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Prospective efficacy of molecular preoperative diagnostics of papillary thyroid carcinoma

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Background. The diagnostic efficacy of the molecular analysis that included determination of papillary thyroid carcinoma (PTC) marker gene expression levels and *BRAF* mutation in fine-needle aspiration biopsy material was evaluated in a prospective study of patients with thyroid nodules.

Materials and methods. Totally, 36 patients (29 females and 7 males) with thyroid nodules were included in the study. The mRNA expression of genes (*SFTPB* and *TFF3*) was estimated in relation to a housekeeping gene level (*KPNA4*) by means of duplex RT-PCR followed by the band intensity measurement. Detection of BRAF mutation was performed by PCR followed by direct sequencing.

Results. In 25/32 (78.1%) cases, results of the molecular test were in agreement with the cytological diagnosis (7/7 PTC and 18/25 non-PTC) further confirmed by histological examination of tissues surgically removed from all seven PTC patients and 10 individuals with benign nodules. In 7/32 patients (21.9%) there was a discrepancy between cytological findings and molecular results, which revealed a benign nodule and a PTC-like pattern, respectively. Upon a repeated examination of five of these patients about one year later, three were cytologically diagnosed with PTC (all patients had been operated on), and the diagnosis of the other two patients remained unchanged.

Conclusions. The results have demonstrated that the molecular analysis of FNAB material is an informative means of the preoperative diagnosis of thyroid nodules as it allows identification of patients with suspected PTC before other diagnostically significant changes take place.

Key words: papillary thyroid carcinoma, fine-needle aspiration biopsy, preoperative diagnosis, molecular test

INTRODUCTION

Papillary thyroid carcinoma (PTC) accounts for approximately 85% of thyroid malignant neoplasms (1, 2). The prognosis of PTC is excellent (10-year survival is more that 90%), although 10–15% of adult patients may manifest an aggressive life-threatening course (3).

Ultrasound-guided fine-needle aspiration biopsy (FNAB) followed by cytological examination is a well-established approach to the primary diagnosis of thyroid nodules (4–6). However, PTC includes numerous morphological variants some of which, particularly those featuring follicular structures, may be difficult for cytology (1, 7). In such cases, correct diagnosis becomes possible only after a histological analysis of the removed tissue. Thus, there is a clear need for the development of additional preoperative PTC diagnostic means.

In our previous work, we studied the expression levels of 8 genes – five over- and three under expressed – in PTC (8) and showed that only a combination of two genes – *SFTPB* (surfactant, pulmonary-associated protein B; upregulated in PTC) and *TFF3* (trefoil factor 3; down regulated in PTC) can be used for the molecular diagnosis of PTC. The sensitivity, specificity and accuracy of the method were 77.8%, 93.3% and 89.7%, respectively. Besides, detection of the somatic point mutation at codon 600 *BRAF* gene, which

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occurs in 29–69% of adult PTCs and is highly specific of this type of thyroid malignancy, has been advocated for the preoperative diagnosis of PTC (9–14). The RAF family of serine / threonine kinases is a key component of the RAS→RAF→MEK→MAP / ERK signaling pathway involved in cell growth and proliferation, which provided a potent mitogenic force-driving malignant transformation. Among the several characterized mammalian RAF kinases, the B type RAF, or BRAF, is the strongest activator of this signaling pathway (10).

Making use of these molecular tests, we applied them to a series of FNAB samples of thyroid nodules and compared the obtained data with the results of ultrasonography, cytological examination and, in some cases, pathological findings to evaluate the prospective efficacy of molecular analysis in PTC diagnosis.

MATERIALS AND METHODS

Aspiration biopsy samples

Totally, 36 FNAB samples of thyroid nodules from 29 females and 7 males (F/M ratio 4.1:1) aged 19.2–65.4 years (mean 34.6 ± 10.8 years) were included in the study. Ultrasound-guided FNAB was performed using a 20 ml syringe with a 22-gauge needle. After preparation of a slide for cytological investigation, the leftover material from inside the needle was washed out into 0.5 ml of an RNAlater (Ambion, USA). The results of ultrasound and cytological examinations were retrieved from medical records. Histology was available in 20 cases (10 PTC and 10 benign nodules) by the end of the study.

The biomaterial and clinical data were received from the Thyroid Cancer Center (Minsk, Belarus). Informed consent was obtained from each patient as appropriate.

Extraction of nucleic acids

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

Reverse transcription was performed using 5 µl of total RNA and MuLV Reverse Transcriptase in the presence of random hexamers (all reagents from Applied Biosystems, Foster City, CA, USA) for 1 hour at 41 °C, followed by heat inactivation of the enzyme at 95 °C for 5 minutes.

