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Mutational and clinico-pathological analysis of papillary thyroid carcinoma in Serbia

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Abstract. Molecular pathogenesis of papillary thyroid carcinoma (PTC) is largely associated with mutational changes in the *BRAF*, *RAS* family and *RET* genes. Our aim was to assess clinico-pathological and prognostic correlations of these PTC-specific gene alterations, with a particular emphasis on the *BRAF* mutation, in a group of 266 Serbian PTC patients, for the first time. The reference center-based retrospective cohort included 201 (75.6%) females and 65 (24.4%) males aged 48.0±16.1 years (8-83 years old, range) diagnosed and treated for PTC during 1993-2008. Follow-up period was 53.1±41.6 months (7-187 months, range). *BRAF* and *RAS* mutations were determined by direct sequencing of genomic DNA. *RET/PTC* rearrangements were analyzed by RT-PCR/Southern blotting. Genetic alterations were detected in 150/266 tumors (56.4%). One tumor displayed two genetic alterations. The $BRAF^{V600E}$ was found in 84/266 (31.6%) cases, *RAS* mutations in 11/266 (4.1%) and *RET/PTC* in 55/266 (20.7%; 42/266 (15.8%) *RET/PTC1* and 13/266 (4.9%) *RET/PTC3*). On multivariate analysis $BRAF^{V600E}$ was associated with the classical papillary morphology ($P = 0.05$), the higher pT category ($P = 0.05$) and advanced clinical stage ($P = 0.03$). In a proportional hazard model, $BRAF^{V600E}$ did not appear to be an independent risk factor for the faster recurrence ($P = 0.784$). We conclude that under the extensive thyroid surgery and limited application of radioiodine ablation $BRAF^{V600E}$ may not be an indicator of poorer disease-free survival during the short to middle follow-up period. However, it has a potential to contribute to patients stratification into high- and low-risk groups.

Key words: Papillary thyroid carcinoma, BRAF mutation, Prognosis, Disease-free survival

THYROID cancer is the most prevalent type of endocrine malignancy. During the past decades its incidence has been increasing in many countries [1]. Papillary thyroid carcinoma (PTC) accounts for more than 80% of all thyroid cancers and for 95% of this increase [2]. About 70% of the newly diagnosed PTCs are small tumors measuring less than 2 centimeters. As illustrated in Fig. 1, a similar trend is seen in Serbia according to the files of the Institute for Oncology and Radiology, a thyroid cancer reference center in the country. The shift towards the small-size tumors is largely due to the introduction of ultrasound examination and fine-needle aspiration biopsy allowing the detection and diagnosis of early-stage neoplasms. Although small papillary thyroid carcinomas are relatively indolent and frequently curable with surgery with or without, depending on the extent of initial surgery and indications, radioiodine ablation and therapy, and hormone therapy,

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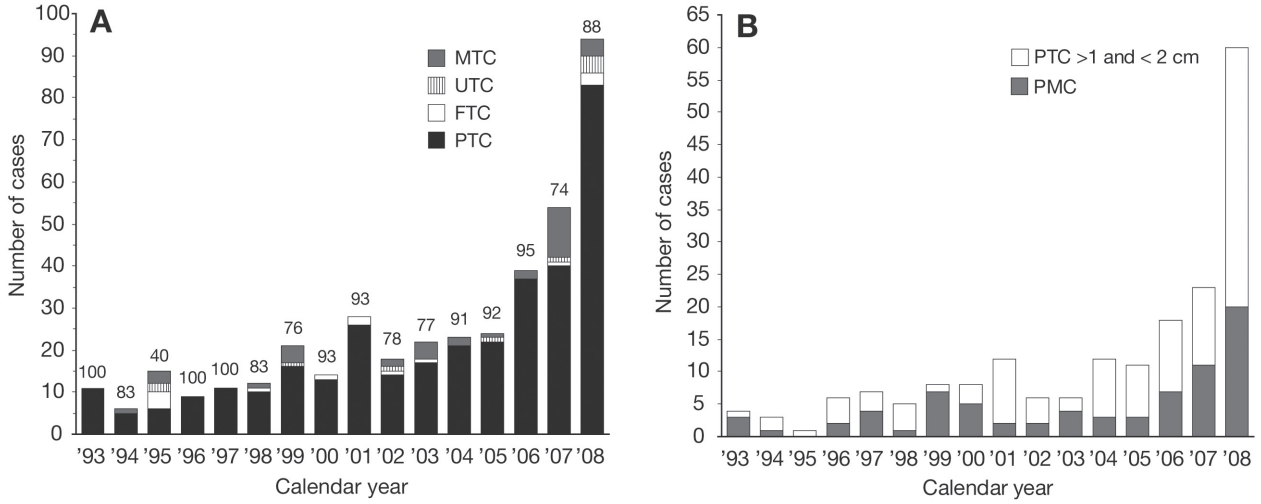

Fig. 1 Thyroid cancer cases treated in the Institute for Oncology and Radiology of Serbia, Belgrade, from 1993 to 2008. (A) Distribution of thyroid cancers by histological type: papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), undifferentiated thyroid carcinoma (UTC) and medullary thyroid carcinoma (MTC). The relative proportion of PTC (%) is indicated above each bar. (B) The distribution of PTC measuring less than 2 cm in the largest diameter. PMC denotes papillary microcarcinoma measuring less than 1 cm.

the number of deaths due to thyroid cancer has been also increasing [3]. This brings up a pressing need of identifying high-risk patients for tailored optimization of clinical management. Moreover, it is also desirable to evaluate risk factors preoperatively in order to facilitate surgical decision-making.

