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Immunohistochemical Expression of NPC2 in Thyroid Lesions and Its Possible Diagnostic Utility

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Abstract

Thyroid nodules are a very common pathology among the population, especially women. Although the majority of them are of benign origin, the incidence of thyroid cancer (TC) is currently rather rising. Thyroid cancer is the most prevalent endocrine malignancy. PTC is the most common primary malignant thyroid tumor and it depends on the presence of classic nuclear features that may be seen in other neoplastic and non-neoplastic thyroid lesions. Immunohistochemical markers may be helpful, but till now there is no 100% consistent markers that distinct between PTC and other follicular thyroid lesions. The Niemann-Pick type C intracellular cholesterol transporter 2 (NPC2) gene encodes the NPC2 protein, which plays an important role in the regulation of intracellular cholesterol homeostasis via direct binding with free cholesterol. NPC2 expression in thyroid has been described, especially with high levels in PTC. Evaluation of the diagnostic value of immunohistochemical markers NPC2 to distinguish papillary thyroid carcinoma from other neoplastic or non-neoplastic mimickers. The current study included 70 randomly selected tissue blocks of formalin-fixed and paraffin embedded benign and malignant thyroid lesions. The 40 selected malignant cases include 30 papillary thyroid carcinomas and 10 follicular carcinomas. The 30 benign thyroid lesions include 20 follicular adenomas and 10 nodular hyperplasia. Among the studied different thyroid lesions, NPC2 high expression was detected in 36 (51.4%) cases. A statistically significant association was detected between NPC2 expression and patient sex (*p-value=0.044)* and histologic subtype (*p*-value *<0.001)*. In PTC cases, NPC2 high expression was detected in 25 (83.3%) cases. A statistically significant association was detected between NPC2 expression and pathological tumor stage (*p-value=0.013)* and PTC versus non-PTC lesions (*p-value <0.001)*. NPC2 is a potentially good immunohistochemical marker in differentiating PTC from other follicular thyroid lesions.

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

Full-length article **Corresponding Author, e-mail: rehamfarouk795@hotmail.com*

1. Introduction

Thyroid nodules are a common entity and are detected in approximately 5% to 7% of the adult population by physical examination alone [1]. Nodules may be solitary, multiple, cystic, or solid. Although more than 90% of detected nodules are benign lesions, thyroid nodules are clinically important as they may represent thyroid cancer in approximately 4.0% to 6.5% of cases [2]. Thyroid cancer (TC) is the most prevalent endocrine malignancy. It ranked ninth for cancer incidence in 2020 and was responsible for 586 000 cases worldwide. Women accounted for most of the cases. In 2020, TC was the fifth most common cancer in women after breast, colorectal, lung and cervical cancers [3,4]. In Egypt, thyroid cancer represents about 1.5% of all cancers and constitutes about 30% of endocrine malignancies based upon results of National Cancer Registry Program (NCRP). The rate in Egyptian females is 3.28% while in males is 0.95% among cancers [5,6].

PTC is the most common primary malignant thyroid tumor and it depends on the presence of classic nuclear features including elongated, crowded, ground glass–like (“Orphan Annie” eye) and grooved nuclei, with inconspicuous eccentric nucleoli. Although majority of PTC cases can be diagnosed and classified on the basis of histopathologic criteria, some thyroid lesions may show nuclear cytologic features or the architecture and growth pattern of PTC, leading to diagnostic problems [7,8]. NPC2 protein is a small soluble glycoprotein that contains a nineteen amino acids signal peptide. It plays an important role in the regulation of intracellular cholesterol homeostasis via direct binding with free cholesterol [9]. NPC2 has an important role in papillae formation and modulates papillary growth [10]. NPC2 expression was found to be up-regulated

in human breast, colon, and lung cancers, while, in contrast, there was down-regulation of its expression in kidney and liver cancers [9]. Also, NPC2 expression in thyroid has been described, especially with high levels in papillary thyroid carcinoma [11].

The aim of the current study is to assess the diagnostic value of immunohistochemical marker NPC2 to distinguish papillary thyroid carcinoma from other neoplastic or non-neoplastic mimickers.

2. Materials and Methods

2.1. Tissue specimens

The present study comprised 70 randomly selected tissue blocks of formalin-fixed, and paraffin embedded benign and malignant thyroid lesions. The 40 selected malignant cases include 30 papillary thyroid carcinomas and 10 follicular carcinomas. The 30 benign thyroid lesions include 20 follicular adenomas and 10 nodular hyperplasia. The available clinicopathological data included: patient age, sex, size of lesion (greatest dimension), site of lesion, background, type of operation, histopathologic type of thyroid lesion, laterality, focality, lymphovascular invasion, capsular infiltration, extrathyroidal extension, lymph node metastasis, pathological tumor Stage (according to the TNM staging of thyroid cancer) and tumor necrosis (Table 1).

Patient's age was classified in two age groups; the first group <55 years and the second age group ≥55 years according to previous studies [12-14]. Regarding tumor size in PTC cases, it was classified in two groups; the first group ≤1 cm and the second group >1 cm according to previous studies [11,15].

2.2. Immunohistochemical (IHC) procedure

Five µm sections were prepared on positive charged slides for immunohistochemistry of NPC2 primary antibody utilizing the avidin biotin-peroxidase complex method with diaminobenzidine (DAB) chromogen detection system. Initially tissue sections on the positive charged slides were deparaffinized and rehydrated. Then the endogenous peroxidase was blocked by immersion in a 3% solution of hydrogen peroxide and incubated for 30 minutes.

Antigen retrieval was performed by immersing the slides in citrate buffer solution (pH 6) for 2 times (10 minutes each) at 750-W. To block nonspecific background staining, the slides were treated by UV block. Primary antibody NPC2 (polyclonal rabbit antibody, 100 µ, concentrated, Cat No FNab10049, Dako; Agilent Technologies, Inc., USA) was then added and tissue sections were incubated for 1 hour at room temperature (dilution 1:100).

Excess reagent was thrown off and the slides were then rinsed gently with buffer solution for 5 minutes. After that Secondary biotinylated antibody was added for each slide for 30 minutes. DAB substrate and chromogen solutions were added to each slide and following that tissue sections were counter stained by Mayer's hematoxylin. The positive control for NPC2 was normal human liver tissue. Negative control tissue sections were processed by omitting the specific primary antibody from the staining procedure and replaced with PBS.

