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CROSS-SECTIONAL STUDY

Fine-needle biopsy of thyroid nodules and the contribution of molecular analysis of BRAF and RAS mutations

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ABSTRACT

BACKGROUND: Thyroid cancer is the most common endocrine malignancy. There is a significant overdiagnosis of thyroid carcinomas that would never clinically manifest, with consequent unnecessary surgical treatment. The fine-needle biopsy and subsequent cytologic examination is of crucial importance in the differential diagnosis of thyroid nodules. On the other hand, a significant portion of the results are indeterminate.

OBJECTIVE: To assess the relationship of BRAF/RAS mutations in biopsy specimens to histological characteristics of thyroid nodules in individuals who undergone fine-needle biopsy and surgery.

METHODS: This cross-sectional study involves 170 subjects with indeterminate cytology analyzed for BRAF/RAS mutations.

RESULTS: Of all 170 patients with indeterminate cytological finding, 103 were indicated for surgery. Of these, 31 were BRAF and 25 RAS positive. Thyroid cancer was diagnosed in 59 patients, while 44 patients had non-malignant thyroid lesions. The BRAF V600E mutation was detected in 30 patients, and the RAS (K-RAS, N-RAS, and H-RAS) mutation in 13 patients with thyroid cancer. In all BRAF-positive nodules, thyroid cancer was histologically confirmed. This means a 100 % positive predictive value of BRAF testing in our study.

CONCLUSION: Stratification of thyroid lesions with uncertain results of fine-needle cytology using genetic markers can help to deliver more tailored medical treatment *(Tab. 6, Ref. 19)*. Text in PDF *www.elis.sk*

KEY WORDS: fine-needle biopsy, thyroid gland, thyroid carcinoma, BRAF, RAS.

Introduction

Thyroid cancer (TC) is the most common endocrine malignancy (1). Well-differentiated papillary carcinoma (PTC) represents the vast majority of TC (80 %). PTC has a good prognosis. Up to 95 % of patients are cured, while the rest usually achieve a well-controlled state (2). Mortality is very low and 10-year survival reaches almost 100 %. Thyroid cancer usually manifests as nodule. Thyroid nodules have a high prevalence, occurring in up to 50 % of ultrasound examinations of individuals over 50 years in the European population (3, 4). The majority of thyroid nodules are benign, cancer is diagnosed in 5 % (5, 6). Fine-Needle Biopsy (FNB) is the most effective and exact pre-operative modality for the routine examination of thyroid nodules. Cytology can distinguish between benign and malignant nodules in 60–80 % cases (6, 7). 20–30 % of FNB results are classified as indeterminate (neither clearly benign nor clearly malignant) according to the Bethesda System for Reporting Thyroid Cytopathology (Bethesda) 2017: Bethesda III – atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS), Bethesda IV - follicular neoplasia or suspicious for follicular neoplasm (FN/SFN) and Bethesda V – suspicious for malignancy (SM). The optimal clinical management for the diagnostic categories mentioned above has not yet been established and ranges from observation through repeated FNB to lobectomy (LT), and total thyroidectomy (TTE). Recently, molecular genetic testing has demonstrated the ability to better stratify thyroid lesions and estimate their malignant potential and clinical behavior (8).

The recent guidelines of the European Thyroid Association regarding thyroid nodule molecular fine-needle aspiration cytology diagnostics also call for an innovative approach – predictive testing and the use of genetic markers in thyroid nodules management (9).

Molecular genetic testing in fine-needle thyroid biopsy

The risk of malignancy in the first two indeterminate cytological categories, Bethesda III and IV, ranges from 10 to 40 %, while in Bethesda V it ranges between 50 % and 75 % (10). Before the introduction of molecular testing, most patients with a cytological result of Bethesda III or Bethesda IV underwent diagnostic LT. In many patients who underwent surgery, histological examination finally showed a benign disease. Currently, there is progress in the possibility of examining genetic mar-

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kers, which provide relevant prognostic information for further decision-making and choosing the most appropriate clinical approach. The evaluation of uncertain FNB results using molecular testing provides better risk stratification and reduces indications for unnecessary thyroid gland operations. However, molecular testing remains limited by its availability and cost.

Genetic markers BRAF and RAS in thyroid carcinoma

Thyroid neoplasms originating from follicular cells are conditioned by various genetic changes that contribute to the determination of their histological pattern, degree of differentiation and clinical features. Genetic mutations can be specific to a given type of tumor on the one hand, while on the other hand, a given mutation can occur in various types and subtypes of tumors.

