Investigation of V600E *BRAF* mutation in papillary thyroid carcinoma in the Polish population

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Abstract

Papillary thyroid carcinoma (PTC) is the most common malignancy of the thyroid gland. The high incidence of *RET/PTC* and *Trk* rearrangements or point mutations in *RAS* and *c-MET* oncogenes are the genetic hallmarks of PTC. Recently, oncogene *BRAF* has become a subject of great interest. The mutation of *BRAF* gene is characteristic for PTC and poorly differentiated and/or undifferentiated cancers derived from PTC. The predominant mutation of this gene, reported in PTC, is a single transversion in exon 15 (T1799A), which results in substitution of valine to glutamate at residue 600 (*BRAF* V600E, formerly position 1796 and residue V599E). It has been proved that the frequency of this mutation in PTC varies within the range of 29% to 69% in different populations. **OBJECTIVES:** The aim of this study was to estimate the frequency of *BRAF* (V600E) mutation in PTC in the Polish population, and to evaluate the possible relationships between the presence of *BRAF* mutation and such parameters, as patient's age, gender, histopathological variant and the clinical staging of PTC. **METHODS**: Analysis of *BRAF* (V600E) mutation was performed by single strand conformation polymorphism (SSCP) analysis and real-time allele-specific polymerase chain reaction (AS-PCR) in tumour tissues from 25 patients with PTC. We compared the sensitivity of real-time AS-PCR, SSCP method and direct DNA sequencing of PCR products. We used 25 PTC tissues (including the follicular variant of PTC – 8 cases, the classic variant of PTC – 14 cases and the tall-cell variant of PTC – 3 cases). **RESULTS:** V600E mutation in *BRAF* gene was detected in 12/25 (48%) cases of PTC. Mutation screening of exon 15 gene *BRAF* revealed three types of mutations, i.e. V600E, V600M, and overlapping mutations V600E/V600K. No correlation was found between *BRAF* mutation and patient's age and sex and particular stage in clinical staging systems (TNM Staging, the University of Chicago clinical class, and Ohio State University Staging). Regarding the histopathological variants of PTC, mutation in *BRAF* gene was more frequent in classic variant of PTC as compared with follicular variant of PTC.

ORIGINAL ARTICLE

CONCLUSION: The real-time AS-PCR method proved to be more sensitive than SSCP and sequencing of PCR products. Our study is the first one in which the frequency of *BRAF* (V600E) mutation in PTC was reported for the Polish population. Similarly to the results obtained by others, there was no coexistence of *BRAF* (V600E) mutation and *RET/PTC* and/or *Trk* rearrangements or *RAS* mutation in PTC tissue. Our results do not confirm the relationship between the *BRAF* (V600E) mutation and the clinical outcome of PTC.

INTRODUCTION

Thyroid cancers have been categorised as the most prevalent endocrine malignancy. Papillary thyroid carcinoma (PTC) is the most common malignancy of the thyroid gland, occurring with the frequency of 60–80% [22]. The main genetic events in PTC development are related either with rearrangements of *RET* and *NTRK*1 oncogenes, point mutations in *RAS* family oncogenes or overexpression of *c-MET* gene [4,8,9,17]. In PTC, this mutation leads to aberrant expression of the genes, coding the kinase domain of RET or NTRK1 tyrosine kinase receptors or activating mutations in *RAS* or *c-MET* oncogene. All these genetic events, resulting in constitutive activation of RAS-RAF-MEK-MAP kinase signalling pathway, transmit a mitogenic signal to the nucleus and promote uncontrolled cell division [16].

The abnormal genetic alternations, mentioned above, i.e., point mutation in RAS family or chromosomal aberrations in PTC, have been shown to play an important role in thyroid tumorigenesis. However, these types of genetic alternations are not sufficiently useful as diagnostic or/and prognostic markers in the clinical practice. Therefore, further studies are needed, confirming the existence of potentially prognostic markers in PTC.

Activating mutation in *BRAF* gene has recently been suggested as an alternative predominant cause of PTC without *RAS* mutation [11]. The *BRAF* gene (the official name: *v-raf murine sarcoma viral oncogene homolog B1*) encodes B-type RAF protein, one of the elements of the RAS→RAF→ MEK→ ERK/MAP kinase pathway that plays a key role in cell proliferation, differentiation and apoptosis. The most frequent genetic event occurring in *BRAF* gene in the course of thyroid cancer is point mutation, i.e., a single transversion in exon 15 (T1799A). This mutation results in substitution of valine to glutamate in the highly conservative segment of the BRAF protein chain, at codon 600 (*BRAF* V600E, formerly position 1796 and residue V599E).

This mutation is believed to mimic phosphorylation in the activation segment because of the contiguity of glutamic acid (glutamate) COOH residue, stimulating the site of regulated phosphorylation at serine 599 [16,11,3].

The presence of *BRAF* mutations have been confirmed in melanoma, gliomas, colon and lung cancers [6]. It has been proven that *BRAF* T1799A transversion is the most frequent (80%) DNA alternation in PTC. Mutation of *BRAF* gene has also been detected in PTC and poorly differentiated or undifferentiated thyroid cancer, derived from PTC. According to various studies, the frequency of *BRAF* mutation in PTC varies within the range of 29% to 69% in different populations [16,3,23,26]. Nikiforova *et al.* [18] confirmed the presence of the *BRAF* mutation in 29% cases of poorly differentiated (insular) cancers and in 60% cases of anaplastic thyroid carcinoma (ATC), arising from PTC. Because of controversial results, concerning the frequency of *BRAF* (V600E) mutation in PTC in different populations and ambiguous evidences on the correlation between the presence of *BRAF* mutation and the clinical outcome, further studies are needed.

The general aim of the present study was to estimate the frequency of *BRAF* (V600E) mutation in PTC in the Polish population and to evaluate the possible relationship between the presence of *BRAF* (V600E) mutation and such parameters as patient's age and gender, the histopathological variant of the studied PTC cases, tumour size and regional lymph node metastases, according to TNM definition (pT_1-pT_3 and N_0-N_1, The TNM Staging System, according to American Joint Committee on Cancer [12].

