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ORIGINAL ARTICLE

Low Prevalence of *TERT* Promoter, *BRAF* and *RAS* Mutations in Papillary Thyroid Cancer in the Greek Population

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Abstract

Thyroid cancer is a common endocrine malignancy and displays a variety of histological patterns ranging from adenoma to malignant tumors. Molecular diagnostics have given significant insights into the genetic basis of thyroid tumorigenesis, known to be linked with signaling pathways affected by oxidative stress. We report for the first time a genotype study of *TERT* promoter combined with *BRAF* and *RAS* mutations in Papillary Thyroid Cancer (PTC) cases in the Greek population. Polymerase Chain Reaction and sequencing were used to identify *TERT* promoter (C228T, C250T, CC243-243TT) mutations, the *BRAF* (T1799A) mutation and mutations in codons 12, 13, 61 of the *HRAS*, *KRAS* and *NRAS* genes. The most common C228T *TERT* promoter mutation was identified in 2 PTC cases co-existing with the *BRAF* mutation. The *BRAF* T1799A mutation was detected in 10 PTC cases, while two different *NRAS* mutations in codon 61 (C181A and A182G) were found in 2 PTC cases. These mutations occur in a mutually exclusive manner. Our results indicate that despite the low frequencies, the study of the specific mutations should be encouraged because they are indicative of aggressive forms of thyroid cancer of the papillary histotype in this patient cohort, thus providing insights towards their therapeutic management.

Keywords TERT · BRAF · RAS · Mutations · Papillary thyroid cancer

Introduction

Thyroid cancer is the most common endocrine malignancy and its incidence has been steadily increasing worldwide. According to histopathological characteristics, thyroid tumors arising from follicular cells are classified into the well-differentiated cancer, including the most common Papillary Thyroid Cancer (PTC) (~80% of the cases) and Follicular Thyroid Cancer (FTC) (~10%), Poorly Differentiated Thyroid Cancer (PDTC) (~2–7%) and the undifferentiated Anaplastic Thyroid Cancer (ATC) (~2–5%). Finally, Medullary Thyroid Cancer (MTC) derived from calcitonin-secreting parafollicular cells is reported to have a prevalence of 2–4% [1]. Additional subtypes exist among these types, including Microcarcinomas, Conventional PTC, the Follicular (PTC-FV) and the Tall Cell Variant of PTC (TCV-PTC) as well as the Hurtle cell neoplasm of FTC [1]. It should be noted that the various types of thyroid cancer have been related to different biological behavior, extra-thyroidal extension and lymph node metastases, leading to different survival rates and, thus, different therapeutic modalities.

The understanding of the molecular pathogenesis and the identification of molecular markers, which will be used for diagnosis and prognosis is of high clinical significance. In this aspect, recent studies have identified genetic mutations, especially ones resulting in the activation of the Mitogen-Activated Protein Kinase (MAPK) signaling pathway, which contribute to the pathogenesis of thyroid cancer. These molecular alterations include point mutations in the *BRAF* and *RAS* genes, the rearrangements of *RET/Papillary Thyroid Cancer* (PTC) and *PAX8/Peroxisome Proliferator-Activated Receptor*

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γ *(PPARγ)* [2, 3] and the recently identified *Telomerase Reverse Transcriptase (TERT)* promoter mutations [4].

Genome stability is maintained by the action of telomerase, which adds tandem repeats of TTAGGG sequence (telomeres) at the end of chromosomes [5]. Telomerase consists of a catalytic protein subunit, telomerase reverse transcriptase (TERT), and an RNA subunit. *TERT* promoter mutations have been recently associated with many types of human cancers [6, 7]. The two common mutations C228T and C250T in the *TERT* promoter correspond to positions 124 and 146 bp, respectively, while the less common CC242-243TT mutation is located at positions 137 and 138 bp upstream of the TERT translation start site. These mutations create an extra E-twenty-six (ETS) binding motif, which increases *TERT* transcriptional activity, resulting in oncogenesis [8, 9]. *TERT* promoter mutations appear to be associated with thyroid cancer, especially with the more aggressive types [4, 10, 11]. In addition, *TERT* C228T mutations combined with *BRAF* mutations demonstrate a synergistic association in increasing PTC tumor recurrence and patient mortality [12–14].

