Preoperative detection of malignancy in fine-needle aspiration cytology (FNAC) smears with indeterminate cytology (Bethesda III, IV) by a combined molecular classifier

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Received 15 January 2020
Revised 18 February 2020
Accepted 1 March 2020
Published Online First 25 March 2020

To cite: Titov S, Demenkov PS, Lukyanov SA, *et al*. *J Clin Pathol* 2020;**73**:722–727.

ABSTRACT

Aims Analysis of molecular markers in addition to cytological analysis of fine-needle aspiration (FNA) samples is a promising way to improve the preoperative diagnosis of thyroid nodules. Previously, we have developed an algorithm for the differential diagnosis of thyroid nodules by means of a small set of molecular markers. Here, we aimed to validate this approach using FNA cytology samples of Bethesda categories III and IV, in which preoperative detection of malignancy by cytological analysis is impossible.

Methods A total of 122 FNA smears from patients with indeterminate cytology (Bethesda III: 13 patients, Bethesda IV: 109 patients) were analysed by real-time PCR regarding the preselected set of molecular markers (the *BRAF* V600E mutation, normalised concentrations of *HMGA2* mRNA, 3 microRNAs, and the mitochondrial/nuclear DNA ratio). The decision tree–based classifier was used to discriminate between benign and malignant tumours.

Results The molecular testing detected malignancy in FNA smears of indeterminate cytology with 89.2% sensitivity, 84.6% positive predictive value, 92.9% specificity and 95.2% negative predictive value; these characteristics are comparable with those of more complicated commercial tests. Residual risk of malignancy for the thyroid nodules that were shown to be benign by this molecular method did not exceed the reported risk of malignancy for Bethesda II histological diagnosis. Analytical-accuracy assessment revealed required nucleic-acid input of ≥5 ng.

Conclusions The study shows feasibility of preoperative differential diagnosis of thyroid nodules of indeterminate cytology using a small panel of molecular markers of different types by a simple PCR-based method using stained FNA smears.

INTRODUCTION

Of all the diagnostic methods for thyroid nodules, fine-needle aspiration cytology (FNAC) is the one used most widely. In a meta-analysis of more than 25,000 FNAC samples of the thyroid gland, for 25% of which the subsequent pathology report was available, the average diagnostic sensitivity of the method was 97%, specificity ~50%, diagnostic accuracy ~69%, the predictive value of a positive result (PPV) ~56% and the predictive value of a negative result (NPV) was ~96%.[1]

Currently, there are several classification systems for cytological diagnoses: British Thyroid Association/Royal College of Pathologists,[2] the Bethesda System for Reporting Thyroid Cytopathology[3] and Italian Consensus for the Classification and Reporting of Thyroid Cytology (Italian AME Consensus).[4] In all cases, these diagnoses are divided into five main categories: nondiagnostic (uninformative), benign, indeterminate, suspected malignancy and malignant. Indeterminate cytology results are obtained in 10%–35% of the cases.

The risk of malignancy (ROM) in nodules with indeterminate cytology varies from 5% to 75% (typically 15%–30%) depending on the classification system.[2 5] Besides, the ROM in nodules with an uncertain cytology result varies significantly among clinical settings. In particular, this state of affairs is characteristic of nodules with a preoperative diagnosis of 'atypia of undetermined significance' (Bethesda category III; ROM of 5%–28%) or 'follicular neoplasm/suspected follicular tumor' (Bethesda category IV; ROM of 15%–40%).[1 3 6 7]

Thus, the uncertain category includes a heterogeneous group in which it is impossible to classify the thyroid lesion as benign or malignant on the basis of cytomorphological characteristics.[3] According to pathology reports, these samples most often correspond to adenomatoid hyperplasia or follicular adenoma (FTA) and less often to carcinoma, Hürthle cell tumours, the follicular variant of papillary thyroid cancer (FVPTC) and others.[2 4]

According to clinical recommendations, most patients with indeterminate cytology (including all those belonging to the Bethesda IV category) are referred to diagnostic surgery or molecular testing.[3] Meanwhile, postoperative histological examination shows that 70%–80% of thyroid nodules turn out to be benign, and the surgical procedure appears to be unnecessary.[2 8] The possible postoperative complications and postoperative hypothyroidism, which requires lifelong hormone replacement therapy, significantly reduce quality of life.[9] Accordingly, a good way to categorise samples with uncertain cytology into benign and malignant groups will reduce the number of surgical interventions and the consequent risk of complications.

