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The value of the repeated examination of BRAF V600E mutation status in diagnostics of papillary thyroid cancer

Wartość powtórnego badania stanu mutacji genu BRAF V600E w diagnostyce raka brodawkowatego tarczycy

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Abstract

Introduction: Nodular thyroid disease is one of the most frequently diagnosed pathologies of the adult population in iodine-deficient regions. Approximately 30% of thyroid aspirates are classified as nondiagnostic/unsatisfactory or indeterminate. However, patients with indeterminate cytology still undergo surgery. The object of this study was to determine the diagnostic value of re-examining the BRAF V600E mutation in papillary thyroid carcinoma patients.

Material and methods: All patients underwent ultrasound guided fine-needle aspiration of a thyroid nodule. They were assigned to one of the four groups (indeterminate or positive for malignant cells) of the Bethesda System for Reporting Thyroid Cytopathology. Genetic investigation of the BRAF V600E mutation was performed for all of the fine-needle aspiration cytology specimens. All of the patients underwent surgery. Subsequently, histological investigation of the removed tissues was performed. Additional analysis of the BRAF V600E mutation from the histology specimen was then performed for the initially BRAF-negative cases.

Results: Two hundred and fourteen patients were involved in the study. One hundred and six (49.53%) patients were diagnosed with thyroid cancer. Of these 106 patients, 95 (89.62%) patients were diagnosed with papillary thyroid cancer. The BRAF V600E mutation was positive in 62 (65.26%) and negative in 33 (34.74%) histologically confirmed papillary thyroid cancer cases. After the genetic investigation, a total of 74 (77.89%) papillary thyroid cancer cases were positive for the BRAF V600E mutation and 21 (22.11%) were negative.

Conclusions: Repeated examination of the BRAF V600E mutation status in the fine-needle aspiration may potentially increase the sensitivity of papillary thyroid cancer diagnostics. **(Endokrynol Pol 2016; 67 (1): 35–40)**

Key word: *BRAF mutation; thyroid cancer; fine-needle aspiration*

Streszczenie

Wstęp: Choroba guzkowa tarczycy to jedna z najczęściej wykrywanych patologii w populacji osób dorosłych w regionach z deficytem jodu. Około 30% aspiratów tarczycy jest klasyfikowanych jako nie do zdiagnozowania/niesatysfakcjonujące lub nieokreślone. Jednakże, pacjenci z nieokreślonym wynikiem cytologii nadal poddawani są operacjom. Celem niniejszego badania było ustalenie wartości diagnostycznej powtórnego badania mutacji genu BRAF V600E wśród pacjentów z rakiem brodawkowatym tarczycy.

Materiał i metody: Wszystkich pacjentów poddano biopsji aspiracyjnej cienkoigłowej guzka tarczycy pod kontrolą USG. Przydzielono ich do jednej z czterech grup (nieokreślona lub dodatnia dla komórek złośliwych) klasyfikacji Bethesda do klasy obrazów cytologicznych. Badanie genetyczne mutacji genu BRAF V600E przeprowadzono dla wszystkich próbek uzyskanych z aspiracyjnej cytologii cienkoigłowej. Wszystkich pacjentów zoperowano. Jednocześnie przeprowadzono badanie histologiczne usuniętych tkanek. Dodatkowa analiza mutacji BRAF V600E z próbki histologicznej została przeprowadzona dla przypadków początkowo BRAF ujemnych.

Wyniki: Dwustu czternastu pacjentów poddano badaniu. U 106 pacjentów (49,53%) zdiagnozowano raka tarczycy. Z tychże pacjentów, u 95 (89,62%) zdiagnozowano raka brodawkowatego tarczycy. U 62 pacjentów (65,26%) mutacja BRAF V600E była dodatnia, u 33 (34,74%) histologicznie potwierdzonych przypadków raka brodawkowatego tarczycy była ona ujemna. Po badaniu genetycznym stwierdzono 74 (77,89%) przypadki raka brodawkowatego tarczycy dodatnich dla mutacji BRAF V600E i 21 (22,11%) ujemnych.

