Immunohistochemical Detection of the BRAF V600E-mutated Protein in Papillary Thyroid Carcinoma

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Abstract: The V600E mutation of the B-type Raf kinase (BRAF) gene is a common event in papillary thyroid carcinoma (PTC) and seems to play a key role in the development and progression of this disease. We evaluated the expression of the mutated BRAF V600E protein in 144 cases of PTC using a novel mutation-specific antibody. Seventy-six PTCs (52.8%) showed unequivocal diffuse cytoplasmic expression of the mutated BRAF protein, and the T1799A point mutation was confirmed by sequencing analysis in selected cases. No statistical difference in V600E BRAF protein expression was seen between microcarcinomas and macrocarcinomas. Further, no significant correlation of V600E expression with clinicopathologic parameters of aggressiveness such as lymph node metastasis, peritumoral infiltration, or perithyroidal infiltration was found. BRAF V600E protein expression was significantly more common in tumors with tall cell or oncocytic features but was less common in tumors with follicular growth pattern. Diffuse sclerosing, solid and follicular variants did not show the mutated BRAF protein. Immunohistochemical detection of the mutated V600E BRAF protein in PTC may facilitate mutational analysis in the clinical setting. Our data show that the expression of the mutated BRAF V600 protein and thus the corresponding BRAF mutation seems not to be per se a marker of aggressiveness but is already seen in clinically indolent microcarcinomas. Nevertheless, the investigation of BRAF V600E protein expression might be of clinical interest especially in therapy-resistant disease, as new therapeutics inhibiting the mutated protein are clinically available.

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Papillary thyroid carcinoma (PTC) is the most common type of thyroid cancer. Mutation of the B-type Raf kinase (BRAF) is detected in 20% to 80% of sporadic PTCs with higher prevalence in conventional PTC than in follicular variants.[36] The T1799A point mutation leading to V600E amino-acid substitution is the only PTC-specific mutation of the BRAF gene detected in PTC and PTC-related poorly and undifferentiated carcinoma.[8,10,16,26,36,37]

The aberrant BRAF protein causes a constitutive activation of the BRAF serine/threonine kinase and thus leads to an activation of the mitogen-activated protein kinase cascade.[9] BRAF V600E also leads to higher expression of matrix metalloproteases and to increased tumor cell motility.[37]

In several clinical studies, PTC with a BRAF T1799A mutation was associated with advanced tumor stage and higher risk of recurrence compared with nonmutated tumors, and thus BRAF mutation analysis has been suggested as an important prognostic parameter.[2,11,24,37] In contrast, a few studies did not show prognostic relevance of the T1799A point mutation of the BRAF gene in PTC.[37] Recently, the selective BRAF V600E inhibitor PLX4032 showed compelling clinical activity in melanomas harboring the T1799A point mutation and was consequently approved by the US Food and Drug Administration (FDA).[12] This drug or other emerging BRAF inhibitors may represent an alternative therapy for otherwise treatment-resistant BRAF T1799A-mutated PTC.

Many different methods for BRAF mutation analyses with different sensitivities have been developed, including single-strand conformation polymorphism, gene sequencing (direct DNA sequencing, pyrosequencing), and mutation-specific polymerase chain reaction. All these methods are more or less laborious and expensive and often complicated by suboptimal DNA preservation in formalin-fixed and paraffin-embedded tissues and by the presence of non-neoplastic thyroid tissue.[13,14,22] Recently, a mutation-specific antibody (clone VE1) was developed, which allows immunohistochemical visualization of the BRAF V600E protein with high sensitivity and

specificity.[3,4] In this study, we investigated the expression of the mutated BRAF protein in a series of PTC including papillary thyroid microcarcinoma with available lymph node status.

MATERIALS AND METHODS

We analyzed 144 cases of histologically proven PTC retrieved from the archives of the Department of Clinical Pathology of the Medical University of Vienna. All cases underwent surgical treatment between 2000 and 2006 in the Section of Endocrine Surgery, Department of Surgery of the Medical University of Vienna. Only cases with clinicopathologic data including sex, age, tumor size, and lymph node status and with sufficient formalin-fixed paraffin-embedded tumor tissue were investigated. For technical reasons, cases in which only previously frozen tissue was available were excluded from the study. Total thyroidectomy was performed in all patients. At least lymph nodes dissected from the central cervical compartment were available for histologic analysis in all cases.

