

Preoperative Diagnostics of Papillary Thyroid Carcinoma by Molecular Analysis of FNAB Materials

Abstract. Papillary thyroid carcinoma (PTC) is the most common type of thyroid cancer. Molecular markers of papillary thyroid carcinoma (PTC) including the BRAF1799A mutation, RET/PTC1,3 rearrangements, and differentially expressed SFTP (up-regulated) and TFF3 (down-regulated) genes were prospectively assessed in FNAB (fine needle aspiration biopsy) material from thyroid nodules.

We examined 73 cases, of them 59 PTC (papillary thyroid carcinoma), five nodular goiters, and nine follicular adenomas.

Concordantly with cytology, molecular diagnosis of PTC by SFTP and TFF3 expression levels was confirmed in 38/41 cases (92.7%); in them, 22/41 (53.6%) had BRAF1799A mutation and 7/41 (17.1%) had RET/PTC rearrangements. For benign nodules, concordance was observed in 10/12 (83.3%) cases. Among 20 cases, including 19 suspicious for malignancy and one inadequate sample, 17 were positive for SFTP/TFF3 test (85.0%). Of these, four were also positive for RET/PTC rearrangements, five were positive for BRAF1799A and all resulted in PTC at histology. The remaining 3 cases were negative for three molecular markers. These nodules showed benign lesions in histology.

Our results demonstrated that specific molecular tests improve the efficacy of preoperative diagnostics of PTC.

Keywords: papillary thyroid carcinoma, preoperative diagnosis, molecular test.

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Introduction

Papillary thyroid carcinoma (PTC) is the most common type of thyroid cancer, with a relative frequency of 70% to 80% [1]. Currently, fine needle aspiration biopsy (FNAB) is the best diagnostic tool for identifying patients with malignant neoplasms of the thyroid. In most cases, PTC is usually easily diagnosed by cytology due to characteristic nuclear features of tumor cells. However, in some situations, papillary carcinoma cannot be found at cytological examination either because of material insufficiency, or else cytomorphological characteristics do not clearly indicate the malignant nature of the lesion [2, 3]. In cases of inadequate sampling, careful follow-up or repeated FNAB procedure is recommended. In cases of suspicion of malignancy, a diagnostic hemithyroidectomy is necessary. Intraoperative analysis of frozen sections is used as a means of the extent of surgical resection indication. Sometimes, the correct diagnosis becomes possible only after histological analysis of the removed tissue. If postoperative diagnosis is established as PTC, in most institutions a thyroidectomy must be completed with a second operation. This two-step surgery has a higher incidence of complications than initial total thyroidectomy [4]. Therefore, additional diagnostic tests are essential to improve the preoperative assessment of thyroid nodules.

It is known that *RET* and *BRAF* protooncogenes play important roles in the pathogenesis of PTC. The tyrosine kinase receptor *RET* is a component of a multiprotein complex, activated by the glial cell line-derived neurotrophic factor family molecules, that plays a crucial role in the development of the enteric nervous system and kidneys. Fusion of the sequence coding for the tyrosine kinase domain of *RET* to the 5' sequence of genes that are expressed in thyroid follicular cells leads to the generation of a number of chimerical oncogenes known as *RET/PTC*. *RET/PTC1* and *RET/PTC3* are the most prevalent variants. The rearrangements result in constitutive activation of *RET*, which is phosphorylated on

tyrosine and translocated from the membrane to the cytoplasm [5]. BRAF is a serine/threonine kinase that receives a mitogenic signal from RAS and transmits it to the mitogen activated protein kinase (MAPK) pathway. The thymine-to-adenine transversion at nucleotide position 1799 of BRAF (*BRAF^{T1799A}*), which translates into valine to glutamate substitution at residue 600, occurs in PTC [6]. Recent studies have shown that mutations *RET/PTC* and *BRAF^{T1799A}* are present in 5–85% and 29–69% in papillary carcinoma, respectively [5, 6]. In view of their selective expression and high prevalence, the oncogenes *RET/PTC* and *BRAF^{T1799A}* are ideal diagnostic hallmarks of PTC.