Wnioski: Powtórne badanie mutacji genu BRAF V600E za pomocą aspiracji cienkoigłowej może potencjalnie przyczynić się do zwiększenia czujności w wykrywaniu raka brodawkowatego tarczycy. **(Endokrynol Pol 2016; 67 (1): 35–40)**

Słowa kluczowe: *mutacja BRAF; rak tarczycy; aspiracja cienkoigłowa*

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Introduction

Thyroid cancer (TC) is the most common endocrine malignancy, with an increasing incidence in the Western world [1]. Papillary thyroid carcinoma (PTC) accounts for 85–90% of all of the thyroid malignancies [2]. Up to the present date, ultrasonography (US)-guided fine-needle aspiration (FNA) and cytological assessment of the aspirate has been considered a standard diagnostic method for the diagnosis of various thyroid nodules detected on US [3, 4]. It is a simple, safe, and cost-effective diagnostic tool [5]. Although most cytology of the aspirates will benefit diagnostically, approximately 30% of thyroid aspirates are classified as nondiagnostic/unsatisfactory (ND/UNS) or indeterminate [6]. Most patients with indeterminate cytology still undergo surgery for definitive evaluation; however, only 8–17% of such surgically removed nodules are malignant [1, 7]. Consequently, for patients with indeterminate aspirates, surgery is performed for both diagnostic and therapeutic purposes. It should, however, ideally be reserved for therapeutic reasons [8]. Recently, molecular testing of FNAB specimens for genetic alterations frequently associated with TC has emerged as another valuable diagnostic tool. A point mutation of the BRAF V600E oncogene, which results in a change from valine to glutamate in codon V600E, has been reported in 30–80% of PTC patients [7]. It plays a role in cell proliferation, differentiation, and apoptosis [6]. Furthermore, some authors state that there is a direct association between the BRAF mutation and aggressive clinical outcomes such as invasion, metastasis, relapse of PTC, or loss of radioiodine avidity in recurrent PTC [9]. Identification of the BRAF V600E mutation has high specificity and a positive prediction value (PPV) for PTC [7]. Nevertheless, it is important to consider that the prevalence of the BRAF V600E mutation depends on its detection methods, geographical factors, and age [10]. The object was to determine the value of reassessing the presence of the BRAF V600E mutation in making the diagnosis of PTC. The results of the FNAB cytology and its genetic analysis were evaluated by means of definite histopathological diagnosis and genetic investigation of initially BRAF V600E-negative TC histology specimens.

Material and methods

Between January 2012 and January 2015 all patients with an US-suspicious thyroid nodule underwent an US-guided FNAB in the Vilnius University Hospital Santariskiu Clinics. Each of the aspirates was transferred to a liquid-based preparation for routine cytological assessment. The results of the cytological investigation were classified based on the Bethesda System for Reporting Thyroid Cytopathology (BSRTC) into the following groups: Nondiagnostic or Unsatisfactory (ND/UNS) cytology, benign, atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS), follicular or oncocytic (Hürthle cell) neoplasm/suspicious for follicular or oncocytic (Hürthle cell) neoplasm (FN/SFN), suspicious for malignant cells (SMC), or positive for malignant cells (PMC) [11]. Four of these diagnoses (AUS/FLUS, FN/SFN, SMC, and PMC) were considered as the object of this investigation, and these patients were invited to participate in the study. No additional pre-selection criteria were used. Genetic investigation of the BRAF V600E mutation was performed for all of the FNAB cytology specimens of these four groups. Surgeons were aware of the genetic investigation results, and it did not have any influence on the extension of the operation. All patients underwent thyroid surgery (lobisthmectomy or thyroidectomy, with or without lymph node dissection). Routine histological examination of the removed thyroid tissue was then performed. Additional analysis of the BRAF V600E mutation from the histology specimen was performed for patients in whom the BRAF V600E mutation was not detected in the initial FNAB cytology specimen but who were diagnosed with TC after the postoperative histological examination.

Genetic investigation of the BRAF V600E mutation

Material from the liquid-based preparation or histology specimen was transferred into 1.5-ml tubes and treated with RBC lysis solution. Genomic DNA was extracted with the GeneJET Genomic DNA Purification Kit (Thermo Scientific, Vilnius, Lithuania). In order to detect the BRAF V600E mutation (GTG > GAG), we developed a Real-Time PCR assay comprised of two types of reactions. The first one served as an internal reference sample control flanking the BRAF codon V600 of the BRAF gene. The second reaction, or the mutation specific reaction, was designed to specifically target the GTG > GAG mutation. Primers were predesigned manually and investigated *in silico* using the OligoCalc web tool [12]. A BRAF V600E mutation specific forward primer was edited manually by introducing a relevant mismatch nucleotide at the 3′ end according to the Amplification Refractory Mutation System primer design approach [13, 14]. Both reactions shared the same reverse primer. Primer sequences were as follows: BRAF_V600E_ Forward gtgattttggtctagctacggag; BRAF_Forward taggtgattttggtctagctacag; BRAF_Reverse CATCCACAAAATGGATCCAGAC. Real-Time PCR reactions were performed in duplicates using a Maxima™ SYBR Green qPCR Master Mix supplemented with 0.4 units per reaction of UNG (both Thermo Scientific, Vilnius, Lithuania), 300 nmol of each primer