The entire thyroid gland was sectioned horizontally in slices of approximately 3 to 5 mm and formalin fixed. Representative slices from the upper, middle, and lower region of each lobe and from every macroscopically suspect lesion were embedded in paraffin.

Histology

All PTCs were classified and subtyped according to the World Health Organization criteria outlined in 2004[10] and staged according to the International Union Against Cancer (UICC) 2009 guidelines.[30] All cases $\leq$1 cm in diameter were regarded as microcarcinoma.

We assessed different growth patterns including exclusively follicular growth and focal (< 50%) or prominent (> 50%) solid growth, and we analyzed cell types including oncocytic cell type and tall cell type.

In addition, extrathyroidal extension, peritumoral infiltration, and vascular invasion were analyzed. Regarding stroma characteristics, calcification, lymphocytes within the tumor stroma, and desmoplastic stroma reaction were analyzed. A tumor was evaluated as positive for calcification whenever a calcification was found intratumoral or peritumoral and was considered positive for lymphocytes within the tumor whenever nests of lymphocytes were seen within the tumor. Desmoplasia, as defined previously,[17,18] was assessed semiquantitatively as follows: negative (−), little (+), moderate (++), strong (+++).

Preexisting thyroid gland was evaluated for nodular goiter disease and lymphocytic thyroiditis. In addition, concomitant neoplastic diseases were recorded.

Immunohistochemistry

For immunohistochemical analysis we used the BRAF V600E mutation-specific antibody VE1. The generation and validation of this antibody have been reported previously.[3,4] In brief, an 11-amino-acid synthetic peptide representing the BRAF V600E-mutated amino-acid sequence from amino acid 596 to 606 (GLATEKSRWSG) was coupled to keyhole limpet hemocyanin (Peptide Specialty Laboratories, Heidelberg, Germany), and 6-week-old female C57BL/6 mice (Charles River, Sulzfeld, Germany) were repeatedly immunized with 20 μg coupled peptide. Lymph node cells of anti-BRAF V600E-seropositive mice were fused with mouse myeloma SP2/O cells by polyethylene glycol fusion. Clones were screened for specificity to BRAF V600E by western blot and immunohistochemistry, resulting in the identification of clone VE1. Clone VE1 has been shown to be highly specific for BRAF V600E in western blot analysis (not detecting wild-type BRAF or other types of BRAF mutations, except a weak binding to BRAF V600D: unpublished observation) and immunohistochemistry.[4] The isotype of VE1 was classified as IgG2a.

One representative block of each tumor was selected, 3-μm sections were cut, and immunohistochemistry was performed using the anti BRAF V600E antibody VE1 as described previously.[4] As control, a tissue microarray containing 3 samples of melanoma was constructed and stained with every batch. Two cases had a V600E mutation detected by capillary sequencing and showed strong and moderate V600E mutant protein expression. The third case was a melanoma without V600 gene mutation and lack of V600E mutant protein expression. As additional negative control, the primary antibody was omitted in case of equivocal staining. A tumor was considered as unequivocally positive for VE1 immunostaining when a distinct, homogenous immunoreaction was seen in the cytoplasm of all cancer cells (Fig. 1). Cases with equivocal or focal staining were subjected to genetic analysis (see below). The immunohistochemical analyses were performed by 2 pathologists (O.K. and P.B.).

BRAF Direct DNA Sequencing

All tumors larger than 2 cm were evaluated by capillary sequencing for the T1799A point mutation of the BRAF gene; in addition, smaller tumors with questionable or focal tumor cell staining were also evaluated. Genomic DNA used as template for sequencing exon 15 of the BRAF gene was extracted from representative formalin-fixed paraffin-embedded tissue blocks. All tumors were at least macrodissected. Tumors < 1 cm in diameter or tumors with focal immunoreactivity, and also tumors with equivocal results of genetic analysis after macrodissection, were microdissected. Tumor blocks were cut at 5 μm thickness onto Leica PEN membrane slides (Leica Microsystems, Wetzlar, Germany). They were deparaffinized, dehydrated, and stained with Haemalaun according to standard procedures. Slides were air-dried quickly and subjected to laser microdissection with a Leica LMD6000 microscope (Leica Microsystems). As positive control, a PTC with known T1799A point mutation was used.

