Clinical and molecular genetic analysis of cytologically uncertain thyroid nodules in patients with thyroid disease

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Background. The current requirement is to establish the preoperative diagnosis accurately as possible and to achieve an adequate extent of surgery. The aim of this study was to define the preoperative clinical and molecular genetic risks of malignancy in indeterminate thyroid nodules (Bethesda III and IV) and to determine their impact on the surgical strategy.

Methods. Prospectively retrospective analysis of 287 patients provided the basis of preoperative laboratory examination, sonographic stratification of malignancy risks and cytological findings. Molecular tests focused on pathogenic variants of genes associated with thyroid oncogenesis in cytologically indeterminate nodules (Bethesda III and IV). The evaluation included clinical risk factors: positive family history, radiation exposure and growth in size and/or number of nodules.

Results. Preoperative FNAB detected 52 cytologically indeterminate nodules (28.7%) out of 181 patients. Postoperative histopathological examination revealed malignancy in 12 cases (23.7%) and there was no significant difference between Bethesda III and IV categories (*P*=0.517). Clinical risk factors for malignancy were found in 32 patients (61.5%) and the presence of at least one of them resulted in a clearly higher incidence of malignancy than their absence (31.3% vs. 10.0%, respectively). Pathogenic variants of genes were detected in 12/49 patients in Bethesda III and IV, and in 4 cases (33.3%) thyroid carcinoma was revealed. The rate of malignancies was substantially higher in patients with pathogenic variants than in those without (33.3% vs. 16.2%, respectively).

Conclusions. Our experience implies that molecular genetic testing is one of several decision factors. We will continue to monitor and enlarge our patient cohort to obtain long-term follow-up data.

Key words: thyroid nodules, cytology, molecular testing, thyroidectomy

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INTRODUCTION

The occurrence of thyroid nodules is frequent. They are revealed by ultrasound examination (USG) in almost two-thirds of the adult population, often as incidental findings, and four times more frequently in women than in men. In up to 90% of cases, they are benign lesions[1-3]. The prevalence of nodules increases with age, body mass index and iodine deficiency[1,2]. The main requirement of the preoperative examination is to determine as accurately as possible the biological nature of the thyroid nodules, and thus facilitate the decision on further treatment[2-4]. Fine-needle aspiration cytology (FNAC) is able to classify 70% to 80% of benign or malignant thyroid nodules and is considered the gold standard of preoperative examination[5-7]. However, 20–30% of nodules are classified as indeterminate lesions – atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS) – Bethesda III category or follicular or Hürthle cell neoplasm or suspected follicular neoplasm (FN/SFN) – Bethesda IV category[5-8]. These findings reflect the limits of cytological examination to identify specific features and characteristics needed for a definitive diagnosis[9,10]. The sensitivity of cytological examination decreases with the nodule size and in cases of multinodular transformation[9,10]. The category of indeterminate modules is a heterogenous group with varying risks of malignancy, which includes approximately 30% of all cases (ranging from 10% to 75%) (ref.[11-14]). The American Thyroid Association (ATA) was the first[8], and subsequently, additional international thyroid associations[15-18], to recommend that along with the evaluation of ultrasound risk factors of malignan-

cy using the ACR TI-RADS (Thyroid Imaging Reporting and Data System of the American College of Radiology) classification system[8], clinical risk factors should also be considered (positive family history, thyreopathy/thyroid carcinoma, radiation exposure to the neck, increase in size >1 cm and/or the number of nodules, elastography-confirmed nodule stiffness, coexistence of chronic autoimmune thyroid disease (AIT), increased ^{18}FDG uptake, age <40/≥60 (ref.[15-19]). The preoperative diagnosis of thyroid nodules increasingly involves identifying molecular markers of thyroid carcinomas[3,20]. The rule-out tests attempt to identify benign nodules by gene expression analysis (e. g. GEC – gene expression classifier testing the mRNA expression of 167 genes). These tests yield high sensitivity and a negative predictive value (NPV) in Bethesda III nodules and can prevent unnecessary surgery[3,20,21]. The second approach is the detection of pathogenic variants of genes associated with thyroid oncogenesis (e. g. *BRAF*, *TERT*, *RET*, *RAS* and fusion genes) that predict malignancy (rule-in test) and are characterized by high specificity and a positive predictive value (PPV) (ref.[3,20,22]). Detection of certain genetic alterations contributes to the optimization of surgical interventions and helps to identify rare and aggressive microcarcinoma variants (<5%) with the risk of extrathyroidal invasion or metastasis, and to determine the prognosis of the disease[3,20,22]. Currently, there is an effort to develop combined testing using new generation sequencing methods (NGS), e. g. Thyroseq panel[3,5,13], but these tests are not available in the Czech Republic. The treatment of indeterminate nodules includes both non-surgical methods such as clinical and USG follow-up, repeated US-FNA (US-guided fine-needle aspiration) after 6 months[11,21], molecular genetic testing, and surgical methods such as diagnostic or therapeutic surgery and histopathological examination[12,22,23].