Besides, in our previous work, we studied the expression levels of 8 genes: 5 over- and 3 under-expressed in PTC [7]. It has been shown, that only a combination of two genes: *SFTPB* (codes surfactant, pulmonary-associated protein B; up-regulated in PTC) and *TFF3* (codes trefoil factor 3; down-regulated in PTC) can be used for molecular diagnosis of PTC. The sensitivity, specificity, and accuracy of the given method were 77.8%, 93.3%, and 89.7%, respectively.

Thus, we investigated detection of *RET/PTC* and *BRAF^{T1799A}* mutations and expression levels of *SFTPB* and *TFF3* genes in FNAB specimens. The obtained data were compared with the results of cytological and pathological examination to evaluate the prospective efficacy of molecular analysis in diagnostics of PTC.

Material and methods

Aspiration biopsy samples

Totally, 73 FNAB samples of thyroid nodules corresponding to 71 patients (55 females and 16 males, aged 19.1–56.6 years) were analyzed. Ultrasound-guided FNAB was performed using a 20 ml syringe with a 22-gauge needle. After the preparation of a slide glass for cytological investigation, leftover material from inside the needle was washed out into 0.5 ml of an RNA-later (Ambion, USA) in a plastic tube and stored at -20 °C until RNA and DNA extraction.

Smears were classified according to [8] as PTC, if aspirates had complex papillary structures and psammoma bodies with distinctive nuclear features such as grooves, pseudoinclusions, and ground-glass appearance; suspicious for malignancy, aspirates of moderate to low cellular component and equivocal malignant atypia such as abundant nuclear folds but no pseudo-inclusions; benign nodule, aspirates of high cellular component without nuclear atypia, the presence colloid and macrophages, inadequate, limited cellular component or poor presentation and fixation.

The biomaterial and clinical data were received from Thyroid Cancer Center (Minsk, Belarus). Informed consent was obtained from each patient as appropriate. The protocols of the study were approved by the Ethical Committees of the Thyroid Cancer Center.

Extraction of nucleic acids

Total RNA isolation from FNAB samples was carried out with the Isogen reagent (Wako, Japan), according to manufacturer's guidelines. DNA was extracted from the interphase and organic phase with a buffer containing from 4M guanidine thiocyanate, 50 mM sodium citrate and 1M Tris (free base). The concentration of nucleic acids was measured with a Nanodrop ND -1000 spectrophotometer.

Reverse transcription (RT) was performed using 5 µl of total RNA (40–60 ng of RNA template from each sample) and MuLV Reverse Transcriptase in the presence of random hexamers (all reagents from Applied Biosystems, USA) for 1 hour at 41°C following by heat inactivation of the enzyme at 95°C for 5 minutes. The integrity of the RNA and efficiency of the RT reaction in each sample was confirmed by polymerase chain reaction (PCR) for porphobilinogen deaminase (PBGD) mRNA (Table 1).

Determination of RET/PTC rearrangements

RET/PTC1 and RET/PTC3 rearrangements were detected by RT-PCR. The reaction mixture (final volume 25 µl) was comprised of 2 µl cDNA, 1.5 mM MgCl₂, 0.2 mM of each of deoxynucleotide triphosphates, 0.5 U of AmpliTaq Gold polymerase (all reagents from Applied Biosystems) and the pair of primers specific for RET/PTC1 or RET/PTC3 fusion genes (See Table 1). The forward primer had a sequence specific for H4 gene (in case RET/PTC1 rearrangement), or RGF (previous name *Ele1*) gene (in case RET/PTC3 rearrangement). The sequence of reverse primer in both reactions was specific to site of *c-ret* gene. The thermal cycling conditions were: 95°C for 10 min, followed by 40 cycles at 94°C for 30 s, 57°C for 30 s (for RET/PTC3) or 56°C for 30 s (for RET/PTC1), 72°C for 30 s and a final extension step at 72°C for 5 min. After PCR amplification, 10 µl of reaction products were separated in 1.5% TAE agarose gel and visualized by ethidium bromide staining.

