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miRNA expression analysis and prognostic significance in thyroid carcinomas

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Romena Laukienė

Skydliaukės karcinomų miRNR analizė ir prognostinė vertė

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ABBREVIATIONS

AUC – area under curve
BTN – benign thyroid nodules
CCLN – central cervical lymph nodes
CI – confidence interval
CLN – cervical lymph nodes
CLNM – cervical lymph nodes metastases
CT – computed tomography
Ct – cycle threshold
DTC – differentiated thyroid carcinoma
Extr.Ex. – extrathyroidal extension
FNAB – fine needle aspiration biopsy
FTC – follicular thyroid carcinoma
HT – Hashimoto thyroiditis
IQR – interquartile range
LCLN – lateral cervical lymph nodes
LNM – lymph nodes metastases
MF – multifocality
miRNA – micro ribonucleic acid
MRI – magnetic resonance imaging
MTS – metastases
PTC – papillary thyroid carcinoma
ROC – receiver operating characteristic
RT-PCR – real-time reverse transcription polymerase chain reaction
SD – standard deviation
TC – thyroid carcinoma

1. INTRODUCTION

Thyroid carcinoma (TC) is the most common endocrine malignancy [1]. Two histological subtypes of differentiated thyroid carcinoma (DTC), papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC), arise from follicular thyroid epithelial cells and account for about 94% of all thyroid carcinomas [2]. The incidence of TC has increased dramatically worldwide over the last 20 years and it is likely that by 2030, TC will be the fourth most common form of malignancy [3]. The increase in the incidence of TC is exclusively driven by the increase in the number of cases of PTC [4][5]. Advances in ultrasound imaging are improving the early detection of thyroid carcinoma, but the possibility of overdiagnosis is also emerging, which leads to unnecessary, aggressive surgery and associated postoperative complications, which reduce the quality of life of patients [6]. International professional organizations, including the European Thyroid Association [7], the American Thyroid Association [8] and the American College of Radiology [9], have developed risk stratification criteria for thyroid nodules to guide the ultrasonographic investigation and management of thyroid nodules [10]. Ultrasonography and fine-needle aspiration biopsy (FNAB) are the "gold standard" for differentiating benign from malignant thyroid nodules. The results of the aspiration biopsy are reported according to the Bethesda system of thyroid cytopathology, which has a high diagnostic specificity [11]. Nevertheless, 10-20% of thyroid nodules remain indeterminate after cytological evaluation [12]. This results in a relatively high number of diagnostic operations.

Although the incidence of PTC is steadily rising, the mortality rate from thyroid carcinoma has changed only minimally over the last five decades [13]. The prognosis of PTC is generally good, with a relatively low mortality rate, but a small proportion of patients develop an aggressive form of the disease, with tumor invasion of the surrounding tissues and metastasis [14]. Metastases (MTS) to the

cervical lymph nodes (CLN) are often observed early in the disease. Usually, PTC metastasizes to the central cervical lymph nodes (CCLN) and less frequently to the lateral cervical lymph nodes (LCLN). Cervical lymph nodes metastases (CLNM) lead to a higher incidence of local recurrence of TC, which is an indicator of a worse prognosis [15][16][17]. Neither ultrasonography of the CLN nor multi-slice computed tomography (CT) or magnetic resonance imaging (MRI) can fully detect the spread of the neoplastic process before surgery [18]. Clear structural and size changes in the lymph nodes usually do not cause diagnostic difficulties. The situation is more complicated with small CLNM up to 1 cm in size. Often, the lack of specific features, even with precise assessment of the short axis of the CLN, its structure, borders, the nature of contrast agent accumulation and the adjacent tissue structures, even with precise CT and MRI, does not allow an accurate evaluation of small CLNM. This hampers the accurate staging of the disease before surgery.

A meta-analysis by Chinese investigators showed that the specificity of ultrasound for the diagnosis of CCLN metastases was 93%, while the sensitivity was only 33%, and the specificity and sensitivity for the diagnosis of LCLN metastases was 84% and 70% respectively [19]. The most challenging diagnostic problem is the verification of retropharyngeal and mediastinal CLNM. Up to 60% of CLNM that were not detected by imaging before surgery are diagnosed histologically [20].

The American Thyroid Association's International Guidelines [21] recommend the removal of CCLN in patients with preoperative abnormalities in the lymph nodes of the neck. However, the value of prophylactic surgery for CCLN removal is controversial [8]. Although prophylactic CCLN lymphadenectomy reduces the risk of local recurrence of PTC, it increases the likelihood of postoperative complications such as transient or permanent laryngeal recurrent nerve injury, transient or permanent hypocalcaemia [22].

Therefore, it is very important to identify the spread of the oncological process before surgery, which will allow the selection of an optimal and individualized treatment for PTC, leading to a lower risk of thyroid carcinoma recurrence. Since available imaging studies do not accurately diagnose the spread of PTC, other clinicopathological risk factors are being sought. Meta-analyses have shown that male sex, young age (<45 years), size of the primary tumor, multifocality and extension beyond the thyroid gland could be independent risk factors for metastasis to the cervical lymph nodes [23][24]. However, even this is not sufficient, the search for new biomarkers to differentiate non-malignant thyroid nodules from malignant ones is important, as well as to identify disease progression and the appropriate choice of treatment.

Micro ribonucleic acids (miRNAs) are non-coding nucleic acid molecules that regulate gene translation or product degradation and are involved in a variety of processes that regulate cellular functions. Research has identified changes in miRNA expression in the pathogenesis of various diseases, including cancer. Therefore, it is important to identify the role of miRNA in the differential diagnosis of thyroid carcinoma and in the detection of metastases in the CLN.

Aim of the study

To assess the role of miRNA expression in the diagnosis and prognosis of papillary thyroid carcinoma.

Main tasks of the study

- To determine the expression of miRNAs in fine needle aspirates of thyroid nodules with suspected PTC.
- To compare miRNA expression in malignant and benign thyroid nodule cells.

- To evaluate the association of miRNA expression with clinicopathological features of PTC.
- To compare miRNA expression in thyroid nodule aspirates in PTC patients with and without CLN metastases.