HYPOTHESIS

Identification of clinical and molecular genetic markers in the preoperative examination makes the determination of the malignancy risk in Bethesda III and Bethesda IV thyroid nodules more accurate.

AIM

The aim of this study was to determine the preoperative clinical and molecular genetic risks of malignancy in thyroid nodules classified as Bethesda III and IV, and to determine their impact on the surgical treatment strategy.

MATERIAL AND METHODS

Prospectively retrospective analysis of 287 patients that underwent surgery at the ENT – Head and Neck Surgery Department of the Na Homolce Hospital by a single thyroid surgeon (J.L.) between 2020 and 2022. Patients received the operation based on preoperative laboratory examination, sonographic risk stratification of thyroid nodule malignancy and cytological evaluation of fine needle aspiration (FNAC). Molecular genetic analysis of patients with cytologically uncertain nodules (Bethesda III and IV) was performed in 49 of the 52 patients having surgery. In two cases, the collected material was not satisfactory and the analysis could not be performed. In the third case, the patient refused molecular genetic testing. Material for molecular diagnostics was initially obtained postoperatively from formalin-fixed paraffin embedded samples (FFPE) (22 patients), and later, preoperatively from FNAC (27 patients). Molecular genetic testing aimed to determine the occurrence of pathogenic variants of genes associated with thyroid oncogenesis. In the FFPE samples, DNA and RNA were isolated using the AllPrep DNA/RNA FFPE Kit (Qiagen), and their concentration was measured using a Quantus fluorometer (Promega). The malignant samples were analyzed for *BRAF*, *HRAS*, *KRAS*, *NRAS* and *TP53* genes using a Nextera XT kit (Illumina) on a MiSeq sequencer (Illumina). Pathogenic variants in the *TERT* gene promoter were examined by allele-specific Real-Time PCR (LC480, Roche). FFPE samples without pathogenic variants were analyzed for 23 fusion genes, including *ALK*, *BRAF*, *GLIS*, *NTRK1*, *NTRK3*, *PPAR* and *RET*, using Real-Time PCR (LC480, Roche). The benign samples were only analyzed for genes that can carry pathogenic variants (*BRAF*, *HRAS*, *KRAS*, *NRAS*) and fusion genes, including *PPARg.*

DNA and RNA were isolated from FNA samples stabilized in an RNA/DNA Shield (ZYMO Research) using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen), and their concentration was measured using a Quantus fluorometer (Promega). Initially, the pathogenic variant V600E in the *BRAF* gene was examined by allele-specific Real-Time PCR (LC480, Roche). BRAF V600E-positive samples were then further analyzed for pathogenic variants in the *TERT* gene promoter by allele-specific Real-Time PCR (LC480, Roche). Samples testing negative for the BRAF V600E variant were analyzed using the NGS (next generation sequencing) Thyro-ID panel (4 bases) targeting 14 genes (*BRAF*, *HRAS*, *KRAS*, *NRAS*, *TERT, PTEN, PIK3CA, TP53, EGFR*, *CDKN2A*, *NOTCH*, *CTNNB1*, *AKT1*, *TSHR*) using a MiSeq sequencer (Illumina). Negative FNA samples were further analyzed for 23 fusion genes, including *ALK*, *BRAF*, *GLIS*, *NTRK1*, *NTRK3*, *PPAR* and *RET,* in Thyro-ID by Real-Time PCR (LC480, Roche).