To overcome the limitations of cytological analysis, several molecular tests for preoperative diagnosis of thyroid nodules were developed in recent

years. Some of these tests involve detection of somatic point substitutions (eg, in genes *BRAF* and *RAS*) and/or translocations (eg, *RET-PTC*, *PAX8-PPARγ*).[10 11] Other approaches involve profiling of protein-coding genes[12] or microRNAs (miRNAs)[13 14] or combine analyses of somatic mutations, mRNA and miRNA levels.[15 16]

At present, all existing solutions for the molecular diagnosis of thyroid nodules have various limitations. Most of the existing tests require a separate biopsy, which does not allow cytological and molecular analysis of the same specimen. Most tests are limited by the analysis of only one molecular marker type, which may be insufficient to achieve high negative or positive predictive values of the test.[17] Finally, modern molecular tests do not allow the typing of malignant thyroid tumours (only Rosetta GX Reveal separately identifies medullary carcinoma).

In our recent work, we described our version of this diagnostic test; this version enables the detection and typing of malignant thyroid tumours via analysis of a small number of molecular markers in FNAC preparations (levels of HMGA2 mRNA and miR-375, -221 and -146b in combination with the mitochondrial-to-nuclear DNA ratio).[18] The results described in this work were obtained from samples belonging to Bethesda categories II and VI. This work was aimed at assessing diagnostic characteristics of the previously developed method on a group of samples of dried FNAC smears of Bethesda categories III and IV (indeterminate cytology): the most relevant ones for molecular testing during the management of patients with thyroid nodules.

MATERIALS AND METHODS

In this study, 122 cytological samples obtained by fine-needle aspiration (FNA) biopsy of thyroid nodules were used. The samples were obtained from Regional Clinical Hospital No. 2 (Krasnodar) (45 samples) and the South Ural State Medical University, Department of General and Paediatric Surgery (Chelyabinsk) (77 samples). The cytological material was obtained in compliance with the laws and regulations of the Russian Federation, and written informed consent was obtained from each patient for the research use of the samples. All the data were depersonalised.

The cytology smears were air-dried without fixation and then stained with May-Grunwald-Giemsa. Only samples belonging to Bethesda categories III and IV and associated with a subsequent pathology report were included in this study. Histological examination was carried out by pathologists of the corresponding institution. Twenty-seven specimens were collected from males aged 50.8±14.7 years (mean±SD), and 95 specimens from females aged 48.6±14.3 years. Paediatric patients were not included in our study population. The distribution of tumour types was as follows: non-neoplastic lesions (benign nodule, BN), 24 samples; FTA, 61 samples; follicular carcinoma (FTC), 10 samples; Hürthle cell carcinoma (HTC), 3 samples; papillary thyroid carcinoma (PTC), 17 samples; and the FVPTC, 7 samples.

The classification method

The classification method described by Titov *et al*[18] was used with a minor modification (see figure 1). During the validation of the method by means of the expanded group of cytological preparations, benign neoplasms with increased miR-146b content (without other markers of malignancy) were revealed, which were apparently associated with lymphocytic infiltration. To exclude erroneously identified malignancy, the criterion 'increased miR-146b content' in the decision tree was replaced by 'the presence of the V600E mutation in the *BRAF* gene'. The introduced modification did not worsen the diagnostic characteristics when a training sample (from the previous study) of FNAC smears of Bethesda categories II–VI was analysed (n=494).