As a result of initiating mutations in the carcinogenesis of follicular cells, well-differentiated thyroid tumors (DTC) most often develop, namely follicular thyroid carcinoma (FTC) and PTC. The gradual accumulation of mutations (increasing of the mutation load) in the tumor can lead to dedifferentiation of a previously well-differentiated tumor via forming of a poorly differentiated thyroid carcinoma (PDTC) to an undifferentiated anaplastic thyroid carcinoma (ATC) (11). The pathomechanisms involved in the development of different histological types of thyroid cancer – differentiated, poorly differentiated and undifferentiated carcinomas, are alternative and the gradual accumulation of key mutations seems to be decisive.

Thyroid cancer originating from follicular cells includes several types of tumors with mutually exclusive driver mutations, such as tumors with BRAF V600E mutation (60 %), BRAF mutations without V600E mutation – including K601E, tumors with RAS mutation (15 %) and mutations or fusions of other receptor tyrosine kinases (12 %), such as RET, NTRK and ALK (8). These driver genes are associated with different histological phenotypes. The histology of thyroid carcinoma with BRAF V600E mutation includes classical PTC and its tall cell variant (TCV), which is more aggressive than classical PTC and manifests with more frequent extrathyroidal spreading and lymph node metastases. Somatic RAS mutations can be observed in FTC with more frequent hematogenous metastases. PTC with BRAF V600E mutation and PTC with RET/PTC rearrangements were more often associated with advanced-stage III/IV disease and recurrence incidence, while RET oncogene rearrangement was additionally associated with a higher incidence of distant metastases (10). The gene rearrangements in follicular cells RET/PTC have generally higher incidence in radiation-induced and younger age TC and PAX8-PPARγ presents mainly in conventional FTC (2).

BRAF mutations

The mutation in codon 600 of the BRAF gene, as the most common mutation in PTC, positively predicts tumor aggressiveness, lymph node metastases, recurrence, severity of prognosis and higher TNM stage (Tumor-Node-Metastasis classification of malignant tumors). It also occurs in papillary microcarcinoma, where it positively correlates with extrathyroidal spread and lymph node metastasis. BRAF V600E mutations are more frequently reported in the PTC subgroup with more aggressive clinical-pathological behavior, and their higher malignancy potential is enhanced by tendency towards dedifferentiation. This is also confirmed by the presence of this mutation in poorly differentiated and anaplastic carcinoma (11).

The European Thyroid Association (ETA) recommends total thyroidectomy for BRAF V600E type PTC in nodes larger than 1 cm and consideration of prophylactic dissection of central lymph nodes (9).

RAS mutations

RAS mutations were among the first to be recognized as mandatory in thyroid oncogenesis (12). However, their presence is not a reliable indicator of malignancy of a thyroid nodule, as they also occur in other thyroid neoplasms such as benign follicular adenomas or non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP). Of the 3 RAS isoforms (H-RAS, K-RAS and N-RAS) present in thyroid cancer, N-RAS and H-RAS are the most frequent (13, 14). RAS mutations are more often present in well-differentiated TCs, especially the follicular variant of PTC and FTC. Compared to PTC BRAF type, this group of tumors behaves less aggressively, although distant metastases are sometimes present. The RAS oncogene is probably responsible for the gradual transformation of thyrocytes and progression from benign to malignant nodules, from differentiated to dedifferentiated and anaplastic tumors.

The European Thyroid Association recommends less radical surgical treatment for RAS type PTC – lobectomy (9).

Patients and methods

During a period of three years, an indeterminate result of cytological examination was found in 170 individuals. The indeterminate result (Bethesda III – V) was analyzed by BRAF and RAS genes mutations examination in relation to the histological characteristics of biopsied lesions and the risk of malignancy (ROM). Sample for BRAF and RAS mutations detection was gained by rinsing the bioptic needle into a small amount of physiological saline (0.5 ml).