A molecular genetic examination was performed at the Department of Molecular Endocrinology at the Institute of Endocrinology. Consent for the molecular genetic testing was confirmed by signing the Informed Consent of the Patient, approved by the Na Homolce Hospital Ethics Committee and the Institute of Endocrinology. We evaluated, along with standard demographic data (age, gender), the presence of clinical risk factors for thyroid nodule malignancies, which include positive family history, radiation exposure, growth in size, and/or the number of nodules. Definitive histopathological examinations of the thyroid were performed by two experienced pathologists from Na

Homolce Hospital (M.S.) and the Institute of Pathology from the 1st Faculty of Medicine, Charles University and Faculty General Hospital in Prague (J.D.).

The statistical analysis presents categorical data using absolute frequencies and percentages and compares groups using Fisher's exact test. Continuous data are presented as medians and ranges. The Mann-Whitney U test was used for group comparisons. Results with *P*-values equal to or lower than 0.05 were considered statistically significant. The data was processed using Stata 14.2 (StataCorp LP, College Station, TX, USA).

RESULTS

Clinicopathological analysis of the whole cohort

The prospectively retrospective clinical analysis included 287 patients undergoing surgery with a median age of 54 years (range 18–89) with a prevalence of females (237; 82.6%). Nodular goiter was diagnosed in 265 patients (92.3%), a solid nodule occurred in 120 patients (41.8%) and multinodular goiter (MNG) in 145 (50.5%) of patients receiving the operation. The incidence of carcinoma was significantly higher in nodules less than 20 mm in size (T1) than in nodules larger than 20 mm (31.0% vs. 18.8%, respectively; *P*=0.030). Diffuse goiter was observed in 22 patients (7.6%). Benign postoperative histopathological findings were present in 211 cases (73.5%), low-risk tumors in 12 cases (4.2%) and malignant tumors in 64 cases (22.3%), of which 58 patients (90.6%) had well-differentiated thyroid carcinoma (WDTC). The remaining 6 patients (9.4%) had other thyroid malignancies – sporadic medullary thyroid carcinoma (MTC) in two cases (both were males), metastasis of clear-cell renal cell carcinoma (CCRCC – Grawitz tumor) in two cases (both were females), and in one case diffuse large B-cell lymphoma (DLBCL) and mucoepidermoid thyroid carcinoma.

Clinicopathological analysis of cytologically uncertain categories (Bethesda III and IV)

According to ACR TI-RADS, the categories were as follows: 2 – 2x (3.9%), 3 – 22x (42.3%), 4 – 23x (44.2%), and 5 – 5x (9.6%). Preoperative FNAC of thyroid nodules was performed in 181 patients (63.1%), 52 of whom had indeterminate lesions (28.7%), and among whom Bethesda III occurred in 27 patients (51.9%) and Bethesda IV in 25 patients (48.0%). The median age of these patients was 53 (range 26–79 years), and the female population was 38/52 (73.1%) (Table 1). MNG was detected by ultrasonography in 21 patients (40.4%) with the median size of the dominant nodule at 16 mm (range 3–45 mm). The remaining 31 patients had a solid nodule (SNG) (59.6%) with the median size of the nodule at 20 mm (range 8–75 mm). The results imply that there was no significant difference between Bethesda III and IV categories in the proportion of SNG and MNG (*P*=0.778), or the size of the dominant nodule (*P*=0.276).

All 52 patients underwent surgery – 38 (73.1%) a total thyroidectomy (TTE) and 14 (26.9%) a hemithyroidectomy (HTE) – and a postoperative histopathological examination. All surgeries were successful, without permanent postoperative complications. Malignant tumors were diagnosed in 12 cases (8 papillary thyroid carcinomas – PTC, 3 follicular thyroid carcinoma – FTC and 1 poorly differentiated thyroid carcinoma – PDTC) (23.7%), low-risk tumors in 4 cases (7.7%) (2 follicular tumors of uncertain malignant potential – FT-UMP, 1 non-invasive follicular thyroid neoplasm with papillary-like nuclear features – NIFTP, and 1 hyalinizing trabecular tumor – HTT). 36 patients had benign findings (69.2%) (Table 1). In the incidence of malignancy, there were no significant differences between Bethesda III and IV categories (*P*=0.517), and neither differed in the types of the detected tumours (*P*=0.735).