Relevance and novelty of the study

In clinical practice, doctors face the challenge of balancing the treatment of papillary thyroid carcinoma in a way that does not overly aggressively treat patients at lower risk of thyroid carcinoma. At the same time, they need to identify patients with more advanced disease, who are at high risk of recurrence and who require a more aggressive treatment approach. Therefore, it is important to look for new biomarkers to help diagnose thyroid carcinoma more accurately and to assess the spread of the disease before surgery. There are studies showing that some miRNAs may be potential biomarkers to predict tumor metastasis, e.g. miR21 in breast carcinoma [25]; miR21 and miR31 in colorectal carcinoma [26]; and miR1207-5p in gastric carcinoma [27]. miRNAs in relation to metastasis of PTC to the cervical lymph node appeared in a publication in 2010 [28]. Although there is a lot of work examining the application of different miRNAs in the diagnosis, progression and risk of recurrence of PTC, the data published so far are conflicting. Most of the studies have been conducted in China. In Lithuania, the number of papers investigating the association of miRNAs with PTC is limited [29][30][31].
Analysis of miRNA expression in fine-needle aspiration specimens of thyroid nodules may improve the preoperative evaluation of patients, which would allow selection of appropriate treatment tactics and operative decision-making. This would reduce post-operative complications and improve the quality of life of patients.

Statements to be defended

- The expression of miRNA in thyroid fine-needle aspirates differs significantly between malignant and benign thyroid nodules.
- miRNA expression in thyroid fine-needle aspirates is significantly different between papillary thyroid carcinomas with different pathological features.
- miRNA expression in thyroid papillary carcinoma fine-needle aspirates is significantly different between subjects diagnosed with metastases to the cervical lymph nodes and those without metastases to the cervical lymph nodes.

2. MATERIALS AND METHODS

Patients

The study was approved by Vilnius Regional Bioethics Committee (Lithuania, approval No. 158200-17-905-414; 2017-03-17). All samples were collected upon obtaining the written informed consent from patients and the prior approval for this study as per the Declaration of Helsinki. From May 2017 to April 2021, all patients who underwent a diagnostic FNAB of a thyroid nodule at Vilnius University Hospital "Santaros klinikos" and were suspected of having thyroid carcinoma (Bethesda cytology grades III-VI) were offered the opportunity to participate in the study. A total of 160 patients were enrolled and subsequently underwent thyroid surgery. Of these, 28 (17.5%) were male and 132 (82.5%) were female; 34 (21.25%) patients underwent thyroid resection (lobectomy or lobisthmectomy) and 126 (78.75%) underwent thyroidectomy. In 59 (36.88%) patients the neck lymph nodes were not removed, 79 (49.38%) underwent prophylactic CCLN lymphadenectomy, 22 (13.75%) underwent therapeutic CLN lymphadenectomy of which 18 (81.8%) underwent CCLN lymphadenectomy, and 4 (18.2%) underwent both CCLN and LCLN lymphadenectomy. 48 (30.0%) patients had histologically confirmed benign thyroid nodules and 112 (70.0%) patients had PTC. The first 60 patients formed the screening cohort and were tested for miRNA expression by next-generation sequencing. These patients were divided into three groups according to the post-operative final histological result: 12 patients with benign thyroid nodules (BTN), 33 patients with malignant nodules without CLNM, 15 patients with malignant nodules with CLNM.

Based on the results of the sequencing, the three miRNAs that were most divergent between the groups – miR4324, miR200B, miR125A – were selected for further analysis. Following the literature review, two more miRNAs, miR146B, miR221, which have been mainly

described for differentiating both PTC with benign thyroid disease and more aggressive forms of PTC, were selected for further investigation. Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed to investigate the expression of selected miRNAs in 100 patients from the validation cohort. These patients were also divided into analogous groups according to the final histological result: 36 BTN, 33 PTC without CLNM and 30 PTC with CLNM.

Fine needle aspiration biopsy

The material for the cytological examination was obtained by percutaneous puncture of the thyroid nodule with fine sterile needles. The procedure was performed under ultrasound guidance. The ultrasound transducer was placed on the neck, in the projection of the thyroid gland, and an image of the nodule to be punctured was displayed on the screen. The transducer was held with one hand and a syringe with a needle in the other free hand, and the needle was inserted perpendicularly to the ultrasound beam at a short distance from the transducer. Following the tip of the needle, the needle was introduced into the desired location and the punctures were made in a sudden pendulum motion. The aspirate was obtained by creating a vacuum in the syringe and the plunger was released 2 to 3 seconds after the start of aspiration. The nodes were punctured 2-3 times during the same procedure to obtain material suitable for the test. Part of the thyroid tissue material obtained during FNAB was injected into a labelled vial containing liquid medium. The remainder of the aspirated material is spread on a marked slide. The swabs were fixed dry (air-dried).

Cytological examination of thyroid nodule aspirate

Cytological examinations of thyroid nodule aspirates were performed at the Vilnius University hospital “Santaros klinikos” Centre of Laboratory Medicine, Laboratory of Hematology and General

Cytology, according to the standardized diagnostic procedure. All aspirates of FNAB samples were evaluated on Papanicolaou stained smears and on smears prepared by the automated BD Prep Stain system. FNAB results were classified as diagnostic (sufficient) and non-diagnostic (insufficient). A sample is considered "diagnostic" when it contains at least six well-preserved groups of thyroid epithelial cells of at least 10 cells each, or when abundant colloid is found. The cytological diagnosis was divided into 6 categories using the Bethesda system [11].

Surgical treatment

The operating surgeon was not informed about the patient's participation in the biomedical research, which did not influence the decision on the indications for surgery or the extent of the surgery itself. Patients underwent surgery to remove a single lobe with an intermediate part or the entire thyroid gland. For those patients with suspected central neck lymph node metastases on ultrasound before surgery, therapeutic central neck lymph node lymphadenectomy was performed. If pathological changes in the lateral neck lymph nodes were detected by ultrasound prior to surgery, a radical modified lymphadenectomy of the lateral neck lymph nodes was performed in parallel. Some patients were not diagnosed with metastases before surgery and underwent prophylactic surgery to remove central lymph nodes on one side or both sides. A small number of patients did not undergo lymphadenectomy. Histological examination of the surgical material was performed at the State Pathology Centre, Department of Autopsy and Biopsy, Vilnius University hospital "Santaros klinikos".

Total RNA isolation

Total RNA was purified from thyroid nodule fine needle aspirates using the commercial Quick-DNA/RNA™ Microprep Plus Kit (Zymo Research, USA). RNA quantification was performed using a

Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). Isolated total RNA samples were stored at -80°C.

Small RNA sequencing

miRNA profiling was performed by small RNA sequencing. Small RNA libraries were prepared using Ion Total RNA-Seq Kit v2 for Small RNA Libraries (Thermo Fisher Scientific, USA) according to the manufacturer's protocol using 1μg of RNA per sample. This step was followed by RNA 3' adapter ligation, RNA 5' adapter ligation, cDNA synthesis, PCR amplification using unique barcode sequences for each sample. Multiplexed libraries were then sequenced using the Ion Torrent PGM (Thermo Fisher Scientific, USA) platform.
Differential expression analyses were performed in *Partek® Flow®* (Partek incorporated, USA).