PTC is associated with constitutive activation of the RET-RAS-RAF-MAPK pathway which transduces potent mitogenic and cell survival signals [4, 5]. Pathway activation is usually caused by *RET/PTC* gene rearrangements or activating point mutations in the *BRAF* or *RAS*-family genes. These molecular alterations occur cumulatively in up to 70% of all PTCs [6, 7]. They are nearly mutually exclusive since activation of any single proto-oncogene confers uncontrolled functioning of downstream effectors.

Rearrangements involving the *RET* gene, referred to as *RET/PTC*, are detected in approximately 20% of PTC in adults, in 40-70% of childhood and adolescent patients with sporadic PTC and 50-86% in radiation-exposed individuals [8]. To date 17 different RET/PTC variants have been described among which *RET/PTC1* and *RET/PTC3* account for 90-100% in different series [9]. The presence of *RET/PTC* rearrangement correlates with some clinicopathological features of PTC such as younger age of patients, tumor morphology and a higher probability of lymph node involvement [10].

In the *BRAF* gene, a thymidine-to-adenine transversion at nucleotide 1799 of exon 15, resulting in a valine to glutamic acid substitution at amino acid residue 600 ($BRAF^{V600E}$), is the most prevalent mutation in PTC with the frequency of 29–83% in adult series [11-12]. It is also detected, to a smaller extent, in poorly differentiated thyroid tumors and anaplastic thyroid carcinoma arising from PTC [13, 14]. $BRAF^{V600E}$ associates with dedifferentiation, genomic instability and increased invasiveness in model cells [15, 16]. Numerous studies have suggested, although equivocally, that *BRAF* mutation associates with the older age, advanced disease, classical papillary or tall cell histotype and poorer prognosis of disease-free and overall survival [17]. It has been also demonstrated that the prevalence of $BRAF^{V600E}$ may depend on iodine intake within one ethnic population [18].

The prevalence of *RAS* gene family mutations in PTC ranges from 0 to 21% (about 11% on average). It is particularly high in the follicular variant of PTC, 43% [19]. Mutations in the RAS genes most frequently affect codon 61 of *H-RAS* and *N-RAS* and less commonly codons 12 and 13 whereas alterations in the *K-RAS* gene and in other codons are rare [20]. Besides of follicular morphology, tumors harboring mutant RAS are frequently encapsulated and display the lower rate of nodal disease which are favorable prognostic factors. In addition, some studies have shown a high rate of *RAS* mutations in benign tumors especially in up to 50% of microfollicular adenomas suggesting that these genetic alterations may represent an early event in follicular thy-

roid tumorigenesis [21]. Interestingly, transgenic mice with thyroid-specific mutant *RAS* expression develop thyroid hyperplasia and malignancy [22]. On the other hand, *RAS* mutations are also found in approximately a half of poorly differentiated and anaplastic thyroid carcinomas and associate with the poor patients' survival [23] suggesting that RAS may have distinct roles in the early and late stage of thyroid cancer.

The aim of our investigation was to assess clinico-pathological correlations between the mutational background and disease phenotype, and to evaluate the prognostic value of *BRAF* mutation in a group of 266 Serbian patients admitted to our reference center for PTC from 1992 to 2008. Our work is the first large-scale study of this kind in Serbia as well as in the Central Balkan region.

Materials and Methods

Patients, clinicopathological characteristics, treatment and follow-up

A total of 266 patients diagnosed and treated for PTC in the Institute of Oncology and Radiology of Serbia, Belgrade, between June, 1992 and December, 2008 were enrolled. The group included 201 (75.6%) females and 65 (24.4%) males (sex ratio 0.32) aged 8-83 years old at diagnosis (48.0 ± 16.1 years old, mean ± SD). None of patients had a history of radiation exposure.

Surgical treatment was classified as total thyroidectomy (n = 255, 95.9%) or less then total (n = 11, 4.1%). Central neck dissections (level VI) were done in 192 (75.2%) patients while therapeutic lateral neck dissection was performed for biopsy-proven clinically involved lymph nodes in levels II-V in 64 (24.8%) (Table 1). Note that radioiodine ablation was done only in 28 (10.5%) patients due to unavailability of this modality in the country for the most period of the study. All patients received TSH suppression therapy.

Pathological diagnosis was based on the WHO standards [24] and confirmed independently by two experienced pathologists (Z.M. and M.N.). Histologically, the series included 218/266 (82.0%) tumors with classical papillary architecture, 45/266 (16.9%) were follicular variant of PTC and 3 of other subtype (1.1%). Pathological information was retrieved from patients' records. Tumor size ranged 1.5 - 190 mm (20.0 ± 19.2 mm, mean ± SD). Multifocality was observed in 88 (33.1%) cases, extrathyroidal invasion in 81 (30.5%), vascular invasion in 54 (20.3%), lymph node metastasis in 175 (71.7% of cases with available information, n = 244), distant metastasis in 9 (3.4% of cases with available information, n = 261).

Tumor staging was according to UICC TNM classification of malignant tumors [25]. The pT category distribution in the series was as follows: 1 – 112 cases (42.1%), 2 – 41 (15.4%), 3 – 77 (28.9%), 4 – 19 (7.1%) and was not available in 17 (6.4%) patients. Clinical

