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Frequency assessment of *BRAF* mutation, *KRas* mutation, and *RASSF1A* methylation in nodular goitre based on fine-needle aspiration cytology specimens

Ocena częstości występowania mutacji genów *BRAF*, *KRas* oraz metylacji genu *RASSF1A* w wolu guzkowym na podstawie badania materiału cytologicznego uzyskanego drogą biopsji aspiracyjnej cienkoigłowej

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Abstract

Introduction: Standard pre-operative diagnosis of nodular goitre is not always conclusive. The decision about nodular goitre surgery is increasingly based on molecular methods.
The aim of the study was to determine *BRAF* T1799A mutation and K*Ras* proto-oncogene mutation, and the analysis of *RASSF1A* promoter methylation level in cytological material obtained from FNAB specimens of thyroid nodules.
Material and methods: The study population consisted of 85 women and 12 men. The study material was genomic DNA isolated from peripheral blood and thyroid bioptates. Pyrosequencing was used for the evaluation of *RASSF1* methylation level. KRas mutation was investigated with Sanger sequencing. BRAF mutation was analysed by standard methods of real-time amplification detection (real-time PCR) with the use of specific starters surrounding the mutated site.
Results: A significant positive correlation was demonstrated between mean methylation of four CpG islands of RASSF1A gene and thyroid tumour volume and its largest diameter ($p < 0.05$). KRas mutation was not detected in any of the 97 patients. In 7/85 subjects (8.2%) BRAF mutation was observed. In 6/7 patients with BRAF mutation, FNAB of thyroid nodules confirmed a benign nature of the lesions; the material was non-diagnostic in one patient, and papillary thyroid cancer was diagnosed on the basis of postoperative histopathology assessment.
Conclusions: The results of genetic tests reported in our study indicate that the presence of BRAF mutation or higher RASSF1A methylation levels in FNAB cytology specimens of benign lesions may be useful in the assessment of oncological risk, while the evaluation of KRas proto-oncogene mutation is not a valuable test in pre-operative diagnosis of nodular goitre. **(Endokrynol Pol 2015; 66 (5): 384–393)**

Key words: *nodular goitre; fine-needle aspiration biopsy; BRAFV600E mutation; KRas mutation; RASSF1A methylation*

Streszczenie

Wstęp: Na podstawie standardowej przedoperacyjnej diagnostyki wola guzkowego nie zawsze uzyskuje się jednoznaczne rozpoznanie. Coraz częściej w kwalifikacji wola guzkowego do zabiegu operacyjnego wykorzystywane są metody badań molekularnych.
Celem pracy było oznaczenie mutacji T1799A genu *BRAF* i mutacji protoonkogenu K*Ras* oraz analiza stopnia metylacji promotora genu *RASSF1A* w materiale komórkowym uzyskanym z guzków tarczycy na drodze biopsji aspiracyjnej cienkoigłowej.
Materiały i metody: Badaniami objęto 85 kobiet i 12 mężczyzn. Materiał do badań stanowił genomowy DNA wyizolowany z krwi obwodowej pacjentów oraz z bioptatów tarczycy. Do oceny stopnia metylacji genu *RASSF1* wykorzystano metodę pirosekwencjonowania. Mutacje genu *KRas* badano metodą sekwencjonowania Sangera. Do oznaczania mutacji *BRAF* użyto standardowej metodologii detekcji amplifikacji w czasie rzeczywistym (*real-time* PCR) z zastosowaniem specyficznych starterów otaczających miejsce zmutowane.
Wyniki: Wykazano, że średnia metylacji czterech wysp CpG w genie *RASSF1A* znamiennie, dodatnio koreluje z objętością guza tarczycy i największym wymiarem guza ($p < 0,05$). U żadnej z 97 osób nie stwierdzono mutacji *Kras*. U 7/85 badanych (8,2%) stwierdzono obecność mutacji genu *BRAF*. U 6/7 osób z obecnością mutacji *BRAF*, *BAC* guzków tarczycy wykazała łagodny charakter tych zmian, u jednej osoby otrzymano materiał niediagnostyczny, a na podstawie pooperacyjnego badania histopatologicznego rozpoznano raka brodawkowatego tarczycy.
Wnioski: Otrzymane wyniki badań genetycznych wskazują, że obecność mutacji genu *BRAF* lub wyższego odsetka metylacji genu *RASSF1A* w materiale cytologicznym z biopsji aspiracyjnej cienkoigłowej zmiany cytologicznie łagodnej może mieć znaczenie w ocenie zagrożenia onkologicznego, podczas gdy ocena mutacji protonkogenu *KRas* nie jest przydatna w diagnostyce przedoperacyjnej wola guzkowego. **(Endokrynol Pol 2015; 66 (5): 384–393)**

Słowa kluczowe: *wole guzkowe; biopsja aspiracyjna cienkoigłowa; mutacja genu BRAFV600E; mutacja Kras; metylacja RASSF1A*

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Introduction

Nodular goitre is the most common pathology of the thyroid gland. Palpable thyroid nodules are found in 4–7% adults and are more frequent in women than in men [1]. The ultrasonography findings showed that the thyroid nodules are visualised in nearly 60% of clinically normal thyroid glands. In the majority of cases, these nodules are benign [2, 3]. In 5% of fine-needle aspiration biopsies a thyroid cancer is diagnosed [4, 5]. Standard pre-operative diagnostic tests of nodular goitre are mainly based on two investigations: a thyroid ultrasound (US) and ultrasound-guided fine-needle aspiration biopsy (US-FNAB). A thyroid FNAB remains the most sensitive and the most specific pre-operative diagnostic test for thyroid cancer. However, its results may not always be conclusive. Patients with non-diagnostic results in two consecutive thyroid FNABs and some patients with indeterminate follicular lesions are candidates for thyroid surgery, although only 10–20% of these tumours turn out to be cancerous [4, 5].