RT-PCR

miRNA expression levels were measured by quantitative reverse transcriptase-polymerase chain reaction (RL-PCR) using TaqMan® Advanced miRNA Assays (Applied Biosystems, USA) kits. The cDNA was synthesized from the purified total RNA using the TaqMan miRNA Reverse Transcription Kit according to the manufacturer's protocol. The synthesized cDNA was amplified using TaqMan miRNA probes. Three PCR reactions were performed for each sample according to the manufacturer's instructions.
Initial processing and normalization of Ct values was performed using Expression Suite release v1.3 (Thermo Fisher Scientific, USA) computer-aided data analysis software. As the expression of none of the miRNAs studied was equally distributed in all samples, global normalization was used to normalize the data, with the calculated average of all miRNAs used as normalizer. Relative expression was calculated using the 2-ΔCt method [32]. The data were converted to

relative units and the data obtained were converted on a logarithmic scale. The processed values were used in further statistical analysis.

Statistical analysis

Data were analyzed using SPSS, Inc., Chicago Illinois, version 23. A p value less than 0.05 was considered as statistically significant.

Variables were tested for normality according to the Shapiro-Wilk test. The results were presented as means ± standard deviation (SD) if the distributions were normal or median and interquartile range (IQR) if the distributions did not meet the criteria of normality. Chi-square ($\chi 2$) test was used for analysis of data when variables were categorical, it was used to analyze the relationship between miRNAs expression and the clinical demographic characteristics.

The expression levels of miRNAs among different groups were compared by nonparametric Mann-Whitney U test or Kruskal-Wallis test, as appropriate. Multivariate logistic regression analysis was performed to assess the associations between different miRNAs and the presence of malignancy and metastases.

Receiver operating characteristic (ROC) curves were used to determine the accuracy of the miRNA expression studies in the classification of malignant and benign thyroid nodules by evaluating the area under the curve.

EpiTools (epidemiological calculators, www.epitools.ausvet.com.au) and GPower 3.1 software were used to determine the sample size. The significance level of the criterion was set at $\alpha = 0.05$. The power of the criterion was considered to be at least 0.8. The sample size chosen for the study meets the requirements.

3. RESULTS

It was hypothesized that FNAB miRNAs could distinguish PTC from benign thyroid nodules and the possible role of miRNAs in the selection of surgical treatment tactics for thyroid nodules with suspected PTC on cytological examination was investigated. In addition, the association between miRNA expression and key clinical demographic parameters was investigated to assess the potential utility of miRNAs in predicting PTC invasion and metastasis.

Characteristics of the study population

The final sample consisted of 160 patients who met the inclusion criteria: 60 in the screening cohort and 100 in the validation cohort. The mean age was similar in both groups, 50.3 (+/- 14.5) and 49.7 (+/- 15.2) years. More than two thirds of the patients were women. The median tumor size on pathology was 12 (7) mm in the screening group and 16.5 (14.25) mm in the validation group. Clinical-demographic characteristics of the study population are shown in the Table 1.

The distribution of the Bethesda system categories in the PTC and BTN groups showed that category IV was significantly more frequent in BTN group, while categories V and VI were more frequent in the PTC group ($p < 0.05$). Category III was not significantly different between the BTN and PTC groups ($p = 0.62$). The frequencies of the Bethesda system categories between groups are shown in Table 2.

In the screening group, 43 (89.5%) patients were diagnosed with classical PTC, three (6.3%) with PTC follicular variant, one (2.1%) with tall cell PTC and one (2.1%) with oncocytic PTC. In validation group 58 (90,6%) patients were diagnosed with classical PTC, 5 (7.8%) with PTC follicular variant, and 1 (1.6%) with solid-trabecular PTC.

Table 1. Clinical-demographic characteristics of the study population.

Characteristic		Screening cohort	Validation cohort
All patients			
Number (n, %)	BTN	12 (20)	36 (36)
	PTC CLNM (-)	33 (55)	34 (34)
	PTC CLNM (+)	15 (25)	30 (30)
Age (years), mean (SD)		50.3 (14.5)	49.7 (15.2)
Sex (n, %)	Male	5 (8.3)	23 (23)
	Female	55 (91.7)	77 (77)
Tumor size mm, median (IQR)		12 (7)	16.5 (14.25)
Patients with PTC			
LNM (n, %)	N0	33 (68.75)	34 (53.1)
	N1	15 (31.25)	30 (46.9)
Extrathyroidal extension (n, %)	No	26 (54.2)	40 (62.5)
	Yes	22 (45.8)	24 (37.5)
Age groups (n, %)	≤ 55 years	32 (66.7)	46 (71.9)
	> 55 years	16 (33.3)	18 (28.1)
Multifocality (n, %)	No	31 (64.6)	41 (64.1)
	Yes	17 (35.4)	23 (35.9)
Hashimoto thyroiditis (n, %)	No	27 (56.25)	42 (65.6)
	Yes	21 (43.75)	22 (34.4)
Tumor size (mm) (n, %)	≤ 10	25 (52.1)	21 (32.8)
	> 10	23 (47.9)	43 (67.2)
T (TNM) (n, %)	T1	32 (66.7)	49 (76.5)
	T2	4 (8.3)	9 (14.1)
	T3	12 (25.0)	5 (7,8)
	T4	0 (0)	1 (1.6)
Surgery	Lobectomy	5 (10.4)	2 (3.1)
	Thyroidectomy	43 (89.6)	62 (96.9)
Lymphadenectomy: Prophylactic		37 (86.0)	39 (73.59)
Therapeutic CCLN		4 (9.3)	11 (20.75)
Therapeutic CCLN and LCLN		2 (4.7)	3 (5.66)

Table 2. Distribution of patients by Bethesda categories

Bethesda category	BTN (n, %)	PTC (n, %)	Total (n, %)	P value
III	17 (10.6)	20 (12.5)	37 (23.1)	0.62
IV	21 (13.1)	3 (1.9)	24 (15.0)	0.001
V	6 (3.8)	23 (14.4)	29 (18.1)	0.001
VI	4 (2.5)	66 (43.8)	70 (43.8)	< 0.001
Total (n, %)	48 (30.0)	112 (70.0)	160 (100)	

miRNA next-generation sequencing results (screening cohort)

Total RNA isolated from thyroid fine needle aspirate material from the first 60 subjects was analyzed for miRNA expression profile by next-generation sequencing. To compare the differences in miRNA expression, two analyses were performed: 1) a group of patients (n = 33) with PTC without CLNM and a group of patients (n = 15) with PTC with CLNM; 2) a group of patients (n = 12) with benign thyroid nodules and a group (n = 48) obtained by combining the two PTC groups PTC with CLNM and PTC without CLNM.