Table 1**Primers used in the PCR analysis**

Primers	Sequence, 5'–3', forward and reverse	Ampicon size, b.p.	Annealing temperature, °C
PBGD	TGCCAGAGAAGAGTGTGGTG AACTGTGGGTCATCCTCAGG	534	60
RET/PTC3	ACCTGCCAGTGGTTATCAAGCT CTCTGCCTTTCAGATGGAA	210	57
RET/PTC1	GCCTGGAGGAGCTCACCAA CTCTGCCTTTCAGATGGAA	255	56
BRAF	ACATACTTATTGACTCTAAGAGGAAAGATGAA GATTTTTGTGAATACTGGGAACTATGA	400	60
SFTPБ	AATTCCCCATTCCTCTCCCCAT GATGCCGCCCGCCAC	137	61
TFF3	TGGTGTTTCAAGCCCCTGCA CAAAGGGACAGAAAAGCTGAGATGA	147	61
KPNA4	AAGTTGTGCAAGTAGTACTCGATGG ATCAATGATCTCATAGGCCAATT	170	61

Determination of RET/PTC rearrangements

RET/PTC1 and RET/PTC3 rearrangements were detected by RT-PCR. The reaction mixture (final volume 25 µl) was comprised of 2 µl cDNA, 1.5 mM MgCl₂, 0.2 mM of each of deoxynucleotide triphosphates, 0.5 U of AmpliTaq Gold polymerase (all reagents from Applied Biosystems) and the pair of primers specific for RET/PTC1 or RET/PTC3 fusion genes (See Table 1). The forward primer had a sequence specific for H4 gene (in case RET/PTC1 rearrangement), or RGF (previous name *Ele1*) gene (in case RET/PTC3 rearrangement). The sequence of reverse primer in both reactions was specific to site of *c-ret* gene. The thermal cycling conditions were: 95°C for 10 min, followed by 40 cycles at 94°C for 30 s, 57°C for 30 s (for RET/PTC3) or 56°C for 30 s (for RET/PTC1), 72°C for 30 s and a final extension step at 72°C for 5 min. After PCR amplification, 10 µl of reaction products were separated in 1.5% TAE agarose gel and visualized by ethidium bromide staining.

Detection of BRAF point mutation

Analysis of a portion of *BRAF* exon 15 was performed by PCR followed by direct sequencing. Genomic DNA (approximately 50–80 ng of DNA template) was amplified using 0.5 U of AmpliTaq Gold polymerase (Applied Biosystems) and specific forward and reverse primer (See Table 1). The cycling conditions were the following: 94°C for 10 min, followed by 40 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 30 s and 72°C for 5 min as a final extension. The PCR products were resolved in 1.5% TAE agarose gel and stained with ethidium bromide. After visualization of a gel in UV-light (final fragment size 400 bp), the remaining PCR products (4 µl) were treated with ExoSAP-IT PCR clean-up reagent (USB Corp., USA) and sequenced on an ABI PRISM 3100 automated capillary sequencer (Applied Biosystems) using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with the aforementioned forward primer as a sequencing oligonucleotide.

Analysis of expression *SFTPB* and *TFF3* genes

SFTPB and *TFF3* expression levels were determined as described by Hamada *et al.* [7]. Briefly, expression of the target genes was estimated in relation to a housekeeping gene level (*KPNA4*) by means of duplex PCR followed by the band intensity measurement using image processing software Gel-Pro ANALYZER (Media Cybernetics Inc., USA).

Statistical analyses

Sensitivity, specificity and accuracy were calculated to assess the diagnostic efficacy of each test. A P-value of less than 0.05 was considered statistically significant.

Results

Cytological and final pathological findings

Cytological examination revealed PTC in 41 cases, suspicions of malignancy in 19, benign nodules in 12, and it was non-informative in 1. Histology was 59 PTC, five nodular goiters, and nine follicular adenomas. All removed nodules during the operation were diagnosed according to the World Health Organization histological typing of thyroid tumors classification [9].

Molecular analysis of FNAB samples

RET/PTC rearrangements were diagnosed in 11 of 73 samples (15.1%). Five cases were positive for *RET/PTC1*, and six were positive for *RET/PTC3*. The point mutation T1799A of the *BRAF* gene was found in 27 cases (37.0%). No cases both *RET/PTC* rearrangements and *BRAF*^{T1799A} mutation were identified. Up-regulated of *SFTPB* and down-regulated of *TFF3* genes, i.e. a PTC-like pattern was documented in 78.1% of FNAB specimens (57/73), including all *RET/PTC*- and *BRAF*-positive cases.

