THE ASSOCIATION BETWEEN LYMPH NODE METASTASIS AND MOLECULAR MARKERS IN DIFFERENTIATED THYROID CANCER

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Abstract

Context. There is no consensus regarding routine usage and benefits of molecular markers for prediction of prognosis and assessment of risk groups in differentiated thyroid cancer (DTC).

Objective. We aimed to investigate NIS, Galectin-3, PTEN, P53 and Ki67 expressions in tumor tissue and metastatic lymph nodes in PTC and their association with lymph node metastasis and prognosis.

Material and Methods. Ninety two papillary thyroid cancer patients who underwent total thyroidectomy and central lymph node dissection were included in this study. NIS, Galectin-3, PTEN, P53 and Ki67 immunohistochemical stainings were performed for all surgical tumor tissues and metastatic lymph nodes of the 38 patients. Age, gender, tumor size, multifocality, capsular invasion, extrathyroidal extension and lymphocytic thyroiditis were assessed retrospectively.

Results. Seventy three females (79.3%) and nineteen males (20.7%) were included in this study. Risk of lymph node metastasis was higher in tumors with capsular invasion and extrathyroidal extension ($p=0.03$ and $p < 0.001$). NIS, PTEN and Galectin-3 protein expressions in tumor tissue were not associated with gender, tumor size, multifocality, extrathyroidal extension, capsular invasion, lymph node metastasis and tumor recurrence. Mean Ki 67 proliferation index was $2.08 \pm 0.95\%$. Ki 67 proliferation index was associated with tumor size ($p=0.012$). Intensity and expression of NIS and PTEN in tumor tissue were concordant with intensity and expression in metastatic lymph nodes ($p<0.001$). Ki 67 proliferation index in tumor was concordant with metastatic lymph nodes ($p=0.02$).

Conclusions. NIS, PTEN, Galectin-3, Ki67 and P53 expressions were not associated with the risk of lymph node metastasis in PTC patients. Routine analysis of these markers does not seem to be favorable. Further studies with new markers are necessary to determine prognostic predictors.

Key words: Differentiated thyroid cancer, NIS, PTEN, Galectin-3, Prognosis, Lymph node metastasis, Ki-67.

INTRODUCTION

Thyroid cancer is the most prevalent endocrinologic malignancy with approximately 1% frequency among all cancers (1). These tumors usually have excellent prognosis with more than 90% survival rate in ten years (2). However, tumor recurrence is reported in up to 20-40% of patients even after a successful initial treatment (3). Metastatic disease may be evident many years later after the onset of disease. Presence of lymph node metastasis is an independent prognostic factor for DTC (4, 5). Ten years survival rate decreases to 49-68% in node positive disease (6). Frequency of lymph node metastasis is 30-65% even in clinically node negative patients and routine prophylactic central lymph node dissection was recommended by certain studies to reduce the need for reoperation (7-10).

Recent studies demonstrated that differential diagnosis of benign and malignant thyroid nodules is possible by identification of molecular rearrangements in many proteins. Definition of molecules with diagnostic value raised questions about the potential markers associated with prognosis. Cytokeratin 19, Galectin-3, Ki67, HBME-1 and BRAF are the molecular markers which have been proved to be useful for preoperative diagnosis of thyroid cancer (11, 12). Previous studies demonstrated associations between Galectin-3, Ki-67, BRAF and prognostic factors just as tumor size, histological subtypes, invasive characteristics and metastasis of DTC (13-15).

Improvement in prognostic utility of molecular markers is expected by the use of molecular panels consisting reliable markers. For this purpose, Galectin-3, PTEN, P53 and Ki-67 proliferation index were investigated in DTCs. Augmentation of P53 protein or mutant P53 was shown to be associated with aggressive histologic variants and anaplastic

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transformation of DTCs (16). However, further studies failed to reveal a significant relationship between P53 expression and prognostic factors such as lymph node metastasis and extrathyroidal extension (17). High Ki67 proliferation index was suggested to be predictive for recurrence and shortened disease free survival in certain studies (18). Studies which evaluated the association between Galectin-3 expression and prognosis of DTC also demonstrated conflicting results (13, 19). Phosphatase and tensin homolog (PTEN) mutations and promoter methylation were reported in both familial and sporadic DTC cases (20). PTEN loss was suggested to be related with greater tumor size, aggressive histological subtypes and extrathyroidal extension (14). Additionally, sodium-iodide symporter expression was observed in metastatic lymph nodes of DTC (21). This finding was insufficient to suggest a relationship between NIS expression and risk of lymph node metastasis but suggested a favorable response to radioiodine ablation therapy in microscopic lymph node metastasis of NIS positive DTCs.