When comparing the PTC with CLNM and PTC without CLNM groups, miRNAs with a mean number of reads greater than 20 in the sample were included in the analysis, with a statistically significant fold change in expression increasing/decreasing > 2, $p < 0.05$. There were 12 miRNAs that were found to have a significant difference in fold change (Figure 1A).

When comparing PTCs with the group of benign nodules, miRNAs with an average read count greater than 0 in the sample were included in the analysis, with a statistically significant fold change with an increase in expression > 2, $p < 0.05$. There were 12 miRNAs that were found to have a significant difference in fold change (Figure 1B).

Of the significantly different miRNAs between PTCs and benign nodules, and between PTCs with CLNM and PTCs without CLNM,

only 6 miRNAs overlapped. Of these, the three most divergent miRNAs, miR4324, miR200B, miR125A, were selected for further validation. After the literature review, two more miRNAs – miR146B, miR221, which have been mainly described for differentiating both PTC with benign thyroid disease and more aggressive forms of PTC, were selected for further testing [33].

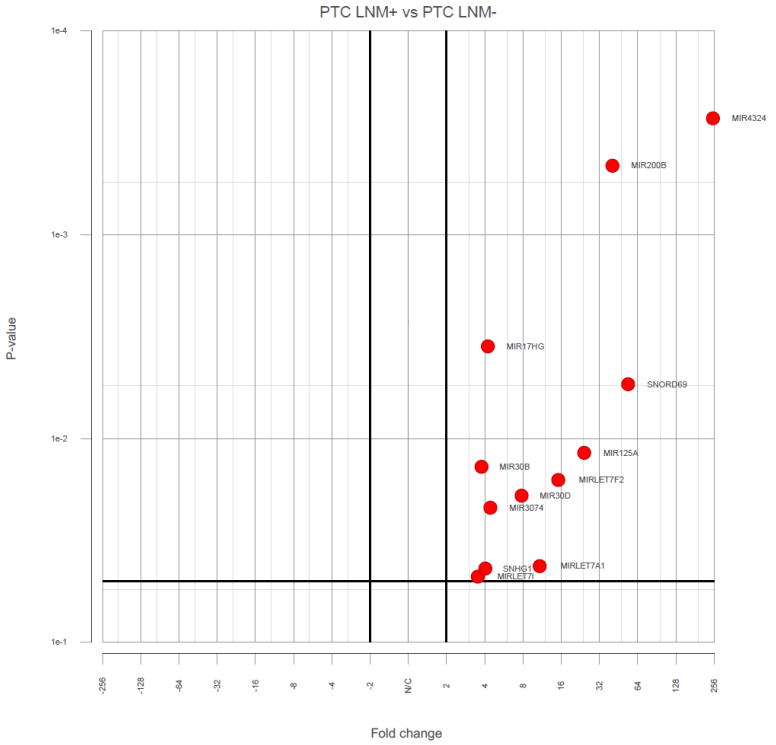

Figure 1A. Next-generation sequencing results of FNA miRNA. The volcano plot shows significantly different miRNAs between PTC with CLNM and PTC without CLNM.

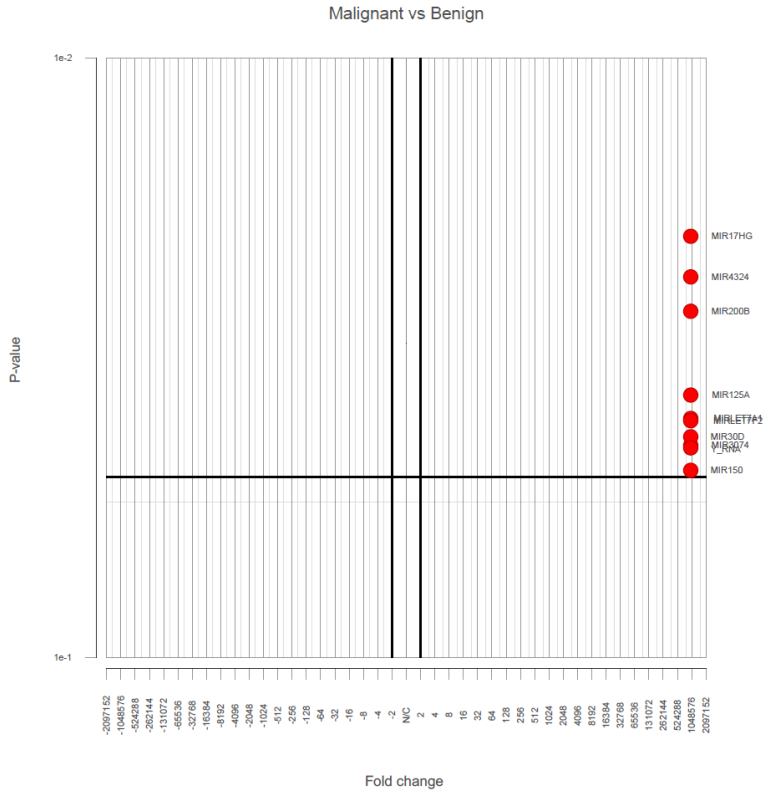

Figure 1B. Next-generation sequencing results of FNA miRNA. The volcano plot shows significantly different miRNAs between PTC and benign nodules.

miRNA expression by real-time PCR results (validation cohort)

Evaluation of selected miRNA relative expression differences based on clinical demographic features of PTC patients.

The analysis of five selected miRNAs expression in association with clinical demographic features of PTC was performed. The relative expression ($log_2 2^{-\Delta Ct}$) of miRNAs are shown in table 3.

Table 3. Comparison of miRNA relative expression between clinical-demographic characteristics of PTC.