Analysis of *SFTPB* and *TFF3* gene expression

SFTPB and *TFF3* expression levels were determined as previously described (8). 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.

Detection of point mutation at *BRAF* gene

Analysis of a portion of *BRAF* exon 15 was performed by PCR followed by direct sequencing. The primers used were: 5'-ACATACTTATTGACTCTAAGAGGAAAGATGAA-3' (forward, located in intron 14 of the BRAF gene) and 5'-GATTTTTGTGAATACTGGGAACTATGA-3'(reverse,located in intron 15). Genomic DNA extracted from FNAB samples (approximately 50–80 ng of DNA template) was amplified using AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA, USA). The cycling conditions were the following: 94 °C for 10 min, followed by 38 cycles of 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. PCR products were resolved in 1.5% TAE agarose gel and stained with ethidium bromide. After visualization in a gel (final fragment 400 bp in length), 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, USA) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with the aforementioned forward primer as a sequencing oligonucleotide.

RESULTS

Nucleic acids were successfully extracted from 32 (88.9%) FNABs. It was impossible to isolate RNA / DNA in 4 (11.1%) specimens. In 25 (78.1%) from 32 analyzable cases, the results of the molecular test were in agreement with the cytological diagnosis (7/7 PTC and 18/25 non-PTC). All seven PTCs (two cases with $BRAF^{V600E}$ mutation) and 10 benign nodules from this group had been surgically treated, and the diagnosis was confirmed by histological examination. The other patients (8 cases of nontoxic nodular goiter) are currently under follow-up.

In the remaining seven (21.9%) cases (5 females and 2 males, age 19.1–43.2 years at the moment of examination) there was a discrepancy between molecular findings and cytological diagnosis. Whereas cytology (as well as ultrasound) follicular adenoma of the thyroid was established in two cases and nodular goiter in five cases, the molecular test revealed an over expression of *SFTPB* and under expression of *TFF3* genes, i. e. a PTC-like pattern. These patients were followed by an endocrinologist and advised additional clinical checkup.

Repeated ultrasound and cytological examination was performed for 10 of 15 patients approximately 12 months later. In three of them, the cytological diagnosis changed for PTC (Table 1, cases 2, 6, 7). Note that repeated molecular testing detected non-cancer thyroid diseases in five cases and PTC-like gene expression pattern also in five. Furthermore, we found a typical $BRAF^{V600E}$ mutation in two of three FNAB samples cytologically diagnosed for PTC (Table 1, cases 2 and 6; Figure). By the moment of writing this paper, all three patients had already been operated on. The histological examination of the tumour confirmed the diagnosis of papillary carcinoma.

Table 1. **Comparison of initial and repeated clinical examinations of patients**

Cases	First consultation			Repeated consultation			Histology
	Ultrasonography	Cytology	Gene test	Ultrasonography	Cytology	Gene test	
1	NG	NG	PTC	–[a]	–	–	
2[b]	NG	NG	PTC	Susp PTC (after 14 months)	Susp PTC	PTC	PTC
3	FA	NG	PTC	–	–	–	
4	NG	NG	PTC	NG (after 14 months)	NG	PTC	
5[d]	FA	NG	PTC	FA (after 12 months)	FA	PTC	
6[b]	NG	NG	PTC	Susp PTC (after 10 months)	PTC	PTC	PTC
7	NG	NG	PTC	Susp PTC (after 11 months)	PTC	PTC	PTC
8	NG	NG	non-PTC	NG (after 15 months)	NG	non-PTC	
9	NG	NG	non-PTC	–	–	–	
10	NG	NG	non-PTC	NG (after 11 months)	NG	non-PTC	
11	NG	NG	non-PTC	NG (after 16 months)	NG	non-PTC	
12	NG	NG	non-PTC	–	–	–	
13	NG	NG	non-PTC	–	–	–	
14	NG	NG	non-PTC	NG (after 11 months)	NG	non-PTC	
15	NG	NG	non-PTC	NG (after 12 months)	NG	non-PTC	

NG – nodular goiter; PTC – papillary thyroid carcinoma; FA – follicular adenoma; Susp – suspected.

[a] data on two patients were not available by the end of this study;

[b] cases with $BRAF^{V600E}$;

[c] period between the first and the second consultations, months;

[d] case with $BRAF^{K601E}$.