Due to conflicting results from previous studies, there is no consensus on the usefulness of these molecular markers for the prediction of prognosis in DTC. Identification of molecular markers associated with tumor prognosis may provide the objective risk categorization, decision of appropriate initial treatment approach, understanding of thyroid cancer pathogenesis and broaden treatment options by development of new targeted therapies.

We aimed to investigate the association between Ki67 proliferation index, NIS, Galectin-3, PTEN, P53 expressions, histopathological tumor characteristics, lymph node metastasis and recurrence risk in DTC.

MATERIALS AND METHODS

Patients

Papillary thyroid cancer patients who underwent total thyroidectomy and prophylactic central lymph node dissection in Ankara University Faculty of Medicine between 2006 and 2014, and followed up in Ankara University Endocrinology and Metabolism Clinic more than two years were included in this study. The study was approved by local Ethical Committee of Ankara University Faculty of Medicine (July 19, 2013, 46004091/302-14).

Demographical characteristics, histopathology reports and follow-up data of 92 patients were evaluated retrospectively. Eighty one classic variant and eleven

follicular variant PTCs were included in the study. Lymph node ratio, defined as the ratio of the number of positive lymph nodes to the total number of lymph nodes removed, was also assessed. Prophylactic central lymph node dissection was defined as resection of at least five central lymph nodes. Thirty eight patients with lymph node positive and fifty four patients with node negative disease whose surgical specimens were enough for immunohistochemical analysis were recruited to study. Patients with anti-thyroglobulin antibodies were excluded during patient selection for reliable definition of remission. Recurrence/persistence of diseases was defined as structural disease detected by imaging techniques (USG/PET-CT/RxWBS/CT) or/and TSH-stimulated serum Tg levels greater than 2 ng/mL. Informed consent forms were obtained from all patients.

Immunohistochemistry

Immunohistochemical analyses for NIS, PTEN, P53, Ki67 and Galectin-3 were performed in tumor tissues of 92 patients (Fig. 1). The tumor with the largest diameter was chosen for staining in multifocal tumors. Metastatic lymph nodes of 38 patients were also examined immunohistochemically (Fig. 2). The lymph nodes with the largest tumor tissue were evaluated. Galectin-3 (QC4, Neomarkers, 1:40 dilution), NIS (Monoclonal Antibody to SLC5A5, ACRIS, 1:600 dilution), PTEN (PTEN / MMAC1 Ab-4, Thermo Scientific, 1:40 dilution), Ki-67 (SP6, Cell Marque, 1:200 dilution) and P53 (DO7, Cell Marque, 1:100 dilution) antibodies were used for immunohistochemistry.

The most appropriate paraffin-embedded tissues for demonstration of histological characteristics of tumor were chosen and sliced into 4- μ m thick sections by microtome. These sections were stained through streptavidin-biotin-peroxidase method with Ventana automated immunostainer (BenchMark XT Staining Module, Ventana Medical Systems). Antigen retrieval consisted of CC1 (EDTA, pH:8) or CC2 (citrate, pH:6) solutions (Ventana Medical Systems). Hematoxylin and eosin was used as basic staining method and diaminobenzidine (DAB) was used as chromogen. Positive control tissues recommended by the suppliers of the antibodies were stained in all procedures.

All immunostainings were evaluated by two independent observers. Galectin-3 and PTEN were evaluated with cytoplasmic staining. Cytoplasmic and basolateral membrane staining was evaluated for NIS. Intensity of staining was classified as; 0: no staining,

Figure 1. Galectin-3, PTEN, P53 and NIS immunoexpression in a papillary thyroid carcinoma tumor tissue; A- Strong and intense Galectin-3 staining of tumor (x5); B- Strong and intense PTEN staining at same part of tumor (x5); C- Negative staining for P53(x5); D- Strong and intense NIS staining at same part of tumor (x5).

1: mild, 2: moderate and 3: strong positivity for these antibodies. Staining extensibility was classified as: 0: no, 1: <50% and 2:>50% staining. Nuclear staining with Ki-67 was regarded as positive and percentage was given. P53 nuclear staining was considered positive if nuclear staining was positive. Cytoplasmic staining of P53 was ignored.

Statistical Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) software, version 20.0 (IBM Corp, NY, and USA). Categorical data were compared using the chi-square Fisher exact test. Group data with a normal distribution were compared using the Student t test or analysis of variance, and nonparametric data were compared using the Mann-Whitney U or Kruskal-Wallis tests. Values were expressed as mean $\pm$ standard deviation or median as appropriate. Spearman correlation test was used for continuous variables without normal distribution. Pearson correlation test was used for continuous variables with normal distribution. Concordance of expressions between tumor tissue and metastatic lymph nodes was assessed with Cohen's kappa coefficient (95% confidence interval). $p < 0.05$ was considered statistically significant.