MATERIALS AND METHODS

The procedures, used in the study, had been approved by the Ethical Committee of the Medical University of Lodz, Poland.

Thyroid tissues

Tumour tissue samples (50–100 mg) were obtained from 25 patients who underwent surgery (total thyroidectomy) for primary PTC (25 cases: 20 females, 5 males) at the Holy Family Municipal Hospital in Lodz, and at the Department of Oncologic Surgery, Centre of Oncology – the MSC Memorial Institute, Gliwice, Poland, during the years 2001–2005. The average age of all studied patients with PTC was 49.32 ± 17.23 years (the range: 16–74 years), and of the individuals who revealed *BRAF* mutation: 49.33 ± 17.81 (range: 16–72 years).

Histopathological variants of PTC were as follows: PTC classic variant – 14 cases, PTC follicular variant – 8 cases, PTC tall-cell variant – 3 cases. Tissue samples were immediately snap-frozen and stored at –70 °C. Histopathological diagnoses, according to WHO classification of tumours [7], were obtained from pathomorphological reports and have been listed in Table 1, together with TNM classification and AJCC stage grouping [24].

DNA isolation and polymerase chain reaction (PCR) of exon 15 of BRAF gene

Genomic DNA extraction was performed, according to the manufacturer's recommendations (Genomic Midi AX, A&A Biotechnology, Gdynia, Poland). DNA concentration and purity were spectrophotometri-

cally quantified by measuring absorbance at 260/280 nm (Spectrophotometer Ultrospec 2000, Pharmacia Biotech, Sweden).

The amplification of exon 15 of *BRAF* gene was performed in a Mastercycler personal (Eppendorf, Hamburg, Germany) in total volume of 25 µl, containing: 1 000 ng DNA, 0.25 µM of each (i.e. forward and reverse) oligonucleotide primers (TIB MOLBIOL, Gdynia, Poland), 25 mM mix dNTP, Taq Polimerase (2 U/µl) (Finnzymes OY, Finland) and 10×PCR buffer (1×10 mM Tris-HCl, pH 8.8, 1.5 mM of $MgCl_2$, 50 mM of KCl, 0.1% Triton X-100) and nuclease-free water. The sequences of primers, used for PCR amplification, were as follows: forward primer – 5'-AAACTCTTCATAATGCTTGCTCTG-3' and reverse primer – 5'-GGCCAAAAATTTAATCAGTGGA-3'. DNA amplification was carried out, according to the following protocol: initial denaturation at 95 °C for 5 min, followed by 35 cycles: denaturation at 96 °C, 1 min, annealing at 60 °C, 1 min, and elongation at 72 °C, 1 min; the final elongation step: 72 °C, 10 min. PCR sample without any addition of DNA (blank sample with nuclease free water instead of DNA) was the control for PCR contamination.

As a result of PCR reaction we obtained 240 bp PCR fragments. Products of PCR amplification were separated and identified on 2% agarose gels, stained with ethidium bromide (0.5 mg/ml) and visualized under UV light (Transiluminator TI 1, Biometra, Germany).

Single strand conformation polymorphism (SSCP)

PCR products (240 bp DNA fragments) were subjected to single strand conformation polymorphism (SSCP) analysis. For SSCP analysis 10 µl of PCR products of exon 15 of *BRAF* gene were diluted (1:1)

Table 1. Clinical and histological characteristics of the studied patients.

Case number	Age	Sex	Histopathological diagnosis	TNM staging system	American Joint Committee on Cancer (AJCC) grouping system
1.	59	F	PTC– tall-cell variant	$pT_3N_xM_x$	III
2.	72	F	PTC– follicular variant	$pT_{2b}N_0M_0$	II
3.	26	F	PTC– follicular variant	$pT_4N_{1a}M_0$	I
4.	47	M	PTC– classic variant	$pT_{2a}N_{1a}M_0$	III
5.	68	F	PTC– follicular variant	$pT_{2b}N_{1a}M_0$	III
6.	74	F	PTC– classic variant	$pT_{1a}N_0M_0$	I
7.	41	F	PTC– follicular variant	$pT_3N_0M_0$	I
8.	16	F	PTC– classic variant	$pT_{2a}N_0M_0$	I
9.	39	F	PTC– classic variant	$pT_4N_0M_x$	I
10.	61	F	PTC– classic variant	$pT_{2a}N_0M_0$	II
11.	30	M	PTC– classic variant	$pT_4N_{1b}M_0$	I
12.	71	F	PTC– classic variant	$pT_{2a}N_0M_0$	II
13.	66	M	PTC– follicular variant	$pT_{4a}N_0M_0$	I
14.	67	F	PTC– tall-cell variant	$pT_{4b}N_0M_0$	I
15.	40	M	PTC– classic variant	$pT_{1a}N_{1a}M_0$	I
16.	40	F	PTC– classic variant	$pT_{2a}N_0M_0$	I
17.	16	M	PTC– follicular variant	$pT_{1a}N_{1a}M_0$	I
18.	32	F	PTC– classic variant	$pT_{2a}N_0M_0$	I
19.	47	F	PTC– follicular variant	$pT_{1b}N_0M_0$	I
20.	56	F	PTC– follicular variant	$pT_{2a}N_{1b}M_0$	IVA
21.	61	F	PTC– classic variant	$pT_{1a}N_0M_0$	I
22.	65	F	PTC– classic variant	$pT_3N_0M_0$	III
23.	49	F	PTC– tall-cell variant	$pT_{2b}N_xM_x$	II
24.	38	F	PTC– classic variant	$pT_1N_0M_x$	I
25.	52	F	PTC– classic variant	$pT_2N_0M_0$	II

with 10 µl of denaturing solution, containing: 95% formamide (Sigma-Aldrich, Saint Louis, USA), 10 mM of NaOH (Applichem, Darmstadt, Germany), 0.25% bromophenol blue (Sigma-Aldrich, Saint Louis, USA) and 0.25% xylene cyanol (Sigma-Aldrich, Saint Louis, USA), placed at 94 °C for 5 min and immediately chilled on ice to prevent reannealing of the single-strand DNA products. Evaluation of single-strand DNA mobility was performed, using 8% acrylamide gel for mutation detection (Sigma-Aldrich, Saint Louis, USA) in 0.6 × Tris –borate-ethylenediamine tetraacetate (TBE) buffer at 4 °C, 90 V for 12 h. We analysed DNA from both PTC and macroscopically unchanged thyroid tissue in parallel, to compare the migration band pattern. Acrylamide gels were visualised by standard DNA silver staining (Silver nitrate 10%, AppliChem, Darmstadt, Germany) according to the manufacturer's instructions.