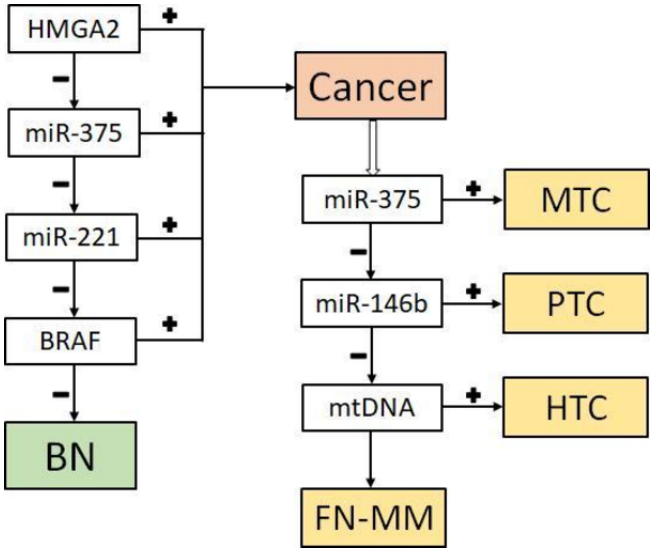

Figure 1 The decision tree for classifying samples into benign and malignant followed by cancer typing. (+) means exceeding the established cut-off or identifying the *BRAF* V600E mutation. BN, benign nodule; FN-MM, follicular neoplasms with markers of malignancy; HTC, Hürthle cell thyroid carcinoma; MTC, medullary thyroid carcinoma; PTC, papillary thyroid carcinoma.

Thus, the proposed classifier subdivides all cytological samples into the following groups: (a) benign, including goitres and follicular neoplasms with no markers of malignancy, (b) malignant, which, in turn, are subdivided into PTC, medullary thyroid carcinoma, HTC, and follicular neoplasms with markers of malignancy.

Anaplastic and poorly differentiated carcinomas are not categorised by this classifier as separate types and are identified simply as 'malignant'.

Total nucleic-acid extraction

The nucleic acids were extracted from FNAC preparations as described by Titov *et al*[19] : the dried cytological preparation was washed into a microcentrifuge tube with three 200 µl portions of guanidine lysis buffer. The sample was vigorously mixed and incubated in a thermal shaker for 15 min at 65 °C. Next, an equal volume of isopropanol was added. The reaction solution was thoroughly mixed and kept at room temperature for 5 min. After centrifugation for 10 min at 14000×*g*, the supernatant was discarded, and the pellet was washed with 500 µl of 70% ethanol and 300 µl of acetone. Finally, the RNA was dissolved in 200 µl of deionised water. If not analysed immediately, RNA samples were stored at −20°C until further use. The concentration of total RNA in each sample was measured on a Qubit fluorimeter (Invitrogen, USA) with the Qubit RNA HS Assay Kit. The RNA concentrations were in the range of 3.3–47.4 ng/µl (15.2 ng/µl on average).

Determination of the amount of RNA sufficient for reliable testing

To this end, 14 extracted RNA samples from FNAC smears with known histological diagnosis were diluted with deionised water prior to reverse-transcription real-time PCR so that 10, 5, 1 or

Table 1 Molecular-testing results on samples with different amounts of input RNA

		Starting input RNA amount	Input RNA amount in diluted samples			
#	Histological diagnosis	11–30 ng	10 ng	5 ng	1 ng	0.5 ng
1	Incapsulated PTC	PTC	PTC	PTC	PTC	PTC
2	PTC	PTC	PTC	PTC	PTC	PTC
3	PTC	PTC	PTC	PTC	PTC	PTC
4	PTC	PTC	PTC	PTC	PTC	PTC
5	Hashimoto's thyroiditis	BN	BN	BN	**N/A**	**N/A**
6	Hashimoto's thyroiditis	BN	BN	BN	BN	BN
7	Hashimoto's thyroiditis	BN	BN	BN	BN	BN
8	Goitre	BN	BN	BN	BN	BN
9	FTA+Hashimoto's thyroiditis	BN	BN	BN	BN	BN
10	FTA	BN	BN	BN	**FN-MM**	BN
11	FTA	BN	BN	BN	**FN-MM**	BN
12	HTC	HTC	HTC	HTC	HTC	**N/A**
13	FTC	FN-MM	FN-MM	FN-MM	FN-MM	FN-MM
14	FTC	FN-MM	FN-MM	FN-MM	**PTC**	**PTC**

Discordant or invalid results are boldfaced.
BN, benign nodule; FN-MM, follicular neoplasms with markers of malignancy; FTA, follicular adenoma; FTC, follicular carcinoma; HTC, Hürthle cell carcinoma; PTC, papillary thyroid carcinoma.