RESULTS

Seventy-three females (79.3%) and nineteen males (20.7%) were included in this study. The mean age at the diagnosis was 39.6 ± 13.8 years. Fifty-six patients (60.9%) were younger than 45 years old. Histopathological features of tumors are summarized

Figure 2. PTEN, Galectin-3, Ki-67 and NIS immunoexpression in a metastatic lymph node; A- PTEN staining of lymph node (x5); B- Galectin-3 staining of same lymph node (x5) ; C- Low (<1%) Ki67 labeling index (x5); D- NIS staining of lymph node (x5).

in Table 1. The mean number of foci was 2.9 ± 2.5 in multifocal tumors.

Lymph node metastasis was not associated with age, gender, tumor size, multifocality and the number of tumor foci. Frequency of lymph node metastasis was higher in tumors with capsular invasion and extrathyroidal extension ($p=0.03$ and $p < 0.001$, respectively). Association between lymph node metastasis, clinical and pathological characteristics are summarized in Table 2.

Histopathologically proven autoimmune thyroiditis was not associated with age, gender, extrathyroidal extension and multifocality ($p=0.07$, $p=0.63$ and $p=0.83$, respectively). Capsular invasion was negatively associated with autoimmune thyroiditis ($p=0.029$).

Recurrence or persistence of disease was diagnosed in 12 out of 92 patients. Mean follow-up period was 62.2 ± 21.2 months (median 30, 30-120 months). Presence of lymph node metastasis at diagnosis and extrathyroidal extension were positively associated with recurrent/persistent disease ($p=0.013$ and $p<0.01$, respectively). Autoimmune thyroiditis was inversely associated with recurrence/persistence of tumor. Capsular invasion, multifocality, tumor size, age, gender and lymph node ratio were not associated with recurrence. None of the molecular markers was associated with recurrence/persistence of tumor.

Three out of ninety two tumors were negative for Galectin-3 immunostaining. Galectin-3 expression was higher than 50% in 81 (88%) tumors and less than 50% in 8 (9%) of tumors (Table 3). Galectin expression was not associated with age, gender, tumor size, multifocality, extrathyroidal extension, capsular

Table 1. General characteristics of patients and histopathological features of tumors

Age at diagnosis	
Mean±SD	39.6±13.8
≥45, n (%)	36 (39.1%)
<45, n (%)	56 (60.9%)
Gender	
Female, n (%)	73 (79.3%)
Male, n (%)	19 (20.7%)
Lymph node metastasis	
Negative, n (%)	54 (58.7%)
Positive, n (%)	38 (41.3%)
Extrathyroidal extension	
Negative, n (%)	68 (73.9%)
Positive, n (%)	24 (26.1%)
Multifocality	
Negative, n (%)	48 (52.2%)
Positive, n (%)	44 (47.8%)
Capsular invasion	
Negative, n (%)	46 (50%)
Positive, n (%)	46 (50%)
Tumor size (maximum diameter)	
Mean±SD (mm)	15.3 ±11
≤ 10 mm, n (%)	38 (41.3%)
> 10 mm, n (%)	54 (58.7%)
Tumor stage	
I, n (%)	76 (82.6%)
II, n (%)	2 (2.2%)
III, n (%)	2 (2.2%)
IV, n (%)	12 (13%)
Risk groups*	
Low-risk patients	47 (51.1%)
Intermediate-risk patients	45 (48.9%)
Autoimmune thyroiditis	
Present, n (%)	40 (43.5%)
Absent, n (%)	52 (56.5%)
Tumor recurrence on follow-up	
Present, n (%)	12 (13%)
Absent, n (%)	80 (87%)

*Risk groups were defined according to three level risk stratification level for assessment of recurrence risk, recommended in "Revised American Thyroid Association Management Guidelines for Patients with Thyroid Nodules and Differentiated Thyroid Cancer, 2009"(64).

invasion, autoimmune thyroiditis (Table 3). Galectin-3 expression was diffuse (>50%) in all tumors (n=38) with lymph node metastasis and in 79.6% (n=43) of tumors without lymph node metastasis (p=0.04). Lymph node ratio or risk of recurrence were not associated with Galectin-3 staining. Galectin-3 staining intensity was mild in 8 (9%), moderate in 10 (11%) and strong in 71 (77%) tumors. Intensity of expression was not associated with age, gender, tumor size, multifocality, extrathyroidal extension, capsular invasion, autoimmune thyroiditis, tumor stage and recurrence of tumor.