BRAF is a serine-threonine kinase that belongs to the family of RAF proteins. BRAF activation is triggered by RAS binding and results in phosphorylation and activation of downstream targets along the MAPK cascade. The most common mutation in *BRAF* in many cancers, including PTC, is the T1799A, which results in a valine to glutamic acid substitution at residue 600 (V600E) [15, 16]. This substitution disrupts hydrophobic interactions between the activation loop and the ATP-binding site, maintaining the active conformation of the protein, irrespectively of upstream signaling [17]. Recently, BRAF V600E and reactive oxygen species generated by the NADPH oxidase NOX4, have been demonstrated to play an important role in the pathogenesis of PTC [18].

The family of human *RAS* genes includes *HRAS*, *NRAS* and *KRAS*. They encode highly related GTPases, which reside in the inner surface of the cell membrane and propagate signals arising from transmembrane receptors along the MAPK and other signaling pathways. In its inactive state, RAS protein is bound to guanosine diphosphate (GDP) and upon activation it releases GDP and binds guanosine triphosphate (GTP). Point mutations in the *RAS* genes, which result in either an increased affinity for GTP (mutations in codons 12 and 13) or inactivation of the autocatalytic GTPase function (mutations in codon 61), leading to a permanent switch of the protein in the active position, are common in different types of human cancers, including certain thyroid types [19, 20].

TERT promoter mutations, alone, or in combination with *BRAF* or *RAS* mutations are recognized as clinically important diagnostic and prognostic genetic markers for thyroid cancer. In this line, the present study focuses for the first time on the identification of *TERT* promoter mutations in thyroid tumors of the most common type in the Greek population. Specifically, the examined cohort includes papillary thyroid cancer cases of the major subtypes (Conventional PTC and PTC-FV), which are further characterized at the molecular level for *BRAF* and *RAS* mutations.

Materials and Methods

Human Biopsies

Samples were collected from patients who underwent partial or total thyroidectomy and were retrieved retrospectively from the files of the Pathology Department of the N.I.M.T.S. Hospital in Athens. The study included 59 samples which represent 24 Conventional PTC (18 female, mean age 45,2 years and 6 male, mean age 45 years) and 35 PTC-FV (31 female, mean age 48,6 years and 4 male, mean age 49,8 years) cases. PTC-FV is given to PTC with an exclusively or almost exclusively follicular pattern of growth [21]. This variant shares many features with Conventional papillary carcinomas: capsule formation is usually absent or incomplete, fibrous septa are common and scattered psammoma bodies may be found in the interfollicular stroma.

Genomic DNA Isolation and Mutation Screening by PCR and Sequencing

Genomic DNA was isolated from sections of formalin-fixed and paraffin-embedded (FFPE) tumor biopsies using the QIAamp DNA FFPE Tissue kit (Qiagen GmbH, Germany). Typically, 30 μm sections/per case were initially treated with xylene/ethanol, then lysed during an overnight incubation with proteinase K (0.4 mg/ml) in a 55 °C rotating incubator, and DNA purification was performed through QIAamp columns, according to the manufacturer's instructions. This protocol yielded DNA of sufficient quantity and quality from all samples. Polymerase Chain Reaction (PCR) was used to amplify the desired regions of genomic DNA using specific primers (Table 1). For all genes except *TERT* reactions were performed in a final volume of 50 μl using as template 100–300 ng of genomic DNA, with 1X buffer including 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 25 pmoles of each (Forward, Reverse) primer and 1 unit of Taq polymerase (Kapa Biosystems). For the detection of *TERT* promoter mutations reactions were performed as outlined above, with the addition of DMSO (5%) and with different amounts of $MgCl_2$ (2.5 mM) and each primer (50 pmoles). Each reaction included a negative control sample, in which DNA template was substituted by water. PCR cycling started with the initial denaturation step at 95 °C for 5 min, followed by 40 cycles of denaturation (95 °C, 30 s), annealing (Tan, 30 s) and extension (72 °C, 30 s) and a last step of 10 min extension at 72 °C. The annealing temperatures (Tan) used for the amplification of the different genetic regions were: 49 °C for *NRAS* codon 61;