Patients with nodular goitre who initially have not been selected for surgery still require periodic US and cytology monitoring of their focal lesions in the thyroid gland. Even though these standard investigations may be performed on a regular basis, they do not always provide sufficient information on the risk of neoplastic transformation in the so-far benign thyroid nodules. The selection of patients for nodular goitre surgery is more and more commonly based on molecular methods evaluating the frequency of mutations in the selected genes and epigenetic changes involved in the neoplastic transformation processes [6].

The aim of this study was to determine BRAF T1799A mutation and *KRas* proto-oncogene mutation as well as the analysis of *RASSF1A* promoter methylation levels in the cytological material obtained from nodular goitres using fine-needle aspiration biopsy.

The authors made an attempt to assess the value of selected molecular methods for pre-operative diagnosis of nodular goitre.

Material and methods

Ninety-seven (97) patients with nodular goitre were included in the study. Hyper- and hypothyroidism were excluded with hormone testing, while the presence of autoimmune chronic inflammatory process involving the thyroid gland was ruled out with antithyroid antibody tests and a thyroid ultrasound. The following investigations were performed for each patient:

— Laboratory tests: serum levels of TSH, fT4, fT3, antithyroglobulin, and antimicrosomal antibodies. TSH (n 0.27–4.2 uIU/mL), fT4 (n 0.9–1.8 ng/dL), and fT3 (n 2.00-4.4 pg/dL) levels were determined with electrochemiluminescence using commercial kits, while antithyroglobulin and antimicrosomal antibody levels were measured with microparticle enzyme immunoassay (MEIA) using commercial kits.
— A thyroid ultrasound was performed with an ALOKA ultrasound system and a 5–12 MHz linear transducer. All thyroid ultrasound investigations were performed by an experienced endocrinologist.
— US-guided fine-needle aspiration biopsy of a focal lesion in the thyroid gland selected on the basis of its US characteristics.
— FNAB cytology. Cytology assessment was based on international Bethesda System with six diagnostic categories for reporting thyroid cytopathology in FNAB samples of thyroid tumour.
— FNAB and peripheral blood samples were preserved for genetic testing including the assessment of RASSF1A gene methylation and the detection of mutations in BRAF (T1799A) and KRas genes. These tests were performed in patients treated at the Endocrine Outpatient Clinic located at our hospital. The subjects included in the study gave their informed consent to take part in this scientific research. The study was approved by the Ethics Committee at Pomeranian Medical University in Szczecin.

The study material consisted of genomic DNA isolated from patients' peripheral blood and thyroid bioptates. Peripheral blood samples were collected into ethylenediaminetetraacetic acid (EDTA) test tubes and stored at –20°C until DNA isolation. The samples of the material obtained from thyroid biopsy were collected into RNA later buffer (Ambion) and stored at –80°C until DNA isolation. QIAamp DNA Blood Mini Kit (QIAGEN) was used for DNA isolation from blood, while DNA was isolated from bioptates with AutoMate Express DNA Extraction System (Applied Biosystems) using a PrepFiler Express Forensic DNA Extraction Kit (Applied Biosystems). Then, DNA isolate levels were measured with a PICODROP (Picodrop) UV/Vis spectrophotometer. All DNA samples obtained from bioptates were modified with sodium bisulphate — Epitect Bisulfite Kit (QIAGEN).

Pyrosequencing was used for the assessment of RASSF1 promoter methylation. Preparation of biotinylated PCR product

RASSF1 fragment (chr3:50374236-50374409) containing four CpG islands was analysed. Nucleotide sequence of analysed *RASSF1* fragment: TCGAACGCGGAAATCGA (CpG islands have been underlined). Having optimised

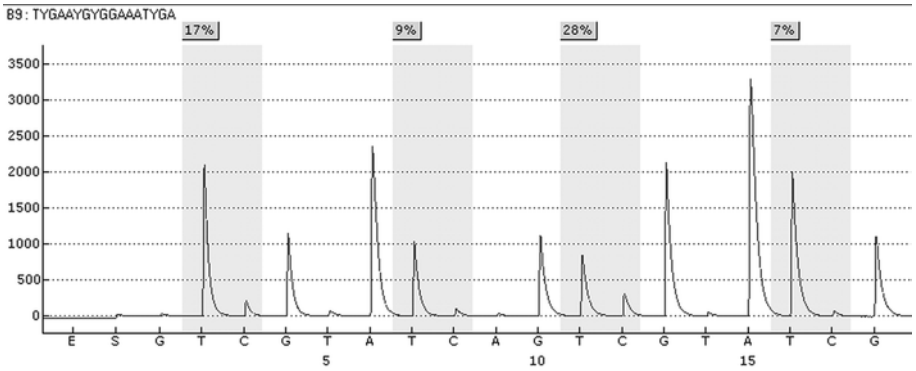

Figure 1. *Example of a sequential pyrogram obtained for RASSF1 gene*
Rycina 1. *Przykładowy pirogram sekwencyjny genu RASSF1*

all PCR conditions, this fragment was amplified in Veriti 96 Well Fast Thermal Cycler (Applied Biosystems) with 60 μL of reaction mixture containing the following components:
— 12 μL bisulphite-treated DNA
— 30 μL 2XPCR Master Mix (MBI Fermentas)
— 3 μL PCR Primer Set [10×] — primer reverse with 5′-biotin modification (PyroMark CpG Assay — Hs_RASSF1_03_PM; QIAGEN)
— 3 μL PCR Primer Set [10×] — starter reverse with 5′-biotin modification (QIAGEN)
— 15 μL H_2O
The following temperature and time profiles were applied in PCR:
Phase I: preliminary denaturation 95˚C — 15 min.
Phase II (50 cycles):
— denaturation: 95˚C — 20 sec.
— hybridization: 56˚C — 40 sec.
— elongation: 72˚C — 40 sec.
Phase III: final elongation 72˚C — 10 min.