2.3. Scoring of NPC2 immunostaining

Evaluation of NPC2 expression was assessed using the widely accepted HSCORE system. Cytoplasmic +/- membranous NPC2 staining was considered as positive. The immunohistochemical scores were obtained by light microscopy (Olympus, Tokyo, Japan) as the staining intensity multiplied by the percentage of positive cells within 5 high power fields (in hot areas). The HSCORE was calculated using the following equation: HSCORE= ∑Pi (I), where (I) represents the staining intensity score (0=no staining,1=weak staining, 2=moderate staining, and 3=strong staining) and (Pi) represents the percentage of stained cells (from 0 to 100%). The final HSCORE ranged from 0 to 300 and NPC2 expression levels were classified as negative and positive using the median of final HSCORE as a cut-off value [16].

2.4. Statistical analysis

The analysis of the data was carried out using the IBM SPSS 28.0 statistical package software (IBM; Armonk, New York, USA). Data were tested for normal distribution using Shapiro-Wilk test. Data were presented as mean ± SD where normally distributed and as median (interquartile range) where data were skewed. Data were expressed both number and percentage for qualitative data and were analyzed by the Chi-square test or Fisher's exact test. A p-value less than 0.05 was considered significant.

3. Results

In the present study, NPC2 was expressed mainly in the cytoplasm +/- membranous accentuation. In normal thyroid tissue, NPC2 expression was either negative or showed only occasionally positive cells. Membranous accentuation, if present, was either basal or apical. In PTC cases, 9/30 (30%) cases showed basal accentuation (Fig. 2), and 21/30 (70%) showed no membranous accentuation. In non-PTC cases, 1/40 (2.5%) case showed basal accentuation, 10/40 (25%) showed apical accentuation (Fig. 5), and 29/40 (72.5%) showed no membranous accentuation. The difference between these patterns was statistically significant (*p*-value <0.0001*)*.

In the current study, 34/70 cases (48.6%) exhibited low cytoplasmic NPC2 expression, whereas 36/70 cases (51.4%) revealed high expression. A statistically significant association was detected between NPC2 expression and patient sex where (56.9%) of cases with female sex showed high expression while (75%) of cases with male sex showed low expression (*p-value=0.044).* A statistically significant association was also detected between NPC2 expression and histologic subtype where 83.3% of PTC (Fig. 1), 50% of FC (Fig. 4), and 30% of FA (Fig. 7) cases displayed high expression, while 16.7% of PTC (Fig. 3), 50% of FC (Fig. 6), 70% of FA (Fig. 8) and 100% of nodular hyperplasia (Fig. 9) cases demonstrated low expression (*P*-value <0.0001). (*p*-value <*0.001)*. No significant association was found between NPC2 expression and patient's age, size of lesion, site of lesion, background, and type of operation (*p-value*= 0.673, 0.081, 0.212, 0.499, and 0.473 respectively).

Regarding NPC2 expression and its association with histopathological features in malignant cases (PTC and FC), 10/40 cases (25%) exhibited low cytoplasmic NPC2 expression, whereas 30/40 cases (75%) showed high expression.

Table 1. Clinicopathological characteristics for all studied patients (N=70)

Variables		Number	%
Age (years)	**Median**	40	100%
	IQR	(29-50)	
Age group	**< 55**	57	81.4%
	≥ 55	13	18.6%
Sex	**Male**	12	17.1%
	Female	58	82.9%
Size of lesion (cm)	**Median**	3	100%
	IQR	(2-4)	
Site of lesion	**Right lobe**	29	41.4%
	Left lobe	24	34.3%
	Both lobes	17	24.3%
Background	**no pathological abnormality**	47	67.1%
	Nodular hyperplasia	14	20.0%
	lymphocytic thyroiditis	9	12.9%
Type of operation	**Total thyroidectomy**	56	80.0%
	Hemi thyroidectomy	14	20.0%
Histologic subtype	**Papillary thyroid carcinoma**	30	42.9%
	Follicular carcinoma	10	14.3%
	Follicular adenoma	20	28.6%
	Nodular hyperplasia	10	14.3%
In malignant cases (PTC and FC) (N=40)			
Laterality	**Unilateral**	33	82.5%
	Bilateral	7	17.5%
Focality	**Unifocal**	31	77.5%
	Multifocal	9	22.5%
Lymphovascular invasion	**Negative**	28	70%
	Positive	12	30%
Capsular infiltration	**Negative**	13	32.5%
	Positive	27	67.5%
Extrathyroidal extension	**Negative**	24	60%
	Positive	16	40%
Lymph node metastasis	**Negative**	17	42.5%
	Positive	23	57.5%
Pathological tumor stage	**pT1**	17	42.5%
	pT2	9	22.5%
	pT3	14	35%
Tumor necrosis	**Absent**	28	70%
	Present	12	30%

Table 2. Association between Cytoplasmic NPC2 expression and Clinicopathological features for all studied patients (N=70)

Variables		NPC2 Expression		*P*-value
		Low Expression (N=34) (48.6%)	High Expression N=36 (51.4%)	
Age groups	**< 55**	27 (47.4%)	30 (52.6%)	0.673
	≥ 55	7 (53.8%)	6 (46.2%)	
Sex	**Male**	9 (75%)	3 (25%)	**0.044***
	Female	25 (43.1%)	33 (56.9%)	
Size of Lesion (cm)	**Median**	3	2.5	0.081
	IQR	(2-4.3)	(1.5-4)	
Site of lesion	**Right lobe**	11(37.9%)	18 (62.1%)	0.212
	Left lobe	12 (50%)	12 (50%)	
	Both lobes	11 (64.7%)	6 (35.3%)	
Background	**No pathological abnormality**	25 (53.2%)	22 (46.8%)	0.499
	Nodular hyperplasia	5 (35.7%)	9 (64.3%)	
	Lymphocytic thyroiditis	4 (44.4%)	5 (55.6%)	
Type of operation	**Total thyroidectomy**	26 (46.4%)	30 (53.6%)	0.473
	Hemi thyroidectomy	8 (57.1%)	6 (42.9%)	
Histologic subtype	**PTC**	5 (16.7%)	25 (83.3%)	**<0.001***
	FC	5 (50%)	5 (50%)	
	FA	14 (70%)	6 (30%)	
	NH	10 (100%)	0 (0%)	
In malignant cases (PTC and FC) (N=40)				
Laterality	**Unilateral**	9 (27.3%)	24 (72.7%)	0.471
	Bilateral	1 (14.3%)	6 (85.7%)	
Focality	**Unifocal**	8 (25.8%)	23 (74.2%)	0.827
	Multifocal	2 (22.2%)	7 (77.8%)	
Lymphovascular invasion	**Negative**	7 (25%)	21 (75%)	1.00
	Positive	3 (25%)	9 (75%)	
Capsular infiltration	**Negative**	3 (23.1%)	10 (76.9%)	0.845
	Positive	7 (25.9%)	20 (74.1%)	
Extrathyroidal extension	**Negative**	5 (20.8%)	19 (79.2%)	*0.456*
	Positive	5 (31.2%)	11 (68.8%)	
Lymph node metastasis	**Negative**	5 (29.4%)	12 (70.6%)	*0.580*
	Positive	5 (21.7%)	18 (78.3%)	
Pathological tumor stage	**pT1**	2 (11.8%)	15 (88.2%)	0.174
	pT2	4 (44.4%)	5 (55.6%)	
	pT3	4 (28.6%)	10 (71.4%)	
Tumor necrosis	**Absent**	7 (25%)	21 (75%)	1.00
	Present	3 (25%)	9 (75%)	