Real-time allele-specific amplification for BRAF mutation V600E

For real-time AS-PCR we used two (2) different forward primers with variations in their 3' nucleotide sequence, such that one of them was specific for the wild-type of *BRAF* gene sequences of exon 15, *BRAF* (Wt): 5'-AGGTGATTTTGGTCTAGCTACAGT-3', and the other was specific for the mutant variant of *BRAF* gene sequences of exon 15, *BRAF* (M): 5'-AGGTGATTTTGGTCTAGCTACAGA-3'; we used one universal reverse primer, *BRAF* (R): 5'-TAGTAACTCAGCAGCATCTCAGGGC-3' according to Jarry *et al.*'s [14] protocol. The amplification reaction in real-time AS-PCR was performed, using two mixes of primers. The first amplification reaction was performed with a mix of the forward primer, specific for the wild type of *BRAF* gene sequences, i.e., *BRAF* (Wt) and universal reverse primer, i.e. *BRAF* (R), to obtain wild type PCR products. The second real-time AS-PCR reaction was performed with different mix primers, *BRAF (*M) and *BRAF* (R), in order to obtain mutant variants of exon 15 of *BRAF* gene. The annealing temperature and primer concentrations were optimised on a Mastercycler gradient (Eppendorf , Hamburg, Germany).

The real-time AS-PCR reactions were performed in the 7500 Real Time PCR System (Applied Biosystems). The reaction mixture included: 25 µl of SYBR Green PCR MasterMix, containing SYBR Green I Dye, AmpliTaq Gold® DNA Polymerase, dNTPs with dUTP, Passive Reference and optimized buffer components (Applied Biosystems, Warrington UK), 3 µl of forward and 3 µl of reverse primers (300 ng of each), 5 µl of genomic DNA (100 ng) and nuclease-free water to volume of 50 µl. Both real-time AS-PCR reactions – the first with *BRAF* (Wt)/ *BRAF* (R) primers, and the second with *BRAF (*M)/ *BRAF* (R) primers – were run for each tumour sample in the same real-time AS-PCR reaction but in separate wells. The cycling conditions were as follows: denaturation for 10 min at 95° C; amplification for 40 cycles, with denaturation for 15 s at 95 °C, elongation for 1 min at 60 °C and annealing for 1 minute at 72 °C. The real-time fluorescence signal, generated by the double-stranded DNA binding dye SYBR Green I Dye was analyzed, using the ABI PRISM 7500 SDS analysis software (Applied Biosystems). A threshold cycle (C_T) was determined for each sample in the exponential growth phase of the amplification curve. The threshold fluorescence level was automatically estimated by the ABI PRISM 7500 SDS analysis software. In order to detect nonspecific amplification (primer-dimers formation), we checked purity of the PCR products by 8% polyacrylamide gels (PAA), independently of the real-time PCR reaction. In order to exclude primer-dimer artifacts, we performed also melt curve analysis of PCR products, using dissociation curves, estimated by computer software. Fluorescence emission data, expressed as C_T values at threshold for each samples, were determined as triplicate C_T values (means), obtained in triple repeats of real-time PCR reaction.

Direct automated fluorescent sequencing

All the studied samples of PTC, in which *BRAF* (V600E) mutations were confirmed by real-time AS-PCR, were submitted after purification (AutoSeq™ G-50 Columns Amersham Biosciences, England) to direct sequencing, using the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit and analyzed in the ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

The data were statistically analyzed, using non-parametric U Mann-Whitney's test in order to correlate the presence of *BRAF* (V600E) mutation in PTC tissue with patient's age, primary tumour size (pT_1, pT_2, pT_3, pT_4) and regional lymph node metastasis (N_1, N_0, N_x), according to TNM definition. Fisher's test was performed in order to correlate the presence of *BRAF* (V600E) mutation with patient's gender and with the histopathological variant of PTC (classic, follicular and tall-cell variant of PTC). Statistical significance was determined at the level of $p<0.05$. The results are presented as mean ± SEM.

RESULTS

As a result of the PCR-SSCP analysis of exon 15 of *BRAF* gene, we detected an altered migration pattern (band shift) of a single DNA strand in 9/25 (36%) of analyzed DNA fragments (Figure 1). For SSCP analysis, we used DNA extracted from PTC tissue, as well as from macroscopically unchanged thyroid tissue that served as a wild-type control. Afterwards, all the 25 studied DNA samples (including those with band shift, suggesting the presence of mutation in exon 15) were analyzed for V600E mutation by real-time AS-PCR analysis. The ABI PRISM 7500 SDS software, provided with the instrument, analyses the fluorescence data, generated during the reaction, and calculates the cycle number at which