DNA was purified using an EZ1 DNA Tissue kit (no. 953034, Qiagen, Hilden, Germany) according to the tissue protocol, followed by automated purification using an EZ1 instrument. Exon 15 of the BRAF gene was amplified using a primer set as described previously[9] for

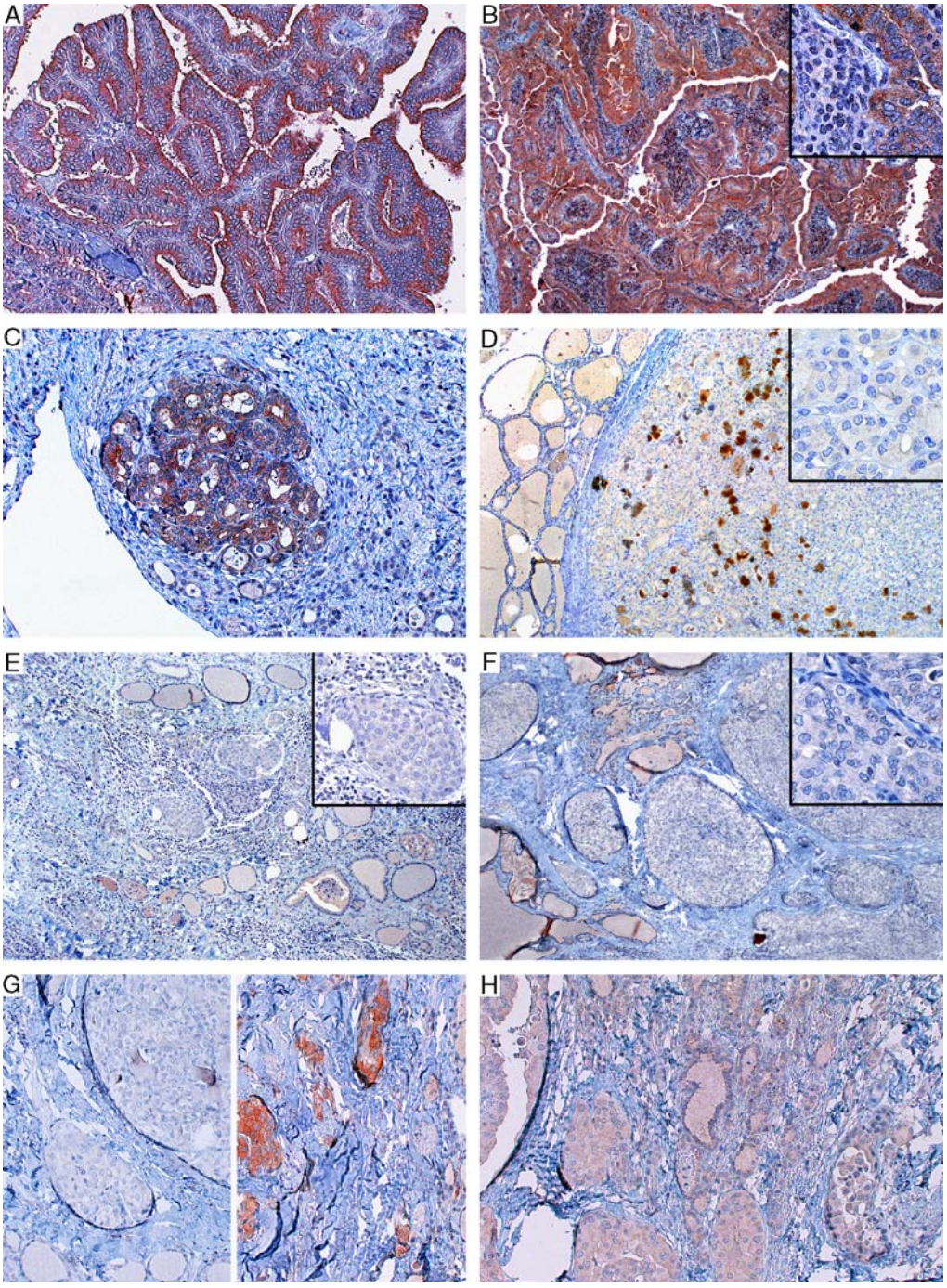

FIGURE 1. Expression of mutated V600E BRAF protein. A–F, Expression of mutated BRAF in different subtypes of PTC. A, Tall cell variant with diffuse expression of the mutated BRAF protein (magnification: ×100). B, Warthin tumor-like variant with diffuse expression of the mutated BRAF protein (magnification: ×40, inset: ×400). C, Papillary microcarcinomas with exclusively follicular growth pattern showing diffuse expression of the mutated BRAF protein (magnification: ×40, inset: ×400). D, Follicular variant, encapsulated type without expression of mutated BRAF protein; note the unspecific staining of the colloid (magnification: ×40, inset: ×400). E, Diffuse sclerosing variant without expression of the mutated BRAF protein. This tumor type often showed focal solid growth besides other growth patterns, for example, papillary and follicular growth (magnification: ×40, inset: ×200). F, Solid variant without expression of the mutated BRAF protein (magnification: ×40, inset: ×400). G and H, Focal staining and inconclusive staining in PTC. G, PTC without expression of mutated BRAF protein (left) except focal staining in a small tumor area (right); microdissection of the stained area did not reveal a T1799A point mutation of the BRAF gene. H, PTC with faint staining indifferent to background staining of preexisting follicles in these cases. These tumors did not reveal a T1799A point mutation of the BRAF gene (magnification: ×100).

40 cycles, gel-purified using ExoSAP-IT (no. 78201; USB, Cleveland, OH), and sequenced using fluorescent dye terminator chemistry (no. 4336774, Big Dye v1.1, Applied Biosystems, Foster City CA) on an ABI 3130 sequencer (Applied Biosystems). All sequencing reactions were carried out in forward and reverse directions. Mutations were identified by visual analysis of the sequence chromatograms using SeqScape 2.5 (Applied Biosystems). GenBank sequence NT_007914 (National Center of Biotechnology Information, NCBI, Bethesda, MD) was used as reference.