**** P - value <0.05 are considered statistically significant according to Chi-Square test and Fisher's exact test.***

Table 3. Association between Cytoplasmic NPC2 expression and Clinicopathological features for patients with papillary thyroid carcinoma (N=30)

Variables		NPC2 Expression		*P*-value
		Low Expression N=5 (16.7%)	High Expression N=25 (83.3%)	
Age groups (year)	**< 55**	3 (12%)	22 (88%)	0.125
	≥ 55	2 (40%)	3 (60%)	
Sex	**Male**	1 (25%)	3 (75%)	0.631
	Female	4 (15.4%)	22 (84.6%)	
Size of Lesion (cm)	**≤1**	1 (14.3%	6 (85.7%)	0.847
	>1	4 (17.4%)	19 (82.6%)	
Site of lesion	**Right lobe**	3 (18.8%)	13 (81.2%)	0.948
	Left lobe	1 (14.3%)	6 (85.7%)	
	Both lobes	1 (14.3%)	6 (85.7%)	
Background	**No pathological abnormality**	2 (14.3%)	12 (85.7%)	0.454
	Nodular hyperplasia	1 (10%)	9 (90%)	
	Lymphocytic thyroiditis	2 (33.3%)	4 (66.7%)	
Type of operation	**Total thyroidectomy**	4 (15.4%)	22 (84.6%)	0.631
	Hemi thyroidectomy	1 (25%)	3 (75%)	
PTC Vs Non-PTC lesions	**PTC**	5 (16.7%)	25 (83.3%)	**<0.001***
	Non-PTC lesions	29 (72.5%)	11 (27.5%)	
Laterality	**Unilateral**	4 (17.4%)	19 (82.6%)	0.847
	Bilateral	1 (14.3%)	6 (85.7%)	
Focality	**Unifocal**	3 (13.6%)	19 (86.4%)	0.460
	Multifocal	2 (25%)	6 (75%)	
Lymphovascular invasion	**Negative**	4 (17.4%)	19 (82.6%)	0.847
	Positive	1 (14.3%)	6 (85.7%)	
Capsular infiltration	**Negative**	3 (23.1%)	10 (76.9%)	0.410
	Positive	2 (11.8%)	15 (88.2%)	
Extrathyroidal extension	**Negative**	4 (20%)	16 (80%)	*0.488*
	Positive	1 (10%)	9 (90%)	
Lymph node metastasis	**Negative**	0 (0%)	7 (100%)	*0.177*
	Positive	5 (21.7%)	18 (78.3%)	
Pathological tumor stage	**pT1**	2 (11.8%)	15 (88.2%)	**0.013***
	pT2	3 (60%)	2 (40%)	
	pT3	0 (0%)	8 (100%)	
Tumor necrosis	**Absent**	4 (17.4%)	19 (85.7%)	0.847
	Present	1 (14.3%)	6 (24%)	

**** P - value <0.05 are considered statistically significant according to Chi-Square test and Fisher's exact test.***

Table 4. The diagnostic characteristics of NPC2 immunohistochemistry for differentiating PTCs from studied non-PTC lesions

Sensitivity	Specificity	PPV	NPV	Diagnostic accuracy
83.33%	85%	80.6%	87.2%	84.3%

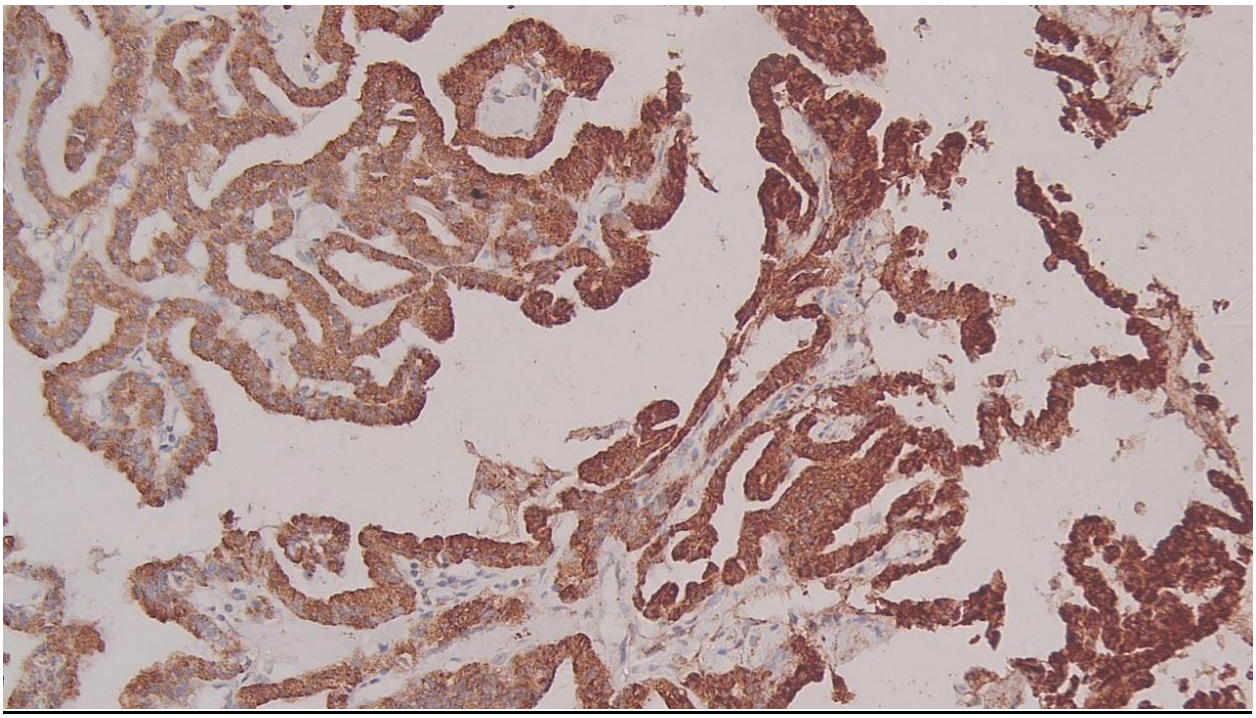

Figure 1. High cytoplasmic NPC2 expression in papillary thyroid carcinoma (IHC, X200)