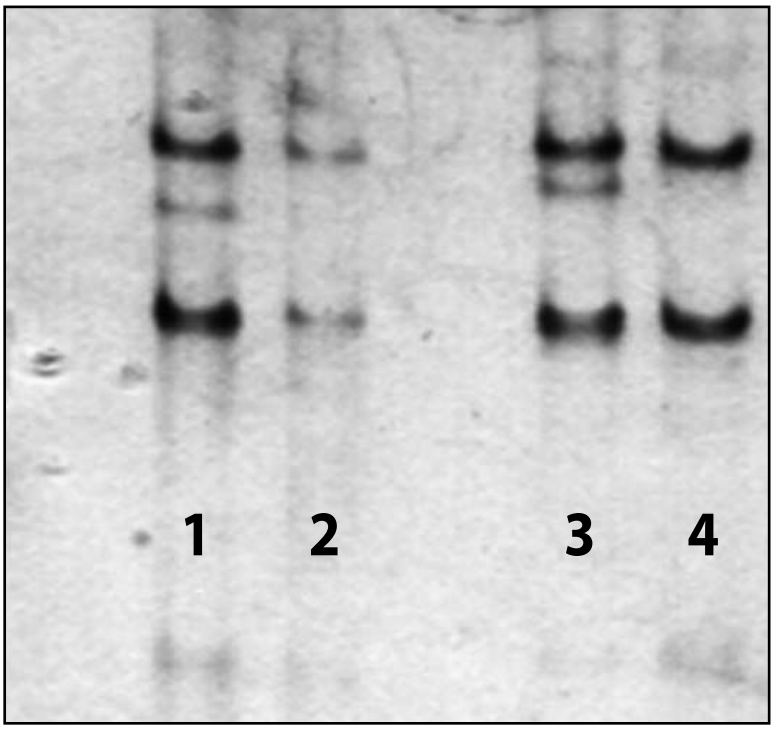

Figure 1. Example of PCR-SSCP analysis of exon 15 of *BRAF* gene.
Lines: 1 and 2 – DNA samples from the same patient (case no. 8); DNA, extracted from tumour tissue, shows a changed pattern of single strand DNA conformation (line 1) and DNA from the macroscopically unchanged thyroid tissue (line 2) has a normal pattern.
Lines: 3 and 4 – DNA samples from the same patient (case no. 19), DNA extracted from tumour tissue shows a changed pattern of single strand DNA conformation (line 3) and DNA from the macroscopically unchanged thyroid tissue (line 4) has a normal pattern.

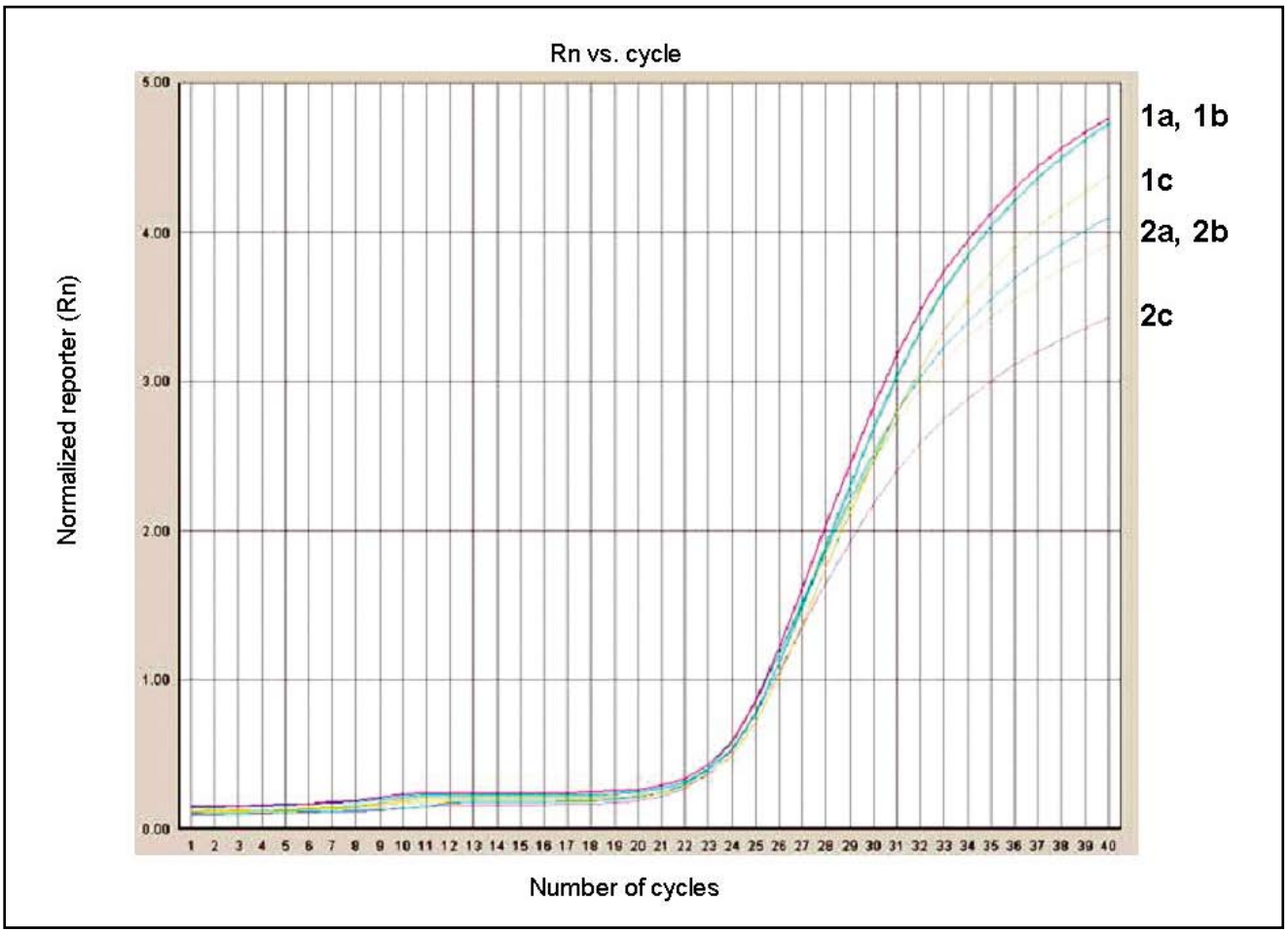

Figure 2. Example of real-time AS-PCR analysis of *BRAF* gene. Representative amplification curves for PTC sample (case no. 8) with presence of V600E mutation (exon 15, T1799A).
Lines 1a, 1b, 1c – triple repeats of real-time AS-PRC reaction, using primers specific for the wild -type allele of gene, C_T value (mean) – 22.988
Lines 2a, 2b, 2c – triple repeats of real-time PRC reaction, using primers specific for the mutant allele gene, C_T value (mean) – 23.417