0.5 ng was added into the reverse-transcription reaction during the analysis of each RNA marker.

Molecular analysis

Assessment of relative levels of *HMGA2* gene expression (normalised to household gene *PGK1*), miRNA-146b, -221 and -375 levels (normalised to the geometric mean content of miRNAs 29b, 23a and 197) and the ratio of mitochondrial to nuclear DNA as well as determination of somatic mutation V600E in the *BRAF* gene were carried out as described earlier.[18]

Data analysis

The data processing was conducted in Excel (Microsoft, USA) or Statistica V.9.1 (StatSoft Inc, USA). Diagnostic characteristics were determined using standard 2×2 contingency tables comparing qualitative, binary molecular test results (positive or negative) relative to the reference standard diagnoses determined by pathology (benign or malignant).

RESULTS

Test results of quantitation of RNA in the reaction

To determine the amount of RNA necessary for this test, 14 FNA smears were subjected to the extraction procedure and were processed with four different initial RNA amounts for the cDNA synthesis reaction. The classification results of undiluted and diluted samples are shown in table 1.

According to the table, when an initial amount of RNA added into the reverse-transcription reaction was ≥5 ng, the classification results did not change with the dilution for the samples of carcinomas, benign tumours and goitres. Therefore, the initial amount of RNA that is necessary for reliable results of the proposed method is 5 ng. All the samples analysed in this work met this criterion. Different RNA extraction methods and storage conditions may lead to various degrees of nucleic-acid degradation (and as a consequence, to a loss of target copies available for amplification). Therefore, C_t obtained for some reference RNA marker can serve as a more reliable criterion for the sample's suitability for analysis. For the proposed method, the criterion of an acceptable amount of RNA was C_t for *PGK1* mRNA not exceeding 33.

Molecular diagnosis of thyroid nodules with indeterminate cytology

The group of cytological preparations with indeterminate cytology results consisted of 122 nodules with a cytological diagnosis of Bethesda III (atypia of undetermined significance) or Bethesda IV (follicular neoplasm/suspected follicular tumour), for each of which the eventual pathology report was available (table 2). Eighty-five samples were classified as benign, including 61 samples classified as FTAs. Out of 37 samples of carcinomas, 7 were classified as the FVPTC, 17 as PTC, 10 as FTC, and 3 samples were assumed to be HTC (cancer prevalence was 30.3%).

Therefore, according to postoperative histological analysis, 69.7% of the samples with indeterminate cytology were benign and 30.3% were malignant. No significant difference in ROM between the Bethesda III and Bethesda IV groups was observed: 4 malignant neoplasms (30.8%) in the Bethesda III group and 33 malignant neoplasms (30.3%) in the Bethesda IV group.

Table 2 Distribution of histological findings among FNAC smears with indeterminate cytology

Histological diagnosis	Bethesda III, n (%)	Bethesda IV, n (%)	Bethesda III+Bethesda IV, n (%)
Benign samples, total number	9 (69.2)	76 (69.7)	85 (69.7)
BN	2 (15.4)	22 (20.2)	24 (19.7)
FTA	7 (53.8)	54 (49.5)	61 (50.0)
Malignant samples, total number	4 (30.8)	33 (30.3)	37 (30.3)
FTC	0	10 (9.2)	10 (8.2)
FVPTC	2 (15.4)	5 (4.6)	7 (5.7)
PTC	2 (15.4)	15 (13.8)	17 (13.9)
HTC	0	3 (2.8)	3 (2.5)
Total	13	109	122

BN, benign nodule; FNAC, fine-needle aspiration cytology; FTA, follicular adenoma; FTC, follicular carcinoma; FVPTC, follicular variant of papillary thyroid cancer; HTC, Hürthle cell carcinoma; PTC, papillary thyroid carcinoma.