In the group of lesions classified as PTC at routine cytology, *RET/PTC* rearrangements were found in 7 (17.1%) cases, and *BRAF*^{T1799A} mutation was found in 22 (53.6%) cases, i.e. 12 cancers were not detected by these molecular tests (29.3%). While the results of the *SFTPB/TFF3* test were in agreement with cytological diagnosis in 92.7% of investigated cases (38/41). The analyses of surgical specimens confirmed the FNAB findings. From 19 FNAB suspicious for malignancy, sixteen cases proved to be papillary carcinoma (84.2%). Among these, four (25.0%) contained *RET/PTC* rearrangements, five (31.2%) contained the thymine to adenine *BRAF* mutation, and sixteen (100%) were positive for *SFTPB/TFF3* test. In this group, three nodules were negative for all molecular markers and showed PTC at histology. Up-regulated of *SFTPB* and down-regulated of *TFF3* genes was also identified in an inadequate biopsy, which showed the absence of *RET/PTC* rearrangements and *BRAF*^{T1799A} mutation. In 12 cases classified as benign lesions at cytology, no oncogenes were detected and a PTC-like pattern was documented in two aspirates (16.7%). However, 11 nodules proved to be benign and one tumor proved to be PTC at histopathology after surgical resection. The results of this study are summarized in Table 2.

Table 2
Results molecular test were compared with cytological and histological diagnosis

Cytology	RET/PTC1,3	BRAF ^{T1799A}	SFTPБ/TFF3		Histology	
			PTC	Non-PTC	PTC	benign
PTC (n=41)	7	22	38	3	41	0
Suspicious for malignancy (n=19)	4	5	16	3	16	3
Benign nodules (n=12)	0	0	2	10	1	11
Inadequate (n=1)	0	0	1	0	1	0
Total (n=73)	11	27	57	16	59	14

PTC; papillary thyroid carcinoma.

Thus, PTC FNAB samples were identified in 69.5% of cases by cytological examination, in 18.6% of cases by RET/PTC1,3 detection, in 45.8% of cases by BRAF^{T1799A} detection, and in 94.9% of cases by expression levels of the marker genes. The diagnostic value for each preoperative method, as calculated from the above-described results, is shown in Table 3. It was established, that specificity of molecular tests for the presence of RET/PTC rearrangements or BRAF^{T1799A} mutation was 100%, but the sensitivity and accuracy of both of them were very low. At the same time sensitivity and accuracy of SFTPБ/TFF3 test were significantly higher than those obtained for the cytological method. The predictive value of the positive result of the given molecular test has made 98.2%, predictive value of the negative result is 81.3%.

Table 3
Efficacy cytological and molecular preoperative diagnostics of papillary thyroid carcinoma

	Cytology (1)	Molecular test		
		RET/PTC1,3 (2)	BRAF ^{T1799A} (3)	SFTPБ/TFF3 (4)
Sensitivity, %	69.5	18.6 ^a	45.8 ^b	94.9 ^c
Specificity, %	78.6	100	100	92.9
Accuracy, %	71.2	34.2 ^a	56.2	94.5 ^c

^ap_{1,2}≤0.0001; ^bp_{1,3}=0.02; ^cp_{1,4}≤0.0005

Discussion

A thyroid nodule is very common in the general population. Approximately 5–10% of adults have palpable thyroid nodules, and 30–50% of them have nodules identified by ultrasound. Although the majority of these neoforations are benign, about 5–7% of thyroid nodules are malignant [10].

FNAB is a very important test for the initial diagnostics of thyroid nodules. However, its effectiveness is highly dependent on the qualification of the operator performing the procedure and the adequacy of the sample for interpretation of the cytological features.

Searching for molecular markers of malignancy in biopsies may increase the accuracy of the cytological method and avoid delayed or incomplete surgical intervention. Several potential genetic biomarkers have been proposed for differential diagnostics of thyroid nodules such as *LGALS3*, *MUC1*, *MET*, and *RAS* [11]. Unfortunately, the results of the studies showed limitations of these markers because of a lack of specificity or sensitivity, or both.