PTEN staining was negative in 29 (32%) tumors. Frequency of PTEN negative tumors was 27.2% (n=22) in classic variant and 63.6% (n=7) in follicular variant PTCs (p=0.02). Fifty one (55%) tumors had

PTEN staining expression higher than 50% and twelve (13%) had PTEN staining less than 50% of tumor. PTEN expression and intensity were not associated with age, gender, tumor size, multifocality, extrathyroidal extension, capsular invasion, autoimmune thyroiditis, presence of lymph node metastasis, lymph node ratio or risk of recurrence (Table 3). Median lymph node ratio was 0.25 (0.1-1) in PTEN expression negative tumors and 0.43 (0.09-1) in PTEN expression positive tumors (p=0.094). When tumors were grouped as PTEN negative and positive, there was no difference between the two groups; regarding age, gender, tumor size, multifocality, extrathyroidal extension, capsular invasion, autoimmune thyroiditis, presence of lymph node metastasis, lymph node ratio or risk of recurrence.

NIS staining was negative in 28 (30.4%)

Table 2. Association between lymph node metastases, clinical and pathological characteristics

Clinical and histopathological characteristics	Lymph Node Metastasis		p
	Present	Absent	
Age at diagnosis			
Mean $\pm$ SD	37.7 $\pm$ 15.5	41.05 $\pm$ 12.4	0.13
$\geq$ 45 years, n(%)	15 (41.7%)	21 (58.3%)	0.9
<45 years, n(%)	23 (41.1%)	33 (58.9%)	
Gender			
Female, n(%)	27 (37%)	46 (63%)	0.09
Male, n(%)	11 (57.9%)	8 (42.1%)	
Extrathyroidal extension			
Negative, n(%)	21 (30.9%)	47 (69.1%)	<0.001
Positive, n(%)	17 (70.8%)	7 (29.2%)	
Multifocality			
Negative, n(%)	18 (37.5%)	30 (62.5%)	0.43
Positive, n(%)	20 (45.5%)	24 (54.5%)	
Capsular invasion			
Negative, n(%)	14 (30.4%)	32 (69.6%)	0.034
Positive, n(%)	24 (52.2%)	22 (47.8%)	
Tumor size (maximum diameter)			
Mean $\pm$ SD (mm)	17.6 $\pm$ 10.8	13.7 $\pm$ 10.9	0.09
$\leq$ 10 mm, n(%)	13 (34.2%)	25 (65.8%)	0.24
> 10 mm, n(%)	25 (46.3%)	29 (53.7%)	
Autoimmune thyroiditis			
Present, n(%)	13 (32.5)	27 (67.5%)	0.098
Absent, n(%)	25 (48.1%)	27 (51.9%)	

tumors. Fifty five (60%) tumors had NIS expression higher than 50% and nine (9.6%) had NIS staining less than 50% of tumor. NIS expression was frequent in tumors of patients older than 45 years when compared to younger patients (86.1% vs. 58.9%; $p=0.005$). Patients whose tumors had positive NIS expression were older (44.9 $\pm$ 14.3 yrs vs. 38.6 $\pm$ 11.8 yrs, $p=0.03$). Intensity or NIS expression was not associated with gender, tumor size, multifocality, extrathyroidal extension, capsular invasion, autoimmune thyroiditis, tumor stage, presence of lymph node metastases, lymph node ratio or recurrence of tumor (Table 3). Median lymph node ratio was 0.25 (0.1-0.87) in NIS expression negative group and 0.43 (0.09-1) in NIS expression positive group ($p=0.072$).

When tumors were grouped as NIS negative and positive, there was no difference between the two groups regarding gender, tumor size, multifocality, extrathyroidal extension, capsular invasion, autoimmune thyroiditis, presence of lymph node metastasis, lymph node ratio or risk of recurrence.

Mean Ki-67 (MIB1) labeling index was 2.08 $\pm$ 0.95 in the whole group. Tumor size was positively associated with Ki-67 labeling index ($p=0.012$). Tumor stage, lymph node metastasis, lymph node ratio and recurrence risk were not associated with Ki-67 index (Table 4).

All tumors and metastatic lymph nodes were

negative for P53 staining.