Table 1 Baseline, cancer and treatment characteristics

Baseline factors	
Age at diagnosis (yr) mean ± SD (range)	48.0 ± 16.1 (8-83)
Sex	
Male	65 (24.4%)
Female	201 (75.6%)
Sex ratio	0.32
Follow-up period (mo) mean ± SD (range)	53.1± 41.6 (7-187)
Cancer characteristics	
pT category [1]	
1	112 (42.1%)
2	41 (15.4%)
3	77 (28.9%)
4	19 (7.1%)
Nodal disease [2]	175 (65.8%)
Distant metastasis [3]	9 (3.4%)
Tumor size (mm) mean ± SD (range)[4]	20.0 ± 19.2 (1.5-190)
≤ 10 mm	61 (23.9%)
> 10 and ≤ 20 mm	86 (33.7%)
> 20 mm	108 (42.4%)
Extrathyroidal extension	81 (30.5%)
Vascular invasion	54 (20.3%)
Tumor multifocality	88 (33.1%)
Tumor capsule	110 (41.4%)
Clinical stage [5]	
l	141(53.8%)
ll	9 (3.4%)
llI	32 (12.2%)
IV	80 (30.5%)
Histopathology variant	
Classic papillary	218 (82.0%)
Follicular	44 (16.5%)
Other	4 (1.5%)
Persistent disease	7 (1.1%)
Treatment modalities	
Extent of thyroid resection	
Total thyroidectomy	255 (95.9%)
Less than total	11 (4.1%)
Central neck dissection (level VI)	192 (75.2%)
Central + lateral neck dissection (levels VI, II-V)	64 (24.8%)
Radioiodine ablation	28 (10.5%)

[1] T category was not available in 17 cases.
[2] N category was not available in 22 cases.
[3] M category was not available in 5 cases with unknown mutation.
[4] Tumor size was not available in 11 cases.
[5] Clinical stage could not be determined in 4 cases.

stage I was registered in 141 patients (53.0%), II – in 9 (3.4%), III – in 32 (12.0%) and IV – in 80 (30.1%) and was not available in 4 cases (1.1%).

Follow-up was performed on a 6-months or an annual basis at the Department of Endocrinology of the Institute and lasted 7–187 months (53.1 ± 41.6, mean ± SD). The follow up examination included serum TSH, FT4, Tg and TgAb measurement, cervical ultrasonography, and consultations by an oncologist and endocrinologist. In case of Tg elevation (in thyroidectomized patients) or suspicious ultrasound findings or neck enlargement on palpation, other imaging means, including X-ray, CT, or PET scanning, were used for diagnosis.

Disease recurrence was defined as a local tumor focus, regional or distant metastasis detected by any diagnostic means not earlier than 6 months after the initial surgery. A total of 20 (12.5%) patients in this series had disease recurrence during the follow-up period.

Seven patients (1.1%) had persistent disease. During the study period, 15 (5.6%) patients died: 6 (2.3%) due to disease progression (2 patients with persistent disease and 4 with cancer recurrence); 1 (0.4%) patient died for cardiac arrest and in 8 (3.0%) patients cause of death was not specified.

The protocols of the study were approved by the Ethical Committees of the Institute of Oncology and Radiology and of Nagasaki University.

Nucleic acid extraction

Thyroid tumor tissues were manually dissected from formalin-fixed paraffin embedded tissue sections obtained from the files of the Department of Pathology, Institute of Oncology and Radiology of Serbia.

DNA was extracted from four 10-μm sections using the Puregene Genomic DNA purification kit (Gentra Systems, Quiagen, Minneapolis, MN, USA). Total RNA was extracted from three 10-μm sections using Recover All Total Nucleic Acid Isolation Kit optimized for FFPE samples *(*Ambion, Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocols. DNA reconstituted in TE and RNA eluted from the columns with the buffer supplied with the corresponding kit were quantified with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, USA).

Determination of BRAF and RAS mutations by direct sequencing

Tumor DNA was PCR-amplified to generate products covering the common mutational hotspots in thyroid tumors, including a portion of *BRAF* exon 15, and codons 12, 13, 31, 60 and 61 of *K-*, *H-* and *N-RAS*. Primers were designed with Primer Express Software ver.1 (Applied Biosystems) to yield relatively short PCR products (<170 bp) suitable for sequencing. The sequences of primers, annealing temperature and amplicon sizes are listed in Table 2. PCR reactions were routinely performed in a final volume of 25 μL with the hot start ExTaq polymerase (Takara, Shizuoka, Japan). The aliquots of amplified products were treated with EXO SAP-IT (USB Corp., Cleveland, OH, USA) and sequenced on an ABI PRISM 3130 automated capillary DNA Sequencer (Applied Biosystems) using BigDye terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Applied Biosystems).

Detection of RET/PTC rearrangements

RET/PTC1 and *RET/PTC3* rearrangements were detected using reverse transcription-polymerase chain reaction (RT-PCR) followed by Southern blotting. Two micrograms or less of total RNA were reverse transcribed with MuLV Reverse Transcriptase (Applied Biosystems) in the presence of random hexamers. cDNA integrity and the efficiency of the RT reaction in each sample was confirmed using the *TUBA1A* gene as a reference (RefSeq accession number NM_006009.2, encodes human alpha 1a tubulin). Amplifications were performed using 2 μL of cDNA as a template (35 cycles), with the primers specific for the *RET/PTC1* and *RET/PTC3* fusion genes (Table 2).

After PCR amplification, 10 μL of reaction products were resolved in 2% agarose gel, denatured, transferred to a Hybond+ nylon membrane (Roche Applied Science, Indianapolis, IN, USA) in 20x SSC and cross-linked using a calibrated UV light source. Membranes were prehybridized at 42°C for 2 h in a buffer containing 0.25 M sodium phosphate, pH 7.2, 1 mM EDTA, 1% BSA, 7% SDS and 15% formamide. The hybridization was performed at 42°C overnight in the same buffer containing specific oligonucleotide probes labeled with digoxigenin-11-ddUTP using DIG oligonucleotide 3'-end labeling Kit (Roche Applied Science, Indianapolis, IN, USA). After appropriate washing and blocking, the bound probe was detected with alkaline phosphatase-conjugated anti-digoxigenin Fab' fragments (Roche Applied Science, Indianapolis, IN, USA) and CDP-Star luminescent substrate (Roche Applied Science, Indianapolis, IN, USA). The detec-