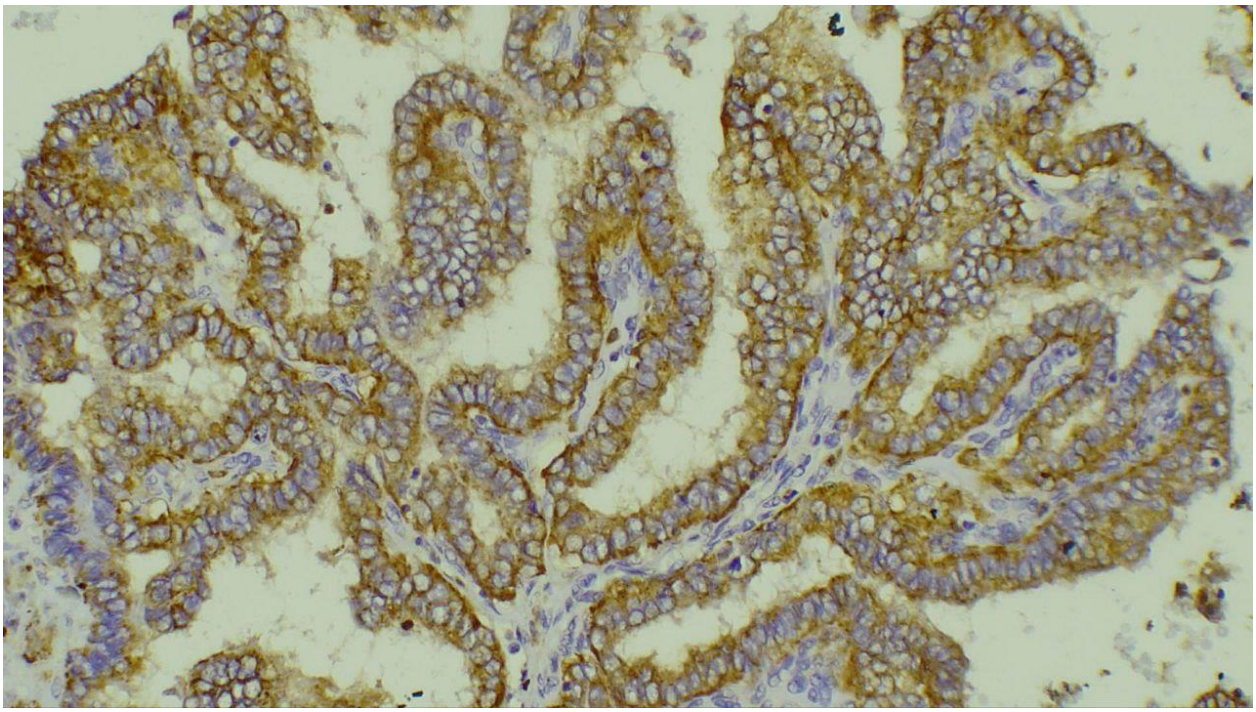

Figure 2. High cytoplasmic NPC2 expression in papillary thyroid carcinoma with basal membranous accentuation (IHC, X200)

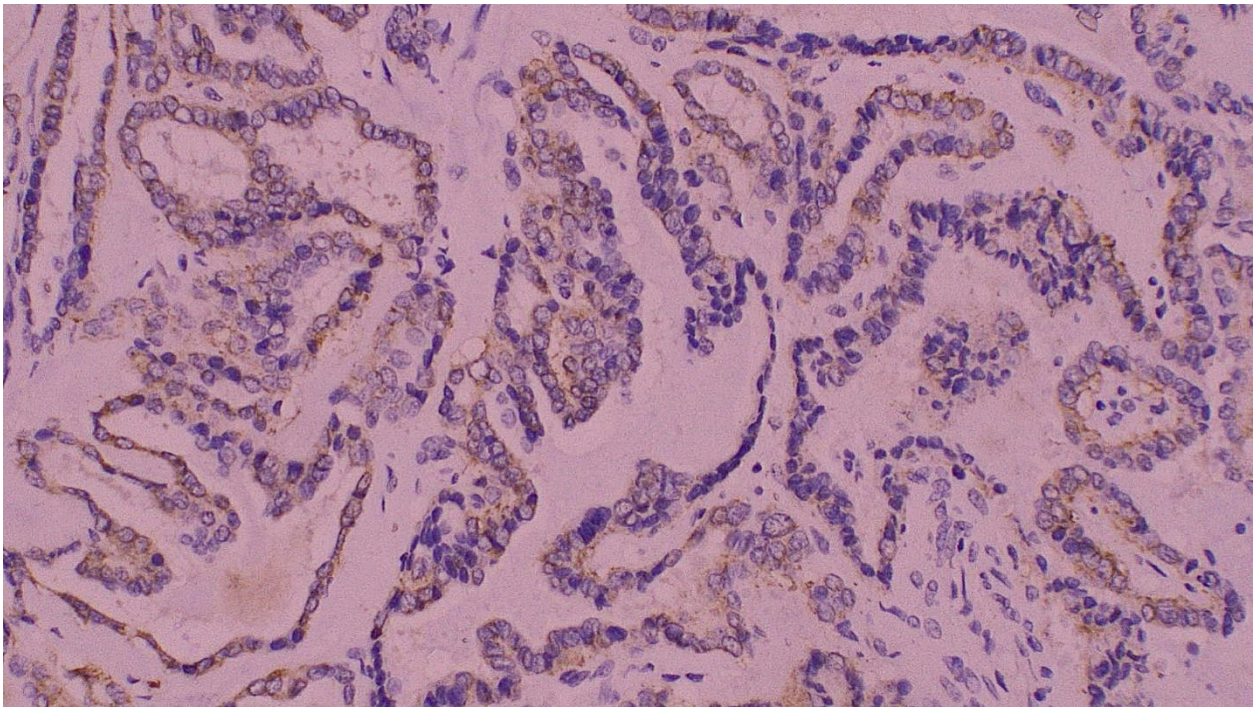

Figure 3. Low cytoplasmic NPC2 expression in papillary thyroid carcinoma (IHC, X200)