Galectin-3 expression and intensity in metastatic lymph nodes were not concordant with tumor tissue ($p=0.7$). There was no association between expression/intensity of Galectin-3 in lymph nodes and tumor recurrence ($p=0.87$ and $p=0.57$). PTEN expression and intensity in metastatic lymph nodes were concordant with tumor tissue (kappa coefficient, 0.51; $p<0.001$). Twenty-three tumors of patients with lymph node metastasis had diffuse (>50%) PTEN expression and 20 of them (86.9%) also had diffuse expression in metastatic lymph nodes. There was no association between expression/intensity of PTEN in lymph nodes and tumor recurrence ($p=0.65$ and $p=0.79$). NIS expression and intensity in metastatic lymph nodes were concordant with tumor tissue (kappa coefficient, 0.43; $p<0.001$). Twenty-five tumors had diffuse (>50%) NIS expression and 20 of them (80%) also had diffuse expression in metastatic lymph nodes. There was no association between expression/intensity of NIS in lymph nodes and tumor recurrence ($p=0.8$ and $p=0.91$). Ki-67 proliferation index in tumor was concordant with metastatic lymph nodes (2.18 $\pm$ 0.95 vs. 2.13 $\pm$ 0.57, $p=0.02$). Ki-67 proliferation index in metastatic lymph nodes was not significantly associated with recurrence of tumor ($p=0.57$). Lymph node ratio was not associated with Galectin-3 expression or intensity in lymph nodes ($p=0.88$ and $p=0.29$, respectively). Intensity of PTEN

Table 3. The associations between PTEN, Galectin-3 and NIS expressions, clinical and pathological features, lymph node metastasis and recurrence

Clinical and pathological features	PTEN Expression			P	Galectin-3 Expression			P	NIS Expression			P
	Negative	<%50	>%50		Negative	< %50	>%50		Negative	<%50	>%50	
Age at diagnosis												
Mean±SD	37.5±13.4	37±14.8	41.5±13.7	0.36 0.42	41.6±15	37.3±7.2	39.8±14.3	0.86 0.24	35.1±12	45.2±14.4	41±14	0.4 0.01
≥45, n(%)	9 (25.0%)	4 (11.1%)	23 (63.9%)		1 (2.8%)	1 (2.8%)	34 (94.4%)		5 (13.9%)	6 (16.7%)	25 (69.4%)	
<45, n(%)	20(35.7%)	8 (14.3%)	28(50%)		2 (3.6%)	7 (12.5%)	47 (83.9%)		23(41.0%)	3 (5.4%)	30(53.6%)	
Gender												
Female, n(%)	21(28.8%)	9 (12.3%)	43 (58.9%)	0.41	3 (4.1%)	5 (6.9%)	65 (89%)	0.42	20(27.4%)	6 (8.2%)	47 (64.4%)	0.2
Male, n(%)	8 (42.1%)	3 (15.8%)	8 (42.1%)		0 (0%)	3 (15.8%)	16 (84.2%)		8 (42.1%)	3 (15.8%)	8 (42.1%)	
Extrathyroidal extansion												
Negative, n(%)	23(33.8%)	6 (8.8%)	39 (57.4%)	0.12	3 (4.4%)	6 (8.8%)	59 (86.8%)	0.85	20(29.4%)	7 (10.3%)	41 (60.3%)	0.93
Positive, n(%)	6 (25.0%)	6 (25.0%)	12 (50.0%)		0 (0%)	2 (8.3%)	22 (91.7%)		8 (33.2%)	2 (8.3%)	14 (58.3%)	
Multifocality												
Negative, n(%)	15(31.3%)	6 (12.5%)	27 (56.2%)	0.74	2 (4.2%)	4 (8.3%)	42 (87.5%)	0.98	15(31.3%)	4 (8.3%)	29 (60.4%)	0.62
Positive, n(%)	13(30.2%)	6 (14.0%)	24 (55.8%)		1 (2.2%)	4 (9.3%)	38 (88.5%)		12(27.9%)	5 (11.6%)	26 (60.5%)	
Capsular invasion												
Negative, n(%)	16(34.8%)	6 (13.0%)	24(52.2%)	0.83	2 (4.3%)	2 (4.3%)	42 (91.3%)	0.44	14(30.4%)	6 (13.0%)	26(56.6%)	0.61
Positive, n(%)	13(28.3%)	6 (13.0%)	27 (58.7%)		1 (2.2%)	6 (13%)	39 (84.8%)		14(30.5%)	3 (6.5%)	29(63.0%)	
Risk Groups												
Low-risk	17(36.2%)	5 (10.6%)	25 (53.2%)	0.88	3 (6.4%)	6 (12.8%)	38 (80.8%)	0.08	16(34.0%)	5 (10.6%)	26 (55.4%)	0.66
Intermediate-risk	12 (26.6%)	7 (15.6%)	26 (57.8%)		0 (0%)	2 (4.4%)	43 (95.6%)		12(26.7%)	4 (8.9%)	29 (64.4%)	
Tumor size												
Mean±SD (mm)	14.9±10.6	15.7±11.8	15.5±11.2	0.96 0.88	17.6±13.6	16.7±6.7	15.1±11.3	0.87 0.58	15.8±12.2	11.4±5.5	15.8±11	0.96 0.73
≤ 10 mm, n(%)	13 (34.2%)	5 (13.2%)	20 (52.6%)		1 (2.6%)	2 (5.3%)	35 (92.1%)		13(34.2%)	4 (10.5%)	21 (55.3%)	
> 10 mm, n(%)	16 (29.6%)	7 (13.0%)	31 (57.4%)		2 (3.7%)	6 (11.1%)	46 (85.2%)		15(27.8%)	5 (9.2%)	34 (63.0%)	
Autoimmune thyroiditis												
Absent, n(%)	19(36.5%)	6 (11.5%)	27 (51%)	0.49	1 (1.9%)	4 (7.7%)	47 (90.4%)	0.68	17(32.7%)	5 (9.6%)	30 (57.7%)	0.86
Present, n(%)	10 (25.0%)	6 (%15.0)	24 (%60.0)		2 (5.0%)	4 (10.0%)	34 (85.0%)		11(27.5%)	4 (10.0%)	25 (62.5%)	
Lymph node metastasis												
Absent, n(%)	18(33.3%)	8 (14.8%)	28 (51.9%)	0.68	3 (5.6%)	8 (14.8%)	43 (79.6%)	0.04	18(%33.3)	6 (%11.1)	30 (%55.6)	0.61
Present, n(%)	11(28.9%)	4 (10.5%)	23 (60.6%)		0 (0%)	0 (0%)	38 (100%)		10(%26.3)	3 (%7.9)	25 (%65.8)	
Tumor recurrence during follow-up												
Present, n(%)	5 (41.7%)	3 (25.0%)	4 (33.3%)	0.17	0 (0%)	0 (0%)	12 (100%)	0.7	5 (41.7%)	2 (16.6%)	5 (41.7%)	0.31
Absent, n(%)	24 (30.0%)	9 (11.3%)	47 (58.7%)		3 (3.8%)	8 (10.0%)	69 (86.2%)		23 (28.8%)	7 (8.8%)	50 (62.4%)	