Clinical risk factors for malignancy were identified preoperatively in 32 patients (61.5%). 11 patients had a positive family history (thyreopathy, thyroid carcinoma – TC), and three of them were diagnosed with PTC and/or FTC variants. Four patients had a personal history of radiation exposure – one patient was diagnosed with widely-invasive FTC, the second with papillary thyroid microcarcinoma (PTMC), and in the other two patients, no malignancy was confirmed. Growth in size and/or number of nodules was observed in 17 patients, six of whom were confirmed by histopathological examination to have TC (1 FTC together with FT-UMP, 1 PDTC, 1 FTC and 3 PTC). In patients with at least one of the studied clinical risk factors in their personal history, the incidence of malignancy was 31.3%, while patients without preoperative clinical risk factors had a 10.0% incidence of malignancy. However, the difference was not statistically significant (*P*=0.099).

Molecular genetic analysis

Pathogenic variants with genes associated with thyroid oncogenesis were detected in 12 out of 49 patients (24.5%), of which 4 patients (33.3%) were diagnosed with thyroid carcinoma. Of the 27 FNAC samples defined as Bethesda III and IV, pathogenic variants were found in 6 cases (22.2%), which were then examined histopathologically – 1 *BRAF* (PTC), 1 *BRAF+TERT* (PTC), 2 *NRAS* (NIFTP, FTC), *HRAS* (benign) and *PTEN* (benign). Of the 22 FFPE samples defined as Bethesda III and IV, pathogenic variants were found in 6 cases (27.3%) – 1 *BRAF* (PTC), 1 *KRAS* (benign), 2 *NRAS* (benign), 1 *HRAS* (benign) and 1 *PAX8/GLIS3* (HTT).

In 37 patients with Bethesda III and IV, no pathogenic variants were detected in the analyzed genes (Table 2). Malignancy was found in 6 (16.2%) of these 37 patients – 4 PTMC, 1 PTC and 1 FTC together with FT UMP (follicular tumour of uncertain malignant potential). 29 (78.4%) patients had benign lesions and two cases were diagnosed with a low-risk tumour (FT UMP). The number of malignant tumours was significantly higher in patients with detected pathogenic variants than in patients without them (33.3% vs. 16.2%, respectively), but the difference was not statistically significant (*P*=0.233). Two of

Table 1. Clinical and pathological findings in patients with Bethesda III/IV.

TBSRTC	Gender	Age	Nodule type and size		Sampling method	No. of pathogenic variants	Surgery	Histopathological examination								
	M/F	median (age)	SNG/ MNG (n)	SNG/MNG (median, mm)	FNA/FFPE	*BRAF*, *RAS*, *TERT* and fusion genes	TTE/HTE (n)	Benign (n)			A low-risk tumour (n)			Malignant (n)		
								NCG	AIT	FA	FT UMP	NIFTP	HTT	PTC	FTC	PDTC
AUS/F LUS (n=27)	8/19 (30%/70%)	54	17/10 (63%/37%)	25/18	13/12 (52%/48%)	7 (28%)	22/5 (81%/19%)	13	4	2	1	1	1	3	1	1
SFN/FN (n=25)	6/19 (24%/76%)	49	14/11 (56%/44%)	20/15	14/10 (58%/42%)	5 (21%)	16/9 (64%/36%)	9	4	4	1	0	0	5	2	0
TOTAL	14/38 (27%/73%)	53	31/21 (60%/40%)	20/16	27/22 (55%/45%)	12 (24%)	38/14 (73%/27%)	22	8	6	2	1	1	8	3	1
P†	0.759	0.627	0.778	0.276	0.776	0.742	0.214	0.462			1.000			0.735		