Modern technologies of genetic analysis allowed for extending knowledge about molecular characteristics of PTC. The thymine-to-adenine transversion at nucleotide position 1799 of *BRAF*, which results in a valine-to-glutamate substitution at residue 600, is the most common genetic event in this type of thyroid cancer. The high prevalence makes *BRAF*^{T1799A} mutation an attractive genetic marker for preoperative diagnostics of PTC [11-13]. In our study, *BRAF* analysis of FNAB has properly identified 45.8% of PTC. There were no false-negative results for T1799A transversion detection in biopsies compared with the analysis of follow-up surgical samples. There were no false-positive results because neither adenomas nor goiters scored *BRAF* as positive.

Translocations of the tyrosine kinase domain of the *RET* gene to a series of other genes are another genetic lesion that are often found in a subset of PTC [5, 11]. We analyzed 73 thyroid aspirates for the presence of either *RET/PTC1* or *RET/PTC3* and found that 18.6% of papillary carcinoma samples were *RET/PTC* positive. Samples bearing chimeric *RET* genes were negative for *BRAF*. Thus, taken together, the detection of *BRAF*^{T1799A} mutation and *RET/PTC* rearrangements in FNAB diagnosed PTC in 38 (64.4%) samples, including 9 (five with *BRAF*^{T1799A} mutation and four with *RET/PTC* rearrangements) of 19 suspicious for malignancy. Of course, the search for *BRAF*^{T1799A} and *RET/PTC* oncogenes in biopsies may increase the accuracy of FNAB but has some important limitations. While the presence of one of these oncogenes is indicative of cancer, their absence will not exclude a malignant lesion. So, the molecular diagnosis of PTC wasn't established in 35.6% of tumors. Besides, in contrast to *BRAF*^{T1799A} mutation, which appears to be restricted to PTC, *RET/PTC* rearrangements can be also present in benign conditions, including trabecular adenomas and Hashimoto thyroiditis [14, 15]. However, these molecular markers have 100% specificity. Therefore, in suspicious nodules, the finding of *BRAF*^{T1799A} mutation or *RET/PTC* rearrangements can support decision-making about the extent of surgical intervention, indicating the need for total thyroidectomy performing rather than hemithyroidectomy.

Recently, novel molecular targets of potentially high diagnostic value have been proposed based on gene expression in normal and malignant tissues [16]. Nowadays, biomarkers for other malignancies, cervical and prostate cancers, have been successfully developed and adopted to cytological specimens similar to FNAB [17, 18]. In this work, we examined of the relative expression levels of the *SFTPB* and *TFF3* genes in thyroid aspirates using a conventional duplex PCR. We received the matched molecular and histological data in the majority of cases (94.9% of cases of PTC and 92.9% of cases of benign nodules). In 20 (27.4%) cases, including 19 suspicious for malignancy and one inadequate sample, results of genetic examination based on the expression levels of the *SFTPB* and *TFF3* genes have allowed establishing the true diagnoses. Only in 5 (6.8%) cases discrepancy between molecular and cytological diagnoses was revealed. In three of five samples with PTC cytological and histological diagnosis, the absence of characteristic expression of the *SFTPB* and *TFF3* genes indicated PTC absence. It is noteworthy, that in two of them not only marker genes low expression, but also a gene of internal control was recorded. Perhaps, it was a principal cause leading to a false-negative result of the molecular test. In the remaining two observations without cytological evidence of malignancy, the molecular test results were interpreted as having a PTC-like pattern. After histological examination PTC was diagnosed in the first patient, but in the second one – the follicular adenoma of oxyphilic cells. Thus, a false-positive result was reported only in one sample, while three cancers were not detected by this molecular method.

Behind exception PTC an up-regulation of the *SFTP3* gene was observed in cases of lung cancer [19]. *TFF3* underexpression has been fixed in follicular thyroid carcinoma [20] and, on the contrary, its over-expression has been found in breast and colon carcinoma [21, 22].

Conclusion

In conclusion, we propose to employ an estimation of the expression levels of the marker genes in thyroid aspirates as a useful tool to improve the efficacy of preoperative diagnostics of PTC. A larger and prospective study will be necessary to confirm the diagnostic utility of FNAB molecular analysis.