expression in lymph nodes was moderately associated with lymph node ratio ($p=0.03$, $r=0.42$). Expression and intensity of NIS expression in lymph nodes had an association with lymph node ratio ($p=0.27$, $r=0.43$ and $p=0.01$, $r=0.48$). Ki-67 labeling index in lymph nodes was not related with lymph node ratio ($p=0.78$).

A subgroup consisting of T1 tumors (tumor size ≤ 2 cm, $n=57$) was evaluated separately. In this subgroup, expression of molecular markers were not associated with lymph node metastasis, recurrence/persistence risk or lymph node ratio. NIS expression was not associated with capsular invasion, multifocality and

autoimmune thyroiditis ($p=0.79$, $p=0.21$ and $p=0.88$, respectively). PTEN expression was not associated with capsular invasion, multifocality and autoimmune thyroiditis ($p=0.45$, $p=0.44$ and $p=0.4$, respectively).

DISCUSSION

Management of differentiated thyroid cancer has been guided by postoperative risk scoring systems (22-24). However, recent studies focused on the molecular markers which have been proved to be useful for distinction of malignant from benign thyroid

Table 4. The association between Ki-67 proliferation index, clinical and pathological features, lymph node metastasis and recurrence

Clinical and pathological features	Ki-67 proliferation index (mean±SD)	P
≥45, <45,	1.91±0.84 2.19±1.08	0.2
Gender		
Female	2.09±0.91	0.64
Male	2.05±1.12	
Extrathyroidal extension		
Negative	2.05±0.92	0.7
Positive	2.16±1.04	
Multifocality		
Negative	2.2±1	0.8
Positive	1.9±0.89	
Capsular invasion		
Negative	1.9±0.8	0.7
Positive	2.1±1.08	
Tumor size		
≤ 10 mm	1.78±0.84	0.01
> 10 mm	2.2±0.98	
AITD		
Absent	2.01±1.03	0.24
Present	2.17±0.84	
Risk Groups		
Low-risk	2.1±1.0	0.84
Intermediate-risk	2.06±0.9	
LNM		
Absent	2.09±1.06	0.65
Present	2.07±0.78	
Recurrence during follow-up		
Present	1.58±0.79	0.06
Absent	2.16±0.96	

tumors and suggested that molecular markers may also be a part of risk stratification systems.