Characteristics	n	miR125A	miR146B	miR200B	miR221	miR4324
Tumor size						
≤ 10 mm	21	0.53 (0.46)	-0.10 (0.42)	-2.35 (1.84)	-1.79 (1.14)	0.49 (0.50)
> 10 mm	43	0.37 (0.43)	-0.21 (0.42)	-2.52 (1.70)	-1.84 (1.62)	0.36 (0.38)
		p = 0.183	p = 0.424	p = 0.426	p = 0.424	p = 0.183
MF						
No	40	0.37 (0.49)	-0.23 (0.44)	-2.68 (2.28)	-2.10 (1.77)	0.37 (2.09)
Yes	24	0.53 (0.64)	-0.10 (0.29)	-2.35 (0.99)	-1.79 (0.81)	0.58 (0.48)
		p = 0.127	p = 0.084	p = 0.258	p = 0.043*	p = 0.107
Extr.Ex.						
No	40	0.40 (0.38)	-0.10 (0.41)	-2.35 (2.16)	-1.79 (1.15)	0.39 (0.40)
Yes	24	0.53 (0.64)	-0.24 (0.48)	-2.35 (1.22)	-1.93 (1.63)	0.47 (0.46)
		p < 0.001*	p < 0.001*	p = 0.164	p = 0.027*	p < 0.001*
Age						
≤ 55y	46	0.40 (0.37)	-0.18 (0.41)	-2.35 (1.72)	-1.94 (1.37)	0.37 (0.35)
> 55y	18	0.53 (0.86)	-0.10 (0.65)	-2.35 (1.70)	-1.79 (1.11)	0.57 (0.56)
		p = 0.931	p = 0.996	p = 0.867	p = 0.268	p = 0.278
HT						
No	42	0.40 (0.47)	-0.14 (0.41)	-2.35 (1.89)	-1.79 (0.99)	0.40 (0.39)
Yes	22	0.47 (0.40)	-0.18 (0.52)	-2.35 (1.78)	-1.90 (1.87)	0.48 (0.51)
		p = 0.849	p = 0.874	p = 0.496	p = 0.985	p = 0.409
Sex						
Male	12	0.41 (0.36)	-0.21 (0.31)	-2.68 (0.93)	-1.81 (1.34)	0.35 (0.56)
Female	52	0.44 (0.46)	-0.10 (0.42)	-2.35 (1.92)	-1.82 (0.72)	0.46 (0.44)
		p = 0.705	p = 0.509	p = 0.286	p = 0.874	p = 0.161

Mann-Whitney U test, relative expression, median and IQR; MF – multifocality; Extr.Ex. – extrathyroidal extension; HT – Hashimoto thyroiditis

The expression levels of all five miRNAs were similar between patients with microcarcinoma (tumor size ≤ 10 mm) and those with tumor size > 10 mm.

Significantly higher relative expression of miR221 (p = 0.043) was observed in patients with multifocal carcinoma compared with those with solitary carcinoma (Figure 2). No other differences in miRNA expression were found between these groups.

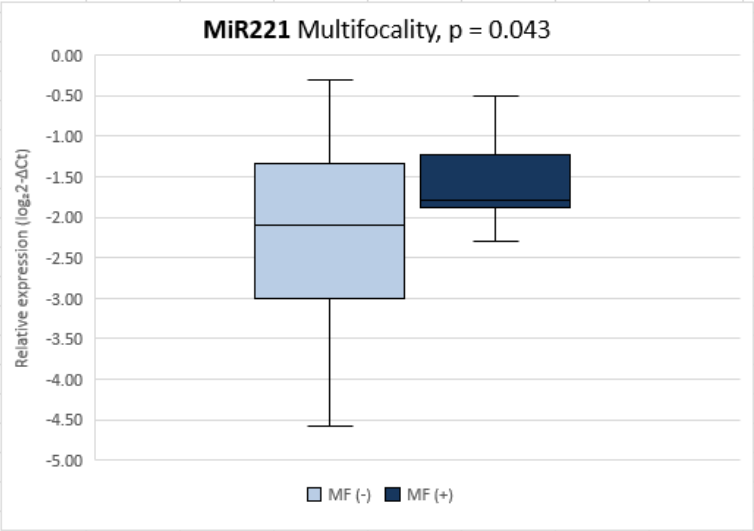

Figure 2. Comparison of miR221 relative expression between solitary and multifocal PTC.

Significantly higher expression of miR125A and miR4324 (p < 0.001) was observed in patients with tumor extrathyroidal extension compared with patients without PTC spreading beyond the thyroid capsule. Significant higher expression of miR146B (p < 0.001) and miR221 (p = 0.027) was found in patient without extrathyroidal tumor extension compared with patients with extrathyroidal PTC extension (Figure 3). No difference in miR200B expression was found between these groups.

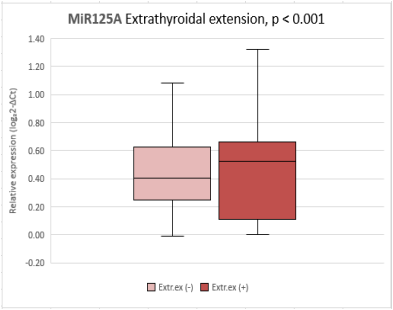

Figure 3A

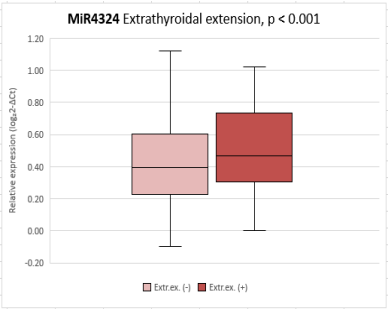

Figure 3B

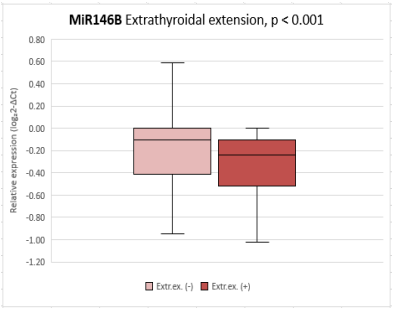

Figure 3C

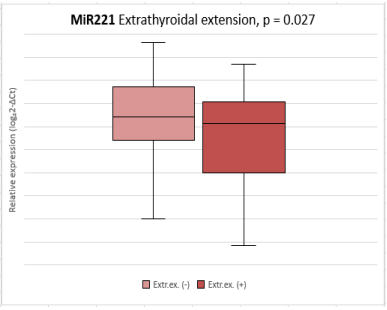

Figure 3D

Figure 3. Comparison of miRNAs relative expression between PTC with and without extrathyroidal extension; miR125A (A); miR146B (B); miR221 (C); miR4324 (D).

No significant differences in miRNA expression were found among other clinical features, such as patients' gender, age group, presence of Hashimoto's thyroiditis.

Evaluation of selected miRNA relative expression differences between benign thyroid nodules, PTC with CLNM and PTC without CLNM groups

The expression levels of selected miRNAs (miR125A, miR200B, miR146B, miR221, miR4324) were analyzed in PTC without CLNM (n = 34), PTC with CLNM (n = 30) and BTN (n = 36) groups. The

expression levels of 4 miRNAs (miR125A, miR146B, miR221 and miR4324) differed significantly between groups ($p < 0.05$). The expression difference of miR200B was not significant ($p = 0.129$). Median and interquartile range (IQR) in expression for all miRNAs are shown in table 4.