Statistical Evaluation

For statistical analysis, SPSS 17.0 (SPSS, Chicago, IL) was used. For statistical analysis the χ^2 and Mann-Whitney tests were applied as appropriate. *P*-values of ≤ 0.05 were considered significant.

RESULTS

Mutated BRAF Protein and Gene Sequencing Analysis

Of the 144 PTCs, 76 (52.8%) tumors showed a homogenous cytoplasmic expression of the mutated BRAF protein in all tumor cells and were scored as unequivocally VE1 positive (Fig. 1). In 3 cases, a weak staining of tumor cells not clearly different from a weak diffuse background staining in non-neoplastic thyreocytes was seen and thus were classified as questionable. In 2 cases, unequivocal but only focal staining of the tumor cells was seen (Fig. 1). No unequivocal staining of preexisting thyroid tissue (including normal tissue, inflammatory diseases, and nodular goiter) could be seen.

Gene analyses were performed on 39 PTCs (all tumors > 20 mm diameter, all tumors with questionable staining or focal staining). All but one tumor with unequivocal expression of the mutated BRAF protein showed the BRAF T1799A point mutation after macrodissection, whereas 1 case showed only a minor signal of the mutated base pair in the sequencing analysis that could not be interpreted. After microdissection of the genetically equivocal case, a clear signal of the T1799A BRAF point mutation was confirmed (data not shown).

None of the 3 cases with questionable staining, apart from the 2 cases with focal staining pattern, revealed the BRAF T1799A point mutation even after repeated microdissection and gene sequencing of the areas with the strongest staining signal.

Correlation of Mutated BRAF Expression With Clinicopathologic/Morphologic Parameters

Patient data, morphologic characteristics of the tumors, and correlations with expression of the mutated BRAF protein are listed in Table 1.

Mutated BRAF V600E protein expression correlated with patients' age and tumor size but not with infiltrative growth, extrathyroidal infiltration, or lymph node metastases. Moreover, none of the tumors with distant metastases at primary diagnosis (n = 4) and none of the pT4 tumors (according to the UICC2009, n = 2) showed mutated BRAF protein expression.