Table 2 Primer sequences, annealing temperature and PCR product sizes

Gene	Exon	Codon	Primer sequence	Annealing temp (˚C)	Ampliconsize (bp)
BRAF	15	600	(F) 5'-GAAATTAGATCTCTTACCTAAACTCTTCATA-3' (R) 5'-GACCCACTCCATCGAGATTT-3'	55	168
H-RAS	1	12/13	(F) 5'-TGAGGAGCGATGACGGAATAT-3' (R) 5'-ATTCGTCCACAAAATGGTTCTG-3'	58	103
H-RAS	2	61	(F) 5'-GCCTGTTGGACATCCTGGATA-3' (R) 5'-TTGTTGATGGCAAACACACAC-3'	56	106
K-RAS	1	12/13	(F) 5'-GTCACATTTTCATTATTTTTATTATAAG-3' (R) 5'-CTGTATCGTCAAGGCACTCTT-3'	50	105
K-RAS	2	61	(F) 5'-TTCTCAGGATTCCTACAGGAAGC-3' (R) 5'-TACTGGTCCCTCATTGCACTG-3'	56	106
N-RAS	1	12/13	(F) 5'-TGATTACTGGTTTCCAACAGGTT-3' (R) 5'-GGATTGTCAGTGCGCTTTTC-3'	55	101
N-RAS	2	61	(F) 5'-TGATTACTGGTTTCCAACAGGTT-3' (R) 5'-GGATTGTCAGTGCGCTTTTC-3'	55	101
TUBA1A	3/4		(F) 5'-AGATCATTGACCTCGTGTTGG A-3' (R) 5'- ACCAGTTCCCCCACCAAA G -3'	60	101
RET/PTC1			(F) 5'-GCAAAGCCAGCGTTACCAT-3' (R) 5'-GCCTTGACCACTTTTCCAAATT-3'	55	106
probe			5'- CCAGCGTTACCATCGAGGATCCAAAGTGG-3'		
RET/PTC3			(F) 5'-AAAAGCAGACCTTGGAGAACA-3' (R) 5'-CTTTTCCAAATTCGCCTTCT-3'	55	102
probe			5'- AGACCTTGGAGAACAGTCAGGAGGATCCAAAG-3'		

tion was performed using a LAS3000 imaging system (Fujifilm, Japan).

Statistical analysis

Demographic and clinicopathological characteristics of patients with the tumors harboring different types of mutations were compared using Fisher's exact test or its extension for categorical data or Wilcoxon rank-sum test for continuous measurements in univariate setting using the FREQ and NPAR1WAY procedures in the SAS system (SAS/STAT User's Guide Version 8. SAS Institute, Cary, NC, USA).

In multivariate setting, $BRAF^{V600E}$ associations with patient's sex (categorical), age (continuous), tumor histology (classic papillary *vs*. other, categorical), tumor size (continuous), extrathyroidal invasion (categorical), the presence of tumor capsule (categorical), vascular invasion (categorical), tumor multifocality (categorical), pT category (3+4 *vs*. 1+2, categorical), nodal disease (categorical), distant metastasis (categorical) and tumor stage (III+IV *vs*. I+II, categorical) were evaluated by logistic regression analysis. The most appropriate model was selected by Akaike information criterion (AIC) starting from the full model. The LOGISTIC procedures in the SAS system were used for the calculations.

For disease-free survival (DFS) analysis of $BRAF^{V600E}$-positive and -negative patients groups, Cox proportional hazard model was applied. The initial full model included all the variables specified above except that patient's age at diagnosis was tested by 10-year increments and, in addition, the *BRAF* status (present *vs*. absent, categorical) was also used as a variable. Model optimization was performed using the AIC. The PHREG procedure in the SAS system was used for calculations. Once the most appropri-

Table 3 Association between genetic alterations and clinicopathological characteristics in the series

Parameter	*BRAF*^{V600E} [1] n=84	*RAS* n=11	*RET/PTC1* n=42	*RET/PTC3* n=13	Unknown n=116	*BRAF*^{V600E} *vs.* all other *P*-value
Age at diagnosis (yr) ± SD	50.9 ± 15.4	44.9 ± 17.7	44.5 ± 17.5	43.6 ± 15.9	49.2 ± 14.9	0.091
Gender						0.065
Male	27 (32.1%)	3 (27.3%)	7 (16.7%)	3 (23.1%)	25 (21.6%)	
Female	57 (67.9%)	8 (72.7%)	35 (83.3%)	10 (76.9%)	91 (78.4%)	
Sex Ratio	0.47	0.37	0.20	0.30	0.27	
Tumor size (cm) ± SD	2.5 ± 1.8	3.2 ± 2.6	2.7 ± 3.2	2.9 ± 4.5	2.8 ± 3.6	0.335
pT category [2]						0.135
1+2	38 (47.5%)	5 (45.5%)	28 (66.7%)	10 (83.3%)	72 (69.2%)	
3+4	42 (52.5%)	6 (54.5%)	14 (33.3%)	2 (16.7%)	32 (30.8%)	
N category [3]						0.219
N0	17 (22.4%)	3 (33.3%)	13 (31.0%)	3 (25.0%)	33 (31.4%)	
N1	59 (77.6%)	6 (66.7%)	29 (69.0%)	9 (75.0%)	72 (68.6%)	
M category [4]						0.475
M0	80 (95.3%)	11 (100%)	41 (97.8%)	13 (100%)	107 (96.4%)	
M1	4 (4.7%)	0	1 (2.2%)	0	4 (3.6%)	
Histological variant						0.016
Classic	76 (90.5%)	6 (54.5%)	37 (88.1%)	11 (84.6%)	88 (75.9%)	
Other	8 (9.5%)	5 (45.5%)	5 (11.9%)	2 (15.4%)	28 (24.1%)	
Vascular invasion	20 (23.8%)	4 (36.4%)	8 (19.0%)	2 (15.4%)	20 (17.2%)	0.331
Tumor multifocality	23 (27.4%)	4 (33.4%)	19 (45.2%)	4 (30.8%)	38 (32.8%)	0.562
Tumor capsule	41 (48.8%)	5 (45.5%)	17 (40.5%)	3 (23.1%)	44 (37.9%)	0.108
Clinical stage [5]						0.001
I+II	36 (42.9%)	8 (72.7%)	28 (66.7%)	10 (76.9%)	68 (60.7%)	
III +IV	48 (57.1%)	3 (27.3%)	14 (33.3%)	3 (23.1)	44 (39.3)	
Extrathyroidal extension	32 (38.1%)	4 (36.4%)	12 (28.6%)	5 (38.5%)	28 (24.4%)	0.085