Table 4. Comparison of miRNA relative expression between the BTN, PTC without CLNM and PTC with CLNM groups.

miRNA	Benign	CLNM (-)	CLNM (+)	P value
miR125A	0.02 (0.38)	0.53 (0.46)	0.37 (0.43)	0.001
miR146B	-1.49 (1.15)	-0.10 (0.20)	-0.41 (0.53)	< 0.001
miR200B	-2.74 (1.55)	-2.35 (1.51)	-2.68 (1.71)	0.129
miR221	-2.34 (1.02)	-1.79 (1.2)	-2.30 (1.68)	0.024
miR4328	-0.12 (0.2)	0.58 (0.43)	0.35 (0.31)	< 0.001

Kruskal-Wallis test, relative expression ($log_2 2^{-\Delta Ct}$), median and IQR.

Next, the relative expression of miRNAs between groups of patients (n = 36) with BTN and patients (n = 64) with PTC was compared. The PTC group was formed by combining the two PTC groups PTC with CLNM and PTC without CLNM. The expression levels of 4 miRNAs (miR125A, miR146B, miR221 and miR4324) differed significantly between groups ($p < 0.05$). The expression difference of miR200B was not significant ($p = 0.135$). The median and IQR of the relative expression of all miRNAs are shown in Table 5.

The results of the miR125A and miR4324 relative expression by RT-PCR confirmed the results of the next-generation sequencing that the relative expression of these miRNAs is higher in the PTC group compared to the BTN group. The difference in expression between the BTN and PTC groups for miR146B and miR221 selected from the literature was also confirmed. The difference in miR200B expression between the BTN and PTC groups as determined by next-generation sequencing was not confirmed by RT-PCR.

Table 5. Comparison of miRNA relative expression between Benign and PTC groups.

miRNA	Benign	Malignant	P value
miR125A	0.02 (0.38)	0.44 (0.43)	< 0.001
miR146B	-1.49 (1.15)	-0.14 (0.42)	< 0.001
miR200B	-2.74 (1.55)	-2.35 (1.79)	0.135
miR221	-2.34 (1.02)	-1.81 (1.10)	0.032
miR4328	-0.12 (0.2)	0.42 (0.40)	< 0.001

Mann-Whitney U test, relative expression ($log_2 2^{-\Delta Ct}$), median and IQR.

ROC curve analysis was performed to assess the diagnostic accuracy of miR125A, miR146B, miR221 and miR4324, which differ significantly between the groups, in differentiating PTC from benign thyroid nodules. It can be concluded that miR146B (AUC = 0.809; $p < 0.001$) and miR4324 (AUC = 0.827; $p < 0.001$) were able to identify patients with PTC very well, while miR125A (AUC = 0.716; $p < 0.001$) was able to identify PTC patients well with a cutoff of 0.8 between good and very good model quality. The sensitivity and specificity of these three miRNAs are shown in table 6. miR221was not evaluated because the model predicts BTN poorly, only 5.6%.

Table 6. Prognostic value of miRNAs in differentiating malignant from benign nodules.

	AUC	p value	95% CI	Sensitivity	Specificity
miR125a	0.716	< 0.001	0.600-0.832	0.667	0.600
miR146b	0.809	< 0.001	0.700-0.919	0.824	0.885
miR4324	0.827	< 0.001	0.790-0.926	0.859	0.750

AUC: area under curve; CI: confidence interval.

The ROC curves for miR146b, miR4324 and miR125A are shown in Figure 4.

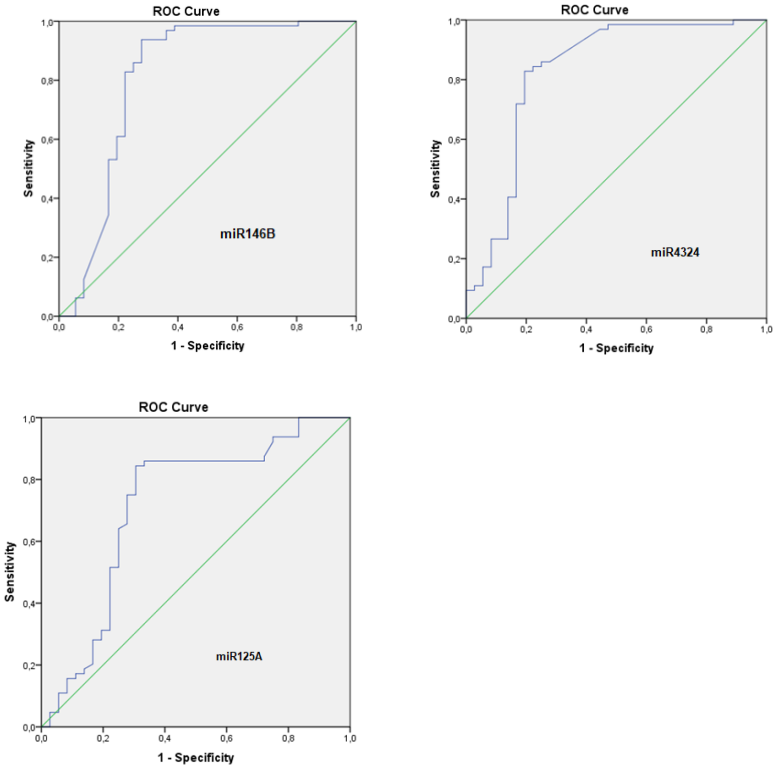

Figure 4. Prognostic value in differentiating malignant from benign thyroid nodules.

Also, the relative expression of miRNAs between groups of patients (n = 34) with PTC without CLNM and patients (n = 30) diagnosed with PTC with CLNM was compared. The median and IQR in the expression of all miRNAs are shown in table 7. Expression analysis of selected miRNAs: miR125A, miR200B and miR4324 between groups by RL-PCR did not confirm the difference in expression between these groups obtained by next-generation sequencing. The difference in expression of the miRNAs (miR221 and miR146B) selected from the literature was also not confirmed. Significantly higher expression of miR146B and miR4324 was

observed in patients with PTC without CLNM compared to patients with PTC with CLNM, which is in contrast to the miRNA expression detected in these groups by next-generation sequencing.

Table 7. Comparison of miRNA relative expression between PTC with CLNM and PTC without CLNM groups.

miRNA	CLNM -	CLNM +	P value
miR125A	0.53 (0.46)	0.37 (0.43)	0.080
miR146B	-0.10 (0.20)	-0.41 (0.53)	< 0.001
miR200B	-2.35 (1.51)	-2.68 (1.71)	0.128
miR221	-1.79 (1.2)	-2.30 (1.68)	0.058
miR4328	0.58 (0.43)	0.35 (0.31)	0.016

Mann-Whitney U test, relative expression ($log_2$2-ΔCt), median and IQR

Multivariable logistic regression was applied to predict the patient's histological category of BTN, PTC without CLNM or PTC with CLNM based on the miRNA. As the logistic regression calculates the ratio of success to failure, the results of the analysis are presented in the form of odds ratios. Two regressors, miR146B and miR4234, were included in the model. The control category was the group of benign thyroid nodules. The model fitted the data well. The correct classification was: PTC without CLNM 70.6%, PSC with CLNM 56.7%, benign thyroid nodules 72.2%, overall correct classification 67%. Nagelkerke's pseudo coefficient of determination $R^2 = 0{,}552$. Model likelihood ratio criterion statistic $\chi^2 = 67.406$, p < 0.01. Judging from the likelihood ratio criteria, the regressors are statistically significant.