†*P* value to compare groups of patients with Bethesda III and Bethesda IV.
M, male; F, female; TBSRTC, The Bethesda System for Reporting Thyroid Cytopathology; AUS/FLUS, atypia of undetermined significance/follicular lesions of undetermined significance; FN/SFN, follicular neoplasm/suspicious for neoplasm; SNG, solitary nodular goiter; MNG, multinodular goiter; FNA, fine needle aspiration (preoperative examination); FFPE, formalin-fixed paraffin embedded samples (postoperative examination), Benign tumours; NCG, nodular colloid goiter; AIT, autoimmune thyroiditis; FA, follicular adenoma, Low-risk tumours – borderline thyroid tumours; FT-UMP, follicular tumour of uncertain malignant potential; NIFTP, noninvasive follicular thyroid neoplasm with papillary-like nuclear features; HTT, hyalinizing trabecular tumour, Malignant tumours: PTC-papillary thyroid carcinoma; FTC, follicular thyroid carcinoma; PDTC, poorly differentiated thyroid carcinoma.

Table 2. Comparison of patients with Bethesda III/IV based on the presence of pathogenic variants of genes associated with thyroid oncogenesis.

Group	TBRSC	Gender	Age	Nodule type and size		Sampling method	Surgery	Histopathological examination								
	III/IV	M/F	median (years)	SNG/ MNG (n)	SNG/MNG (median, mm)	FNAB/FFPE	TTE/HTE	Benign			Low-risk tumour			Malignant		
								NCG	AIT	FA	FT UMP	NIFTP	HTT	PTC	FTC	PDTC
With pathogenic variant (n=12)	7/5 (58%/42%)	4/8 (33%/67%)	43.5	9/3 (75%/25%)	25/31	6/6 (50%/50%)	8/4 (67%/33%)	3	1	2	0	1	1	3	1	0
Without pathogenic variant (n=37)	18/19 (49%/51%)	9/28 (24%/76%)	53	21/16 (57%/43%)	20/14,5	21/16 (57%/43%)	27/10 (73%/27%)	18	7	4	2	0	0	5	1	0
Gen. analysis. Not performed (n=3)	2/1	1/2	54	1/2	8/14	–	3/0	1	0	0	0	0	0	0	1	1
P-value†	0.742	0.708	0.306	0.323	0.034	0.747	0.721	0.482			0.333			1.000		

†*P* value to compare groups of patients with and without the pathogenic variant.
M, male; F, female; TBSRTC, The Bethesda System for Reporting Thyroid Cytopathology; SNG, solitary nodular goiter; MNG, multinodular goiter; FNA, fine needle aspiration (preoperative examination); FFPE, formalin-fixed paraffin embedded samples (postoperative examination), Benign tumours; NCG, nodular colloid goiter; AIT, autoimmune thyroiditis; FA, follicular adenoma, Low-risk tumours – borderline thyroid tumours; FT-UMP, follicular tumour of uncertain malignant potential; NIFTP, noninvasive follicular thyroid neoplasm with papillary-like nuclear features; HTT, hyalinizing trabecular tumour, Malignant tumours: PTC-papillary thyroid carcinoma; FTC, follicular thyroid carcinoma; PDTC, poorly differentiated thyroid carcinoma.

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the three patients who did not undergo molecular genetic testing were diagnosed with malignant tumours (PDTC and FTC) (Table 2).

DISCUSSION

In Bethesda III/IV thyroid nodules, it is not possible to exclude malignancy based on preoperative USG and cytological examination[4,5,7]. Furthermore, the classification of indeterminate nodules in the Bethesda system is, to a certain degree, biased by the subjectivity of the evaluators[3,10,12]. FNA – obtained material and its parallel use in the detection of genetic markers has become an increasingly important malignancy risk stratification[3,20,21,24]. In the case of preoperative findings of pathogenic variants of BRAF V600E, in the *TERT* gene promoter or *RET/PTC* fusion genes, TTE is recommended in nodules exceeding 1 cm due to the 100% risk of malignancy[24]. Fusion genes with *RET* and *NTRK1/3* genes are often associated with the occurrence of lymph node metastases[25]. Some pathogenic variants also have prognostic and therapeutic significance[26]. In PTC, the coexistence of BRAF V600E and TERT mutations has a strong synergistic effect on disease recurrence and mortality (22.7%), regardless of the clinical risk factors for malignancy[27]. In some pathogenic variants, tyrosine kinase inhibitors (e. g. larotrectinib in *NTRK* fusion genes and selpercatinib in *RET* fusion genes) may be used for the treatment of progressive radioiodine-refractory cancers[28].

