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ORIGINAL ARTICLE

Associations Between Nucleus Size, and Immunohistochemical Galectin-3, Cytokeratine-19 and Hbme-1 Markers in Thyroid Papillary Carcinoma: A Morphometric Analyze

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Abstract This study aimed to evaluate the morphometric measurements in cases with papillary thyroid carcinoma, and determine a cut-off value to support diagnosis. Fifty cases with a diagnosis of papillary thyroid carcinoma (PTC) were included in the study with their Galectine-3, CK-19 and HBME-1 immunohistochemical staining results. Demographic and clinical data gathered from pathology reports, which included demographic information such as patients' sex, age, macroscopic tumor size, number of tumor focuses; prognostic parameters such as lenfovascular invasion, perineural invasion, thyroid capsule invasion; and results of immunohistochemical CK-19, Galectin-3 and HBME-1 staining. Longest nuclear diameters of 150 tumor cells and 150 normal thyrocytes of each case were manually measured in an image analysis software, and mean longest nuclear diameters (MLND-TC and MLND-NC), and also tumor cell/normal cell longest nuclear diameter ratio (TC/NC-LNDR) were calculated. MLND-TC was higher than MLND-NC. The cases with higher MLND-TC had increased risk of capsule invasion in case of a negative staining with Galectine-3, HBME-1, or CK-19. When TC/NC-LNDR was high, number of tumor focus tended to be multiple and lymphovascular invasion risk was also increased. Subtypes of PTC were not differed regarding staining patterns. And finally, increased TC/NC-LNDR was associated with increased risk of having poor prognostic factors. The results of this study suggest that MLND-NC, MLND-TC, and TC/NCLNDR are valuable and easy-to-use measures, which can assist routine histology practice.

Keywords Papillary thyroid carcinoma · Nucleus diameter · Morphometric analyze

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Introduction

Thyroid gland is the most frequent site of malignancy among endocrine organs. Thyroid cancers constitute 1% of all malignancies. Papillary carcinoma is the most frequent type with a frequency of more than 70% [17].

Papillary thyroid carcinoma (PTC) is diagnosed histomorphologically. Typical histologic characteristics of papillary carcinoma are nuclear transparency, nuclear grooves and inclusions, nuclear congestion, and papillary configuration. But histomorphologicalpatterns are not such apparent in all cases. Additionally, these findings are frequently seen in papillary carcinoma, but they might also be observed in some benign entities. For instance, cells with transparent nucleus may be seen focally in diffuse hyperplasia and follicular adenoma. Moreover, intranuclearpseudoinclusions are rare but may be present in follicular adenomas [2, 13].

Papillary changes similar to thyroid papillary carcinoma can be seenin hyperplasic circumstances like Graves disease. But in these cases, epithelium lining of papillary structures is single layered at the base and normochromatographic, and papillary structures do not include fibrovascular core. Despite this, papillary structures with multi-layered, crowded, and oval nuclear cells that surround a fibrovascular core can be seen in papillary thyroid carcinoma. Papillary structures in papillary hyperplasia are much more similar to papillary structures in PTC. This situation complicates the differential diagnosis from papillary carcinoma, and currently many immunohistochemical studies are conducted on this topic. Cytokeratine-19, Galectin-3, and HBME-1 are the most widely used and useful immune markers [22]. Cytokeratine-19 stains focally and poorly in benign thyroid lesions and normal thyroid tissues, but enhances strongly and diffusely in PTC [1]. Galectin-3 expression is found in papillary thyroid carcinomas, but not seen in normal thyroid tissue [15]. HBME-1 is primarily important in determination of malignancies originated from mesothelium cells. But it is also used in diagnosing thyroid carcinomas. It is the most specific (97.9%) marker of follicle epithelium originated malign thyroid tumors, particularly PTC [22]. Significant membranous or luminal staining can be seen in PTC [1].

These three immunohistochemical markers are quite useful, but cannot be utilized in hospitals with inadequate laboratory conditions, and they require extra cost. Even if they are used in adequate laboratories, some validity and reliability problems may also be encountered. As a consequence, morphological evaluation still keeps its validity in the diagnosis of PTC. In this study we have applied morphometric measurements in cases with PTC, and tried to find a proportion to support diagnosis.