A 16.0-fold increase in the value of miR146B increases the probability (odds ratio) that the disease will be cancerous without metastasis rather than benign (95% CI [4.188; 61.148]). A 4.2-fold increase in the value of miR146B increases the odds (odds ratio) that the disease will be malignant with metastasis rather than benign (95% CI [1.692; 10.366]). A 31.9-fold increase in miR4324 value increases the odds (odds ratio) that the disease will be malignant without

metastases rather than benign (95% CI [5.966; 170.765]). A one-fold increase in miR4324 value increases the odds (odds ratio) that the disease will be malignant with metastasis by 8.3 times more than non-metastatic (95% CI [2.155; 31.977]).

4. DISCUSSION

Differences in miRNA expression between groups of patients with benign and malignant thyroid nodules.

Ultrasound and FNAB with cytological examination are the "gold standard" for differentiating malignant from benign thyroid nodules.

Of the 160 patients in the study, 112 (70%) had histologically confirmed PTC and as many as 48 (30%) had histologically unconfirmed thyroid carcinoma and were diagnosed with BTN. This means that as many as 30% of the patients could have avoided surgery. These data suggest that ultrasound examination of thyroid nodules and the Bethesda cytology system alone do not allow a sufficiently precise differentiation between benign and malignant nodules.

In the first 60 patients (12 with BTN, 33 with PTC without CLNM and 15 with PTC with CLNM), the first step was to investigate the expression of miRNAs in fine-needle biopsy specimens by next-generation sequencing. After sequencing analysis, three miRNAs (miR125A, miR200B, miR4324) with the most significant differences between malignant and benign nodules and between the PTC without CLNM and PTC with CLNM groups were selected and subsequently analyzed in the validation group by RT-PCR. Two more miRNAs (miR146B, miR221) were selected for further study based on literature data and included miRNAs most commonly associated with papillary thyroid carcinoma and its more aggressive course. The study compared the difference in expression of the selected miRNAs between patients with BTN and those with PTC. The expression of four miRNAs (miR125A, miR146B, miR221 and miR4324) was significantly different between the groups ($p < 0.05$). The relative expression of all these four miRNAs was significantly higher in the PTC group. The results of RT-PCR for miR125A and miR4324 confirmed the results of next-generation sequencing that the relative expression of these miRNAs is significantly higher in the PTC group than in BTN group.

The difference in miR200B expression between the BTN and PTC groups as determined by next-generation sequencing was not confirmed by RT-PCR. The significance of miR125A and miR4324 in differentiating malignant from benign thyroid nodules has not been described in the literature so far.

The difference in expression of miRNAs: miR146B and miR221 selected from the literature between the BTN and PTC groups was also confirmed [34][29][30][35][36][37][38]. The expression of these two miRNAs was significantly higher in the PTC group.

ROC curves were used to determine the accuracy of the miRNA expression assays in the classification of PTC and BTN by evaluating the area under the curve. miR146B (AUC = 0.809; $p < 0.001$) and miR4324 (AUC = 0.827; $p < 0.001$) were able to identify patients with PTC very well, whereas miR125A (AUC = 0.716; $p < 0.001$) was able to identify patients with PTC – well.

The results showed that the expression of miR125A, miR146B and miR4324 could improve the accuracy of FNA of thyroid nodules, which would reduce the number of indeterminate cytology samples and allow better differentiation of malignant nodules from BTN.

Differences in miRNA expression according to clinicopathological risk factors for papillary thyroid carcinoma.

The study evaluated the association of miRNAs with risk factors for PTC aggressiveness.

Multifocality, tumour size, and extension beyond the thyroid capsule are important risk factors for aggressiveness of PTC [8][24]. In this study the expression levels of all five miRNAs were similar between patients with microcarcinoma (tumour size $\leq$ 10 mm) and those with tumour size >10 mm ($p > 0.05$).

The significantly higher relative expression of miR221 ($p = 0.043$) was found in patients diagnosed with multifocal carcinoma compared with those diagnosed with a single PTC focus. This confirms the data reported in the literature that miR221 expression is significantly higher in multifocal PTC [39][29][30][40][41]. No other differences

in miRNA expression were found between the solitary and multifocal PTC groups in the present study.

According to the literature, an important risk factor for aggressiveness in PTC is thyroid capsule overgrowth. Patients with PTC extension beyond the thyroid capsule have a higher risk of CLNM (OR = 2.03; 95% CI 1.78-2.31). It is also important to mention that patients with a large extension beyond the thyroid capsule are at higher risk of recurrence than those with a small extension found only histologically [24].

In the present study, patients were not differentiated according to the degree of extension beyond the thyroid. In an analysis of the expression of five miRNAs by RT-PCR, a significantly higher relative expression of miR125A ($p < 0.001$) and miR4324 ($p < 0.001$) was observed in patients with tumour spread beyond the thyroid capsule compared with patients with no PTC spread beyond the thyroid capsule. In contrast, significantly higher relative expression of miR146B ($p < 0.001$) and miR221 ($p = 0.027$) was observed in patients whose PTC did not extend beyond the thyroid capsule. There was no difference in miR200B expression between these groups of patients. The results did not support previous reports in the literature suggesting that miR146B and miR221 may be associated with the spread of PTC outside the thyroid capsule [29][39][34][30]. miR125A and miR4324 expression is not reported in the literature before.

Differences in miRNA expression between groups of patients with papillary thyroid carcinoma with and without metastasis to the neck lymph nodes.

The American Thyroid Association International Guidelines [8] recommend that patients with preoperative findings in the cervical lymph nodes should undergo lymphadenectomy. However, the importance of prophylactic surgery for the removal of CLN is controversial due to the increased likelihood of postoperative complications. Existing diagnostic imaging tests do not accurately determine the extent of the neoplastic process before surgery [18]. Up

to 60% of CLNM are histologically diagnosed and are not detected by imaging studies before surgery [20].

Of the 160 patients in the study, 138 (80.25%) did not have preoperative ultrasound findings of CLNM. 59 (36.87%) had no removal of the CLN and 79 (49.38%) underwent prophylactic lymphadenectomy for CLN, 22 (13.75%) underwent therapeutic lymphadenectomy of CLN, 18 (81.8%) of these underwent CCLN lymphadenectomy and 4 (18.2%) underwent both CCLN and LCLN lymphadenectomy.