(Metabion, 82152 Planegg/Steinkirchen, Germany), and 0.005–0.05 μg of DNA in a 20 μl reaction volume. Real-Time PCR reaction conditions on a Bio-Rad CFX96 system (Bio-Rad Laboratories, Hercules, CA, USA) were as follows: 2 minutes at 50°C, 95°C 10 minutes, and 37 cycles of 95°C 15 seconds 60°C 1 minute. The first 20 BRAF V600E-positive and 20 — negative cases were confirmed by sequencing the histology specimens and a sequencing assay published elsewhere [15]. The sensitivity of the Real-Time PCR assay was tested by serial dilutions of previously sequenced highly positive BRAF V600E DNA in negative DNA. The Real-Time PCR assay proved to detect mutant DNA down to a 2.5 log dilution in wild-type background.

Statistical analysis

The genetic analysis results of the cytology and histology specimens were considered as true positive (TP) when the BRAF V600E mutation was detected and the patients were diagnosed with PTC in the final histological examination. Results were defined as true negative (TN) when BRAF V600E mutation was not identified and histological investigation revealed benign or other non-papillary type of TC. False negative (FN) results included cases that were negative for the BRAF V600E mutation but were diagnosed with PTC in final histology. False positive cases (FP) were defined as positive for the BRAF V600E mutation but, after further histological investigation, benign or non-papillary type TC was confirmed. The following computational formulas were used: sensitivity = [TP/(TP + FN)] × 100, specificity = = [TN/(TN + FP)] × 100, positive predictive value (PPV) = [TP/ (TP + FP)] × 100, negative predictive value (NPV) = [TN/ (TN + FN)] × 100. Statistical analysis was performed using SPSS software (SPSS Inc., Chicago, IL). Continuous variables were analysed by means of Student's *t*-test. Categorical variables were analysed with the use of Pearson Chi-square test. Two-sided P values of less than 0.05 were considered to indicate statistical significance.

Results

US-guided FNAB, cytological assessment with genetic investigation of the aspirated thyroid material, and histological investigation of the surgically removed thyroid tissue was performed for a total of 214 patients. All of the 214 (100%) patients were Europeans, citizens of Lithuania. Thirty (14.02%) of them were male and 184 (85.98%) were female. The average age of the patients was 54.76 ± 13.26 years. Sixteen (7.48%) of the 214 FNABs were classified as AUS/FLUS, 109 (50.93%) as FN/SFN, 40 (18.69%) as SMC, and 49 (22.90%) as PMC. The BRAF V600E mutation was revealed in 62 (28.97%) of the FNABs. After the final histological investigation, a total of 106 (49.53%) patients were diagnosed with TC. Ninety-five (89.62%) of them were diagnosed with PTC, eight (7.55%) with follicular thyroid cancer (FTC), two (1.89%) with medullary (MTC), and one (0.94%) with anaplastic thyroid cancer (ATC). Forty-four cytologically BRAF V600E-negative TC cases underwent further genetic investigation of the histological specimen. Twelve (27.27%) of them were positive for the BRAF V600E mutation. All 74 (100%) BRAF positive cases were diagnosed with PTC: 71 (95.95%)- classical type, 3 (4.05%)- follicular type of PTC (Table I).

Detection of the BRAF V600E mutation in papillary thyroid cancer

Out of the 106 patients with histologically confirmed TC, the majority (95 (89.62%) cases) were diagnosed with PTC. Out of these 95 PTC cases, 88 (92.63%) were classified as the classical type and 7 (7.37%) as follicular type.

During routine cytological examinations 95 PTC cases were classified as follows: AUS/FLUS — 4 (4.21%), FN/ /SFN — 18 (18.95%), SMC — 27 (28.42%), and PMC — 46 (48.42%). The BRAF V600E mutation was positive in 62 (65.26%) and negative in 33 (34.74%) FNABs. Detection of the BRAF V600E mutation in the FNA cytological specimen, when confirming PTC, was considered true positive in 62 cases, true negative in 119, false positive in 0, and false negative in 33 cases. The sensitivity and specificity of this mutation detection for making the diagnosis of papillary thyroid carcinoma were 65.26% and 100%, respectively. Positive predictive value of the BRAF V600E mutation was 100%; negative predictive value was 78.29%.