The quality of the PCR product obtained was verified during electrophoresis in 2% agarose gel stained with ethidium bromide. In order to do that, 8 μL of PCR product with the addition of 2 μL of bromophenol blue was applied to the gel. The separation was performed in 1xTBE buffer [0.089 M Tris, 0.089 M boric acid, 2 mM EDTA], at 20˚C and a voltage of 75 V. The length of PCR product was determined with DNA size marker — pUC Mix Marker 8 (MBI Fermentas). The final stage was to take photographs documenting the gel using Polaroid DS-34 Direct Screen Camera, in UV light (Transiluminator 4000, Stratagene).

Immobilization of biotinylated PCR product onto streptavidin

80 μL of reaction mixture was applied on a 96-well PCR plate. The reaction mixture consisted of:
— 37 μL of PCR product
— 3 μL of streptavidin–coated sepharose beads
— 40 μL of PyroMark Binding Buffer (QIAGEN)
The plate was foil-wrapped and placed in a thermomixer (800 rpm, room temperature) for 10 minutes.

Preparing PyroMark Q96 HS Plate

12 ml of the mixture was dispensed into a PyroMark Q96 HS Plate. The mixture consisted of:
— 1.2 μL Sequencing Primer [10×] (PyroMark CpG Assay - Hs_ RASSF1_03_PM; QIAGEN)
— 10.8 μL PyroMark Annealing Buffer (QIAGEN)

Then, both plates (PyroMark Q96 HS Plate and PCR plate) were placed in a PyroMark Q96 Vacuum Workstation. After gentle aspiration of PCR products, the filters were flushed sequentially in 70% ethanol (10 sec.), Denaturation Solution (10 sec.), and Wash Buffer (10 sec.). Once the flushing had been completed, the filters were immersed in a PyroMark Q96 HS Plate and then the plate was wrapped in aluminium foil and placed in a PyroMarkQ HS Sample Prep Thermoplate in the incubator at 80˚C. Plate incubation at 80˚C lasted for 10 minutes. The plate was left undisturbed for 30 minutes to cool down to room temperature.

Analysis with a Pyromark MD pyrosequencer (Biotage)

After cooling down to room temperature, the PyroMark Q96 HS Plate was loaded onto a Pyromark MD pyrosequencer (Biotage) to perform a pyrosequencing reaction. Pyrosequencing was performed with the reagents included in a PyroMark Q96 Gold Reagents kit (QIAGEN). The resultant sequential pyrograms (Fig. 1) were analysed with Pyro Q-CpG Software (Biotage).

Detecting KRas mutation

Mutations at KRas codons 12 and 13 (exon 2) and 61 (exon 3) were determined (Table I).

The mutations were detected using Sanger sequencing method with direct sequence identification in a capillary analyser. Two pairs of starters anchored

Table I. *KRas codons and mutations detected with sequencing method*

Tabela I. *Kodony i mutacje KRas wykrywane metodą sekwencjonowania*

Gly12Asp (GGT > GAT)
Gly12Val (GGT > GTT)
Gly12Cys (GGT > TGT)
Gly12Ser (GGT > AGT)
Gly12Ala (GGT > GCT)
Gly12Arg (GGT > CGT)
Gly13Asp (GGC > GAC)
Gln61Leu (CAA > CTA)

Table II. *Starter sequence for KRas amplification*

Tabela II. *Sekwencja starterów do ampilfikacji KRas*

Starter sequence*
A 5′ tgtaaaacgacggccagtTATTTGATAGTGTATTAACCTTATGTGTG 3′
B 5′ caggaaacagctatgaccGAAACCTTTATCTGTATCAAAGAATG 3′
C 5′ tgtaaaacgacggccagt TGTGACATGTTCTAATATAGTCACATT 3′
D 5′ caggaaacagctatgacc ACCAGTAATATGCATATTAAAACAAGA 3′
E 5′ tgtaaaacgacggccagtAGGTGCACTGTAATAATCCAGA 3′
F 5′ caggaaacagctatgaccCTATAATTACTCCTTAATGTCAGCTTATT 3′
G 5′ tgtaaaacgacggccagt GACTGTGTTTCTCCCTTCTCA 3′
H 5′ caggaaacagctatgacc AGCTTATTATATTCAATTTAAACCCAC 3′

*lower-case letters used to denote a sequence complementary to M13 starters; upper-case letters used for KRAS exons. A-D starters: exon 2, E-H starters: exon 3.

outside the sequenced exon region were used for each sequenced exon 2 and exon 3. One of the starter pairs used for the sequencing of each exon allowed for the amplification of short DNA fragments (less than 200 base pairs), so that the identification of mutations was possible even for partially degraded genetic material. Starter sequences used for amplification are listed in Table II; standard M13 starters (M13 F 5′ TGTAAAACGACGGCCAGT 3′, M13 R 5′ CAGGAAACAGCTATGACC 3′) were used for sequencing.