[1] One cases with double mutation (*BRAF*^{V600E} and *RET/PTC3*) was included into the *BRAF*^{V600E} -positive subgroup.
[2] T category was not available in 4 cases with *BRAF*^{V600E} mutation, 1 case with *RET/PTC3* and 12 cases with unknown mutation.
[3] N category was not available in 8 cases with *BRAF*^{V600E} mutation, 2 cases with *RAS* mutations, 1 case with *RET/PTC3* and 11 cases with unknown mutation.
[4] M category was not available in 5 cases with unknown mutation.
[5] Clinical stage could not be determined in 4 cases with unknown mutation.

ate model was determined, the maximum likelihood estimates of the respective parameters and their Wald-type 95% confidence intervals were calculated. DFS rates in *BRAF*^{V600E}-positive and -negative tumors were compared using the log-rank test (LIFETEST procedure in the SAS system). SPSS Statistics 17.0 (SPSS Inc., Chicago, IL, USA) was used to plot the Kaplan-Meier estimates of survival functions.

The *P*-value less than 0.05 was regarded as indicating statistical significance.

Results

The prevalence of BRAF and RAS mutations and of RET/PTC rearrangements

BRAF exon 15, codons 12, 13, 31, 60 and 61 of *K-, H- and N-RAS* and the occurrence of *RET/PTC1* and *RET/PTC3* rearrangements were studied in 266 paraffin-embedded tumor tissues. Cumulatively, mutations were found in 150/266 tumors (56.4%) of which 1/266 (0.4%) had two genetic alteration (Table 3).

The *BRAF*^{V600E} was detected in 84/266 (31.6%) of analyzed samples. Specifically, it occurred in 75/218 (34.4%) of typical papillary PTCs, 6/44 (13.6%) of follicular variant, 2/3 (66.7%) of tall cell variant and 1/1 (100%) of Hurthle cell variant of PTC. All mutations were the heterozygous T>A transversions at nucleotide 1799 (V600E). Representative sequencing chromatograms for a portion of *BRAF* codon 15 are shown in Fig. 2A.

RAS mutations were identified in 11/266 (4.1%) tumors. The *N-RAS* codon 61 CAA> CGA (glutamine to arginine) mutation was the most frequent, 8/266 (3.0%). Mutant *K-RAS* was found in 3/266 tumors