Materials and Methods

Cases

Cases with a diagnosis of papillary thyroid carcinoma whom were undergone total thyroidectomy operation between 2012 and 2014 were identified from the archives of Pathology Departments of Necmettin Erbakan University Meram Faculty of Medicine and Antalya Research and Training Hospital. Study population included 50 of these cases with Galectine-3, CK-19 and HBME-1 immunohistochemical staining results (Fig. 1). Data gathered from pathology reports included demographic information such as patients' sex, age, macroscopic tumor size, number of tumor focuses; prognostic parameters such as lenfovascular invasion, perineural invasion, thyroid capsule invasion; and results of immunohistochemical CK-19, Galectin-3 and HBME-1 staining.

Immunohistochemistry

Tumor specimens were fixed in 10% neutral-buffered formalin for 24 h, embedded in paraffin, and then cut into 4-μm sections. Antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) using microwave irradiation (400 W) at 95 °C for 40 min. After quenching endogenous peroxidase activity, sections were incubated with primary anti-bodies(paranteze HBME-1 (DAKO, Clone HBME-1, 1:50), CK19 (DAKO, Clone RCK 108, 1:50), Galectin-3 (R&D SYSTEMS, Clone 194,804, 1:100) for 60 min at room temperature. Following the wash, the secondary antibody was administered(40 min). Following the wash, the samples were closed. More than 10% stainings were considered positive.

Histomorphological Evaluation

Photographs from tumoral and normal tissues of each case were taken with 40× objective ofan Olympus BC51 light microscope, which was connected toa Pixera 150ES-CU camera. Largest nuclear diameters of 150 tumor cells and 150 normal thyrocytes of each case were manually measured in an image analysis software (BAB Bs200Pro Image Analysis Software).

Mean largest nuclear diameter of tumor cells (MLND-TC, Fig. 2) mean largest nuclear diameter of normal cells (MLND-NC), and tumor cell/normal cell largest nuclear diameter ratio (TC/NC- LNDR) were calculated for each case.

Data Analysis

Statistical analyses were performed with SPSS (Statistical Package for Social Sciences) version 15.0 software. Descriptive statistics were presented as mean, standard deviation (SD), and range for quantitative data; and as frequency, and percent for qualitative data. Normal distribution of numerical variables was tested with Kolmogorov-Smirnov test. Differences between frequencies in qualitative data were tested with Chi-square test, and Fisher's exact test was used when assumptions of chi-square were not met. Significant results in multi-group tables were evaluated with Bonferroni correction to determine the significantly different group. Mann-Whitney U test was used for comparing two non-normally distributed quantitative data. Comparisons between non-normally distributed more than two groups were done with Kruskal-Wallis test, and post-hoc pairwise comparisons were done with Mann-Whitney U test and Bonferroni correction. Normally distributed quantitative data in more than two groups were compared with ANOVA test, and post-hoc evaluations were done with Tukey test, since homogeneity of variances were determined with Levene's test. Comparisons of two consecutive measurements were performed with paired