At least 20 ng of genomic DNA was used for amplification, in the conditions described below, using AmpliTaq Gold polymerase on an ABI 9700 thermocycler (Applied Biosystems, USA). Once the amplification products had been purified (ExoSAP enzyme), the sequencing reaction was prepared (see the conditions below) using standard M13 starters; each sequencing was performed in two directions (forward and reverse). After the sequencing reaction and purification of the products with BigDye XTerminator enzyme (Applied Biosystems, USA), product separation and detection with capillary electrophoresis were performed, and consequently, typical chromatograms were obtained. ABI 3500 (4-capillary) sequencer was used for electrophoresis.

Reaction conditions:

Amplification:

— reagents
- AmpliTaq Gold® PCR Master Mix 25 μL
- Starer 1 (A/C/E/G) (5 pM) 5 μL
- Starter 2 (B/D/F/H) (5 pM) 5 μL
- genomic DNA (minimum 20 ng)
- H2O to obtain the volume of 50 μL

— reaction conditions

Phase I: preliminary denaturation 96°C — 5 min.

Phase II (35 cycles):

— denaturation: 94°C — 30 sec.

— annealing: 58°C — 45 sec.

— elongation: 72°C — 45 sec.

Phase III: final elongation 72°C — 10 min.

Sequencing:

— reagents:

BigDye® Terminator Cycle Sequencing Kit v3.1 Master Mix 4 μL

starter M13 (0.8 pmol/μL) 4 μL

buffer 5X 2 μL

purified amplification product: (10 ng)

H2O to obtain the volume of 20 μL

— reaction conditions:

Phase I: preliminary denaturation: 96°C — 1 min.

Phase II (25 cycles):

— denaturation: 96°C — 10 sec.

— annealing: 50°C — 5 sec.

— elongation: 60°C — 2 min.

Phase III: final elongation 72°C — 10 min.

The assembly of sequences based on the obtained chromatograms was performed with Recall on-line tool (http://pssm.cfenet.ubc.ca/home/show) (Fig. 2) and reference sequences for exon 2 (ATGACTGAATATAAACTTGTGGTAGTTGGAGCTGGTGGCGTAGGCAAGAG TGCCTTGACGATACTCGAAATTCAGAATCATTTTGTGGACGAATATGATCCAACAATAGAG) and exon 3 (GATTCCTACAGGAAGCAAGTAGTAATTGATGGAGAAACCTGTCTCTTGGA TATTCTCGACACAGCAGGTCAAGAGGAGTACAGTGCAATGAGGGACCAGT ACATGAGGACTGGGGAGGGCTTTCTTTGTGTATTTGCCATAAATAATACT AAATCATTTGAAGATATTCACCATTATAGG).

Identifying BRAF mutation

BRAF mutation was identified with standard methods for the real-time fluorescence detection of amplifica-

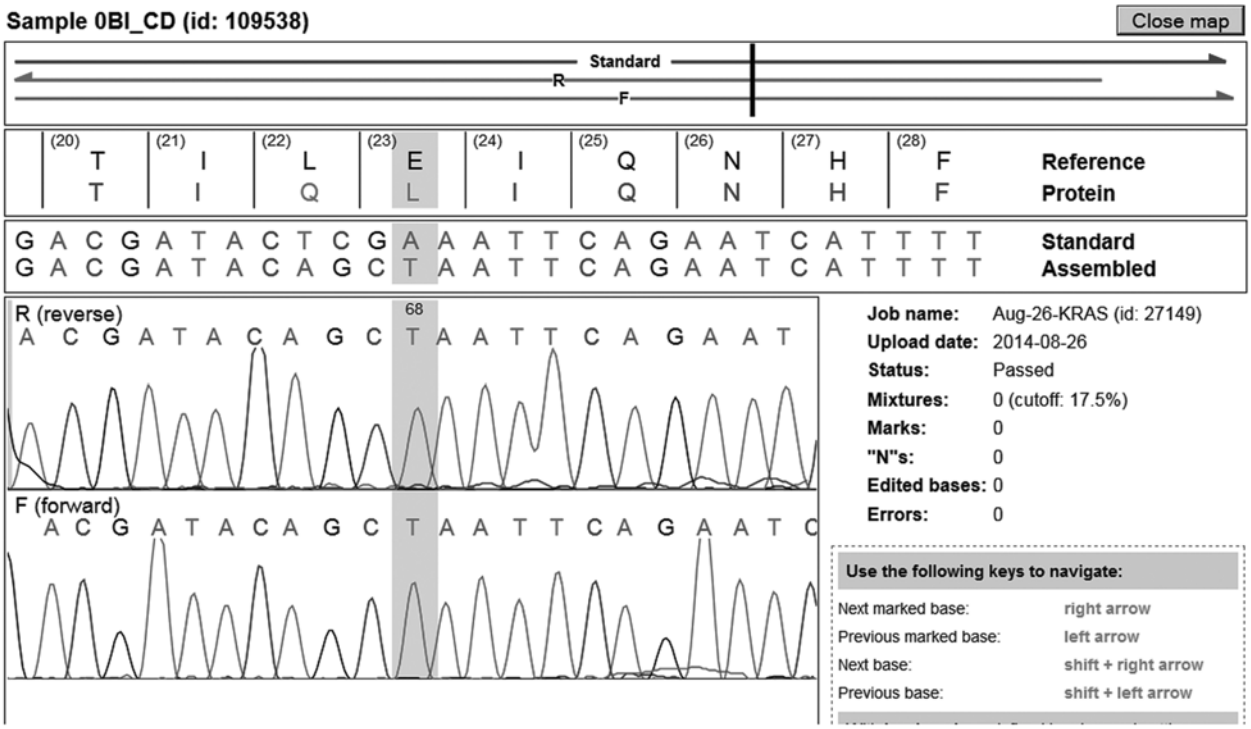

Figure 2. *A sample chromatogram fragment obtained for KRas exon 2 (Recall on-line tool)*
Rycina 2. *Przykładowy fragment chromatogramu dla egzonu 2 KRas (Recall on-line tool)*