After the additional genetic investigation of the postoperative histological specimens in cytologically BRAF V600E-negative PTC cases, 12 additional BRAF V600E positive cases were identified. A total of 74 (77.89%) PTC cases were positive for the BRAF V600E mutation and 21 (22.11%) were negative (Table II). Investigation of the histological specimen increased true positive cases from 62 to 74 and decreased the amount of false negative cases from 33 to 21. The sensitivity of this mutation detection for making the diagnosis of papillary thyroid carcinoma increased from 65.26% to 77.89%. Likewise, the negative predictive value increased to 85% from 78.29% (Table III).

Discussion

Nodular thyroid disease is one of the most frequently diagnosed pathologies, which affects up to 50% of the adult population in iodine-deficient regions [16]. It is less frequent amongst young people and increases progressively with age [10]. Frequent use of sensitive

Table I. *Main data (n = 214)*
Tabela I. *Dane główne (n = 214)*

	AUS/FLUS (a) (n = 16)	FN/SFN (b) (n = 109)	SMC (c) (n = 40)	PMC (d) (n = 49)	p value
Mean age (years)	62.50 ± 12.65	53.03 ± 13.45	55.28 ± 12.83	55.67 ± 12.72	0.5 a + b *vs.* c + d
Gender					
Male	3 (18.75%)	18 (16.51%)	6 (15%)	3 (6.12%)	0.17
Female	13 (81.25%)	91 (83.49%)	34 (85%)	46 (93.88%)	a + b *vs.* c + d
Histology					
Benign	12 (75%)	86 (78.90%)	8 (20%)	2 (4.08%)	< 0.01
Malignant	4 (25%)	23 (21.10%)	32 (80%)	47 (95.92%)	a + b *vs.* c + d
BRAF V600E status (Cytology specimen)					
Positive	1 (6.25%)	6 (5.50%)	16 (40%)	39 (79.59%)	<0.01
Negative	15 (93.75%)	103 (94.50%)	24 (60%)	10 (20.41%)	a + b *vs.* c + d
BRAF V600E status (Cytology + Histology specimen)					
Positive	3 (18.75%)	8 (7.34%)	21 (52.50%)	42 (85.71%)	< 0.01
Negative	13 (81.25%)	101 (92.66%)	19 (47.50%)	7 (14.29%)	a + b *vs.* c + d

Table II. *BRAF status detection in different thyroid cancer types(n = 106)*
Tabela II. *Wykrywanie statusu BRAF w różnych typach raka tarczycy (n = 106)*

TC type /BRAF V600E status	Cytology specimen		Cytology + Histology specimens		p value
	BRAF V600E positive	BRAF V600E negative	BRAF V600E positive	BRAF V600E negative	
Papillary n = 95	**62 (65.26%)**	33 (34.74%)	**74 (77.89%)**	21 (22.11%)	0.05
Follicular n = 8	0 (0%)	8 (100%)	0 (0%)	8 (100%)	> 0.99
Medullar n = 2	0 (0%)	2 (100%)	0 (0%)	2 (100%)	> 0.99
Anaplastic n = 1	0 (0%)	1 (100%)	0 (0%)	1 (100%)	> 0.99

Table III. *Specifications of BRAF mutation status detection in papillary thyroid cancer (n = 214)*
Tabela III. *Specyfikacje wykrywania statusu mutacji BRAF dla raka brodawkowatego tarczycy (n = 214)*

Specification/ Specimen type	Cytology specimen	Cytology + Histology specimen
TP	62	74
TN	119	119
FN	33	21
FP	0	0
Sensitivity	65.26%	77.89%
Specificity	100%	100%
PPV	100%	100%
NPV	78.29%	85%

diagnostic techniques such as high-resolution ultrasonography, computerised tomography, and magnetic resonance or positron emission tomography may be responsible for incidental detection of thyroid tumours. Furthermore, true increases in incidence rates of thyroid cancers can also be explained by increased environmental radiation, use of medical radiation, iodine intake, the Chernobyl disaster, carcinogens, and environmental, ethnic, and genetic factors or combinations of them [9].

Due to its technical simplicity and low cost, US-guided fine-needle aspiration (FNA) is reported to be the first choice diagnostic procedure in the clinical management of nodular thyroid disease [5]. FNAB has been reported to have high sensitivity (65–99%) and specificity (72–100%) in making the diagnosis of thyroid malignancies [17]. Even though the criteria for