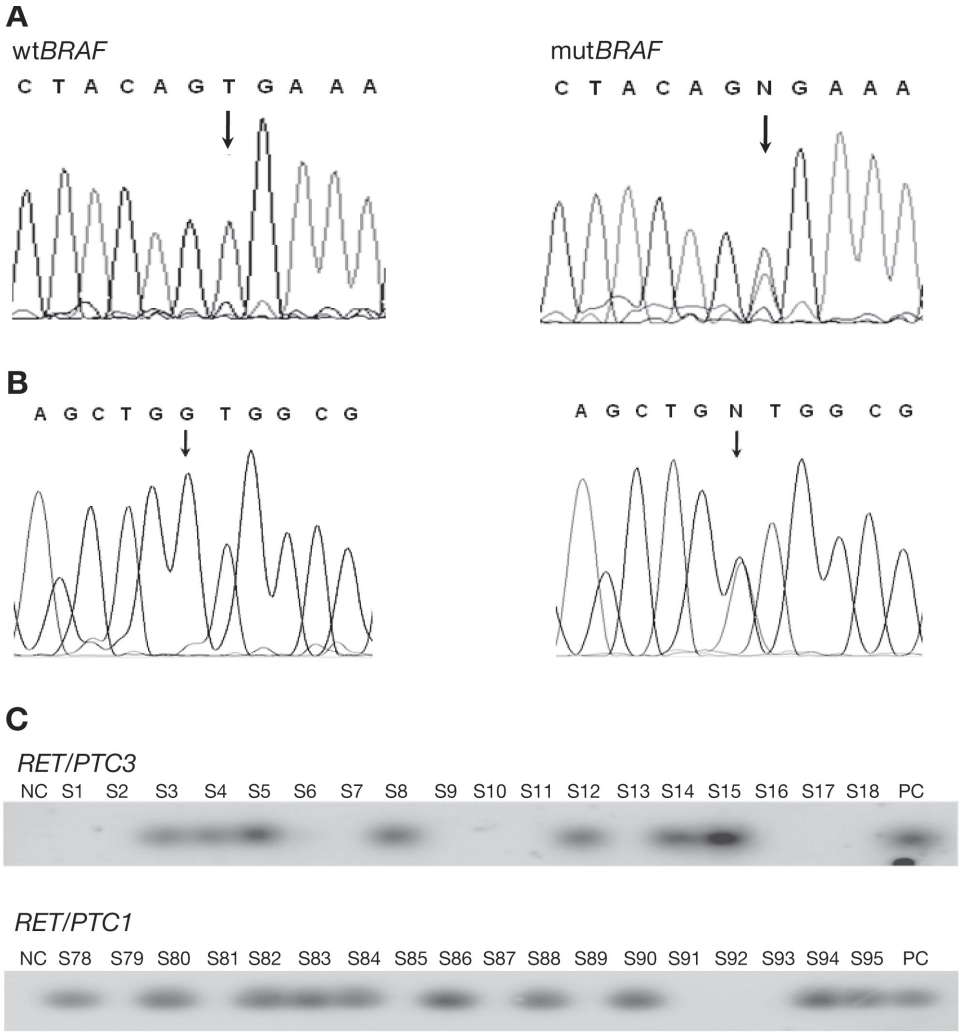

Fig. 2 Detection of point mutations in the (A) *BRAF* and (B) *K-RAS* genes by direct sequencing, and of (C) *RET/PTC* rearrangements by Southern blotting. In (C), PC and NC stand for the Positive and Negative controls, respectively. For *RET/PTC3* analysis, cDNA of PTC from an irrelevant patient previously found to harbor this rearrangement was used as a positive control. For *RET/PTC1* analysis, cDNA of TPC1 cell line was used as a positive control. In both analyses, cDNA from the thyroid of an irrelevant patient with Basedow's disease was used as a negative control.

(1.1%). All mutations were the G>A transitions (glycine to aspartic acid) at codon 12. None of the samples harbored *H-RAS* alterations. According to the histological type, mutant *RAS* occurred in 6/218 (2.8%) of typical papillary tumors and in 5/44 (11.4%) of follicular variant of PTC. Representative sequencing chromatograms for *K-RAS* codon 12-13 are shown in Fig. 2B.

RET/PTC rearrangements were found in 55/266 (20.7%) cases, of them 42/266 (15.8%) were positive for *RET/PTC1* and 13/266 (4.9%) for *RET/PTC3*. Among the *RET/PTC1*-harboring PTCs, 37/218 (17.0%) were typical papillary tumors, 4/44 (9.1%) follicular variant and 1/3 (33.3%) tall cell variant. Of the 13 PTCs with *RET/PTC3*, 11/218 (5.0%) had typical papillary structure, 1/44 (2.3%) was follicular variant and 1/3 (33.3%) was tall cell variant. An example of Southern blotting analyses for *RET/PTC1* and *RET/PTC3* are shown in Fig. 2C.

Only one tumor with tall cell growth pattern displayed both $BRAF^{V600E}$ and *RET/PTC3*.

Correlation between $BRAF^{V600E}$ and clinicopathological characteristics

Since *BRAF* mutation has been associated with cer-

Table 4 Multivariate analysis of the correlation between $BRAF^{V600E}$ and clinicopathological characteristics

Parameter	Comparison	Odds ratio	95% CI	*P*-value
Histological variant	Classic *vs.* other	2.36	1.00–5.55	0.05
pT category	3 + 4 *vs.* 1 + 2	1.93	0.99–3.72	0.05
Clinical stage	III +IV *vs.* I+II	2.05	1.07–3.96	0.03

Table 5 Risk factors for disease–free survival of PTC patients

Factor	Comparison	Hazard ratio	95% CI	*P*-value
Age at surgery	By 10-year increment	1.85	1.28–2.69	0.001
pT category	3+4 *vs*. 1+2	4.69	1.23–17.92	0.024
N category	N1 *vs*. N0	7.59	0.95–60.64	0.056
$BRAF^{V600E}$	Present *vs*. Absent	1.15	0.42–3.19	0.784

tain clinical and demographic parameters of PTC in previous works, we evaluated its correlations in our series.

As shown in Table 3, the univariate analysis demonstrated that $BRAF^{V600E}$ associated with the classic papillary histotype of the tumor ($P = 0.016$), and the strongest association was with the more advanced clinical stage of disease (i.e. III+IV *vs*. I+II, $P = 0.001$).

All other clinico-pathological features, including tumor size, pT category, nodal disease, distant metastasis, vascular invasion, tumor focality and the presence of tumor capsule did not differ significantly between $BRAF^{V600E}$-positive cases and all other.

These results suggested that $BRAF^{V600E}$ mutation may be a potential marker of high-risk patients. To further address this correlation, a multivariate logistic regression analysis was performed. Three parameters, i.e. classical papillary variant (OR = 2.36, 95% CI 1.00 – 5.55, $P = 0.05$), high pT category (OR = 1.93, 95% CI 0.99 – 3.72, $P = 0.05$) and the advanced clinical stage (OR = 2.05, 95% CI 1.07 – 3.96, $P = 0.03$) but not any other tested were independently associated with $BRAF^{V600E}$ (Table 4).

Note also that $BRAF^{V600E}$ was found in 4/7 (57.1%) of patients with persistent disease.

Effect of $BRAF^{V600E}$ on disease-free survival

The total number of registered recurrences in the series was 20 of which 2 (10%) were regional, 13 (65%) regional and local, 3 (15%) regional only and distant (the lung), and 2 (10%) distant only (the lung).

We investigated, using the proportional hazard model, an association between disease-free survival (DFS) and potential clinicopathological risk factors, including *BRAF* status, in a subset of 110 patients in whom the follow-up period could last for not less than 24 months, i.e. those treated from June, 1992 to December, 2006, inclusively. The follow–up period in this subgroup was 62.9 ± 41.4 (mean ± SD) months ranging from 8 to 187 months. As shown in Table 5, among all parameters tested only three, i.e. the older age at surgery (HR = 1.85, 95% CI 1.28 – 2.69, $P = 0.001$), the advanced pT category (HR = 4.69, 95% CI 1.23 – 17.92, $P = 0.024$) and, marginally, nodal disease (HR = 7.59, 95% CI 0.95 – 60.64, $P = 0.056$) increased the risk for the faster recurrence.