tion (real-time PCR) using specific starters surrounding the mutated site. For each sample, separate reactions were performed with BRAF mutation-specific probe and the probe specific for the absence of BRAF mutation. TaqMan Mutation Detection Assay kit (Applied Biosystems, the USA) was used for the detection, with BRAF_475_mu and BRAF_476_mu reagents as well as specific reagents detecting non-mutated sequences. The amplification was performed using a StepOne system (Applied Biosystems, Foster City, CA, USA) at standard conditions of the reaction. The final volume of reaction mixture (20 μl) for each reaction contained 20 ng of genomic DNA, 900 nM of each starter, 300 nM of each probe, and 10 μl of Genotyping Master Mix (Applied Biosystems, Foster City, CA, USA). Reaction conditions: Preliminary activation: 10 min. at 95˚C followed by 40 amplification cycles (denaturation at 95˚C for 15 seconds and annealing /elongation at 60˚C for 1 min. with the acquisition of fluorescence signal).

The direct result — the presence or absence of mutation in a given sample — was based on the presence of fluorescence signal.

Statistical analysis

Since distributions of most quantitative variables were significantly different from normal distribution (Shapiro-Wilk test, $p < 0.05$), we used non-parametric Mann-Whitney test to compare values between groups, and Spearman rank correlation coefficient to analyse correlations between parameters. The general linear model (GLM) was used to find independent factors associated with methylation. Variables with right-skewed distributions (mean methylation, tumour volume, and maximal diameter) were transformed logarithmically before inclusion into the model. $P < 0.05$ was considered statistically significant. Statistica 10 software was used for calculations.

Results

Study population characteristics

The study group consisted of 97 subjects, including 85 women (87.6%) and 12 men (12.4%). Mean age of study subjects was 51 ± 12.2 years (median age: 54; range: 21–69 years).

Thyroid ultrasound findings

Mean thyroid volume in the study population was 16.5 mL (median volume: 14.25 mL range: 4.6–51 mL). The volume of each biopsied thyroid nodule was calculated using spheroid volume formula: volume [mL] = width [cm] × depth [cm] × length [cm] × ϖ/6. Mean tumour volume was 1.8 mL (median volume:

0.624 mL; range: 0.06–26.25 mL). The analysis of the maximum diameter of the biopsied nodules yielded the mean value of 16.1 mm (median value: 14 mm; range: 2–60 mm). In 21.6% (21/97) of patients, one focal lesion was found in the thyroid gland; 38.2% (37/97) of study subjects had two focal lesions, while three and more lesions were found in 40.2% (39/97) of patients. Fine-needle aspiration biopsy was performed on 97 focal lesions, including 68 solid focal lesions (70.1%), 26 mixed solid and fluid-filled focal lesions (26.8%), and 3 fluid-filled focal lesions (3.1%). The analysis of nodule echogenicity showed that the lesions with reduced echogenicity were predominant (47.4%), while 44.3% of lesions were normoechogenic, and there were 6.2% lesions with non-homogenous echogenicity and 2.1% hyperechogenic lesions. A halo was not observed in 93.8% of focal lesions. Micro-calcifications were found in 12.4% of focal lesions, and macro-calcifications were seen in 14.5% of lesions.

Thyroid FNAB findings

The cytology findings of thyroid nodules included: benign lesions in 83.5% (81/97) of subjects (group II), non-diagnostic material in 14.4% (14/97) of subjects (group I), indeterminate follicular lesion in one patient (group III), and a lesion suspicious for follicular neoplasm in one patient (group IV). Out of the whole group of 97 patients, five underwent thyroid surgery. Histological pathology report showed a papillary thyroid cancer (PTC) in three of these patients (3.1%) and a benign lesion in the remaining two subjects. Among patients with the diagnosis of papillary thyroid cancer, two had non-diagnostic cytology material. These patients were selected for surgery because of sonographic features of the focal lesions in the thyroid gland. In one person with papillary thyroid cancer, a cytology report of indeterminate follicular lesions was obtained.

Molecular tests

***RASSF1A* gene methylation**

Mean methylation levels of four CpG islands included in the analysis and the methylation of each individual CpG island of *RASSF1A* gene in cytology specimens of thyroid nodules are presented in Table III.

Table III. *Mean methylation levels and the methylation of individual CpG islands of RASSF1A gene in the cytology specimens of thyroid nodules*

Tabela III. *Odsetek średniej metylacji i metylacji poszczególnych wysp CpG genu RASSF1A w materiale cytologicznym guzków tarczycy*

	n	Mean ± SD (%)	Median (range) (%)
Mean methylation	97	10.3 ± 4.6	9.75 (2.25–23.75)
Methylation of 1CpG	97	11.0 ± 6.2	10 (2–33)
Methylation of 2CpG	97	6.7 ± 3.2	6 (2–17)
Methylation of 3CpG	97	16.7 ± 10.1	15 (2–53)
Methylation of 4CpG	97	6.5 ± 4.9	6 (2–44)

Mean methylation levels and the methylation of each individual CpG island of RASSF1A gene in cytology specimens of thyroid nodules in patients with PTC diagnosed in histopathology material are presented in Table IV.