Table 1 Primer sequences used for the screening of the specific genetic mutations

Gene	Primer sequence (5′ → 3′)	Mutation site	Product size (bp)
TERT	F: CACCCGTCCTGCCCCTTCACCTT	C228T, CC242-243TT, C250T	194
	R: GGCTTCCCACGTGCGCAGCAGGA		
BRAF	F:CATAATGCTTGCTCTGATAGGAA	codon 600 (GTG)	244
	R:AGTAACTCAGCAGCATCTCAG		
HRAS	F: CAGGAGACCCTGTAGGAG	codons 12–13 (GGC-GGT)	225
	R: TATCCTGGCTGTGTCCTG		
	F: TGTCCTCCTGCAGGATTC	codon 61 (CAG)	189
	R: GTACTGGTGGATGTCCTC		
NRAS	F: AAAGTACTGTAGATGTGGCTC	codons 12–13 (GGT-GGT)	224
	R: GTGAGAGACAGGATCAGG		
	F: GATTCTTACAGAAAACAAGTG	codon 61 (CAA)	157
	R: ATGACTTGCTATTATTGATGG		
KRAS	F: AACCTTATGTGTGACATGTTC	codons 12–13 (GGT-GGC)	216
	R: TCCTGCACCAGTAATATGC		
	F: AATCCAGACTGTGTTTCTCC	codon 61 (CAA)	217
	R: TTAAACCCACCTATAATGGTG		

51 °C for *KRAS* codons 12 and 13 and codon 61; 53 °C for *BRAF* codon 600 and *NRAS* codons 12 and 13; 54 °C for *HRAS* codon 61; 62 °C for *TERT* promoter mutations. For the amplification of *HRAS* including codons 12 and 13 a touchdown PCR was used; specifically the initial denaturation step (5 min at 95 °C) was followed by 10 touch-down cycles, during which the annealing temperature was reduced by 2 °C every 2 cycles, from 68 °C to 60 °C, then followed by 40 cycles of denaturation (95 °C, 30 s), annealing (58 °C, 30 s) and extension (72 °C, 30 s) and a last step of 10 min extension at 72 °C. The specific PCR products were verified by 2% agarose gel electrophoresis and were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions. The purification of the PCR products included mostly the PCR clean-up protocol, which cleaned and concentrated DNA directly following the amplification reaction, and in some cases the extraction of DNA from fragments excised from agarose gels, in an effort to reduce background signal during sequencing. The purified products were sequenced using ABI3730xl automatic DNA Sequencer and Big Dye chemistry (Applied Biosystems, v.3.1). Sequences were displayed as chromatograms using the software BioEdit v7.2.5 (developer Tom Hall, Ibis Biosciences, Carlsbad CA 92008) and were screened for genetic alterations in comparison with the online NCBI's BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Detection of NRAS Mutations by Real-Time PCR

Following genomic DNA isolation, the presence of the CGA mutation at codon 61 of the *NRAS* gene was verified by real-time PCR using the 'The *NRAS* Mutation Analysis Kit' (EntroGen Inc., California, USA). PCR cycling started with a denaturation reaction at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s and 64 °C for 60 s and was performed on a 7500 Fast Real – Time PCR System (Applied Biosystems, USA). The detection of the amplification signal was performed using the ABI Prism 7500 Instrument (SDS Software version 2.0.3).