Table 3 Molecular versus histological classification of thyroid nodules

Molecular testing results	Histological type						
	BN	FTA	FTC	FVPTC	PTC	MTC	HTC
BN	23	56	**2**	0	**2**	0	0
PTC	**1**	**2**	2	4	12	0	0
FN-MM	0	**3**	5	3	3	0	0
MTC	0	0	1	0	0	0	0
HTC	0	0	0	0	0	0	3

Mismatched results are boldfaced.
BN, benign nodule; FN-MM, follicular neoplasms with markers of malignancy; FTA, follicular adenoma; FTC, follicular carcinoma; FVPTC, follicular variant of papillary thyroid cancer; HTC, Hürthle cell carcinoma; MTC, medullary thyroid carcinoma; PTC, papillary thyroid carcinoma.

Table 3 represents stratification of the FNAC smears using our molecular classifier. Thirty-three out of 37 samples with indeterminate cytology that were classified as malignant by histology reports were also classified as malignant by the molecular testing, that is, cancer was detected with 89.2% sensitivity. Two false negatives were histologically diagnosed as PTCs, and the two other false negatives as FTCs. Out of 85 histologically benign samples, 79 were categorised as benign by the molecular classifier, that is, the accuracy of identification of benign nodes was 92.9%. Of the 6 samples that were assumed to be false positive according to the molecular diagnosis, 5 were histological FTA and 1 was BN.

The calculated diagnostic characteristics for Bethesda III and Bethesda IV groups of samples are summarised in table 4 together with the characteristics calculated earlier for Bethesda II–VI samples via the same method.[18]

DISCUSSION

The impossibility of detection of malignancy in thyroid nodules of indeterminate cytology necessitates additional methods for preoperative diagnosis. Recent studies suggest that molecular markers can be successfully applied to address this issue. In the case of preoperative diagnosis, for the detection of malignancy, NPV of a test is apparently more important than PPV. For thyroid nodules that are considered benign after molecular testing, it is also desirable that the residual ROM does not exceed the ROM for the Bethesda II histological diagnosis (3%–6%).[3] It is this indicator that will determine the relative safety of clinical observation as compared with a surgical operation. In our study, for Bethesda III and IV samples characterised by the molecular test as benign, the ROM was 4.8%. Taking into account the cancer prevalence (~30% for each of these categories) in the tested group of 122 FNAC preparations, 79 patients could have avoided an unnecessary surgical intervention if the decision about the operation had been based on the results of molecular testing. This finding corresponds to a potential ~14 fold decrease in the number of unnecessary operations. In addition, four false negative results were obtained for samples belonging to Bethesda IV. Although with our test these patients would not have been referred immediately to surgery, they would have been kept under observation and would have undergone the operation later if needed.

There is currently no consensus on the minimum acceptable PPV for preoperative identification of malignancy. Patients with indeterminate cytology will be assigned to repeated biopsy or surgery anyway, that is, the priority is to reduce the number of unnecessary operations. This observation implies that, as mentioned earlier, the additional diagnostic methods are characterised by stricter requirements for NPV, whereas the requirements for the test's PPV are not so stringent yet. The possibility of more aggressive surgical interventions (thyroidectomy instead of lobectomy) is being discussed in relation to patients with some identified molecular markers of malignancy, especially oncogenic mutations.[20] Nonetheless, proving the clinical validity of this approach requires additional research.

Of course, readers must keep in mind that PPV and NPV depend not only on sensitivity and specificity of a test but also on cancer prevalence. The cancer prevalence in our study may be influenced by sample selection bias; besides, cancer prevalence in Bethesda categories III and IV varies among different institutions. To evaluate these biases, we calculated PPV and NPV with different pretest cancer probability using Bayes' theorem and the sensitivity and specificity observed in this study (figure 2). It turned out that within the most probable range of thyroid cancer prevalence (20%–40%), NPV can vary from 97% to 93%, and PPV from 76% to 89%.