In contrast, pathogenic variants in *RAS* genes are associated with a lower risk of malignancy, as they can occur in all types of thyroid cancers. They can be detected in benign tumours, low-risk tumours, PTC (mainly its follicular variant), FTC, sporadic RET-negative medullary carcinoma and aggressive poorly differentiated or anaplastic carcinoma. Therefore, their clinical significance remains unclear. Pathogenic variants in *RAS* genes are thought to be an early transformation, and in benign tumours, they may be predisposed to progression to carcinoma[25,28,29]. The European Thyroid Association (ETA) recommends lobectomy if pathogenic variants in *RAS* genes are detected, especially in the case of unifocal nodules[24,29,30]. However, TTE is a justified surgical procedure in case of the presence of bilateral thyroid nodules, an enlarged contralateral lobe with a large nodule, positive family history of thyroid carcinoma, radiation exposure, and in patients who do not agree to a second surgery, i. e. removal of the contralateral lobe in multifocal and/or extra-thyroidal carcinomas[8,15,16]. Almquist and other authors suggest that experienced thyroid surgeons guarantee that there is little risk of postoperative complications in TTE (ref.[12,29]). Low-risk tumours (NIFTP, follicular tumours with unclear malignant potential – FT UMP, WDT UMP and hyalinizing trabecular tumour – HTT) have a low risk of malignancy and are not considered carcinomas, but neoplasms[4,24,27]. However, suspicion regarding these tumours requires surgical intervention (hemithyroidectomy), and a detailed histopathological examination is necessary to establish a definitive diagnosis, which is very difficult at the cytological level[4,23]. These tumours are very often cytologically classified as indeterminate – Bethesda III (31%) or Bethesda IV (26.6 %) (ref.[4,27]). Our cohort included three indeterminate cases in the category Bethesda III and one Bethesda IV. Along with *RAS* mutations, other non-specific pathogenic variants can be observed in low-risk tumours, such as *BRAF K601E* mutation, *PAX8/PPARγ* fusion genes, and the *THADA* fusion gene in NIFTP (ref.[4,21,28]). In low-risk tumours, the only specific genetic marker is the *PAX8/GLIS* fusion gene in HTT (ref.[31]).

CONCLUSION

In our cohort, malignant tumours were diagnosed in 23.1% of the cases of Bethesda III and Bethesda IV. We found no significant difference in the incidence of malignancy between these two categories. The incidence of carcinomas was significantly higher in nodules smaller than 20 mm than in nodules greater than 20 mm. During the course of the work on this cohort, we gradually shifted from the postoperative genetic examination of FFPE material to preoperative FNA. In some cases, pathogenic variants were detected, an indication leading to TTE. Thus, total thyroidectomy may be an appropriate treatment option even for thyroid nodules classified within the indeterminate Bethesda III and IV categories, and advances in molecular genetic diagnostic will help to refine further treatment for these categories. Our experience implies that molecular genetic testing is one of many decision factors, however, our cohort of patients with indeterminate lesions is relatively small. There is also a lack of long-term follow-up data for these patients, so we will continue to monitor and enlarge our patient cohort.

ABBREVIATIONS

AUS, atypia of undetermined significance; CCRCC, clear cell renal cell carcinoma; DLBCL, diffuse large B-cell lymphoma; FDG, fluoro-deoxy-ribose; FLUS, follicular lesion of undetermined significance; FN, follicular neoplasm; FNA, fine needle aspiration; FNAC, fine needle aspiration cytology; FT-UMP, follicular tumour of uncertain malignant potential; HTT, hyalinizing trabecular tumour; MNG, multinodular goitre; MTC, medullary thyroid carcinoma; NCG, nodular colloid goitre; NGS, Next Generation Sequencing; NIFTP, non-invasive follicular tumour with papillary-like nuclear features; NPV, negative predictive value; PDTC, poorly differentiated thyroid carcinoma; PPV, positive predictive value; PTC, papillary thyroid carcinoma; PTMC, papillary thyroid microcarcinoma; SFN, suspicious for a follicular neoplasm; TI-RADS, Thyroid Imaging Reporting and Data System; USG, ultrasonography; WDTC, well-differentiated thyroid carcinoma.

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