DNA was isolated from cells using the QIAamp DNA Micro Kit (Qiagen) and then the concentration was determined using the Quantus fluorometer (Promega). The analysis was performed using the EasyPGX ready Thyroid kit (Diatech Pharmacogenetics), which detects mutations in codons 12, 13, 61 of K-RAS, N-RAS and H-RAS genes and in codons 600 and 601 of BRAF gene by Real-Time PCR method. The Real-Time PCR reaction in the EasyPGX qPCR instrument 96 analyzer (Diatech Pharmacogenetics) for one sample takes place in 8 tubes (each tube determines a different set of mutations, and each tube also determines the presence of a control gene to control sample validity), 5–10 ng DNA is added to each tube. After the analysis is completed, the result is evaluated using EasyPGX analysis software.

Results

The average age of the observed individuals was 49 ± 14 years, of which 136 were women aged 16 to 81 years and 34 men aged

Tab. 1. Demographic characteristics of individuals with an indeterminate result of cytological examination and distribution according to the presence of BRAF/RAS mutations.

		Bethesda III – V	Bethesda III	Bethesda IV	Bethesda V	BRAF V600E +	RAS +
Gender (count)	Total	170 (100%)	98 (58%)	35 (20%)	37 (22%)	31 (18%)	25 (15%)
	Women	136 (80%)	81 (48%)	27 (16%)	28 (16%)	23 (14%)	18 (11%)
	Men	34 (20%)	17 (10%)	8 (5%)	9 (5%)	8 (5%)	7 (4%)
Age (years)	Total	49±14	50	48	47	48	44
	Women	48±13	49	46	46	45	43
	Men	55±14	58	53	51	55	49

Tab. 2. Overview of patients with TC divided by molecular-genetic examination according to the presence of BRAF/RAS mutation.

	TC	BRAF V600E +	RAS +
Bethesda III – V	59	30 (51%)	13 (22%)
Bethesda III	18 (30%)	9 (15%)	2 (3%)
Bethesda IV	14 (24%)	5 (8%)	5 (8%)
Bethesda V	27 (46%)	16 (27%)	6 (10%)

Tab. 3. Representation of BRAF/RAS mutations across malignancies of follicular origin.

	TC	PTC	FTC	PDTC
Total	59	55 (93%)	3 (5%)	1 (2%)
BRAF V600E +	30	29 (97%)	1 (3%)	0
RAS +	13	12 (92%)	1 (8%)	0
BRAF V600E/RAS –	16	14 (88%)	1 (6%)	1 (6%)

30 to 77 years. Cytological examination with the conclusion of Bethesda III had 98, Bethesda IV 35 and Bethesda V 37 individuals. The BRAF V600E mutation was present in 31 cases, in 23 women and 8 men. The RAS mutation was detected in 25 cases, in 18 women and 7 men (Tab. 1).

Histopathological examination was performed in 103 individuals, 66 were not indicated for surgery based on negative result of molecular genetic testing. TC was diagnosed in 59 patients (43 women and 16 men), 44 individuals had a benign result. BRAF mutation was present in 30 (22 women, 8 men) and the RAS mutation in 13 patients (7 women, 6 men) with carcinoma (Tab. 2). 1 patient with a present BRAF V600E mutation refused surgery.

Three patients had FTC, one PDTC and the rest PTC (Tab. 3). 73 % of patients with TC had a BRAF/RAS mutation present, in 16 the contribution of these mutations in the carcinogenesis process was not confirmed (Tab. 4). BRAF PTC was shown in 53 % and RAS PTC in 22 % of all cases of papillary carcinomas.

Tab. 4. Typing of diagnosed tumors according to their BRAF/RAS status.

		BRAF a RAS +	BRAF V600E +	RAS +	BRAF V600E a RAS –
TC	59	43 (73%)	30 (51%)	13 (22%)	16 (27%)
PTC	55	41 (55%)	29 (53%)	12 (22%)	14 (45%)
FTC	3	2 (66%)	1 (33%)	1 (33%)	1 (33%)
PDTC	1	1 (100%)	1 (100%)	0	0

Tab. 6. Presence of BRAF/RAS mutation and extent of primary tumor (T).

	T – total (100%)	T1	T2	T3	T4
Total TC	59	39 (67%)	9 (16%)	10 (17%)	0
BRAF V600E +	30	18 (60%)	3 (10%)	9 (30%)	0
RAS +	13	5 (38%)	4 (31%)	4 (31%)	0
BRAF V600E/RAS –	16	14 (88%)	2 (12%)	0	0

Tab. 5. Molecular stratification of thyroid nodules according to malignancy status.