fluorescence crosses the threshold value (C_T value). Triplicate amplifications of the sample produced nearly identical, overlapping curves, from which C_T values were calculated. Figure 2 illustrates typical assay results (amplification curves) generated from PTC sample with *BRAF* V600E mutation. In our study, we assumed that C_T values should be less than 26 to be considered an interpretable specimen. Higher C_T values may indicate mostly insufficient template quantity, but also template quality and/or the presence of inhibitors. According to this assumption, we included 12 cases with the C_T values for mutant-type primer ranging from 22.051 to 25.964 (Table 2) into the group of PTC with V600E mutation. PTC samples with C_T values for the mutant-type primer ranging from 26.910 to 35.391, were considered as not possessing V600E mutation (Table 2).

Using real-time AS-PCR assay, we confirmed the presence of V600E mutation in exon 15 of *BRAF* gene in five (5), out of nine (9) cases of the studied PTC samples which presented alterations in single-strand DNA conformation. Moreover, using real-time AS-PCR assay, we detected the presence of V600E mutation in additional seven (7) cases of the studied PTC, which did not present any alterations (band shift) in SSCP analysis. In total, using the real-time AS-PCR method, we proved the presence of V600E mutation – T1799A substitution in codon 600 of *BRAF* gene – in 12/25 (48%) of the studied PTC cases (Table 3).

Table 2. Real-time AS-PCR results calculated by C_T values.

Number of cases	Mean C_T value for wild-type primer	Mean C_T value for mutant-type primer
12 (considered to have V600E mutation in *BRAF* gene)	21.330 (19.548–23.457)	23.215 (22.051–25.964)
13 (considered as not possessing V600E mutation in *BRAF* gene)	22.764 (20.341–25.464)	31.107 (26.910–35.391)

Table 3. The results of mutation screening in exon 15 of *BRAF* gene

Mutation identification method		Number of detected cases
Real-time AS- PCR		12/25
SSCP		9/25
Direct sequencing	Total	4/25
	V600E mutation (T1799A)	1
	V600M mutation (G1798A)	1
	V600K/V600E mutation (GT1798-99AA/T1799A)	2

Additionally, mutation screening of exon 15 of *BRAF* gene, using the direct sequencing method, revealed very interesting results. Namely, we observed three types of mutation in exon 15 of *BRAF* gene, i.e., V600E, V600K/V600E and V600M (Table 3). Sequencing revealed two (2) cases of V600E mutation, as a result of T→A base change at nucleotide position 1799, but only one (1) of those cases was unequivocal (Figure 3A). The other case represented overlapping mutations V600K/V600E (Figure 3B). V600K mutation is a 2-bp change (GT1799-1799AA) in codon 600 of *BRAF* gene. In both cases, the presence of V600E mutation was consistent with the results obtained by real-time AS-PCR. Additionally, we observed a single nucleotide substitution G1798A in the first nucleotide position of the codon 600 of *BRAF* gene in one (1) PTC case (Figure 3C). This single nucleotide substitution results in V600M mutation in the protein chain. Most of our samples with mutations, proved by direct sequencing to be V600E, V600K/V600E or V600M, showed wild-type alleles, as well as weaker signals of mutant-type alleles. The remaining cases of PTC with suspected V600E mutation (based on real-time AS-PCR results) were not proved by direct sequencing, because no definite interpretations was made of V600E mutation presence. In PTC samples with no V600E mutation, found in real-time AS-PCR results, direct sequencing revealed the presence of wild-type alleles (Figure 3D).

No correlation was found between the presence of V600E mutation in *BRAF* gene, confirmed by real-time AS-PCR assay and such characteristics, as patient's age at diagnosis (p=0.849), gender (p=0.540), primary tumour size (pT_1, pT_2, pT_3, pT_4) (p=0.934) and regional lymph node metastases (N_1, N_0, N_x) according to TNM definition (p=1.000). In relation to histopathological variants of PTC, p value in χ^2 test was 0.025, indicating the relationship between the groups, but the results may be false because of small number of cases. Fisher's test for follicular and classic variants of PTC revealed the statistical significance (p=0.011) between the presence of mutation in *BRAF* gene and PTC classic variant.

DISCUSSION

The presence of *BRAF* mutation has been confirmed in melanoma, gliomas, colon and lung cancers. The aim of our study, the first one in the Polish population, was to detect the most frequent genetic event in *BRAF* gene in thyroid cancers, i.e., point mutation, being a transversion of thymine to adenine at nucleotide 1799 (T1799A). It is known that this type of mutation is a hallmark of PTC, however, its frequency reported in different studies and for different populations, varies depending on the studied population, as well as on applied identification methods. Because of very controversial results reported by others, concerning the frequency of V600E mutation and correlation between its presence and the clinical stage of disease, the histhopathological variant of PTC and such characteristics as patient's age or gender, we