The tests showed a significant positive correlation between mean methylation level of four CpG islands in RASSF1A gene and the volume of thyroid tumour as well as the largest tumour diameter ($p < 0.05$). Table V presents mean methylation levels of four CpG islands of RASSF1A gene in three groups distinguished by the largest tumour diameter (< 10 mm, 10–20 mm, ≥ 20 mm). No statistically significant correlation was found between mean methylation levels of four CpG islands and subjects' age, sex, the number of thyroid nodules ≤ 2, and the volume of the thyroid gland. A negative correlation was observed between mean methylation level and the number of thyroid nodules exceeding two (the result was at the border-line of statistical significance, $p = 0.054$). There was a statistically significant, positive correlation between mean methylation level and the presence of a mixed solid and fluid-filled focal lesion. No correlation was found between mean methylation level of analysed CpG islands and the presence

Table IV. *Mean methylation levels and the methylation of individual CpG islands of RASSF1A gene in the cytology specimens of thyroid nodules sampled from patients with histopathology-diagnosed papillary thyroid carcinoma*

Tabela IV. *Odsetek średniej metylacji i metylacji poszczególnych wysp CpG genu RASSF1A w materiale cytologicznym guzków tarczycy u pacjentów z rakiem brodawkowatym rozpoznanym w materiale histopatologicznym*

No.	Sex	AGE (years)	Mean methylation (%)	Methylation of 1CpG (%)	Methylation of 2 CpG (%)	Methylation of 3CpG (%)	Methylation of 4CpG (%)
1	F	66	4	8	2	3	2
2	F	62	9	10	6	15	5
3	F	48	11	12	7	20	6

Table V. *Mean methylation levels of four CpG islands of RASSF1A gene in three subgroups by tumour size*

Tabela V. *Odsetek średniej metylacji czterech wysp CpG genu RASSF1A w trzech grupach utworzonych w zależności od wielkości guzka*

	n	Mean ± SD	Median (range)
Mean methylation level (%) in tumours < 10 mm	20	9.15 ± 4.2	8.3 (2.25–16.75)
Mean methylation level (%) in tumours 10 mm to < 20 mm	51	9.4 ± 4.3	8.75 (2.25–21)
Mean methylation level (%) in tumours ≥ 20 mm	26	12.8 ± 4.7	12.25 (6–23.75)

of a solid or fluid-filled focal lesion; likewise, there was no correlation between mean methylation level and lesion echogenicity.

Multifactorial models including subjects' age and sex as well as the presence of more than two nodules and logarithmic nodule size (given as the volume and the largest diameter of the nodule) showed that larger and fewer (< 2) nodules are independent factors associated with higher (logarithmic) methylation levels of CpG islands.

***KRas* gene mutation**

KRas mutations were investigated in DNA of cytology specimens sampled from thyroid nodules of 97 patients, and no such mutations were found.

***BRAF* gene mutation**

The investigations of BRAF mutations in DNA of thyroid nodule cells were carried out in 85 subjects (in 12 patients, the amount of DNA was insufficient to obtain reliable results). In seven subjects (8.2%), BRAF mutation was found in cytology material and, consequently, peripheral blood DNA was investigated in these patients, and BRAF mutation was found in one of them. Mean methylation levels of four analysed CpG islands and the methylation of each island separately, the characteristics of the biopsied focal lesions in the thyroid gland and cytology findings in patients with BRAF mutation are presented in Table VI. In six patients with BRAF mutation, the FNAB findings in the thyroid nodules included benign lesions, and in one subject non-diagnostic material was reported. The latter patient underwent thyroid surgery, and papillary thyroid cancer was diagnosed based on histological pathology.

Discussion

Ultrasound-guided fine-needle aspiration biopsy is currently the best method used in the diagnosis of thyroid nodules. However, the results of studies show that in 10–20% of biopsies the sampled material is insufficient to establish the diagnosis and approx. 20% thyroid nodule biopsies require additional histopathological assessment of post-operative material to establish a final diagnosis [4, 7, 8]. The FNAB findings may be non-diagnostic because of insufficient follicular cellularity, poor fixation, or poor preservation of the material.

The value of FNAB in the pre-operative diagnosis of nodular goitre may increase significantly when molecular DNA tests of cytology material obtained from biopsied thyroid nodules are included in the standard cytology diagnosis.

Ras proto-oncogene mutations and *BRAF* gene mutations, as well as aberrant DNA methylation in the regions of promoter genes regulating the cell cycle, play an important role in the initiation of the neoplastic transformation of thyrocytes.

The most common *BRAF* gene mutation observed in the papillary thyroid carcinoma (40–70% of PTC cases) is the thymidine to adenine transversion at position 1799 (T1799A) in exon 15 of the *BRAF* gene, resulting in the valine (V) to glutamic acid (E) substitution at residue 600 (V600E) [9–15]. Many studies demonstrate that this mutation is observed mainly in papillary thyroid carcinoma and in 25% of anaplastic thyroid cancers [15–17]. T1799A BRAF oncogene is found at all stages of PTC progression; it is detected even in the early stages of its development [15, 18–20].

In our study, BRAF mutations were observed in 8.2% of analysed subjects (7/85) in FNAB cytology specimens of thyroid nodules. In 6/7 subjects with T1799A BRAF mutation, FNAB findings showed that the lesions were benign, while in one female patient the specimen was non-diagnostic. This woman was ultimately diagnosed with papillary thyroid carcinoma based on histopathology assessment of postoperative material.