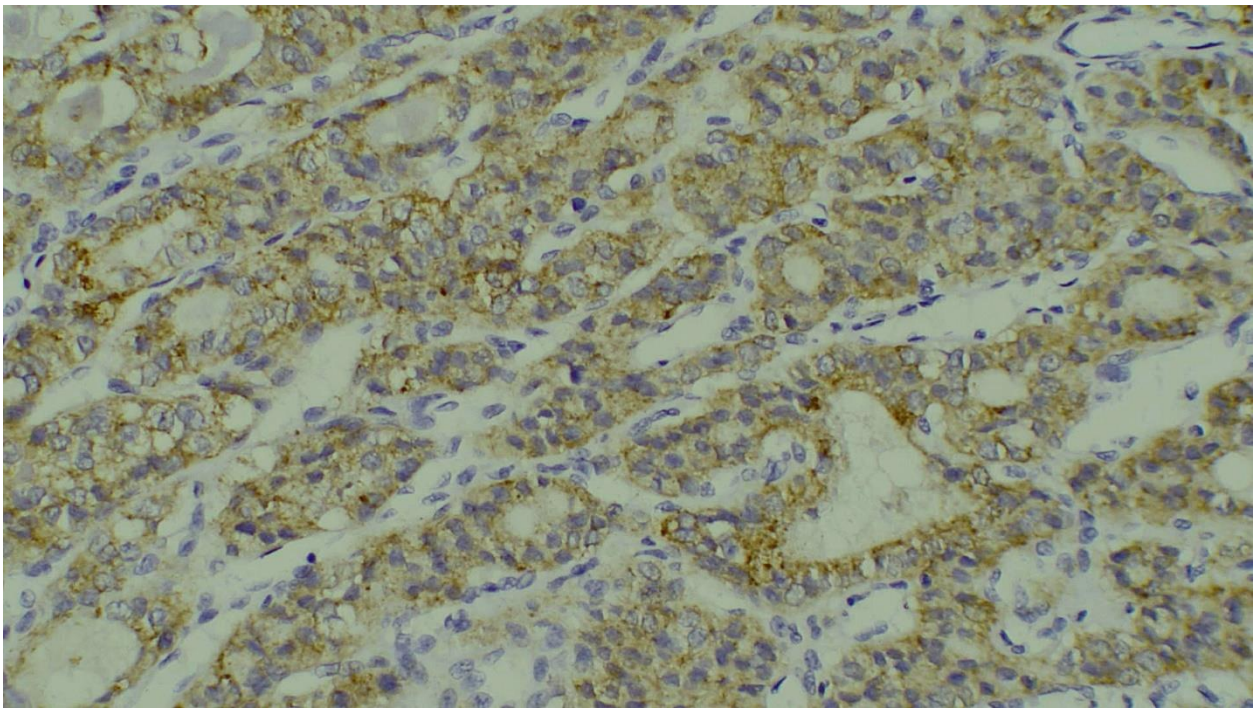

Figure 4. High cytoplasmic NPC2 expression in follicular carcinoma (IHC, X200)

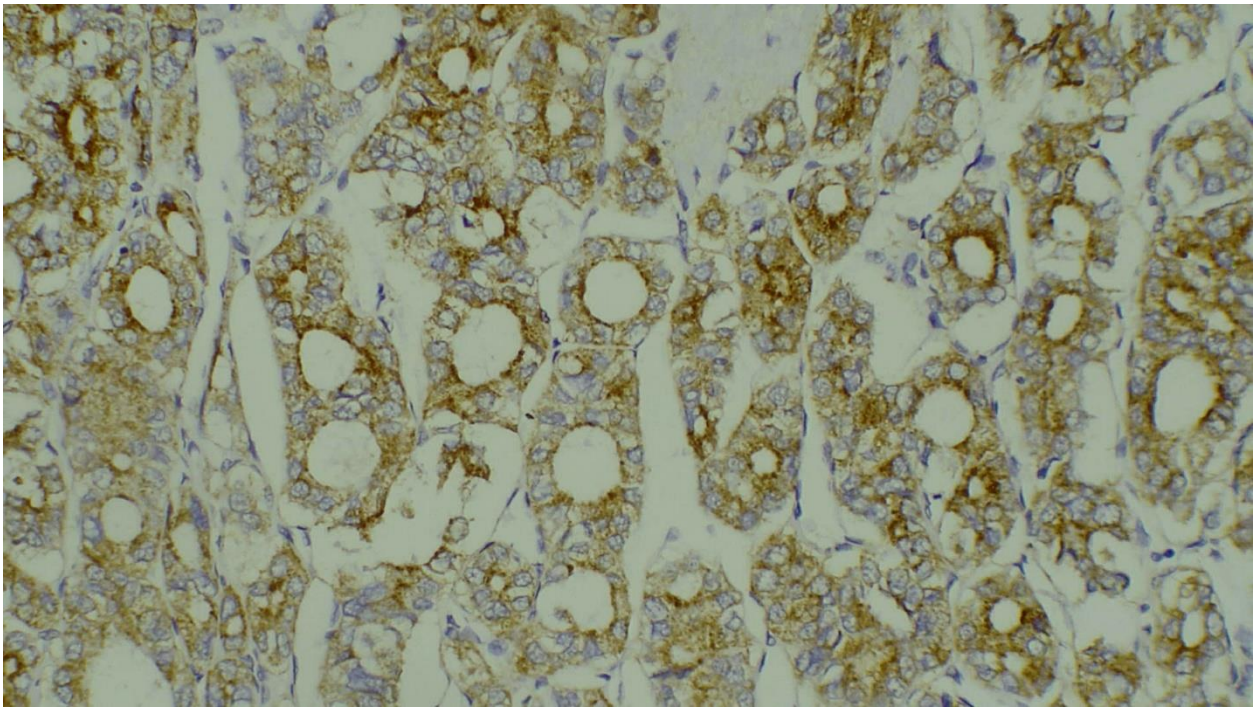

Figure 5. High cytoplasmic NPC2 expression in follicular adenoma with apical membranous accentuation (IHC, X200)