Results

The present study focuses on the molecular characterization of the most common type of thyroid cancer in the Greek population. In addition, based on previous findings supporting that molecular alterations and their frequencies differ between thyroid cancer subtypes [15], we distinguished between Conventional PTC and PTC-FV cases. A representative example of hematoxylin and eosin (H & E) staining of each subtype is given in Fig. 1a, b.

TERT promoter mutations were identified in two cases of thyroid tumors, with a prevalence of 3.4% within PTCs. Specifically, the C228T mutation was found to be heterozygous in PTC-FV tumors, which belong to female patients (Fig. 1c). This common *TERT* promoter mutation is known to increase *TERT* transcriptional activity, leading to oncogenesis. In agreement with previous reports supporting that *TERT* promoter mutations are mutually exclusive, the two C228T-positive bearing tumors did not carry any other *TERT* mutations [10]. Table 2 summarizes the clinicopathological characteristics of the PTC patients carrying the selected mutations. Interestingly, both *TERT* C228T mutations co-existed with the *BRAF* V600E mutation in large tumors of patients with extrathyroid extension and lymph node metastasis (Table 2).

Moreover, we identified 10 cases of *BRAF*-positive thyroid tumors showing 17% prevalence within PTCs. Specifically,

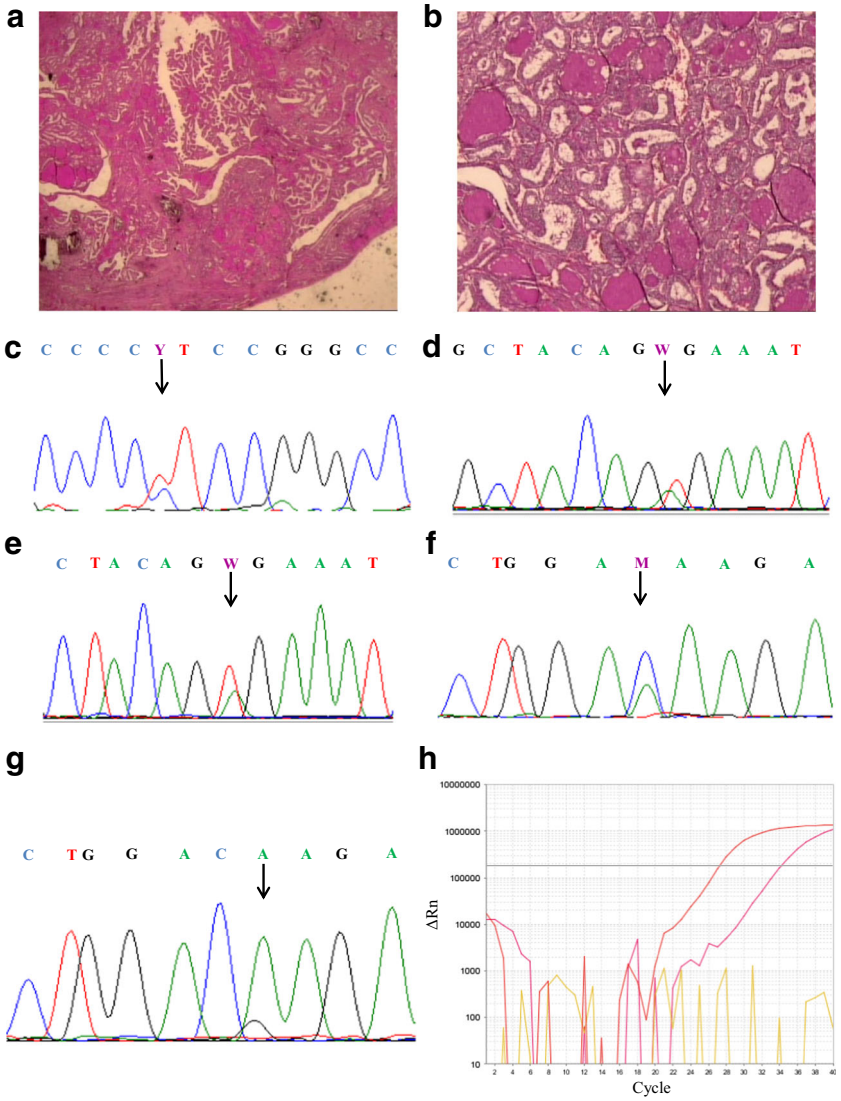

Fig. 1 Histological aspects of PTC cases and the chromatograms of their *BRAF*, *NRAS* and *TERT* promoter mutations. **a** Conventional Papillary Carcinoma of the Thyroid (PTC) with scattered psammoma bodies (H&E staining, × 40). **b** Follicular Variant of Papillary Carcinoma of the Thyroid (H&E staining, × 100). **c** Sequencing chromatogram of the *TERT* promoter locus of a PTC-FV case, showing a C to T transition at nucleotide position 228 in the *TERT* promoter. **d**, **e** *BRAF* Forward chromatogram showing overlapping peak at nucleotide position 1799. Mutant peak demonstrates a T to A transversion. Panel **d** belongs to a conventional PTC case and panel **e** belongs to a PTC-FV case. **f** *NRAS* Forward chromatogram of a PTC-FV case showing overlapping peak at nucleotide position 181. Mutant peak demonstrates a C to A