In a study conducted by Zhang B. et al., BRAF mutation was present in cytology material obtained from 30 out of 42 (71.43%) thyroid nodules with papillary thyroid carcinoma, while this mutation was not detected in any of the patients with benign lesions confirmed with biopsy [4]. On the other hand, Xing M. et al. [14] investigated BRAF mutation in the FNA-biopsied thyroid nodules of 40 patients and in the postoperative thyroid tissue specimens. Histopathology findings included: papillary thyroid carcinoma (16 patients), follicular thyroid carcinoma (five patients), Hurthle cell carcinoma (one patient), adenoma or hyperplasia (14 patients), metastatic clear cell renal cell carcinoma (one patient), Graves' disease (one patient), Hashimoto's disease (one patient), and amiodarone-induced thyrotoxicosis (one patient). The analysis of these results

Table VI. *Mean methylation of 4 CpG islands of RASSF1A gene and the methylation of each individual island, the characteristics of biopsied focal lesions in the thyroid gland, and cytology findings in patients with BRAF mutation*

Tabela VI. *Średnia metylacji 4 badanych wysp CpG genu RASSF1A i metylacja każdej z tych wysp, charakterystyka bioptowanych zmian ogniskowych w tarczycy oraz ich wynik cytologiczny u pacjentów z obecnością mutacji genu BRAF*

No.	Sex	Age (years)	Greatest diameter of tumor [mm]	Tumor structure	Tumor echogenicity	Halo	Micro-calcifications	Mean methylation (%)	1 CpG (%)	2 CpG (%)	3 CpG (%)	4 CpG (%)	Cytology findings	Histopathology findings **
1	F	68	6	Mixed solid and fluid-filled	Normoecho-genic	–	–	5	4	5	6	5	Group II	
2	F	63	10	Solid	Hypoechogenic	–	+	7	6	9	7	7	Group II	
3	F	66	15	Solid	Normoecho-genic	+	–	4	8	2	3	2	Group I	PTC
4	M	45	10	Solid	Normoecho-genic	–	–	11	5	4	30	4	Group II	
5	F	53	19	Mixed solid and fluid-filled	Normoecho-genic	–	–	13	15	7	24	8	Group II	
6	F	22	20	Mixed solid and fluid-filled	Normoecho-genic	+	+	7	4	3	15	4	Group II	Benign lesion
7*	F	41	8	Solid	Hypoechogenic	–	–	9	4	4	24	3	Group II	

*No. 7: additionally, BRAF mutation was detected in peripheral blood DNA; **To date, thyroid surgery has been performed in 2 out of 7 these 7 subjects

showed that BRAF mutation was present in 50% of nodule specimens obtained from pre-operative fine-needle biopsy (8/16) which turned out to be papillary thyroid cancer in post-operative histopathology. On the other hand, BRAF mutation was not detected in any other samples for which different histopathology diagnoses were established. In addition, BRAF mutation was detected in cytology specimens sampled from two patients with indeterminate FNAB results, and final histopathology findings supported the diagnosis of papillary thyroid carcinoma, just as it was the case in our material.

There are many reports dedicated to genetic tests on FNAB cytology specimens, focusing mainly on non-diagnostic material, indeterminate follicular lesions, and the lesions suspected of follicular neoplasm [1, 4, 5, 8, 14, 21]. The presence of BRAF mutation in a FNAB cytology specimen of thyroid nodule is an indication for nodular goitre surgery. It should be remembered, however, that there are 0.2–5.7% of false positive BRAF T1799A results in the nodule and 1.9–5.8% of false negative results [22, 23]. A negative BRAF mutation result in the thyroid nodule may be the consequence of an insufficient number of cells with mutated BRAF genes in some FNAB cytology specimens. At present, there are reasons to repeat FNAB BRAF mutation test after 6–12 months, particularly when suspicious sonographic features are observed and cytology findings are non-diagnostic or signs of atypia are detected [24].

Jia Y. et al. conducted a meta-analysis of literature data demonstrating that the BRAF mutation test was a valuable adjunctive diagnostic tool in the assessment of nodules that are difficult to evaluate with currently available methods [1]. Koh J. et al., on the other hand, analysed the indications for BRAF mutation tests additional to cytology in the diagnosis of nodular goitre, confirming the value of these tests in pre-operative assessment of thyroid nodules with suspicious sonographic features [21]. Rossi M. et al. demonstrated that BRAF mutation tests in FNAB cell specimens enhance the diagnostic sensitivity of cytology by 28% ($p < 0.01$) in the diagnosis of thyroid cancer [25].

As in traditional cytological assessment, the analysis of genetic mutation in cytology specimens may be difficult because of insufficient cellularity, despite the use of genomic DNA amplification techniques. This was also observed in some of our cases.

Ras mutations are detected in both benign and malignant thyroid neoplasms, more frequently in follicular cancers (< 50%) than in papillary cancers (5–20%), but literature reports are greatly inconsistent on the incidence of these mutations, particularly in adenomas (in general < 30%) [9, 10, 26, 27]. A follicular adenoma with Ras mutation may be a precursor of both follicular cancer and a follicular variant of papillary carcinoma. Recent studies indicate that Ras mutation is a valuable diagnostic marker in nodules in which the diagnosis cannot be established on the basis of FNAB cytology

specimens, particularly in follicular variant of papillary carcinoma as well as in follicular carcinoma and adenoma [6,28].