A separate analysis of survival functions in $BRAF^{V600E}$-positive and -negative patients showed that patients $BRAF^{V600E}$ tended to display a higher chance for recurrence but the difference did not reach significance threshold ($P = 0.061$ by the log-rank test, Fig. 3) during the considered follow-up period.

Discussion

Our study assessed the prevalence and clinicopathological correlations of PTC-specific genetic alterations in an ethnically and geographically homogenous group of 266 patients from Serbia. All patients were diagnosed and treated according to a uniform protocol in a single center. Since the Institute of Oncology and Radiology of Serbia, Belgrade, is one of the reference centers for thyroid cancer in the Central Balkan area, we assume our data may be representative of the distributions of histopathological variants and of molecular abnormalities in PTC for this region of Europe.

Cumulatively, $BRAF^{V600E}$ and *RAS* point mutations, and *RET/PTC* rearrangements were detected in 150/266 tumors (56.4%). $BRAF^{V600E}$ was the most prevalent

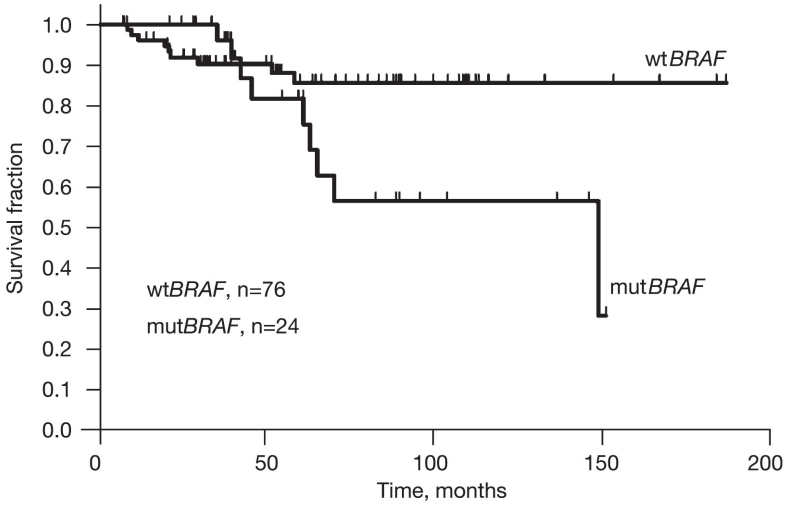

Fig. 3 Disease-free survival of PTC patients with $BRAF^{V600E}$ (mut*BRAF*) or with any other genetic alteration (wt*BRAF*). Patients carrying *BRAF* mutation display a tendency to the higher chance for recurrence but the difference is not significant (P = 0.061) by the log-rank test.

(84/266, 31.6%), *RET/PTC* rearrangements occurred in 55/266 (20.7%) cases, the *RAS* genes mutations were the least frequent (11/266, 4.1%).

The frequency of $BRAF^{V600E}$ mutation in our series is comparable to that in different geographical areas that ranges from 29 to 83% being 44% on average [12, 26]. The majority of $BRAF^{V600E}$ mutations (90.5%) were detected in patients with the classic papillary variant of PTC, in line with the previous reports of the higher prevalence of $BRAF^{V600E}$ in conventional PTCs and its low frequency in the follicular variant of PTC [27].

The prevalence of *RAS*-family gene mutations also corresponds well to that of 0 - 45% described previously [20]. Perhaps such variations are due in part to different proportions of histological variants included in different studies. In the present work *RAS* mutations were detected in 5/44 (11.4%) of follicular variant of PTC and in 6/218 (2.8%) of tumors with classical papillary morphology. Most *RAS* mutations involved codon 61 of *N-RAS* (8/11, 72.7%); 3/11 (17.3%) mutations were at codon 12 of *K-RAS*. Both these observations, i.e. the difference in the frequency of *RAS* mutations between classical papillary and follicular variant of PTC, and the higher prevalence of *N-RAS* codon 61 alterations than any other hotspot mutations are in agreement with the earlier reports.

RET/PTC1 and *RET/PTC3* account for the vast majority of *RET* rearrangements in PTC [28]. Our investigation was therefore focused on these two variants which were found cumulatively in about one-fifth of tumors in the series. *RET/PTC1* was more prevalent than *RET/PTC3*, 42 and 13 cases (15.8% and 4.9%, respectively). These results are consistent with an average frequency of *RET/PTC* rearrangements of about 20% as well as with the higher prevalence of *RET/PTC1* observed in adult patients with sporadic PTC according to different studies [9].

Molecular alterations analyzed in our work were virtually exclusive with only one tall-cell PTC harboring simultaneously $BRAF^{V600E}$ and *RET/PTC3*. The vast majority of studies of typical oncogenic mutations in PTC have found little to no overlap between those. The coexistence of more than one mutation within one tumor could be explained by a technical error, sample cross-contamination or by the polyclonal malignancy. In the latter case a tumor nodule may be developing from two closely localized independent precursors which upon progression form a single tumor. While we cannot rule out any of the above possibilities, our results as whole are in line with the idea that one oncogenic alteration is sufficient to give rise to PTC.

During the last five years, $BRAF^{V600E}$ has been extensively explored for clinicopathological associations. In particular, it has been proposed, although not always unequivocally, to associate with the more aggressive clinical course and poorer outcome of PTC ([29] for review).