transversion. **g** *NRAS* Forward chromatogram of a Conventional PTC case showing overlapping peak at nucleotide position 182. Mutant peak demonstrates the A to G transversion. **h** Real time-PCR amplification plot of the Conventional PTC case sequenced in panel **g** using the probe CGA at position of codon 61 of the *NRAS* gene. Positive control is shown with red, negative control with orange and sample with purple

BRAF mutations were detected in 6 Conventional PTC and 4 PTC-FV cases. All mutations were heterozygous as exhibited by the presence of both the mutant and the wild-type allele (Fig. 1d, e). All *BRAF*-positive thyroid tumors carried the most common *BRAF* T1799A mutation, leading to V600E substitution, which results in the constitutive activation of the BRAF kinase. Within the population of PTCs, it appears that, qualitatively, there was a trend for higher frequency of BRAF V600E in male (3 out of 10) versus female (7 out of 49) patients. In addition, although it was not statistically significant, PTCs carrying BRAF mutations exhibited extrathyroidal extension and lymph node metastasis (7 BRAF-positive vs. 3 BRAF-negative cases) and relatively large tumor size (6 BRAF-positive vs. 4 BRAF-negative cases). No other previously reported rare *BRAF* mutations were identified, including mutations in codon 601 or small in-frame insertions or deletions surrounding codon 600. Finally, all *BRAF*-positive cases did not carry any mutations in codons 12, 13 and 61 of all the *RAS* genes, supporting the fact that these molecular alterations recorded in thyroid cancer are mutually exclusive.

The analysis of codons 12, 13 and 61 of all the *RAS* genes identified two *RAS*-positive cases. Both correspond to NRAS

Table 2 Relation of the examined mutations with clinicopathological characteristics of PTC

Clinicopathological characteristics		Mutations				
		TERT $n=2$	BRAF $n=10$	NRAS $n=2$	BRAF + TERT $n=2$	Wild-type $n=47$
Age at diagnosis (y)	< 45	1	7	1	1	23
	> 45	1	3	1	1	24
Gender	Female	2	7	–	2	42
	Male	–	3	2	–	5
Tumor size (mm)	< 10	–	–	1	–	14
	10–40	–	4	1	–	26
	> 40	2	6	–	2	7
Cancer stage	I/II	2	10	2	2	47
	III/IV	–	–	–	–	–
Extrathyroid extension	No	–	3	2	–	47
	Yes	2	7	–	2	–
Lymph node metastasis	No	–	3	1	–	41
	Yes	2	7	–	2	1
	ND	–	–	1	–	5