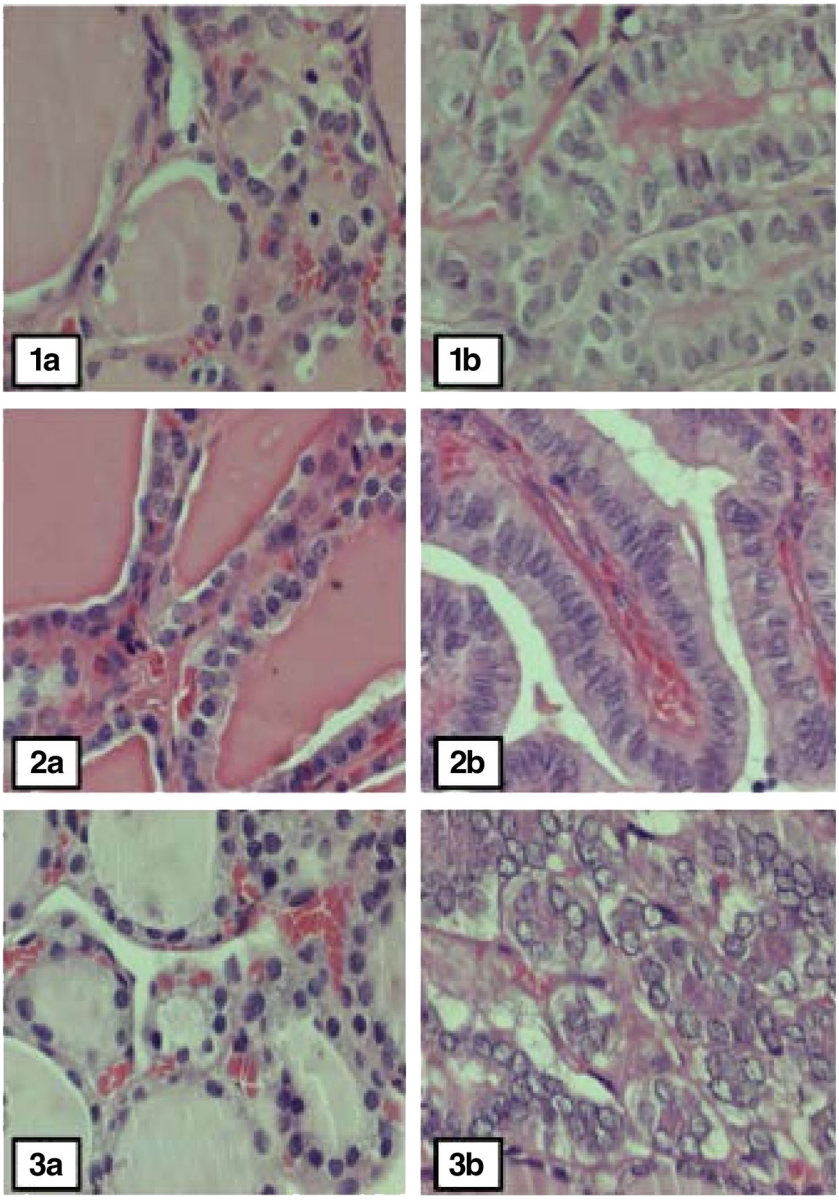

Fig. 1 Normal thyrocytes (1a, 2a, 3a) and tumor cells (1b, 2b, 3b). Normal and tumor cells (a, b) belong to same case, and figure illustrates samples from three different cases (1, 2, 3)

samples t-test. Correlations between discrete and non-normally distributed data were done with Spearman correlation test, and correlations between numerical and normally distributed data were done with Pearson correlation analyses. Multivariate analyses in this study included variables from previous analyses and literature data, and level of agreement was evaluated by Ordinal Logistic Regression analyze. Diagnostic values of statistically significant factors were analyzed by using ROC curve analyses. When a significant cut-off level was obtained, sensitivity, specificity, positive predictive value, and negative predictive values of this level were computed. Statistical significance was considered as a p value of <0.05. The level of significance was considered as $p < 0.017$ (in three groups), and $p < 0.0125$ (in four groups), when a Bonferroni correction was used.

Results

Descriptive data of 50 thyroid papillary carcinoma cases are presented in Table 1. Eight cases with negative stains had only negative result with one stain type in the study. These cases were diagnosed with PTC, because they had positive expression with remaining two immunohistochemical staining, and they met the classical histomorphologic criteria of PTC.

The largest nuclear diameter measurements of 150 tumor and 150 normal cells of each case were shown in Table 1. Paired samples t-test results revealed that MLND-TC was significantly higher than MLND-NC ($p < 0.001$). MLND-TC size was found to be approximately two-times bigger than MLND-NC and an erythrocyte. Evaluation of associations between numerical data such as age, macroscopic size,

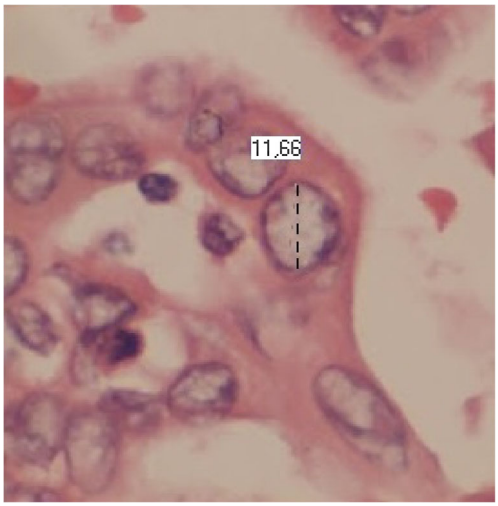

Fig. 2 A measurement of tumour cell in an image analysis software (BAB Bs200Pro Image Analysis Software). ×40, H.E

MLND-TC and MLND- NC revealed that there were no significant correlations between these variables ($p > 0.05$). The hypotheses of differences between MLND-NC, MLND-TC, and MLND-TC/NC ratio with other variables of the study were summarized in Table 2.