We addressed clinical correlations of $BRAF^{V600E}$ in our series. On univariate analysis $BRAF^{V600E}$, besides of classic papillary histotype, strongly associated with

the more advanced clinical stage (P = 0.001, Table 3). Perhaps this was due in part to the two important parameters that tended to be different between $BRAF^{V600E}$-positive and -negative subgroups and which are taken into consideration for staging. These are a somewhat older age of patients with $BRAF^{V600E}$ (50.9 ± 15.4 *vs*. 47.2 ± 15.6 years old, P = 0.091) and a higher frequency of extrathyroidal tumor spread in $BRAF^{V600E}$-positive cases (32/84, 38.1% *vs*. 49/182, 26.9%, P = 0.085). This notion is supported by the results of several works, which also suggested that $BRAF^{V600E}$ association with the more advanced clinical stage of disease may reflect the older age of the patient and the higher frequency of extrathyroidal tumor spread [30, 31]. On multivariate analysis $BRAF^{V600E}$ concordantly associated with the classical papillary variant (OR = 2.36, 95% CI 1.00 – 5.55, P = 0.05), high pT category (OR = 1.93, 95% CI 0.99 – 3.72, P = 0.05) and the advanced clinical stage (OR = 2.05, 95% CI 1.07 – 3.96, P = 0.03). Thus, our results confirm $BRAF^{V600E}$ correlation with the more advanced disease and support its usefulness in identifying high-risk patients.

To assess the prognostic significance of $BRAF^{V600E}$, a subset of 110 patients with the expected follow-up not less than 2 years was analyzed for recurrence risk factors. The proportional hazard model showed that the older age at surgery (HR = 1.85, 95% CI 1.28 – 2.69, P = 0.001), the advanced pT category (HR = 4.69, 95% CI 1.23 – 17.92, P = 0.024) and, to somewhat extent, lymph node involvement at presentation (HR = 7.59, 95% CI 0.95 – 60.64, P = 0.056) increased the risk. The older age of patients, the greater tumor size, nodal disease and extrathyroidal tumor spread are well-established risk factors of PTC recurrence [32-34]; our study confirms these findings.

However, $BRAF^{V600E}$ was not identified as a risk factor for the faster recurrence (HR = 1.15, 95% CI 0.42 – 3.19, P = 0.784) in contrast to some studies [12, 35] and in line with others [36]. Note that, from the clinical point of view, our series is characterized by three particular qualities, i.e. the short to medium follow-up period (32.0; 53.5; 89.6 months, the 25%, 50%, and 75% quartiles, even though we set the expected duration of follow-up of ≥24 months as inclusion criterion for DFS analysis), the high frequency of total thyroidectomy and neck dissections, and the low number of patients who received radioiodine ablation after surgery.

The short follow-up was proposed to be a reason for discordant findings of $BRAF^{V600E}$ association with PTC recurrence in some works [29, 31]. In different studies involving a relatively large number of patients, the proportion of recurrences varies from some 3% to 30%, usually in relation with follow-up duration [32, 34] being on average about 20% after a 10-year follow-up period and 30% after at 30 years [2]. Our study corresponds well with these claims (20/266, 12.5% patients with recurrence) for the short to medium follow-up.

Extent of surgery is another well-established factor to affect DFS in the way that limited surgery and not performed neck dissection generally increase the risk for recurrence [34, 37]. Total thyroidectomy performed in 95.5% cases and at least central neck dissection performed in 100% of patients in our series taken together with the sensitive serum thyroglobulin test (which was used to detect recurrence) would unlikely lead to underdiagnosed recurrence in our study.

Note that radioiodine ablation was done only in 28/266 (10.5%) patients in our series which is in contrast to 38-100% rate in other works [26, 32, 34]. $BRAF^{V600E}$ has been shown to suppress the expression of key genes whose products mediate the uptake and metabolic conversion of iodine in PTC or in model cell systems, such as *NIS* [38], *TPO* [39], *SLC26A4* (pendrin) [40], *TG* [39] and *TSHR* [15]. The anticipated lower efficacy of radioiodine ablation/therapy of $BRAF^{V600E}$-positive tumors could not be demonstrated in our series because of insufficient number of patients receiving this modality that resulted in a low power of statistical test (data not shown). We therefore cannot rule out that $BRAF^{V600E}$ was not identified as a risk factor for recurrence due to this particularity of our series.

With regard to patients' survival, the quality of information did not allow adequate statistical assessment of $BRAF^{V600E}$ implication (in 8/15, 53.3% cases, cause of death was unknown). It, however, is worth noting that among 15 deceased patients 10 (66.7%) harbored $BRAF^{V600E}$. The poorer survival of PTC patients with mutant *BRAF* was reported in a large group of Italian patients [41]. Our observation (i.e., $BRAF^{V600E}$ prevalence of 10/15, 66.7% in the deceased patients *vs*. 74/251, 29.5% in alive patients) may be interpreted, with reservation, as an argument in support of the greater risk for lethal outcome in patients with $BRAF^{V600E}$.

In summary, our study found non-extreme frequencies of the three types of PTC-specific mutations in the *BRAF*, *RAS* and *RET* genes in Serbian patients as could

rather be expected for the geographical area with an optimal iodine sufficiency (average urine iodine excretion of 158 μg/L according to the WHO survey (www.who.int/vmnis/iodine/data/database/countries/srb_idd.pdf). Among the genetic alterations tested, $BRAF^{V600E}$ was the most prevalent. The assessment of $BRAF^{V600E}$ correlation with clinical characteristics confirmed its association with the classic papillary histotype and advanced stages of disease. For disease-free survival, $BRAF^{V600E}$ did not appear to be an independent predictor of the higher risk for faster recurrence. We propose that under extensive thyroid surgery and limited availability of radioiodine ablation (which is a common situation in developing countries) $BRAF^{V600E}$ may not be an indicator of poorer DFS during the short to middle follow-up period. Longer follow-up, currently in progress, is required to evaluate its prognostic significance.

As a whole, our findings support the notion that $BRAF^{V600E}$, which can be detected preoperatively in fine-needle aspiration biopsy material, has a potential to contribute to patients stratification into high- and low-risk groups.

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Appendix

Declaration on Conflict of Interests

All authors declare that there is no conflict of interest.

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