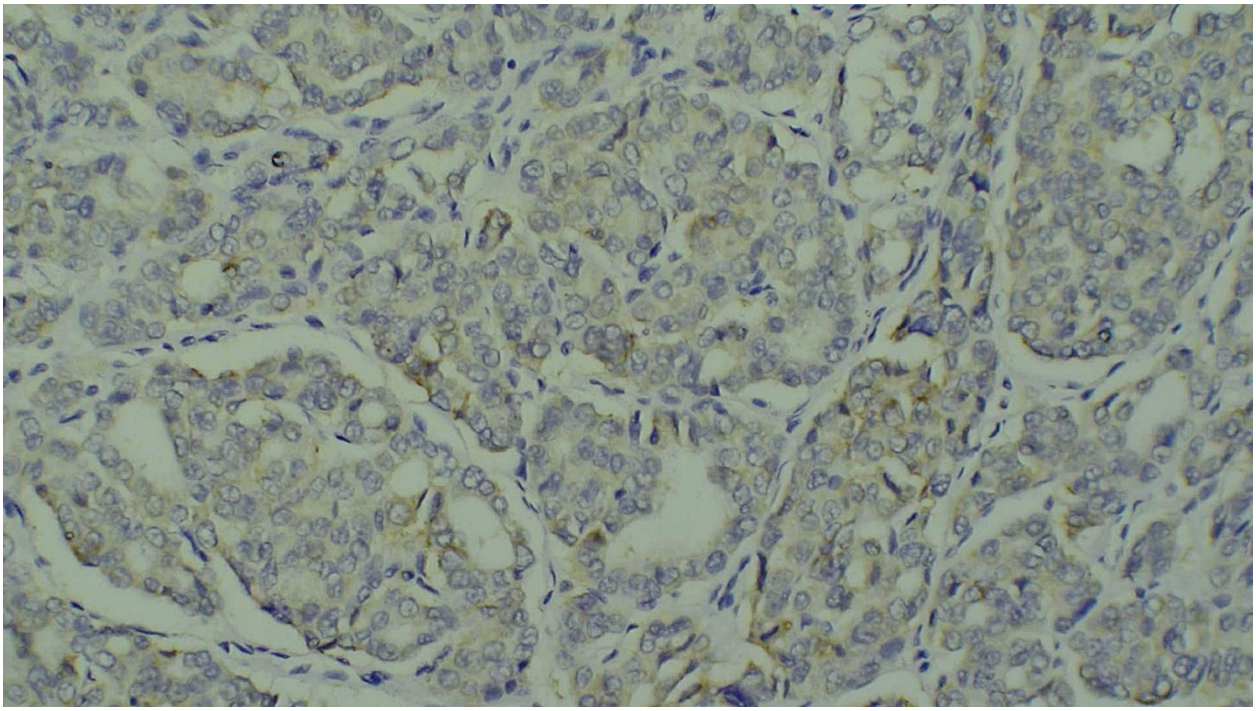

Figure 6. Low cytoplasmic NPC2 expression in follicular thyroid carcinoma (IHC, X200)

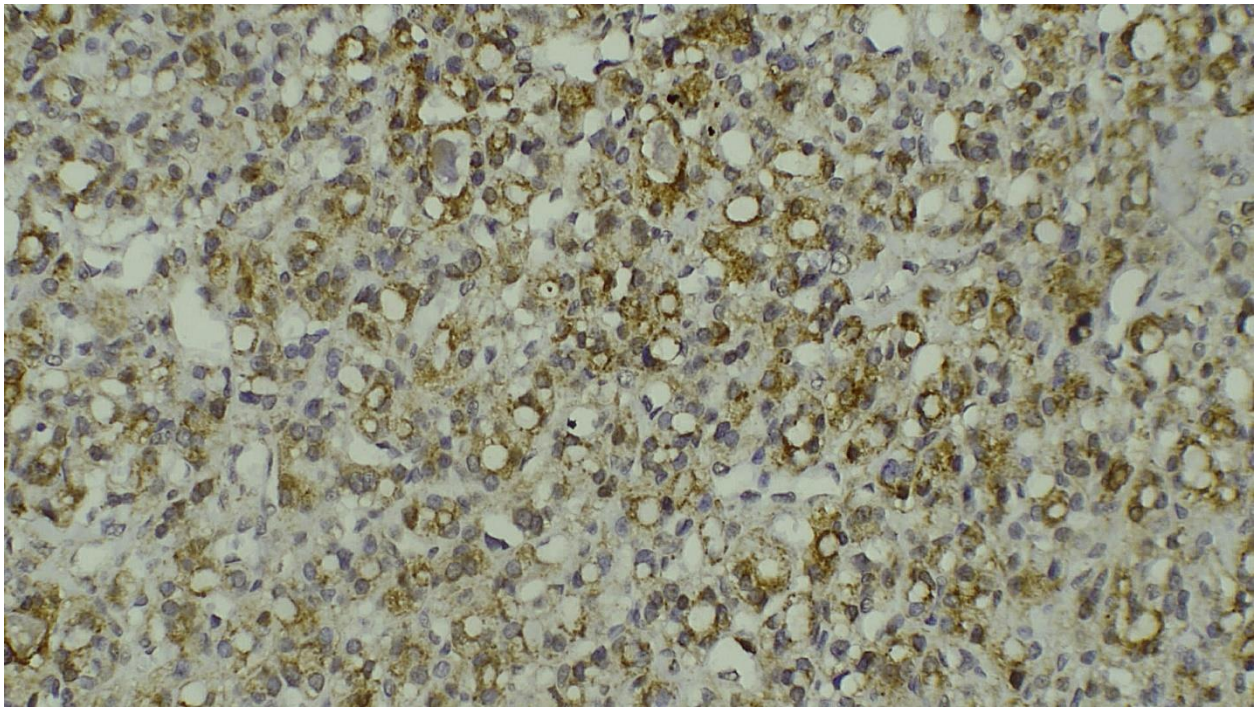

Figure 7. High cytoplasmic NPC2 expression in follicular adenoma (IHC, X200)