When cases were divided into three groups according to their histological subtypes as papillary, follicular, and others,there were no significant differences regarding staining patterns of HBME-1 ($p = 0.486$), Galectin-3 ($p = 0.572$), and CK-19 ($p = 0.280$) between groups. Prognostic factors were compared between cases with one negative staining (8 cases), and without negative staining results. Accordingly, number of tumor focus ($p = 1.000$), lymphovascular invasion (p = 1.000), perineural invasion ($p = 0.297$), TC/NC-LNDR ($p = 0.412$), and histological subtype ($p = 0.184$) were similar between cases with and without negative staining results. But, capsule invasion was significantly higher in cases with negative staining ($p = 0.013$).

Results of logistic regression analyses for determining the risk factors regarding capsule, lypmhovascular, and perineural invasion patterns were presented in Table 3. According to the findings, capsule invasion risk was increased by 13.38 times with Galectin-3 negativity, and 4.16 times with 1 μm increase in MLND-TC; lymphovascular invasion risk was increased by 3.36 times with 1 cm increase in macroscopic tumor size; and perineural invasion risk was increased by 111.45 times with HBME-1 negativity.

Associations between numbers of poor prognostic factors and TC/NC-LNDR were also evaluated by using difference, correlation, and estimation methods. Numbers of poor prognostic factors were ranged between 0 and 3 in the study group. When cases were grouped accordingly, TC/NC-LNDR was significantly different between groups with different numbers of prognostic factors ($\chi = 14.616$; $p = 0.002$). When numbers of poor prognostic factors were accepted as numerical variables, correlation analyses revealed that there was a significant correlation between number of prognostic factors and TC/NC-LNDR (rho = 0.426; $p = 0.017$), which showed that number of poor prognostic factors were increased with increasing TC/NC-LNDR. Finally, predictive role of TC/NC-LNDR for estimating number of poor prognostic factors was evaluated with ordinal logistic regression analyze. Results revealed that

Table 1 Demographic and clinical data of the cases

	Mean ± SD (range)
Age (year)	47.24 ± 12.62 (19–72)
Macroscopic tumor size (cm)	1.78 ± 1.44 (0.30–6.70)
MLND-TC (μm)	10.27 ± 0.75 (8.32–11.61)
MLND-NC (μm)	5.43 ± 0.84 (3.87–7.29)
TC/NC-LNDR	1.73 ± 0.36 (1.19–2.67)
	n (%)
Sex	
Male, n (%)	9 (18.0)
Female, n (%)	41 (82.0)
Histological subtype	
Papillary	36 (72.0)
Follicular	9 (18.0)
Oncocytic	3 (6.0
Columnar	1 (2.0)
Solid	1 (2.0)
Number of tumor focus	
Single	29 (58.0)
Multiple	21 (42.0)
Capsule invasion	
Positive	17 (34.0)
Negative	33 (66.0)
Lymphovascular invasion	
Positive	7 (14.0)
Negative	43 (86.0)
Perineural invasion	
Positive	2 (4.0)
Negative	48 (96.0)
HBME-1 staining	
Positive	48 (96.0)
Negative	2 (4.0)
Galectin-3 staining	
Positive	45 (90.0)
Negative	5 (10.0)
CK-19 staining	
Positive	49 (98.0)
Negative	1 (2.0)

MLND-TC Mean longest diameter of nucleuses of tumor cells
MLND-NC Mean longest diameter of nucleuses of normal cells
TC/NC-LNDR Tumor cell/normal cell longest nuclear diameter ratio