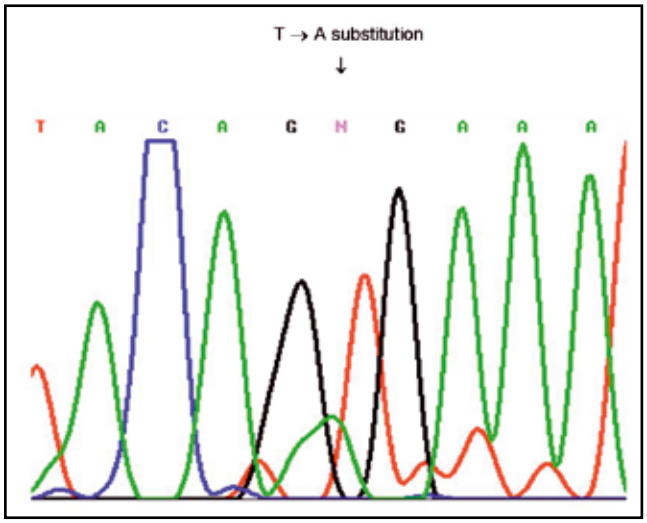

Figure 3A. Mutation analysis of *BRAF* gene (exon 15) by direct sequencing. Heterozygous missense mutation T1799A (V600E) in PTC sample (case no. 8)

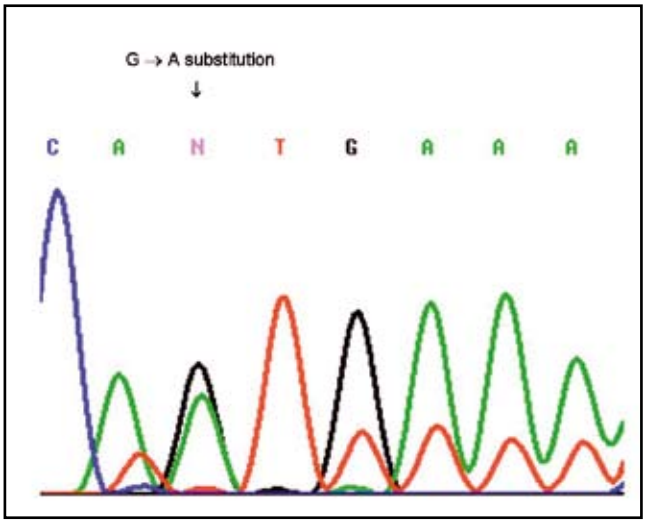

Figure 3C. Mutation analysis of *BRAF* gene (exon 15) by direct sequencing. Heterozygous missense mutation V600M G1798A in PTC sample (case no. 4).

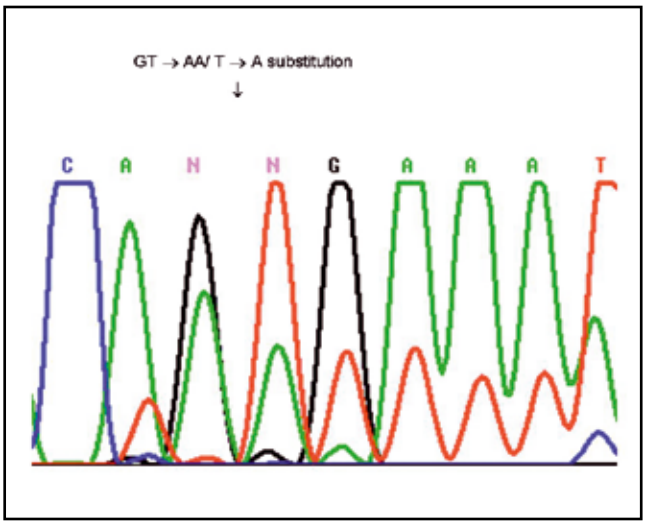

Figure 3B. Mutation analysis of BRAF gene (exon 15) by direct sequencing. Heterozygous missense mutation GT1798-99AA(V600K)/ T1799A (V600E) in PTC sample (case no. 9).

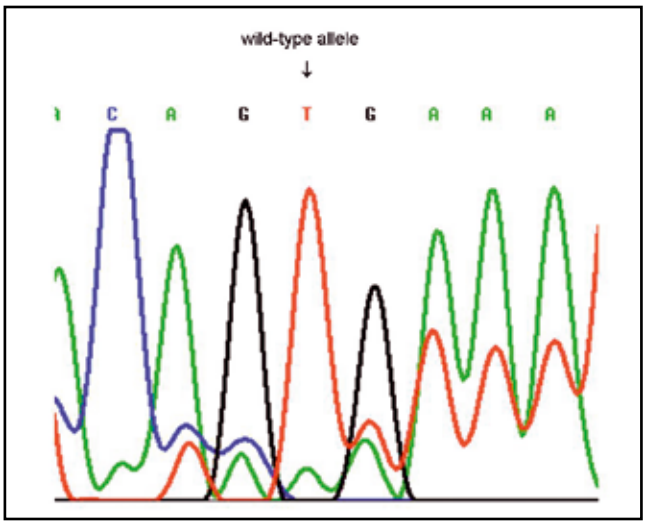

Figure 3D. Mutation analysis of *BRAF* gene (exon 15) by direct sequencing. The part of wild-type sequence of exon 15 *BRAF* gene without mutation (case no. 16).

performed screening studies in search for that mutation, using different molecular methods. In order to determine whether different methods detecting V600E mutation, had different sensitivity and to assess which methods were the most sensitive, we chose SSCP, direct sequencing and the real-time AS-PCR assay.

Based on our findings (see Table 3), we proved that the AS-PCR real-time assay was the most sensitive and rapid method of mutation identification, working even in case of those samples, where the presence of mutation was not detected by direct sequencing. The sensitivity of many molecular methods, including the direct sequencing, depends on the quantity of cells with mutated DNA in the studied sample. We would like to emphasize that – according to Sapio *et al.*'s observation [20] – clearly detectable mutation in sequencing analysis, as well as changed single-strand DNA conformation in the SSCP method, was possible when the content of DNA in the studied samples was at the level of 60% or more (which is equivalent to 30% of mutated DNA, assuming heterozygosity of the alleles).