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FNAB материалдарын молекулалық талдау арқылы папиллярлы қалқанша безінің қатерлі ісігінің операция алдындағы диагностикасы

Аңдатпа. Қалқанша безінің папиллярлық карциномасы (ҚБПК) қалқанша безінің қатерлі ісігінің ең көп таралған түрі болып табылады. BRAF1799A мутациясын, RET/PTC1,3 қайта құруларын және SFTPВ (жоғары реттеу) және TFF3 (төмен реттеу) гендерінің дифференциалды экспрессиясын қоса алғанда, қалқанша безінің папиллярлық карциномасының (ҚБПК) молекулалық маркерлері FNAB (жұқа ине аспирациялық биопсия) проспективті түйінді бездер түрде бағаланды.

Зерттелген 73 жағдайдың 59-ы ҚБПК (қалқанша безінің папиллярлық карциномасы), 5-і түйінді зоб және 9-ы фолликулярлық аденома.

Цитологияға сәйкес 38/41 жағдайда (92,7%) SFTPВ және TFF3 экспрессия деңгейлері бойынша ҚБПК молекулалық диагностикасы расталды; олардың 22/41 (53,6%) BRAF1799A мутациясына және 7/41 (17,1%) RET/PTC қайта құруларына ие болды. Қатерсіз түйіндер үшін сәйкестік 10/12 (83,3%) жағдайда байқалды. 20 талдау жұмысы жасалды, оның 19-ында қатерлі ісік қаупі байқалды, 17-де SFTPВ/TFF3 сынағы оң болды (85,0%). Қайта өңдеу үшін 4 RET/PTC, 5 BRAF1799A оң көрсеткіштегі гистологияда ҚБПК нәтижесін берді. Қалған 3 жағдай үш молекулалық маркер үшін теріс болды. Бұл түйіндер гистологияда қатерсіз зақымдануды көрсетті.

Біздің нәтижелеріміз арнайы молекулалық сынақтар ҚБПК операция алдындағы диагностикасының тиімділігін арттыратынын көрсетті.

Түйін сөздер: қалқанша безінің папиллярлық ісігі, операция алдындағы диагностика, молекулалық тест.

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Предоперационная диагностика папиллярного рака щитовидной железы методом молекулярного анализа материалов FNAB

Аннотация. Папиллярная карцинома щитовидной железы (ПКЩЖ) является наиболее распространенным типом рака щитовидной железы. Молекулярные маркеры папиллярной карциномы щитовидной железы (ПКЩЖ), включая мутацию BRAF1799A, реаранжировки RET/PTC1,3 и дифференциальную экспрессию генов SFTPB (повышенная регуляция) и TFF3 (пониженная регуляция), были проспективно оценены в материале FNAB (тонкоигольная аспирационная биопсия) от узлов щитовидной железы.

Обследовано 73 случая, из них 59 случаев ПКЩЖ (папиллярная карцинома щитовидной железы), 5 узловых зобов и 9 фолликулярных аденом.

В соответствии с цитологией молекулярная диагностика ПКЩЖ по уровням экспрессии SFTPB и TFF3 была подтверждена в 38/41 случае (92,7%); из них 22/41 (53,6%) имели мутацию BRAF1799A и 7/41 (17,1%) имели реаранжировки RET/PTC. Для доброкачественных узлов конкордантность наблюдалась в 10/12 (83,3%) случаях. Среди 20 случаев, в том числе 19 с подозрением на злокачественность и один неадекватный образец, 17 были положительными на тест SFTPB/TFF3 (85,0%). Из них четыре были также положительными в отношении реаранжировки RET/PTC, пять были положительными в отношении BRAF1799A, и все они привели к ПКЩЖ при гистологическом исследовании. Остальные 3 случая были отрицательными по трем молекулярным маркерам. Эти узелки показали доброкачественные поражения в гистологии.

Наши результаты показали, что специфические молекулярные тесты повышают эффективность предоперационной диагностики ПТК.

Ключевые слова: папиллярный рак щитовидной железы, предоперационная диагностика, молекулярный тест.

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