Table 2 Associations between morphometric measures and clinical variables

	MLND-NC	MLND-TC	TC/NC-LNDR
Sex	z = −0.086, p = 0.931	z = −1.232, p = 0.218	z = −0.378, p = 0.705
Age	r = 0.129, p = 0.371	r = −0.022, p = 0.879	r = −0.113, p = 0.434
Macroscopic tumor size	r = −0.189, p = 0.188	r = −0.264, p = 0.063[c]	r = 0.261, p = 0.067[e]
Histological subtype	χ2 = 5.232, p = 0.264	χ2 = 4.317, p = 0.364	χ2 = 2.606, p = 0.625
Number of tumor focus	z = −3.214, p = 0.001[a]	z = −0.183, p = 0.854	z = −2.784, p = 0.005[d]
Capsule invasion	z = −0.689, p = 0.490	z = −2.561, p = 0.008[b]	z = −1.789, p = 0.073[e]
Lymphovascular invasion	z = −1.721, p = 0.085	z = −1.687, p = 0.091[c]	z = −2.516, p = 0.012[d]
Perineural invasion	z = 0.032, p = 0.974	z = −1.364, p = 0.172	z = −0.572, p = 0.567
HBME-1 staining	z = −1.611, p = 0.107	z = −1.589, p = 0.114	z = −1.844, p = 0.065[e]
Galectin-3 staining	z = −0.566, p = 0.571	z = −0.716, p = 0.473	z = −0.096, p = 0.923
CK-19 staining	z = −0.479, p = 0.631	z = −1.034, p = 0.301	z = −0.213, p = 0.831

MLND-TC Mean longest diameter of nucleuses of tumor cells

MLND-NC Mean longest diameter of nucleuses of normal cells

TC/NC-LNDR Tumor cell/normal cell longest nuclear diameter ratio

[a] MLND-NC was smaller in cases with multiple tumor foci

[b] MLND-TC was higher in cases with capsule invasion

[c] MLND-TC was correlated with marginal insignificance with tumor macroscopic size and lymphovascular invasion, which may be explained by our small sample size

[d] MLND-TC/NC was higher in cases with multiple tumor foci and lymphovascular invasion

[e] MLND-TC was higher with marginal statistical significance in cases with greater macroscopic tumor size, capsule invasion, and positive HBME-1 staining

1 unit increase in TC/NC-LNDR increased risk for poor prognosis by 15.41 times (RR = 15.41; 95% CI = 3.03-8235; p = 0.001).

Univariate analyses revealed that lymphovascular invasion was associated with macroscopic tumor size and TC/NC-LNDR, capsule invasion was associated with MLND-TC, and number of tumor focus was associated with TC/NC-LNDR. These associations were also evaluated by ROC analyses to determine an optimal cut-off value of the variables. Accordingly, when a cut-off value of 1.15 cm was used for macroscopic tumor size for estimating lymphovascular invasion, AUC was 0.754 (95% CI = 0.60-0.90; p = 0.032). A cut-off value of 10.48 μm of MLND-TC for estimating capsule invasion revealed an AUC of 0.734 (95% CI = 0.61-0.89; p = 0.012). When a cut-off value of 2.07 was used for TC/NC-LNDR to predict lymphovascular invasion, AUC was found to be 0.807 (95% CI = 0.69-0.91; p = 0.012). The sensitivity, specificity, and positive and negative predictive values of relevant analyses were presented in Table 4.

Table 3 Logistic regression analyzes for estimating the risks of prognostic factors

	RR	95% CI	p
Capsule Invasion			
Histological subtype	3.19	0.46-21.95	0.238
Galectin-3 Staining	13.38	1.15-155.47	0.038
MLND-TC	4.16	1.24-16.21	0.031
Lymphovascular Invasion			
MLND-TC	8.64	0.51-121.52	0.213
TC/NC-LNDR	116.19	0.77-18,213.64	0.085
Macroscopic tumor size	3.36	1.09-10.36	0.034
Perineural Invasion			
HBME-1 Staining	111.45	1.62-7645.06	0.029
Macroscopic tumor size	3.36	1.09-10.36	0.179

Discussion

Pathology is a morphological science that based on disease classification, but importance of quantitative morphometric analyze is also known for many years [3]. Detailed cytological and histological evaluations for increasing the reliability of grading compromises subjectivity between pathologists, and moreover, addition of more sophisticated, versatile and semi-quantitative methods cannot decrease this subjectivity adequately. For this reason, shifting from subjective and qualitative methods to as far as possibly objective and quantitative techniques will increase repeatability and improve the reliability between evaluators [19]. On the other hand, despite the rapid advances in molecular basis of cancers, pathologists should primarily conduct morphological and morphometric evaluations, and should focus on nuclear size and variation of this measure in histological evaluations, which reflect the nuclear anaplasia.