the diagnosis of the most common thyroid malignancies have been well described, in a large number of samples cytomorphological examination of FNA alone does not differentiate well between non-neoplastic and neoplastic lesions [18, 19]. The well-differentiated nature of most thyroid cancers and their morphologic overlap with benign nodules result in the placement of 10% to 26% of thyroid cytology cases in one of the three indeterminate diagnostic categories. Diagnostic thyroidectomy is still often required for such patients in order to make a definitive evaluation of the suspicious thyroid nodule [7, 18, 20]. Ali and Cibas estimated the risk of malignancy as follows: 5% to 15% for AUS/FLUS, 15% to 30% for FN/SFN, and 60 to 75% for SMC [20]. Repeated FNA, core biopsy, and even intraoperative frozen sections are often unsuccessful at clarifying the nature of these ambiguous nodules [6]. However, it has been observed that during the histopathological evaluation of the excised thyroid piece more than two thirds of initially indeterminate significance lesions are, in fact, benign [5]. Our study reflects literature findings; 64.24% of the operated cases with an indeterminate cytological diagnosis were histologically found to be benign. For these patients, thyroid surgery is unnecessary; undergoing such surgery exposes them to a 2–10% risk of serious surgical complications, and for most a lifetime of levothyroxine replacement therapy [8]. Moreover, patients with malignant tumours and indeterminate FNA cytology typically undergo a limited surgery, i.e. thyroid lobectomy. After the diagnosis of the malignancy has been established by pathological examination of the removed nodule, these patients require a second operation to complete the thyroidectomy, which is associated with additional costs and increased rate of morbidity [21].

Out of all thyroid cancer types PTC is the most frequent, accounting for approximately 85–90% of all TC, whereas FTC accounts for about 10% or less, and ATC is very rare (approximately 1–2%) [22]. We have found similar frequency rates of TC types: PTC — 89.62%, FTC — 7.55%, MTC — 1,89%, and ATC — 0.94%. PTC is characterised by slow growth and long existence, thus patients generally have good 5- and 10-year survival rates [23, 24]. The favourable survival is partly due to PTC being a normal finding; rather than being called carcinoma, some authors say it should be called a benign papillary tumour [25]. However, 20 to 30% of these patients develop local recurrence or distant metastasis, and 1% of the cases are lethal [26].

Recently, knowledge of molecular genetics of thyroid cancer has started to be transferred into clinical practice. The main goal of recent studies is to improve the accuracy of FNA biopsy assessment and to predict tumour behaviour. A number of genetic mutations are known to occur in thyroid cancer. PTC may carry point mutations of the BRAF V600E and RAS genes, RET/pTC, and TRK rearrangements [1]. The BRAF V600E mutation is a thymine-to-adenine transversion at nucleotide 1799 (T1799A), leading to the substitution of valine to glutamic acid at residue 600 of the protein (V600E), triggered by the binding of RAS and resulting in the tumorogenetic activation of the cascade of MEK and ERK of the MAPK pathway via phosphorylation [1, 3]. It is the most commonly known genetic alteration, found in 30–80% of papillary thyroid carcinomas [27]. However, there are some demographic differences — with regions in East Asia (in particular Korea) reporting a higher incidence of approximately 90% [20, 28, 29]. We have found that 77.89% of PTC cases were positive for the BRAF V600E mutation. It is reported that preoperative testing for the BRAF V600E mutation may increase the accuracy of the diagnosis of PTC in up to 30% of thyroid FNABs that were initially diagnosed as indeterminate or suspicious for malignancy [18]. The mutation is highly prevalent in the tall cell variant of PTC, where it occurs in 70–80% of cases, and in tumours with classic papillary growth (60%). In contrast, BRAF V600E is found only in about 10% of follicular variants of PTC [25]. Additionally, the BRAF V600E mutation occurs most commonly in aggressive subtypes of PTC [9]. In a histopathological examination, the presence of the BRAF V600E mutation is associated with higher clinical stage, aggressive biological behaviour, such as extra thyroidal extension, angiolymphatic invasion, metastases to the lymph nodes, and loss of responsiveness to radioiodine [30]. Most importantly, PTC patients who were positive for the BRAF V600E mutation had a worse outcome (persistent disease and lower survival rate) during a 15-year follow-up [31]. Finally, because targeted therapy for thyroid cancers with multikinase inhibitors is under rapid development, the detection of mutations in the FNA material may be helpful in the future to guide mutation-specific targeted therapies that can be initiated preoperatively or for those patients who are not surgical candidates [21].

To sum up, PTC accounts for the majority of all thyroid malignancies and has a relatively benign nature. The BRAF V600E mutation is reported to have high specificity for PTC. Furthermore, comparison of the genetic investigations in cytology and histology specimens showed that the BRAF V600E status follow-up can help reveal initially BRAF V600E negative PTC cases.

Conclusions

Repeated examination of the BRAF V600E mutation status in the FNA may potentially increase the sensitivity of diagnosing papillary thyroid cancer. Even though the results are encouraging, larger scale prospective trials are needed.

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