Galectin-3 is a β -galactoside-binding protein with critical roles in cell growth, proliferation, cell to cell and matrix adhesion, transformation, tumorigenesis and angiogenesis (25-28). This marker was also shown to be related with tumor progression and chemotherapy sensitivity in certain types of cancer, suggesting a molecular target to prevent metastasis (27, 29-32). Galectin-3 expression has been accepted as a diagnostic marker for malignant thyroid lesions (33, 34). Consistent with our study, previous studies demonstrated that Galectin-3 was expressed in more than 90% of classic variant of PTCs (34-36). However, effect of Galectin-3 expression alterations in histopathological features, lymph node metastasis and prognosis of DTCs remained controversial. Kawachi *et al.* showed increased Galectin-3 expression in PTCs, higher expression in primary tumors with lymph node metastasis and lower expression in metastatic nodes

(13). Authors concluded that decreased Galectin-3 expression in later stages of disease may promote metastasis by loosening the connection of tumor cells with extracellular matrix. Türköz *et al.* reported that Galectin-3 expression was decreased in PTCs with lymph node metastasis, invasive edges of tumors and in metastatic nodes (19). On the contrary, a recent study revealed high expression of Galectin-3 in DTCs with lymph node metastasis compared to non-metastatic (37). However researchers did not observe any difference between Galectin-3 expression in primary tumors and metastatic nodes. Another study of 142 DTCs demonstrated increased expression of Galectin-3 in peripheral cells of tumors and confirmed the previous studies suggesting a relation between higher expressions of Galectin-3 with tumor aggressiveness (38). In our study, we observed high Galectin-3 expression in all primary tumors with lymph node metastasis (n=38, p=0.04) and in 20/38 of lymph nodes, whereas seven metastatic nodes were negative for Galectin-3 immunostaining. Our results were consistent with previous data suggesting an association between Galectin-3 expression in primary tumors and lymph node metastasis in PTC, whereas no specific Galectin-3 expression was observed in metastatic nodes. On the other hand, 76% of tumors without lymph node metastasis had diffuse Galectin-3 expression, supporting the role of this molecule in tumorigenesis. Consistent with previous data, we did not find any relation between Galectin-3 expression/intensity and clinicopathologic features except for its diagnostic value in DTCs (17, 39, 40).

Phosphatase and tensin homolog (PTEN) is a tumor suppressor gene and a negative regulator of the PI3K (Phosphatidylinositol-4,5-bisphosphate 3-kinase) pathway. Molecular alterations of PTEN are found in many types of cancer including those of breast, endometrium, and prostate (41-43). Cowden syndrome is an autosomal dominant familial cancer syndrome caused by germline PTEN mutations and DTC is a part of Cowden syndrome. PTEN mutation (loss of heterozygosity or deletion) was also reported in sporadic DTCs (44, 45). Nuclear PTEN immunostaining was shown to be weak in DTCs when compared to normal thyroid follicular cells and follicular adenomas (46). In mouse models, thyroid gland proliferation due to activation of mechanistic target of rapamycin (mTOR) by PI3K was demonstrated. Also, the activation of PI3K caused by PTEN loss was reported to result with inhibition of tricarboxylic acid cycle (TCA) and activation of compensatory glycolysis independent

of defective cell proliferation (47). A recent study demonstrated that fibroblast migration and proliferation were induced by PTEN loss and these changes in extracellular matrix caused the progression of follicular thyroid carcinomas. Progression of follicular thyroid carcinoma, increased frequency of lung metastasis and decreased survival were reported in PTEN deficient mouse models (48). However, a Korean study showed that PTEN loss among PTCs was more frequent than previously reported (more than 50% of PTCs) and was associated with both lymph node metastasis and extrathyroidal extension regardless of histological subtype (14). Their results also revealed that frequency of PTEN loss was similar between follicular and classic variant. A study of 63 metastatic and 50 non-metastatic thyroid tumors evaluated the predictive values of 16 molecular markers for metastasis. PTEN and P53 protein expressions were not found to be useful for prediction of metastasis (49). Ates *et al.* showed that PTEN expression was not associated with capsular invasion, lymphovascular invasion, and risk of recurrence whereas PTEN loss was associated with multifocality in both FTCs and PTCs (50). In our study, loss of PTEN expression was demonstrated in 32% of PTCs. The frequency of PTEN loss was 63.6% among follicular and 27.2% in classic variant of PTCs. Our results were concordant with Liang *et al.*, as PTEN expression was not associated with any of the prognostic features including lymph node metastasis and risk of recurrence. Also, consistent with the results of Jung *et al.*, prevalence of PTEN loss was relatively high in our series of PTCs, supporting the previous studies suggesting that the PI3K/Akt pathway was also altered in PTC (20).