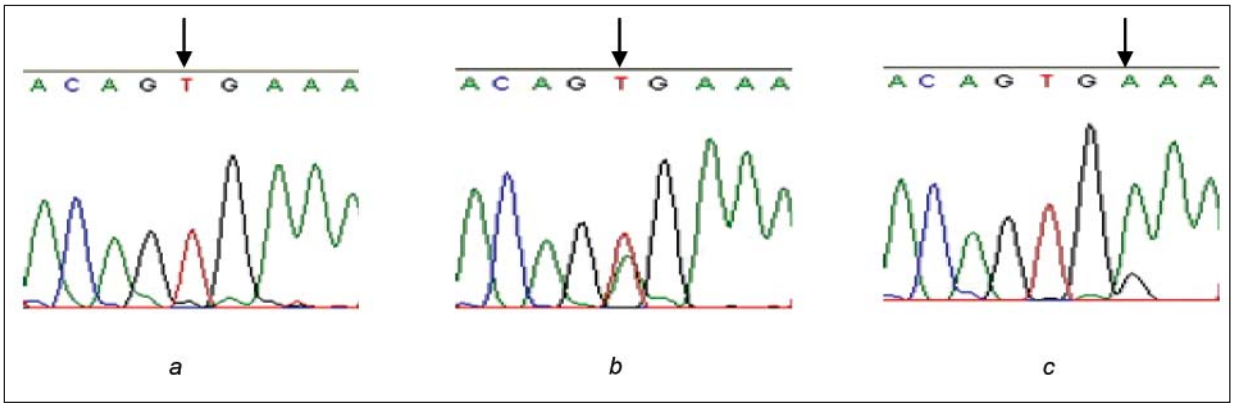

Figure. Detection of mutations in exon 15 of the *BRAF* gene by direct sequencing: *a* – wild-type *BRAF* sample; *b* – specimen with a heterozygous $BRAF^{V600E}$ mutation; *c* – sample with a heterozygous $BRAF^{K601E}$ mutation

In two other cases displaying a PTC-like gene expression pattern, no negative dynamics was noted by ultrasound examination and, after repeated biopsy, in one patient a nodular goiter and in the other a follicular adenoma were diagnosed. The follow-up of these patients is continued. Interestingly, in one of these cases (Table 1, case 5) we revealed a rare mutation ($BRAF^{K601E}$) of the *BRAF* gene (Figure).

For the remaining five patients, results of the molecular test were in agreement with repeated ultrasound and cytological diagnosis.

In Table 2, we have summarized the prevalence of genetic alterations in tumours with different morphological features. The $BRAF^{V600E}$ mutation was present in 75.0% of PTC with papillary and in 25.0% of PTC with mixed papillary / follicular structures. The expression of *SFTPB* and *TFF3* genes was found in tumours of a various morphological architecture: papillary (30.0%), follicular (10.0%) mixed papillary / follicular (40.0%) and mixed papillary / follicular / solid structures (20.0%).

Table 2. **Association of genetic alteration with morphological structures of PTC (n = 10)**

Tumour structures	$BRAF^{V600E}$	*SFTPB / TFF3*
Papillary	3 (75.0%)	3 (30.0%)
Follicular	0	1 (10.0%)
Mixed papillary and follicular	1 (25.0%)	4 (40.0%)
Mixed papillary, follicular and solid	0	2 (20.0%)
Total	4	10

DISCUSSION

Modern technologies of genetic analysis allowed expanding knowledge about the molecular characteristics of PTC. Several potential genetic biomarkers of this type of cancer have been identified and applied to FNAB samples. For example, an activating mutation in *BRAF* is associated with PTC (9–14) and would be an ideal molecular diagnostic candidate provided it occurred in an overwhelming number of PTC cases; unfortunately, this mutation is only present in approximately 45% of papillary carcinoma. Other genetic alterations found in PTC, such as *RET / PTC* and *TRK* rearrangements or point mutations in the *RAS* gene family, are not too specific or their prevalence is not high enough to confirm the molecular diagnosis (12, 15, 16). Therefore, it is necessary to continue the search of a more universal marker of this tumour.

In this prospective study, we estimated the efficacy of molecular analysis of FNAB material in a series of patients with thyroid nodules. Results concordant with cytological diagnosis were obtained in all PTCs and in the majority of benign nodules. However, among the cases that appeared benign on cytology, there were seven samples which according to the results of the molecular analysis were interpreted as PTC. However, this subgroup of patients did not display the clinical features allowing their discrimination from other individuals with non-cancer thyroid nodular diseases included in the study. Since at that moment the positive predictive power of the molecular test was unknown, we decided to follow-up these patients.

