CK19, which share the similar characteristics of positively staining both PTCs and non-PTC lesions [13-15]. Because of the positive staining of non-PTC lesions, studies and reviews regarding the use of immunohistochemistry in practice urge caution in the interpretation of results and the need to use a panel of multiple antibodies [6-8, 13, 14]. The same points may apply to NPC2 immunostaining; however, we found that the immunohistochemistry of NPC2 was different from the other markers. Specifically, unlike the immunohistochemistry of the other known mark-

Table 3. Clinicopathologic characteristics of papillary thyroid carcinomas including non-follicular variant and follicular variant

		PTC Staining Pattern	Non-PTC Staining Pattern	*p* value
Mean age (years)		49.9	56.3	0.02*
Sex	Male (n)	11	6	0.29**
	Female (n)	79	24	
Tumor size	≤1 cm (n)	56	16	0.39**
	>1 cm (n)	34	14	
Lymph node	No metastasis (n)	38	16	0.03**
	Metastasis (n)	47	7	
Tumor multiplicity	Solitary (n)	48	18	0.53**
	Multiple (n)	42	12	
Follicular vs. Non-follicular	Non-follicular PTC (n)	71	2	0.000***
	FVPTC (n)	19	28	
FVPTC with or without nodal metastasis	FVPTC with nodal metastasis (n)	5	7	0.63**
	FVPTC without nodal metastasis (n)	14	14	

PTC indicates papillary thyroid carcinoma; FVPTC, follicular variant PTC. *Independent T-test, **Chi-square test, ***Fisher's exact test.

ers [6-8, 13, 14], NPC2 immunostaining uniquely exhibited different subcellular localization of the immunohistochemical signal in PTCs compared to that in non-PTC lesions. In addition to granular cytoplasmic staining, many of the PTC specimens also showed basal membranous accentuation (**Figure 1A**). In comparison, many of the non-PTC lesions presented apical membranous accentuation (**Figure 1B-F**, **1K** and **1L**). Based on these findings, we grouped the general staining patterns into two distinct categories that differentiated PTC and non-PTC lesions.

The subcellular localization of NPC2 based on immunohistochemistry provided insight into contrasting histopathologic morphology between the papillary pattern of PTCs and follicular pattern of non-PTC lesions. The reversed cellular polarity can be determined from the findings obtained regarding the subcellular localization of the immunohistochemical signal. Most of the non-PTC lesions were composed of follicular structures with apical membranous accentuation of NPC2 immunostaining (**Figure 1B-F**, **1K** and **1L**). This was contrary to PTCs which showed basal membranous accentuation (**Figure 1A**). A previous study on papillary structure formation noted that NPC2 expression is important in papillae formation [16]. Our current finding further elaborates on the expression of NPC2 regarding how and where the protein is differently expressed in papillary and non-papillary tumors.

As a diagnostic marker of PTC, NPC2 immunohistochemistry demonstrated better overall performance in diagnostic characteristics with approximately 95% or greater sensitivity, specificity, positive predictive value, and negative predictive value compared to 82-87% sensitivity of galectin-3, HBME1, and CK19 [14, 15, 17]. However, since interpretation of the subcellular localization of NPC2 using immunohistochemistry required careful examination and not all staining showed membranous accentuation, we speculate that using a standardized protocol in practice would require a panel approach and caution in interpretation of results.

We categorized the PTC specimens into two groups, which included FVPTC and non-follicular PTC lesions. Non-follicular PTCs were regarded as a standard disease group while the FVPTCs were evaluated separately, as we suspected FVPTCs were heterogeneous by nature and/or overdiagnosis. FVPTCs are known to differ genetically from classical variants with higher rates of *RAS* mutations and lower rates of *BRAF* mutations [18]. The heterogeneity of FVPTC is also increased due to the tendency for overdiagnosis [19]. Regardless of the source, our results demonstrated its heterogeneity with the contrast in NPC2 staining patterns between FVPTC and non-follicular PTC specimens. On review of FVPTC for this study, many cases were believed to be overdiagnosed nodular goiter or adenoma with nuclear atypia. To

test this hypothesis, we analyzed the data by comparing PTC with nodal metastasis and PTC without nodal metastasis. We determined that PTC with nodal metastasis showed significantly higher proportions of the PTC staining pattern. However, when the rare occurrence of malignant clinical behavior of atypical follicular nodules is considered [20-22], the true nature of the FVPTC cases in this study may not be easily determined.

The association of age differences and NPC2 staining patterns was significant. The significant difference was attributed to etiological differences between PTC and non-PTC tumors. As the incidence of PTC peaks earlier relative to other histologic types of thyroid tumors [23], the tumors exhibiting the PTC staining pattern, in this case, PTCs, might correlate with significantly lower average patient age than tumors exhibiting the non-PTC staining pattern.

NPC2 immunohistochemistry appeared to be a prominent diagnostic immunohistochemistry for PTC. However, there are some limitations of the study. The NPC2 antibody shares the same shortcoming as the other antibodies currently used for immunostaining of thyroid lesions; positive staining of non-PTC lesions. We were not able to correlate the molecular pathological results of other studies, such as *BRAF* or *RAS* mutations, with our NPC2 staining, especially in FVPTC.

In conclusion, this study demonstrated that NPC2 immunohistochemical signal differed between PTC and non-PTC specimens. Based on our current findings, NPC2 may be used as a diagnostic marker for PTC with better performance than other immunohistochemical markers currently used. In addition, the subcellular localization of the immunohistochemical signal provides insight into the morphologic differences between papillary carcinoma and other follicular structure lesions. We anticipate that future studies using NPC2 immunohistochemistry in conjunction with molecular genetic analyses will provide deeper insight into the pathophysiology of PTC.

Disclosure of conflict of interest

None.

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