In our study, the SSCP method confirmed the altered single-strand DNA conformation in 9/25 (36%) of the studied cases. This method appears to be a useful selective molecular tool as the first step in mutation screening. Sequencing results revealed the presence of mutation in exon 15 of *BRAF* gene in 4/25 (16%) cases, including: 1 case of V600E mutation, 1 case of V600M mutation and 2 cases of overlapping V600K/ V600E mutations. In the latter 2 cases, the results of

sequencing were ambiguous. V600K mutation has not been described in thyroid tumours but it has recently been reported in human malignant melanomas [13] as the second, regarding the frequency, after V600E mutation. It is suggested that this *BRAF* activating mutation, resulting from a 2-bp change, probable occurs on the same chromosome and is characteristic for invasive and metastatic melanoma. Interestingly enough, we found V600K/V600E mutation in patients with large tumour (pT_4N_0Mx and $pT_3N_0M_0$). V600M mutation, found in one case of our patient group, has not yet been described in thyroid tumours, but it has been reported in colorectal cancers [21]. It should be stressed that V600K and V600M mutations have very recently been described in different human carcinomas [13,21,15], using more and more sensitive identification methods (e.g., real-time PCR, ion- pairing reversed phase high pressure liquid chromatography – IPRP-HPLC, mass spectrometry). Moreover, exon 15 of *BRAF* gene is a hot spot region for *BRAF* mutations.

In our study, the new real-time AS-PCR method provided the most reliable results. By using that method, we proved the presence of V600E mutation in 12/25 (48%) of the studied cases. The obtained frequency is in the middle of reported frequency values for other studied populations, where it was assessed as 29–69% [16,3,23,26]. We would like to emphasize that, to our knowledge, this is the first study concerning the frequency of *BRAF* mutation in PTC in the Polish population.

It is important that PTC is associated with *RET* and *NTRK1* oncogene rearrangements and *BRAF* mutation. An analysis of the incidence of *RET* and *NTRK1*gene rearrangements in PTC in the Polish population has recently been performed at our Laboratory [2]. Rearrangements of the *RET* protooncogene (*RET/PTC1*, *RET/PTC2* and *RET/PTC3*) were detected in 7/33 (21%) of PTCs and rearrangements of *NTRK1* [*Trk-T1* and *Trk* (TPM3)] were detected in 4/33 (12%) of the examined samples. Rearrangements of *RET* and *NTRK*1 oncogenes occur in PTC with different frequency, depending on the studied population and/or the used methodology. The frequency of *RET*/*NTRK*1 oncogene rearrangements in PTC in the Polish population was found, with a relatively average incidence.

According to various studies, mutations of the *RAS* family of oncogenes have been identified in 0% to 10% of PTC cases in different populations, while in the Polish population they have been found in 6.8% of studied cases [5]. Furthermore, our studies have not confirmed any coexistence between *RAS* and *BRAF* gene mutations in thyroid carcinoma, either. It is likely that not only does the mutation of *BRAF* gene cause uncontrolled stimulation of the kinase pathway but it also most probably diminishes the sensibility of the stimulation to RAS protein. Additionally, there is no overlapping between *BRAF* and *RET/PTC* rearrangements [9,16,23,19]. However, in contrast, Xu *et al*. [26] have suggested that *BRAF* cooperates with *RET/PTC*. The explanation of this finding could be based on the fact that both BRAF and RET proteins are related with the same proliferation pathway in cells but – evidently – the activity of these genes occurs at different levels, as it leads to regulation of different genes. Mutual exclusivity among *BRAF* and *RAS* mutations and *RET*/PTC rearrangements in thyroid cancer supports the idea that each of the three genetic alterations alone is sufficient to cause thyroid tumorigenesis.

In our study, we also evaluated the relationship between *BRAF* mutation and the histopathological variant and such characteristics, as patient's age and sex or tumour size and lymph node metastases (TNM Staging system). The results of other investigators' studies are controversial. Regarding the correlation with the histopathological variant in our study, *BRAF* mutation was found mainly in the classic variant of PTC, but also in the follicular and the tall-cell variant, and this is also consistent with the results of other authors [18]. Generally, *BRAF* mutation is more frequent in classic variant of PTC and – in our study – it was statistically significant as compared with follicular variant of PTC.

In relation to the clinical stage of the disease, the presence of *BRAF* mutations was more often confirmed in tumours diagnosed in older patients, with extrathyroidal extensions, lymph node metastases and distant metastases, therefore, presenting III and IV clinical stage of the disease [18]. According to Fugazzola *et al*. [10], V600E mutation tended to be associated with bigger volume and extension of the tumour and with lymph node metastases; on the other hand, that mutation did not seem to be correlated with patient's age, sex, or with the stage or the recurrence rate of the disease. Trovisco *et al*. [25] have proved that the mean age of patients with the classic variant of PTC harbouring V600E *BRAF* mutation is higher, when compared with the age of patients bearing PTC without V600E *BRAF* mutation. Similarly to the last mentioned study, our results do not confirm the relationship between the *BRAF* point mutation and the particular stage of tumour in TNM clinical staging systems, although V600E mutations were found in patients with large size of tumours. Additionally, we could not confirm any relationship between the presence of *BRAF* mutation and patients' age or gender.

The detection of the *BRAF* mutation in early stages of the disease and the use of appropriate therapy can prolong patients' life. Inhibitors of the BRAF protein can be beneficial for patients with PTC [21,1]. Hopefully, this will be confirmed by future research *in vivo*. Mutation of the *BRAF* gene can prove to be an important determiner of the histological and clinical phenotype of PTC, becoming a valuable prognostic and diagnostic marker, differentiating PTC from other cancers. Studies, based on the use of BRAF protein inhibitors, extremely selective to cancer cells, give hope for further improvement and for treatment optimisation in patients with thyroid cancer.