ND, not determined; *y*, years; *mm*, millimetres

mutations in codon 61 and they are detected in heterozygosity in PTC tumors of male patients. Specifically, one mutation identified in a PTC-FV tumor carried the CAA → AAA conversion leading to a glutamine to lysine change (Fig. 1f). Another mutation found in a Conventional PTC case carried the CAA → CGA substitution, resulting in a glutamine to arginine change (Fig. 1g). Because of the small peak that was present in the chromatogram (Fig. 1g) the authenticity of this mutation was additionally verified by real-time PCR (Fig. 1h). Both mutations are known to result in constitutive activation of the NRAS protein. *NRAS* mutations occurred in PTCs of relatively small size (<40 mm), without extrathyroidal extension and lymph node metastasis (Table 2). Finally, NRAS61-positive cases were negative for *TERT* promoter mutations and did not carry additional *RAS* and *BRAF* mutations.

Discussion

TERT promoter mutations have been recently identified to play an important role in the pathogenesis of thyroid cancer [4]. In this study, we identified two cases of thyroid tumors carrying only the most common C228T mutation, and no tumors with C250T or the less common CC242-243TT mutations. This was in agreement with previous studies, which demonstrated that the two common *TERT* promoter mutations occur in a mutually exclusive manner, with C228T being far more common than C250T in most cancers [7, 10]. Especially for thyroid cancer, *TERT* promoter mutations have a high prevalence (38–46%) in aggressive types of thyroid cancer, such as ATC and PDTC, and a low prevalence (7.5%–25%) in PTC and FTC [4, 7, 10, 11]. Amongst PTC variants, *TERT* promoter mutations exhibit a prevalence of 10% in Conventional PTC and 8% in PTC-FV [10]. In our study, *TERT* promoter mutations were identified with an overall low prevalence of 3.4% within PCT, and specifically 5.7% in PTC-FV and 0% in Conventional PTC tumors. Furthermore, *TERT* promoter mutations co-existed with the BRAF V600E mutation in large primary tumors linked with extrathyroidal extension and lymph node metastasis, in agreement with previous reports, which have established that these mutations cooperate in driving the clinicopathological aggressiveness of PTC [12–14]. It would be ideal to follow the clinical course of the patients carrying these mutations, which is not possible for the present study, partly due to the short time period following the diagnosis. However, we present other variables, such as lymph node metastasis, extrathyroidal extension, and tumor size (Table 2), which are important aspects of the clinical behavior of these tumors.

The molecular characterization of the studied cohort was further focused on *BRAF* and *RAS* mutations. A low (17%) prevalence of *BRAF* mutations within PTCs was identified. *BRAF* is the most common mutated gene in thyroid cancer of the papillary histotype, with an average worldwide prevalence of ~ 45% [2, 15]. However, a wide frequency of BRAF V600E has been shown, ranging from 27.3 to 87.1% [22, 23]. Such differences in *BRAF* mutation frequencies can be attributed to various reasons. PTC is classified into several histological distinct subtypes and the distribution of *BRAF* mutation displays a clear subtype-related pattern, with a high prevalence in Conventional PTC (60%) and low in PTC-FV (~12%) [15]. Therefore, differences in the prevalence of *BRAF* mutation

can be explained by the fact that different subtype compositions of PTC are analyzed without subtype stratification. It should be noted that our study is enriched for PTC-FV cases, in agreement with a recent study, which demonstrated an increase in the number of PTC-FV cases in last decades [24]. Although we distinguish between tumor subtypes, the calculated prevalence of the *BRAF* mutation (17%) is lower than the estimated prevalence (~30%) we would predict based on the number of analyzed PTC subtypes. Therefore, we can conclude that *BRAF* mutation is detected with the lowest worldwide frequency in our study subjects, which could be attributed to differences in their genetic background or to more favorable clinicopathological characteristics of the randomly selected tumors. Various theories have been proposed regarding the mechanisms underlying differences in *BRAF* mutation frequencies, including iodide uptake, environmental factors active in certain geographical areas and analysis of ethnically diverse groups [25–27]. However, these factors do not apply to this study. Age is another critical factor in the context of *BRAF* mutations. Our results demonstrate that qualitatively there is a higher frequency of BRAF V600E in younger versus older patients in the Greek population, in contrast to other reports studying different patient cohorts [28–30]. *BRAF* mutations were detected in large PTC tumors, with the majority exhibiting extrathyroidal extension and lymph node metastasis, which are characteristics of aggressive clinical outcomes.