In our previous work,[18] the sensitivity and PPV of malignancy detection were slightly better (see table 4). This discrepancy can be attributed to the following: (1) these diagnostic characteristics were calculated for the training sample (ie, this is the maximum possible) and 2) the other study mainly involved Bethesda II and VI samples, with predominance of PTC.

Table 4 Diagnostic characteristics of the molecular classifier for malignant tumours (including a 95% CI) in different categories of cytological preparations

	Bethesda III–IV (n=122)	Bethesda II–VI (n=494)
Specificity	92.9% (85.3%–97.4%)	97.6% (94.4%–99.2%)
Sensitivity	89.2% (74.6%–97.0%)	94.1% (90.7%–96.5%)
Accuracy	91.8% (85.4%–96.0%)	95.5% (93.3%–97.2%)
PPV	84.6% (71.6%–92.3%)	98.2% (95.8%–99.2%)
NPV	95.2% (88.6%–98.0%)	92.2% (88.2%–94.9%)

Consequently, the molecular classifier makes it possible to fairly and accurately identify malignancy in Bethesda III and IV samples. The residual ROM was 4.8% and 84.6% for the samples designated by the molecular testing as benign and malignant, respectively (table 6).
.NPV, predictive value of a negative result; PPV, predictive value of a positive result; ROM, risk of malignancy.

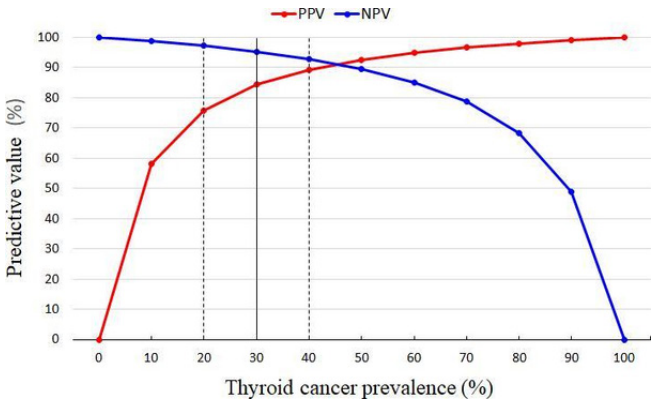

Figure 2 Expected PPV and NPV according to the sensitivity and specificity observed in this study and thyroid cancer prevalence in a given test population. The solid vertical line denotes the cancer prevalence observed in this study. The dashed vertical lines indicate the most likely range of cancer prevalence. NPV, predictive value of a negative result; PPV, predictive value of a positive result.

Table 5 Diagnostic characteristics of various molecular tests for confirmation of malignancy in cases of indeterminate cytology (Bethesda III and IV)

	Specificity, %	Sensitivity, %	PPV, %	NPV, %
Afirma-GEC	52–53	90	37–38	94–95
ThyroSeq v2	92–93	89–90	78–83	96
ThyGenX/ThyraMIR	80–85	89–94	68–74	94–97
RosettaGX Reveal	74	74–85	43–59	92
This study	93	89	85	95

NPV, predictive value of a negative result; PPV, predictive value of a positive result.

Table 6 Residual ROM among FNAC samples for different results of molecular testing

Molecular testing result	Histologically benign, n	Histologically malignant, n	ROM, %
BN	79	4	**4.8**
PTC	3	18	85.7
FN-MM	3	11	78.6
MTC	0	1	100
HTC	0	3	100
All malignant	6	33	**84.6**

BN, benign nodule; FNAC, fine-needle aspiration cytology; FN-MM, follicular neoplasms with markers of malignancy; HTC, Hürthle cell carcinoma; MTC, medullary thyroid carcinoma; PPV, papillary thyroid carcinoma; PTC, papillary thyroid carcinoma; ROM, risk of malignancy.