This problem has been investigated by Lee S.R. et al., who analysed 59 historical FNAB reports with histological pathology diagnosis of follicular variant of papillary carcinoma. FNAB cytology findings in these patients included: a benign lesion in 5% of subjects, atypic cells in 19% of subjects, a follicular neoplasm or suspected follicular neoplasm in 14% of subjects, a suspected papillary cancer in 29% of patients, and a papillary cancer in 34% of patients. In the above-quoted study, the mutations of three Ras proto-oncogenes in FNAB cytology specimens were observed in 18 (33%) nodules, including NRas in 22%, HRas in 6%, and KRas in 6% of Ras-positive nodules. Lee S.R. et al. showed Ras mutation in 67%, 56%, and 63% of biopsies with the following cytology findings: a benign lesion, atypia/indeterminate follicular lesion, and a follicular neoplasm, respectively. The mutations of three Ras genes were found in codon 61, while they were not observed in codons 12 and 13. Lee S.R. et al. concluded that the investigations of Ras proto-oncogene mutations were useful for the improvement of sensitivity in false-negative cytology material, although these authors do not recommend Ras mutational analysis in all patients with cytology reports of benign thyroid nodules [29].

Out of the three Ras proto-oncogenes (NRas, HRas, and KRas), the most common mutation is the one in NRas and HRas codon 61. Mutations in codons 12 and 13 of KRas gene are much less common, which has been confirmed by Lee S.R. et al. [29–31]. We investigated mutations in codons 12, 13, and 61 of KRas gene in the DNA of cytology specimens of thyroid nodules sampled from 97 subjects — no mutation was detected in any of the subjects. Although our findings are different from those obtained by Lee S.R. et al., we made similar conclusions regarding the value of KRas proto-oncogene mutational analysis in the cytology-diagnosed benign lesions.

The role of epigenetic mechanisms in neoplastic transformation of the thyroid gland has also been discussed in recent years. Epigenetic silencing of suppressor gene *RASSF1A* through promoter hypermethylation may lead to the loss of control of thyroid cell differentiation and proliferation. *RASSF1A* gene hypermethylation is observed both in benign and malignant thyroid neoplasms, and it is particularly frequent in follicular cancer [32, 33].

In our study, we did not find any association between mean methylation levels of four CpG islands and patients' age, sex, the number of nodules ≤ 2, thyroid volume, or the echogenicity and structure of a focal lesion (solid and fluid-filled). Although the possibly cancerous nature of a thyroid nodule is indicated by sonographic features of nodule malignancy such as nodule echogenicity, structure, the presence of microcalcifications or irregular contours rather than its size, larger size of the nodule is associated with higher risk of malignant transformation. We demonstrated that mean methylation levels of four analysed CpG islands in RASSF1A gene correlated positively with the volume of the nodule and its largest diameter, which may be significant for neoplastic transformation of the so-far benign focal lesion in the thyroid gland. No information has been found in the available literature on the association between RASSF1A methylation level in cytology specimens of thyroid nodules and their sonographic characteristics. Most published studies on RASSF1A methylation analyse thyroid tissue specimens obtained from surgical samples [33–35].

In a study of thyroid tissues obtained from surgical samples, Hing M. et al. found that 44% of benign adenomas, 75% of follicular thyroid cancer tumours, and 20% of papillary thyroid cancer tumours showed promoter methylation in more than 25% of RASSF1A alleles [33]. An interesting report has been published by Kunstman et al., who analysed RASSF1A methylation in thyroid tissues obtained during surgery from 41 patients with PTC and from 18 patients without thyroid pathology. These authors also analysed the correlation between RASSF1A methylation in patients with PTC and disease progression (lymph node metastases, extracapsular invasion), multifocality of the cancer, and PTC variant (classic, follicular, tall cell PTC). Kunstman et al. found that mean methylation was 4.2 times greater in PTC than in normal thyroid tissue ($p < 0.05$) and mean methylation was higher in multifocal than unifocal PTC ($p < 0.05$) [36].

In the study by Zhang B. et al. quoted above, along with BRAF mutation in thyroid nodule FNAB cytology specimens, the authors also investigated the methylation of five selected genes, including RASSF1A. They found significantly lower RASSF1A methylation levels in PTC than in benign lesions ($p = 0.003$) and an inverse correlation between BRAF mutation and RASSF1A methylation [4]. Similarly, a female patient with confirmed PTC and BRAF mutation included in our study displayed the lowest mean methylation levels of four CpG islands (Table VI). Zhang B et al. suggest that the study of suppressor gene methylation in FNAB cell specimens of thyroid nodules has considerable restrictions, but the authors still believe that combined testing of BRAF mutation and DNA methylation in selected genes would enhance the diagnostic value of pre-operative assessment of nodular goitre [4].

Since the role of epigenetic mechanisms in the development of thyroid cancer has been documented and

RASSF1A methylation can be detected at an early stage of carcinogenesis, further epigenetic studies of FNAB cell specimens of thyroid nodules are necessary. These studies will improve the diagnostic accuracy of thyroid nodules, and in cases of suppressor gene promoter methylation, they will be helpful in making a decision about total thyroidectomy in order to avoid two-stage surgery, which increases the number of post-operative complications and the costs of treatment.

Conclusions

The results of genetic tests reported in our study indicate that the presence of BRAF mutation or higher RASSF1A methylation level in FNAB cytology specimens of benign lesions may be useful in the assessment of oncological risk, while the evaluation of KRas proto-oncogene mutation is not a valuable test in preoperative diagnosis of nodular goitre.

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