In regard to the *RAS* mutation screening, we identified two NRAS61 mutations, A182G and C181A, in a Conventional PTC and a PTC-FV case, respectively. This finding agrees with previous reports demonstrating that the most common mutations in thyroid tumors are detected at codon 61 of *NRAS* and *HRAS* and at codons 12 or 13 of *KRAS* [19, 20, 31]. *RAS* mutations are more prevalent in FTC (~10–55%) and are also associated with 10–15% of the Follicular Variant of PTC [19, 24, 32]. As *RAS* mutations have been mostly connected to the follicular architecture it was a rather unexpected finding to identify a Conventional PTC case harboring an *NRAS* mutation. However, in contrast to the other reported markers, *RAS* mutations are not restricted to a particular histological subtype of thyroid tumor and they have also been identified in follicular adenomas [19]. On the other hand, it should be noted that NRAS mutations at codon 61 have been significantly associated with distant metastasis [33], pointing to aggressive clinical outcomes.

This study, which revealed low prevalence of the *BRAF* mutation and lack of *KRAS* mutations, appears to contradict a previous study that investigated BRAF600 and KRAS12 mutations in PTC cases in a Greek population [23]. In the previous study, the authors used an enriched PCR-restriction fragment length polymorphism method (PCR-RFLP), but they do not specify which mutation they identified, i.e. which nucleotide substitution [23]. Using a similar number of PTC cases (55) compared to our study, and without subtype stratification, the authors also reported the lowest frequency (27.3%) of BRAF600 in PTC compared to previous reports [15, 22, 34]. In contrast, they detected two-fold higher frequency of KRAS12 compared to BRAF600 within PTC (54.4% versus 27.3%) and more than 10% double-positive PTC cases. All these findings are in contrast to our study and to previous reports [2, 22, 31]. This high frequency of mutations could be attributed to false positive results, as a two step PCR-RFLP was performed, which was not further confirmed by direct sequencing. In the present study the authenticity of the mutation was confirmed by performing at least 3 independent PCR reactions, using genomic DNA isolated from different sections of the same biopsy and repeated bidirectional DNA sequencing using two different primer sets (given in Table 1).

Molecular diagnostics has contributed significantly in the prognosis, surgical management and therapeutic treatment of thyroid tumors [2, 3]. *TERT* promoter mutations are a major indicator of poor outcome in PTC and have also been associated with increased mortality in PTC [35, 36]. *BRAF* mutation has been associated with PTC treatment failure, recurrence and patient mortality and BRAF is considered an effective therapeutic target in thyroid cancer [2, 37–39]. NRAS mutations at codon 61 have been significantly associated with distant metastasis [33]. The coexistence of *BRAF* and *TERT* promoter mutations appears to define PTC with the worst clinicopathologic outcomes [12–14]. It is believed that, either mutation alone and mostly *TERT* promoter mutations in combination with *BRAF* or *RAS* mutations could provide unique prognostic implications. This appears to have limited application for thyroid cancer patients in the Greek population because of the low frequencies of the mutations.

In conclusion, the novelty of the present study lies to the report of *TERT* promoter mutations in the Greek population, in combination with the most common *BRAF* and *RAS* genetic alterations. The most intriguing finding is that despite the low frequencies, the detected mutations are indicative of aggressive forms of thyroid cancer of the papillary histotype in this patient cohort. Therefore, their study should be encouraged because it will potentially provide insights towards their therapeutic management.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interest regarding the publication of this article.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. For this type of study formal consent is not required.

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