To date, several diagnostic molecular tests have already been developed: 4 of them are characterised the most: Afirma-GEC (Veracyte, USA), ThyroSeq v2 (CBLPath, USA), ThyGenX/ThyraMIR (Interpace Diagnostic, USA) and RosettaGX Reveal (Rosetta Genomics, USA). These data are summarised in table 5. As readers can see, the sensitivity of cancer detection in samples with indeterminate cytology is 90%–95% for Afirma-GEC, ThyroSeq v2 and ThyGenX/ThyraMIR and 74%–85% for RosettaGX Reveal. The specificity of ThyroSeq v2 and ThyGenX/ThyraMIR is 92% and 80%–85%, respectively, which is higher than that of RosettaGX Reveal (74%) and Afirma-GEC (53%). NPV does not differ significantly among the four tests and ranges from 92% to 96%. PPV values of ThyroSeq v2 and ThyGenX/ThyraMIR are comparable (74%–78%), unlike those of Afirma-GEC and RosettaGX Reveal, where this parameter is 37% and 43%–59%, respectively.[14 17 21 22]

It is noteworthy that the tests with the lowest specificity and PPV are based on mRNA (Afirma-GEC) or miRNA (RosettaGX Reveal) quantitation, whereas the highest values of these parameters belong to the test that identifies a large number of mutations and translocations by next-generation sequencing (ThyroSeq v2). On the other hand, the assay based on simultaneous analysis of several mutations and miRNA levels (ThyGenX/ThyraMIR) yields an intermediate result. Even though our test also quantifies several types of molecular markers, it yielded results similar to those of ThyroSeq. Thus, in this study, the relatively simple molecular test (quantifying several miRNAs, *HMGA2* mRNA, and the mtDNA/nuclear DNA ratio and detecting the *BRAF* V600E somatic mutation) allowed us to identify malignancy in FNAC samples of Bethesda III and IV categories as well as the type of malignant tumour, with acceptable diagnostic characteristics.

Some limitations of our study should be emphasised. First, sample size was relatively small; second, the study population did not include medullary and anaplastic cancers, which rarely fall into Bethesda III and IV categories; third, the study was confined to only two institutions. The limited sample size did not let us estimate the accuracy of tumour typing owing to the insufficient numbers of samples of each type of malignant tumour. An exception was the PTC group, where the typing accuracy was found to be 76.2%. Prospective studies are needed to determine the diagnostic capabilities of the proposed methodology.

Thus, in this work, we demonstrate the feasibility of malignancy detection in FNAC smears of Bethesda III and IV categories by a simple real-time-PCR-based technique combining assays of several molecular markers.

Key messages

- In our recent work, we proposed a diagnostic test that enables the detection of malignant thyroid tumours. In this study, we validated this approach using fine-needle aspiration cytology samples of Bethesda categories III and IV.
- Our test, based on the analysis of several types of molecular markers (miRNAs, mRNA, the mtDNA/nuclear DNA ratio, and BRAF V600E mutation) has a predictive value of a positive result of 84.6% and a predictive value of a negative result of 95.2%, which are comparable with those of more complicated commercial tests.
- The study shows the feasibility of preoperative diagnosis of thyroid nodules using a panel of different types of molecular markers.

Handling editor Runjan Chetty.

Acknowledgements The English language was corrected and certified by shevchuk-editing.com.

Contributors ST and MKI were involved in the study conception; ST and YAV conceived and designed the experiments; SAL, SVS and GAK collected the samples from patients; YAV conducted the experiments; ST and PSD participated in the data analysis and interpretation; ST and SAL wrote the manuscript; MKI, PSD and SVS revised the manuscript.

Funding The work was supported by Russian state budget projects No. 0324-2019-0040-C-01 and 0310-2019-0013. The funding body was not involved in the study design, data acquisition, interpretation of the results or writing of the manuscript.

Competing interests None declared.

Patient consent for publication Not required.

Ethics approval The study protocol was approved by the ethics committee of the South Ural State Medical University.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information.

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