REFERENCES

1 Alessandrini A, Namura S, Moskowitz MA, Bonventre JV. MEK1 protein kinase inhibition protects against damage resulting from focal cerebral ischemia. Neurobiology. 1999; **96**: 12866–9.
2 Brzeziańska E, Karbownik M, Migdalska-Sęk M, Pastuszak-Lewandoska D, Włoch J, Lewiński A. Molecular analysis of the *RET* and *NTRK1* gene rearrangements in papillary thyroid carcinoma in Polish population. Mutat. Res. 2006; **599**: 26–35.
3 Cohen Y, Xing M, Mambo E, Guo Z, Wu G, Trink B, Beller U, Westra WH, Ladenson PW, Sidransky D. BRAF mutation in papillary thyroid carcinoma. J. Natl. Can. Inst. 2003; **95**: 625–7.
4 Cyniak-Magierska A, Brzeziańska E, Januszkiewicz-Caulier J. Pastuszak-Lewandoska D, Lewiński A. Increased expression of mRNA specific for *c-MET* oncogene in human papillary thyroid carcinoma. Arch. Med. Sci. 2007; **3**: 31–6.
5 Cyniak-Magierska A, Brzeziańska E, Pastuszak-Lewandoska D, Januszkiewicz J, Lewiński A. Prevelance of *RAS* point mutations in papillary thyroid carcinoma; a novel mutation at codon 31 of K-*RAS*. Exp. Clin. Endocrinol. Diabet. 2007 (in press).
6 Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, *et al*. Mutations of the BRAF gene in human cancer. Nature. 2002; **417**: 949–54.
7 DeLeilis RA, Lloyd RV, Heitz PU, Eng C. Pathology and genetics of tumours of endocrine organs. WHO classification of tumours. Lyon: IARC Press. 2004.
8 Espana CT, Johnson SJ, Kendall-Taylor P, Lennard TW, Harris PG. Prevalence of ras mutations in thyroid neoplasia. Clin. Endocrinol. (Oxf). 1995; **50**: 529–35.
9 Frattini M, Ferrarion C, Bressan P, Balestra D, De Cecco L, Mondellini P, *et al*. Alternative mutations of *BRAF*, *RET* and *NTRK1* are associated with similar but distinct gene expression patterns in papillary thyroid cancer. Oncogene. 2004; **23**: 7436–40.
10 Fugazzola L, Mannavol D, Cirello V, Vannucchi G, Muzza M, Vicentini L, *et al*. BRAF mutations in an Italian cohort of thyroid cancers. Clin. Endocrinol. 2004; **61**: 239–43.
11 Fukushima T, Suzuki S, Mashiko M, Ohtake T, Endo Y, Takebayashi Y, *et al*. *BRAF* mutations in papillary carcinomas of the thyroid. Oncogene. 2003; **22**: 6455–7.
12 Greene LF, Page DL, Fritz A, Batch M, Haller DG, Morrow M. AJCC. Chapter 8: Thyroid. In: American Joint Committee on Cancer (AJCC), Cancer Staging Manual, 6th ed., Springer, New York. 2002; pp. 89–98.
13 James MR, Dumeni T, Stark MS, Duffy DL, Montgomery, Martin NG, *et al*. Rapid Screening of 4000 individuals for germ-line variations in the BRAF gene. Clin. Chem. 2006; **52**: 1675–8.
14 Jarry A, Masson D, Cassagnau E, Parois S, Laboisse C, Denis MG. Real-time allele-specific amplification for sensitive detection of BRAF mutation V600E. Mol. Cell. Probes. 2004; **18**: 349–52.
15 Jeuken JWM, van der Maazen RWM, Wesseling P. Molecular diagnostics as a tool to personalize treatment in adult glioma patients. Techn. Cancer Res. Treat. 2006; **5**: 215–30.
16 Kimura ET, Nikiforova MN, Zhu Z, Knauf JA, Nikiforov YE, Fagin JA. High prevalence of *BRAF* mutations in thyroid cancer: genetic evidence for constitutive activation of RET/PTC-RAS-BRAF signalling pathway in papillary thyroid carcinoma. Cancer Res. 2003; **63**: 1454–7.
17 Lewiński A, Ferenc T, Sporny S, Jarząb B. Thyroid carcinoma: diagnostic and therapeutic approach; genetic background. Endocr. Regul. 2000; **34**: 99–113.
18 Nikiforova MN, Kimura ET, Gandhi M, Biddinger PW, Knauf JA, Basolo F, *et al*. *BRAF* mutations in thyroid tumors are restricted to papillary carcinomas and anaplastic or poorly differentiated carcinomas arising from papillary carcinomas. J. Clin. Endocrinol. Metab. 2003; **88**: 5399–404.
19 Puxendu E, Moretti S, Elisei R, Romei C, Pascucci R, Martinelli M, *et al*. BRAF V599E mutation is the leading genetic event in adult sporadic papillary thyroid carcinomas. J. Clin. Endocrinol. Metab. 2004; **89**: 2414–20.
20 Sapio MR, Posca D, Troncone G, Pettinato G, Palombini L, Rossi G, *et al*. Detection of BRAF mutation in thyroid papillary carcinomas by mutant allele-specific PCR amplification (MASA). Eur. J. Endocrinol. 2006; **154**: 341–8.
21 Sebolt-Leopold JS, Dudley DT, Herrera R, Van Becelaere K, Wiland A, Gowan RC, *et al*. Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. Nature Med. 1999; **5:** 810–6.
22 Sherman SI. Thyroid carcinoma. Lancet. 2003; **361**: 501–11.
23 Soares P, Trovisco V, Rocha AS, Limo J, Castro P, Preto A, *et al*. BRAF mutations and RET/PTC rearrangements are alternative events in etiopathogenesis of PTC. Oncogene. 2003; **17**: 4578–80.
24 Thyroid. In: American Joint Committee on Cancer. AJCC Cancer Staging Manual. 6th ed. New York, NY: Springer. 2002, pp. 77–87.
25 Trovisco V, Soares P, Preto A, de Castro IV, Lima J, Castro P, *et al*. Type and prevalence of BRAF mutations are closely associated with papillary thyroid carcinoma histotype and patients' age but not with tumour aggressiveness. Virch. Arch. 2005; **446**: 589–95.
26 Xu X, Quiros RM, Gattuso P, Ain KB, Prinz RA. High prevalence of BRAF gene mutation in papillary thyroid carcinomas and thyroid tumor cell lines. Cancer Res. 2003; **63**: 4561–7.