	Patients after surgery	Benign	Malignant – TC
Total	103	44	59
BRAF +	30 (29%)	0	30 (50%)
BRAF –	73 (71%)	44 (100%)	29 (50%)
RAS +	22 (21%)	9 (20%)	13 (22%)
RAS –	81 (79%)	35 (80%)	46 (78%)

In the group with a benign finding, a RAS mutation was present in 9 individuals, a BRAF mutation did not occur (Tab. 5). The BRAF mutation was shown in half and the RAS mutation in slightly over one fifth of all TC cases.

TC with a present BRAF/RAS mutation were most often detected in the lowest T category of the TNM classification of malignant tumors 8th edition (Tab. 6).

Of the 48 patients with autoimmune thyroiditis (AIT), 19 had concomitant TC, of which BRAF V600E and RAS mutation were detected in almost every second or third patient (10 and 5 cases, respectively).

Discussion

When obtaining uncertain cytology, it is useful to perform additional molecular testing, which can be helpful in considering the indication and extent of surgical treatment.

Molecular stratification of papillary thyroid carcinoma has additional prognostic value (15), allowing for personalized therapeutic approach in terms of preventing overtreatment, but also choosing a more radical approach where the benefit of more aggressive treatment for the patient's quality of life outweighs its iatrogenic potential.

Molecular-genetic analysis of 170 cases of nodular goiter identified the presence of BRAF V600E mutation in 31 individuals, 30 had subsequently histologically confirmed thyroid carcinoma originating from follicular cells, 1 refused surgery. The presence of BRAF point mutations is thus a clear benefit in predicting ROM. Testing for RAS did not yield such convincing results, as the number of individuals with histologically malignant nodes only moderately exceeded the number of those with benign histological findings (13 versus 9 cases). Determining RAS mutations remains controversial, as in the population of individuals with this mutation, it is often associated with benign follicular lesions such as benign adenoma. On the other hand, some

studies have pointed to the role of RAS mutation in the process of tumorigenesis and as an early transformational event associated with the transition from adenoma to carcinoma – the so-called histological continuum from benign to malignant lesions (16, 17).

The positivity of RAS mutations plays beneficial role in the presence of mutations of other oncogenes when it increases the risk of tumor aggressiveness.

Despite the demonstrated positive predictive value of BRAF mutation, it was associated with a smaller extent of tumor according to TNM (8th edition). The cause is probably the use of more intensive and enhanced diagnostic methods - especially the availability and high-resolution sensitivity of ultrasound examination.

The presence of AIT was associated with a higher prevalence of TC, while BRAF mutations were as common among these carcinomas as in carcinomas without AIT. This observation supports the hypothesis that different underlying mechanisms of carcinogenesis are involved in the development of PTC in AIT patients (18).

Due to the high positive predictive value of molecular-genetic testing of BRAF V600E mutation, the presence of a positive result can weigh in clinical decision-making in favor of TTE as a first-choice therapeutic approach. On the other hand, almost every third carcinoma from follicular cells of the thyroid gland does not contain either BRAF/RAS mutation, so the absence of mutation cannot exclude malignancy, which points to a low negative predictive value of testing.

Radioiodine treatment is more effective in PTC with RAS mutation than in tumors with BRAF mutation, probably because BRAF mutation reduces the expression of genes that are necessary for the transport and metabolism of iodine, which ultimately reduces the uptake of radioiodine (19).

Stratification of thyroid lesions using genetic markers provides a direct impact on the extent of oncological treatment such as total thyroidectomy, subsequent adjuvant radioiodine treatment and suppressive thyroxine treatment in clinical management. Such complex treatment was usually indicated in all patients with DTC. Genetic markers thus appear to be beneficial parameters determining the extent not only of surgical treatment of small differentiated carcinomas without clinically evident nodal metastases (T1,2 N0) with low risk of recurrence.

Conclusion

The use of genetic markers of thyroid gland carcinogenesis represents new horizons in the diagnosis and treatment of thyroid tumors. Such innovative molecular-genetic approaches will be useful especially in cases of indeterminate results of fine-needle cytology, whose positive predictive value is significantly increased. In combination with traditional diagnostic procedures, they could shift towards more tailored therapeutic approach, particularly the extent of surgical treatment. For example, to perform TTE without the need for prior diagnostic LT in histologically malignant lesions. Applying BRAF/RAS mutation detection in the diagnostic-therapeutic process of nodular goiter can contribute to more effective management of patients with thyroid carcinoma and to more rational use of financial resources in healthcare, mainly in the conditions of specialized centers.

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