Of the 22 therapeutic lymphadenectomies, 19 (86.4%) were histologically confirmed and 3 (13.6%) were unconfirmed as CLNM. Of the 79 patients who underwent prophylactic lymphadenectomy, 26 (32.9%) were found to have metastases and 53 (67.1%) were free CLNM. These data suggest that ultrasonography of the CLN is not sufficiently sensitive for the diagnosis of CLNM. As many as 53 patients, representing 33.13% of the total number of patients and 67.1% of the patients who had not been diagnosed with CLNM by ultrasound prior to the operation, could have had a reduction in the extent of the operation, i.e. no lymphadenectomy.

As currently available imaging and cytology tests are not able to accurately diagnose thyroid carcinoma and its spread to the CLN, it is important to search for new biomarkers that can more accurately diagnose and assess the spread of disease.

In the last decade, many studies have been carried out to identify miRNAs as a diagnostic biomarker for PTC. Several miRNAs have also been associated with PTC metastasis [33].

In the present study, a comparison of selected miRNAs between PTC with and without CLNM yielded contradictory data between the groups of patients. miR125A, miR200B and miR4324 expression detected by next-generation sequencing in PTC with CLNM was significantly higher ($p = 0.01$, $p < 0.001$, $p < 0.001$, respectively) compared with PTC without CLNM. Meanwhile, the expression of miR4324 detected by RT-PCR was significantly higher ($p = 0.016$) in patients with PTC without CLNM than in patients with PTC with

CLNM, while the expression of miR200B and miR125A was not significantly different between the groups ($p > 0.05$). Multivariable logistic regression showed that a one-fold increase in miR146B increased the odds of a node being malignant without metastasis by 16.0-fold and of a node being malignant with metastasis by 4.2-fold. In contrast, a one-unit increase in miR4324 increases the odds of having PTC without metastases by 31.9 times and PTC with metastases by 8.3 times compared to BTN.

The results of the study did not confirm the difference in expression between the groups of miRNAs (miR221 and miR146B) selected from the literature [39][34][29][30][40][42][43][44] as the miRNAs most frequently associated with metastatic PTC. The difference in miR221 expression between the groups was non-significant ($p = 0.058$), whereas miR146B expression was significantly higher ($p < 0.001$) in the group of patients without CLNM.

There were several limitations of the study. This study was conducted at a single institution. The group of patients studied was relatively small.

What could be the reasons why the results were so controversial between the groups of subjects with metastatic and non-metastatic PTC. Firstly, the time elapsed between tumour onset and diagnosis in each group is not known. As 16 (47.1%) patients in the PTC without CLNM group were diagnosed with a microcarcinoma, we can assume that there was not enough time for metastases to occur. Second, the study included patients who underwent both prophylactic and therapeutic lymphadenectomy. This heterogeneous group of patients may also have influenced the results. Thirdly, the study included patients with different subtypes of PTC, i.e. classical, follicular variant, oncotic, solid-trabecular, high cell, which have different degrees of aggressiveness. The vast majority of the patients included in the study had classical PTC, and the small number of patients with other subtypes of PTC precluded a well-founded subgroup analysis for each disease subtype.

5. CONCLUSIONS

1. miRNA expression in fine needle aspirates of thyroid nodules helps differentiate malignant thyroid nodules from benign ones. The study showed significantly higher relative expression of four miRNAs (miR125A, miR146B, miR221 and miR4324) in malignant thyroid nodules ($p < 0.05$) compared to benign thyroid nodules.

2. PTC with different pathological features have different miRNA expression in fine-needle aspirates of thyroid nodules:

- the relative expression of miR125A and miR4324 is significantly higher in PTC extending beyond the thyroid capsule compared to PTC not extending beyond the thyroid capsule ($p < 0.001$);

- the relative expression of miR221 is significantly higher in multifocal PTC ($p = 0.043$) than in solitary PTC.

3. Significantly higher relative expression of miR4324 and miR146B was found in PTC without metastases compared to PSK with metastases in the study groups ($p = 0.016$ and $p < 0.001$, respectively).

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PUBLICATIONS

1. R. Laukienė, A. Padlipskaitė, E. Žurauskas. Inflammatory myofibroblastic tumour of the thyroid gland with follicular carcinoma: a rare clinical case report. Laboratorinė medicina. 2017, Vol. 19, No. 1(73), pp. 61-65.
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PRESENTATIONS

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1. R. Laukienė. “Gerai diferencijuotos skydliaukės karcinomos klinikinių, citologinių ir molekulinių tyrimų diagnostinė reikšmė”. Conference of Vilnius Society of Endocrinology “Endokrininių ligų diagnostikos ir gydymo aktualijos”. Vilnius, 31 May 2018.
2. R. Laukienė. “Skydliaukės TIRADS. Pirmieji rezultatai”. Conference of Ultrasound Society “Ultragarsinės diagnostikos vasaros mokykla: teorija ir praktika”. Dubingiai, 27-29 July 2018.
3. R. Laukienė. “Daugybinės endokrininės neoplazijos sindromai: diagnostika, gydymas ir priežiūra”. Conference of Lithuanian Society of Endocrinology “Endokrininių susirgimų genetika”. Kaunas, 10 May 2019.
4. R. Laukienė, A. Beiša. “Skydliaukės vėžys: endokrinologo ir chirurgo tandemas – kas, kaip, kada?”. Conference of Vilnius Society of Endocrinology “Onkologija endokrinologijoje”. Vilnius, 29 April 2021.

Poster presentations

1. Laukiene R., Padlipskaite A., Zurauskas E. “Concomitant thyroid inflammatory myofibroblastic and follicular tumours”. 19th European Congress of Endocrinology, Lisbon, 20-23 May 2017.
2. Laukiene R., Cimbalistiene L., Utkus A. “Thyroid microRNA analysis and prognostic value”. Life Sciences Baltics. Vilnius, 26-27 September 2018.
3. Laukiene R., Sestakauskaite J., Visockiene Z. “Risk factors of cervical lymph node metastasis of differentiated thyroid carcinoma”. 21th European Congress of Endocrinology. Lyon, 18-21 May 2019.

4. Laukiene R., Miseviciute K. “Primary Thyroid Lymphoma: Could Surgery Be Avoided?”, Congress of Endocrine Society ENDO 2021. 20-23 March 2021.
5. Laukiene R., Ambrozaityte L. “Association of microRNA Expression with Cervical Lymph Node Metastases of Thyroid Carcinoma”. 23rd European Congress of Endocrinology, 22-26 May 2021.
6. Laukiene R., Ambrozaityte L. “Analysis and prognostic significance of miRNAs in papillary thyroid carcinoma”. 24th European Congress of Endocrinology, Milan, 21-24 May 2022.

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