Sodium iodide symporter (NIS) is an internal plasma membrane glycoprotein which is mostly expressed in thyroid follicular cells and mediates active transport of iodine. An association between BRAFV600E and inhibition of NIS expression was demonstrated previously. Romei *et al.* observed that BRAF V600E caused low expression of both NIS and thyroid peroxidase (51). Loss of NIS expression by up-regulation of DNA methyltransferase 1 via BRAFV600E mutation was suggested as a possible mechanism of radioiodine resistance in PTC (52). However, we observed that 60% (n=55) of patients which experienced recurrence on follow-up had diffuse NIS immunoexpression. Previously, it was suggested that low NIS expression was associated with recurrence risk in pediatric PTC patients (53). Morari *et al.* suggested that NIS expression was lower in tumors of

patients older than 45 years but it was not a favorable marker of prognosis (54). On the other hand, Cordioli *et al.* showed that NIS expression intensity was not different between children and adults (55). Authors concluded that investigation of other genetic factors, which may have a role in various course of disease in different age groups, should be warranted as they could not demonstrate an association of thyroid-specific genes with age groups. A meta-analysis of nine studies demonstrated that NIS expression was increased in DTC compared to normal thyroid tissue. Regarding these data, concerns raised about the role of NIS expression in the development of radioiodine resistant DTC (56). Our data demonstrated that NIS expression or intensity was not associated with risk of lymph node metastasis and recurrence but it was positively associated with age ($p=0.03$). NIS immunoexpression negative tumors were observed in 13.9% of patients older than 45 years and in 41.1% of younger patients ($p<0.01$). Recently, Bastos *et al.* revealed that NIS expression was significantly lower in PTCs greater than 10 mm and in BRAFV600E positive tumors (57). Contrary to their findings, we observed that NIS expression distribution was not different between <10 mm and ≥ 10 mm tumor groups. We also found that NIS expression in tumor tissue was concordant with lymph nodes as 25 primary tumors with metastasis had diffuse NIS immunoexpression and 80% (n:20) of them also had diffuse expression in metastatic nodes. These data were inconsistent with previous reports which suggested that NIS expression decreased during metastatic cell dedifferentiation (58). A study by So. *et al.* revealed similar results with our study. NIS expression was frequent in both primary tumors and metastatic LNs in micro-PTCs (21). This result suggests that subclinical metastatic nodes may be sensitive to radioiodine ablation therapy.

Ki-67 (MIB-1) proliferation index is a marker of cell proliferation which was suggested as a reliable marker for prognostic classification of thyroid cancer (15). Müssig *et al.* showed that Ki-67 proliferation index was associated with tumor staging and clinical outcome but also pointed out that a modest increase was not useful to predict aggressive disease (18). Recently, Zhou *et al.* reported that Ki-67 proliferation index was significantly associated with tumor size and lymph node metastasis in thyroid microcarcinomas (59). We did not observe a relationship between Ki-67 proliferation index and prognostic factors with the exception of tumor size. However Ki-67 indices of tumors included in our study were relatively low (1-5%) and our results may be affected by this factor.

Point mutation in P53 tumor suppressor gene is one of the most frequent genetic alterations which predispose to malignant tumors. P53 mutation is often associated with anaplastic thyroid carcinoma but rarely it may be detected in DTCs (60, 61). Previously, p53 was suggested as a diagnostic marker of aggressive thyroid carcinoma. Balta *et al.* reported that P53 mutation was associated with extrathyroidal extension and LNM (62). Another report by Marcello *et al.* suggested that P53 expression was associated with better prognostic factors and authors concluded that results of Balta *et al.* could be influenced by patient selection as dominantly high risk and male patients were included (63). In our study none of the primary tumors or metastatic nodes had positive P53 staining although 47 low-risk, 45 intermediate-risk and predominantly female (79.3%) patients were included.

The major limitation of this study was the limited number of the tumors.

In conclusion, NIS, PTEN, Galectin-3, Ki67 and P53 expressions were not predictive of lymph node metastasis or risk of recurrence in PTC. Expression of NIS with high amounts in metastatic lymph nodes may be suggestive of response to radioiodine ablation for micrometastasis. Routine analysis of these markers does not seem to be favorable. Further studies with new markers are necessary to determine prognostic predictors and risk scoring systems.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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