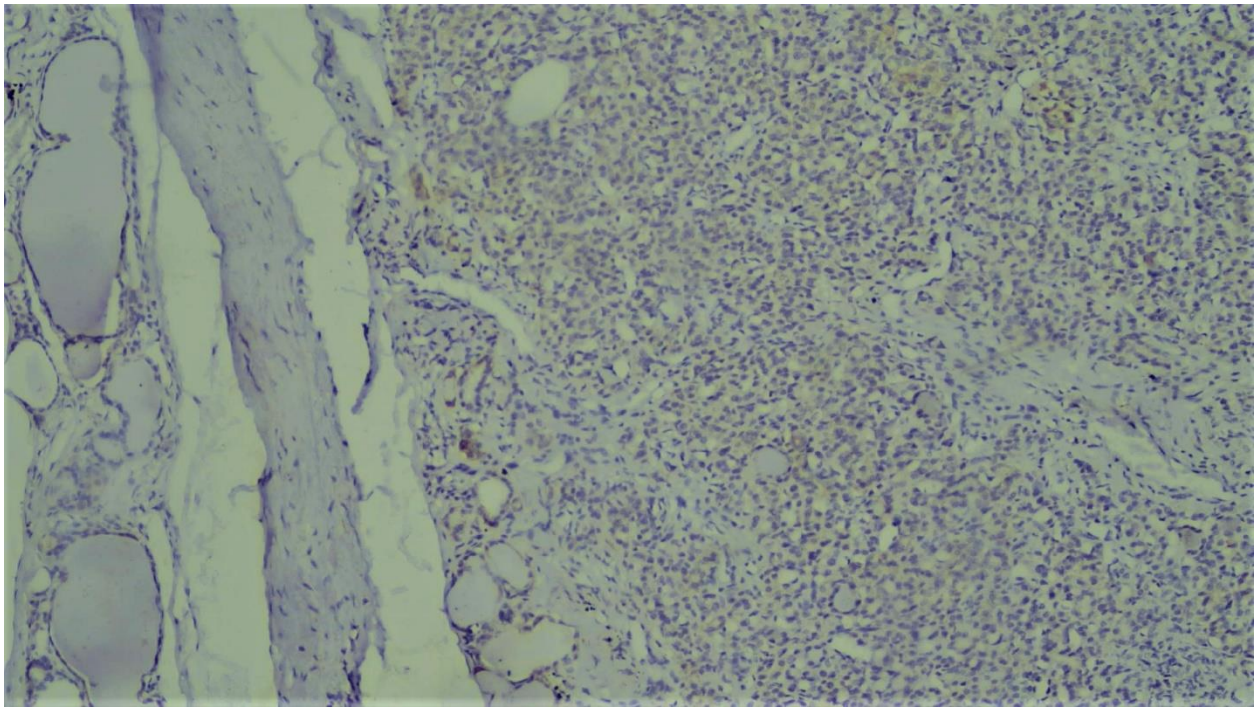

Figure 8. Low cytoplasmic NPC2 expression in follicular adenoma (IHC, X100)

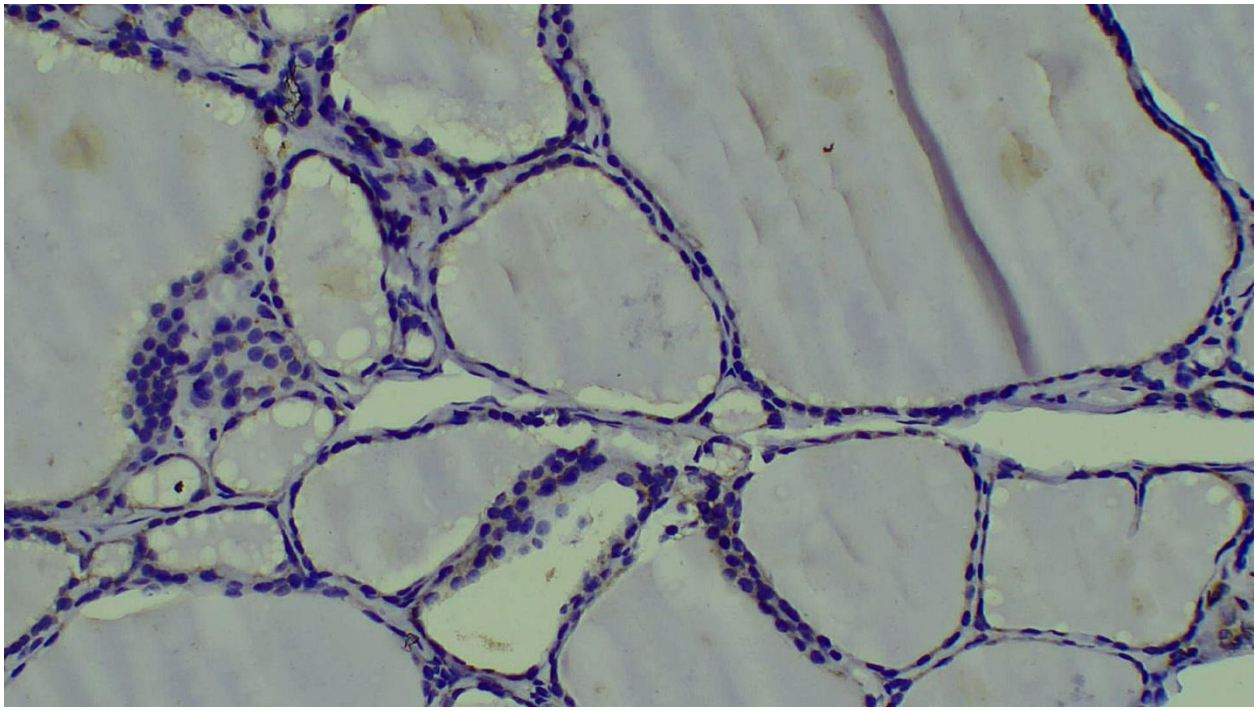

Figure 9. Low cytoplasmic NPC2 expression in nodular hyperplasia (IHC, X200)