Upon a repeated examination of five from seven patients, performed about a year later, three of them were cytologically diagnosed with PTC. Our molecular analysis once again showed a PTC gene expression profile; in addition, in two of these three patients we detected also the $BRAF^{V600E}$ point mutation which is specific of PTC (9–12). Surgical treatment was provided for these patients, and the diagnosis of papillary carcinoma was verified by histological analysis. The period between the positive molecular test and repeated consultation was 10, 11 and 14 months, respectively.

For the remaining two patients with the molecular diagnosis of PTC, the cytological diagnosis was not changed to cancer after the repeated consultation. In one of these cases, the $BRAF^{K601E}$ mutation was detected. This mutation had been found before in 2% follicular adenoma of the thyroid (12) and in 7% of the follicular variant of PTC (13). Cytologically (and ultrasonography) this patient was diagnosed with follicular adenoma, but considering the results of the expression level of *SFTPB* and *TFF3* genes (positive molecular test), it was impossible to exclude the follicular variant of PTC. Till the moment of publication of the paper, a continuing growth of thyroid nodule had been observed in this patient, but no malignant transformation was diagnosed at cytological examination. The further follow-up of this and other patients is necessary to clarify the diagnosis.

Association between the point mutation V600E in the *BRAF* gene and thyroid tumour morphology was demonstrated by several groups documenting PTCs with papillary growth pattern as a principal *BRAF* mutation carrier (9, 10, 12). Furthermore, one of the cited works reported a negative association between *BRAF* mutation occurrence and the follicular histotype of PTCs (9). In our series, $BRAF^{V600E}$ mutation prevailed in tumours with papillary and mixed papillary / follicular structures, while the expression of *SFTPB* and *TFF3* genes did not depend on tumour morphology. Thus, *SFTPB* and *TFF3* genes are universal diagnostic markers of PTC.

Molecular testing has several advantages: it does not require additional sampling, is relatively simple, inexpensive and quite accurate. Also, even though there is a chance of a false positive result, molecular finding *per se* is not an indication for surgery and would not harm patients. On the other hand, based on evidence presented here, molecular analysis may help to determine the group of patients with thyroid nodules, who are at risk of cancer.

As a whole, our investigation demonstrates that the results of molecular study of FNAB samples allow suspecting PTC several months earlier before this diagnosis will be confirmed by clinical evidence. This allows us to assume that the analysis of marker gene expression by the method of duplex PCR is more sensitive and informative in comparison with the cytological study of aspiration material and can be successfully used as an adjunctive means of PTC preoperative diagnosis.

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PRIEŠOPERACINĖ MOLEKULINĖ PAPILINIO SKYDLIAUKĖS VĖŽIO DIAGNOSTIKA

S a n t r a u k a

Įvadas. Prospektyviniu tyrimu įvertintas molekulinės analizės diagnostinis efektyvumas nustatant papilinės skydliaukės karcinomos molekulinių žymenų ekspresijos lygį, taip pat *BRAF* mutaciją skydliaukės mazgų mėginiuose aspiracine biopsija plona adata.

Medžiaga ir metodai. Į tyrimą buvo įtraukti 36 pacientai (24 moterys ir 9 vyrai), turintys mazgų skydliaukėje. Dvigubu RT-PCR metodu buvo nustatyta *SFTPB* ir *TFF3* genų mRNA ekspresija atsižvelgiant į bendrinių genų lygį (*KPNA4*); vėliau atliktas juostinio intensyvumo matavimas. BRAF mutacija buvo nustatyta PCR metodu tiesiogiai atskleidžiant seką.

Rezultatai. Molekulinio testo rezultatai atitiko citologinę diagnozę 25 iš 32 (78,1 %) atvejų (7 iš 7 – papilinis skydliaukės vėžys ir 18 iš 25 – nepapilinis skydliaukės vėžys), vėliau patvirtintą histologiškai po 7 pacientų skydliaukės vėžio operacijos ir 10 pacientų gerybinių mazgų operacijos. Citologiniai radiniai ir molekulinis testas nesutapo 7 iš 32 pacientų (21,9 %). Po vienerių metų pakartotinai ištyrus 5 pacientus, 3 citologiškai buvo diagnozuota papilinė skydliaukės karcinoma (2 iš jų buvo operuoti), kitų dviejų pacientų diagnozė nepakito.

Išvados. Rezultatai rodo, kad biopsijos plona adata medžiagos molekuliniai testai yra informatyvi priešoperacinė skydliaukės mazgų priemonė, padedanti diagnozuoti įtariamą papilinę skydliaukės karcinomą ir kitus diagnostiškai reikšmingus pokyčius.

Raktažodžiai: papilinė skydliaukės karcinoma, aspiracinė biopsija plona adata, priešoperacinė diagnozė, molekuliniai testai