Table 4 Results of ROC analyses

	Cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Macroscopic tumor size (cm) for estimating lympovascular invasion	1.15	1.15	51	25	100
MLND-TC (μm) for estimating capsule invasion	10.48	76	66	54	84
TC/NC-LNDR for estimating lymphovascular invasion	2.07	86	74	35	96
TC/NC-LNDR for estimating number of tumor focus	1.81	76	64	59	78

PPV Positive predictive value; NPV Negative predictive value

Under the nuclear morphometry heading nuclear diameter, circumference and area are conventional morphometric measures, which have been used for estimating average nuclear volume in many studies. Currently, various evaluation procedures and techniques have been developed by the aid of digital technologies; such as automatized or semi-automatized imaging analyses to estimate the most accurate three dimensioned volumes. Moreover, manual stereological estimation methods, which are being used for deriving three-dimensional from two-dimensional measurements, are applicable in daily practice [19]. Increasing number of reports is showing that nuclear volume that trying to be achieved by all these parameters is associated with poor differentiation.

PTC is the most frequent endocrine organ malignancy with an increasing incidence [17]. Despite its histopathological characteristics are well defined, there may be discrepancies even between experienced pathologists. One of the main causes of this conflict is presence of papillary structures and ground-glass appearance also in benign entities [7, 13]. The contribution of routinely used immunohistochemical staining is limited in some cases only, and as a consequence morphometry gains importance. In our study, tumorous and normal tissues were evaluated histologically by measuring MLND-NC, MLND-TC, and MLND- TC/NC ratio, which were described in methods. Because both normal and tumorous cell nucleuses of the same cases were compared, our study both provided quantitative measurements about differentiation, and also an opportunity for evaluating the associations between nuclear volume differentiation, other prognostic factors, and staining patterns. This study is the first one that evaluated all these variables at the same design.

MLND-TC values were significantly higher than MLND-NC, which already fits the basic pathology principles about anaplasia [6, 23]. In a study by Salmon et al. that includes papillary, follicular, and medullary thyroid cancer series; anaplastic thyroid tissues and normal thyroid tissues were compared, and nuclear area was found to be significantly higher in anaplastic cells when compared to normal cells [16]. In a more recent study by Kefeli and colleagues, cellular nuclear morphometric parameters were compared between benign lesions, follicular carcinoma, and papillary carcinoma; and majority of the measurements including nuclear diameter were found to be significantly higher in papillary carcinoma, despite follicular carcinoma, than benign lesions [5].

Primary hypothesis in our study was that there was an association between tumor-related prognostic factors, specific immunohistochemical staining patterns, and nucleus diameter that used for estimating the nuclear volume. When the relationships between MLND-NC and these variables were evaluated, cases with multiple tumorous foci, which is a prognostic factor, had lower MLND-NC values. When previous reports about multifocal PTC histopathology were reviewed in the literature, no morphometric data of non-tumorous normal cells were found. At this point, this is the first study that reports the correlation between number of tumor foci and nuclear diameters of normal cells.

MLND-TC was higher in case of capsule invasion. Ordinal logistic regression model for estimating the capsule invasion risk revealed that 1 μm increase in MLND-TC multiplied the risk 4.16 times. When a cut-off value of 10.48 μm was used in ROC analysis, negative predictive value below this cut-off was 84%. A previous study by Lee et al. that evaluated the effects of nuclear diameter of malignant cells on biological behaviors of thyroid carcinomas reported that nuclear diameter and capsule invasion was statistically significantly correlated [8]. The results of our study are compatible with these previous reports.