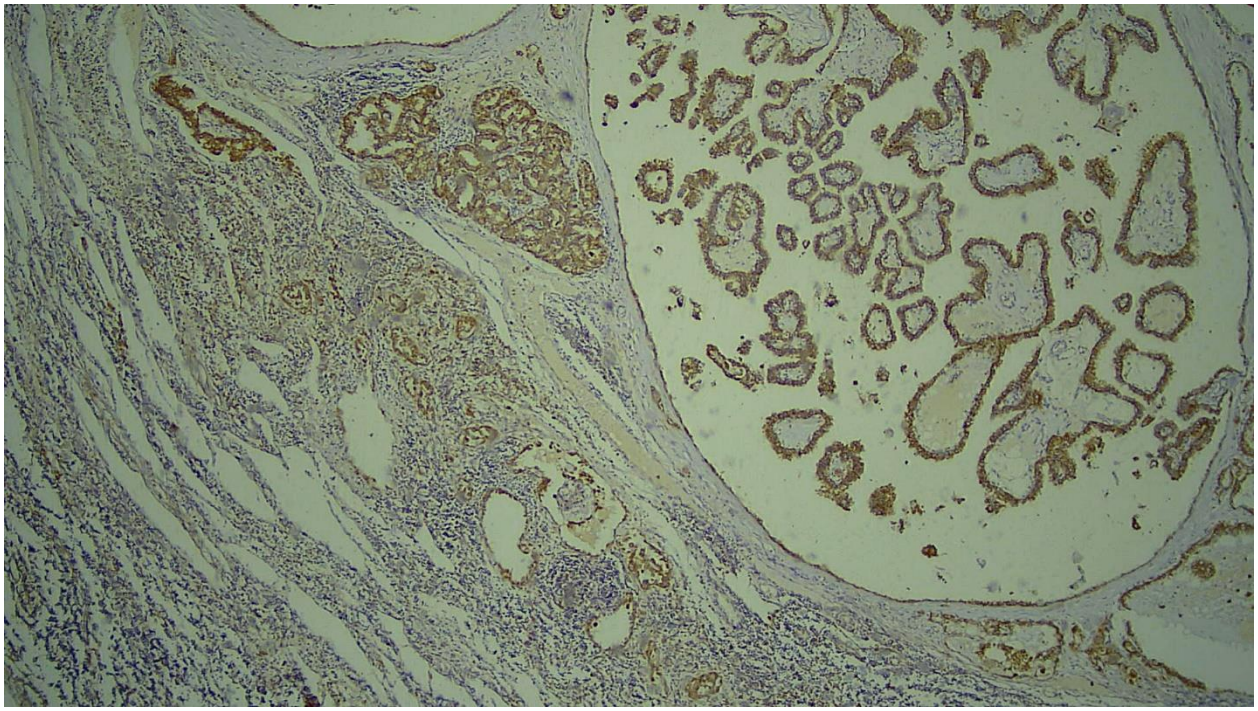

Figure 10. High cytoplasmic NPC2 expression in lymph node infiltrated by papillary thyroid carcinoma (IHC, X)

No significant association could be detected with the studied histopathological variables; laterality, focality, LVI, capsular infiltration, ETE, LN metastasis, pathological tumor stage and tumor necrosis (*p-value*= 0.471, 0.827, 1.00, 0.845, 0.456, 0.580, 0.174 and 1.00 respectively) (Table 2).

As regards NPC2 expression and its association with clinicopathological features in PTC cases, 5/30 cases (16.7%) exhibited low cytoplasmic NPC2 expression, whereas 25/30 cases (83.3%) revealed high expression. A statistically significant association was detected between NPC2 expression and pathological tumor stage where (88.2%) of pT1 cases, (40%) of pT2 cases and (100%) of pT3 cases showed high expression (*P-value* =0.013). Also, a statistically significant association was detected between NPC2 expression in PTC cases and other studied thyroid lesions (non-PTC lesions) where (83.3%) of PTC cases showed high expression while (72.5%) of non-PTC lesions exhibited low expression (*p*-value <0.001). No significant association could be detected between NPC2 expression and other studied clinicopathological variables among PTC cases (Table 3).

The diagnostic characteristics of NPC2 immunohistochemistry for differentiating PTCs from non-PTC lesions (FC, FA, NH) were a sensitivity of 83.33%, specificity of 85%, positive predictive value (PPV) of 80.6%,

negative predictive value (NPV) of 87.2%, and diagnostic accuracy of 84.3% (Table 4).

4. Discussion

In our study, we analyzed the diagnostic utility of NPC2 in discriminating papillary thyroid carcinoma from other thyroid lesions with mimicking features. Regarding NPC2 expression among different thyroid lesions, we found that 51.4% of cases showed high cytoplasmic NPC2 expression whereas 48.6% of cases revealed low expression. Membranous accentuation, if present, was either basal or apical. In PTC cases, 30% of cases showed basal accentuation, while 25% of non-PTC cases showed apical accentuation and the difference between these patterns was statistically significant (*p*-value <0.001*)*.

This was in agreement with *Chae and Kim, 2021*, the only study that evaluated immunohistochemical expression of NPC2 in different thyroid lesions up to our knowledge [(11)]. They reported that most of PTC cases displayed diffuse strong to moderate NPC2 expression with or without basal membranous accentuation while non-PTC cases showed diffuse to focal, weak to moderate NPC2 expression with or without apical membranous accentuation (*P*-value=0.000). Different subcellular localization of NPC2 immunohistochemical signal in PTCs compared to that in non-PTC lesions provided insight into contrasting histopathologic morphology between PTC and non-PTC lesions and requirement for further research on how and where NPC2 protein is differently expressed in papillary and non-papillary lesions.

Moreover, a previous study on papillary structure formation noted that NPC2 expression is essential in papillae formation [10]. On studying the relation between NPC2 immunoexpression and various clinicopathological variables among different thyroid lesions, we found a statistically significant association between NPC2 expression and patient sex (*P-value* =0.044). This was in contrast to what was reported by *Chae and Kim, 2021* who found no significant difference [(11)]. We also found a statistically significant association between NPC2 expression and the histologic subtype in the studied cases where most cases of PTC displayed high expression, while most cases of FA and all cases of nodular hyperplasia demonstrated low expression (*P*-value <0.001), this was in line with *Chae and Kim, 2021* who also found a significant difference [11].

No significant association could be detected between NPC2 expression and other studied clinicopathological variables. On studying the relation between NPC2 immunoexpression and various clinicopathological variables among PTC cases, 83.3% of cases revealed high cytoplasmic NPC2 expression, whereas 16.7% exhibited low expression. we found a statistically significant association between NPC2 expression in PTC cases and other studied thyroid lesions (non-PTC lesions) where most PTC cases showed high expression while most cases of non-PTC lesions exhibited low expression (*p*-value <0.001), and this coincides with what was noted by *Chae and Kim, 2021* who also reported a significant association [11].

Up to our knowledge, this is the first study that evaluated the association between NPC2 expression and the pathological tumor stage in PTC cases. A statistically significant association was detected between NPC2 expression and pathological tumor stage where high expression was detected more in higher stages (*P-value* =0.013). The possible explanation for this finding is that disturbance in NPC2 expression was found to influences cell proliferation and tumor growth through regulation of ERK1/2 MAPK phosphorylation in fibroblast cells [17]. No significant association was found between NPC2 expression and other studied clinicopathological variables.

The diagnostic characteristics of NPC2 immunohistochemistry for differentiating PTCs from non-PTC lesions (FC, FA, NH) were a sensitivity of 83.33%, specificity of 85%, positive predictive value (PPV) of 80.6%, negative predictive value (NPV) of 87.2%, and diagnostic accuracy of 84.3%. This was similar to what was reported by *Chae and Kim, 2021* who reported sensitivity of 97.3%, specificity of 96.9%, PPV of 94.7%, NPV of 98.4%, and diagnostic accuracy of 97% [11]. In conclusion, this study evaluated the diagnostic role of NPC2 immunohistochemical expression in differentiating PTC from other mimicking lesions. Based on current findings of our study, NPC2 may be used as a diagnostic marker for PTC. Furthermore, different immunohistochemical signals reflect the morphological differences between PTC and other lesions. We hope that future studies using NPC2 immunohistochemistry together with molecular analyses will give a deeper insight into the pathophysiology of PTC.

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