The TC/NC-LNDR was used for the first time in this study, and it was found to be significantly higher when the number of tumorous focus was multiple, and when the lymphovascularinvasion was positive. This was thought to be related with the negative association of MLND-NC with number of tumorous focuses. Also, it was higher with marginal significance when macroscopic size of tumor was high, capsule invasion was present, and HBME-1 staining was positive. Additionally, a ROC analysis for estimating lymphovascularinvasion usingTC/NC-LNDR with a cut-off value of 2.07 revealed that negative predictive value below this level was 96%. When the same analyze was performed for estimating number of tumor focus (single/multiple) with a cut-off value of 1.81 for TC/NC-LNDR, negative predictive value below this level was 78%. According to these results, the number of factors predicted by TC/NC-LNDR was higher than MLND-NC and MLND-TC. As a consequence,

utilization of TC/NC-LNDR in laboratory practices should be more beneficial.

HBME-1, Galectin-3 and CK-19 are in use for differentiation of PTC and follicular carcinoma [22]. Moreover, particularly HBME-1 is powerful in differentiation of PTC follicular variant from follicular adenoma and follicular carcinoma [12, 20]. There is no data in the literature about the differentiating properties of these staining methods in any PTC sub-variant. And these immunohistochemical methods had no distinct staining patterns in our study. Ordinal logistic regression analyses for estimating risks of capsule invasion and perineural invasion revealed that Galectin-3 negativity increased capsule invasion risk 13.38 times, and HBME-1 negativity increased perineural invasion risk 111.45 times. This was suggested that cases with poor differentiation might have staining loss. But, a previous study by Liu et al., which evaluated the PTC behavior reported that there was no association between Galectin-3 and capsule invasion [10]. Despite this study, Weber and colleagues reported in their study that Galectin-3 staining was stronger in cases with capsule invasion [21]. Another study by Seok et al. that evaluated prognostic value of HBME-1 in PTC reported that extrathyroidal involvement was associated with positive staining [18]. And finally, Mai et al. reported that decreased expression of HBME-1 was associated with PTC hurtle cell (oncocytic) variant, which has high invasion potential [11]. But in our study, two cases with negative HBME-1 staining were not oncocytic variant.

The 8 cases with negative staining patterns were negatively stained with only one method. These cases were regarded as PTC; because, remaining two stainings were positive and due to their histopathological characteristics. In these 8 cases, capsule invasion rate was higher. These cases were similar for the type of negative staining.

There was no correlation between the TC/NC-LNDR and the negative staining. Additionally, when the PTC sub-variants were classified as papillary/follicular/other, there was no association between these subtypes and negative staining pattern. In a previous study that evaluated the thyroid peroxidase with addition to these three staining in aggressive behavior in PTC, none of these staining methods were found to be associated with capsule invasion [10]. In another study, Dencic and colleagues evaluated the predictor value of CK-19 in PTC behavior, and they found that positivity of this staining is related with extrathyroidal dissemination [4]. In this point of view, our study had provided a third alternative to these data, and this is the first study that evaluated the negative staining cases as a separate group.

When another prognostic variable, the macroscopic tumor size was increased, the TC/NC- LNDR was also increased with a marginal statistical significance. When a cut-off value of 1.15 cm for macroscopic tumor size was used in ROC analysis to predict lymphovascular invasion, negative predictive value below this cut-off was 100%. There are several studies about the association of tumor diameter and nuclear diameter in the literature [8]. Also, associations between macroscopic tumor size and average nuclear area, and lymphatic invasion and lymphovascular invasion were shown by previous reports [9, 14]. Our results are also confirmative of this prevalent finding.

When the associations between TC/NC-LNDR and "number of poor prognostic factors", which includes indicators of poor prognosis (number of tumor focus, capsule invasion, lymphovascular invasion, and perineural invasion) were evaluated, a statistically significant correlation was found. According to the logistic regression analyses, every 1 unit of increase in TC/NC-LNDR values between 1.24 and 2.78 increased the "number of poor prognostic factors" by 10.3 times. There is no literature data about the association of number of poor prognostic factors with nuclear morphometric measurements. From this aspect, this is the first study that evaluated this association.

This study has some limitations such as the low number of samples to adequately reflect the histological sub-variants of PTC, and narrow confidence intervals for estimating the nuclear volume by morphometric measurements. But it has also some valuable outcomes such as raising the awareness about prognostic factors by MLND-NC, MLND-TC, and TC/NC-LNDR, which are easy to use in routine histology practice.

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