PTC Subtypes and Mutated BRAF Protein

Subtypes of the PTC and expression of the mutated BRAF protein are listed in Table 2.

There was no significant difference in the frequency of expression of mutated BRAF protein in microcarcinoma versus macrocarcinoma (42/72 vs. 34/72, respectively; $P = 0.243$, the Fisher exact test). With regard to histologic subtypes, none of the PTCs of the diffuse sclerosing variant (n = 6), the solid variant (n = 2), and the follicular variant (n = 10; including 1 macrofollicular variant and 2 diffuse types) scored positive for mutated BRAF protein. In contrast, 5/6 oncocytic variants (including all warthin tumor-like variants, n = 4) and 2/3 cases of the tall cell variant showed unequivocal expression of the mutated BRAF protein (Fig. 1).

DISCUSSION

The T1799A BRAF mutation is the most frequent gene alteration observed in PTC and associated with histologic subtypes and poor prognosis.[36,37] Its detection in fine needle aspirations of thyroid gland lesions has been proposed as an additional diagnostic tool, improving the specificity of the cytodiagnostic procedure.[7,38]

A high variation of the incidence of BRAF mutations in PTC (20% to 80%) has been observed in the literature.[10] This variation may reflect locoregional differences in the pathogenesis of PTC[23] but may also be due to different materials (eg, frozen tissue vs. paraffin-embedded tissue) and/or different analyzing methods (eg, macrodissection, microdissection, direct sequencing, pyrosequencing, and so on) used for the detection of the mutation.[13,14,22]

Our data show that the antibody VE1[4] directed against the V600E BRAF protein reliably identifies PTC harboring the T1799A BRAF mutation. This is in good concordance to our previous study investigating 1120 tumor specimens (metastases and primary tumors) from 874 patients with brain metastases of various tumor types comprising melanoma, lung cancer, and breast cancer among others.[3] Most of the PTCs (96.5%) could be clearly diagnosed as negative or positive for the BRAF mutation by means of immunohistochemistry, indicating that this method may substantially facilitate analysis of the BRAF status in PTC. VE1 immunohistochemistry appears particularly valuable for evaluation of microcarcinomas. In such cases, microdissection is usually required for reliable genetic analyses, thus making this method suboptimal for the clinical setting. In contrast, VE1 immunohistochemistry is able to highlight only minute mutation-bearing tumor cell aggregates embedded in non-neoplastic tissue. However, in few cases we observed ambiguous weak or focal immunostaining that may cause uncertainty in the diagnostic setting. In such cases, additional genetic analysis may be required to clarify the BRAF status.

TABLE 1. Clinicopathologic/Morphologic Parameters and V600E Mutated BRAF Protein Expression

	PTC Total n (%)	With Mutated BRAF n (%)	Without Mutated BRAF n (%)	Significant
	144 (100%)	76 (52.8%)	68 (47.2%)	*P*
Age (y)	49.9 ± 16.2 (9-85)	53.5 ± 14.6 (21-85)	45.9 ± 17 (9-80)	0.007
Sex				
Male	54 (37.5%)	33 (22.9%)	21 (14.6%)	NS
Female	90 (62.5%)	47 (23.6%)	43 (29.9%)	
Tumor diameter (mm)	16.3 ± 17 (0.5-75)	13 ± 13.7 (0.5-60)	20 ± 19.5 (1-75)	0.018
T-stage*				
pT1a	66 (45.8%)	37 (25.7%)	29 (20.1%)	NS
pT1b	18 (12.5%)	9 (6.3%)	9 (6.3%)	
pT2	16 (11.1%)	7 (4.9%)	9 (6.3%)	
pT3	42 (29.2%)	23 (16%)	19 (13.2%)	
pT4	2 (1.4%)	0 (0%)	2 (1.4%)	
Lymph node metastasis	70 (48.6%)	34 (23.6%)	36 (25%)	NS
Distant metastasis	4 (27.8%)	0 (0%)	4 (27.8%)	0.041
Multifocality	62 (43.1%)	36 (25%)	26 (18.1%)	NS
Desmoplasia				
No	13 (9%)	6 (4.2%)	7 (4.9%)	NS
Little	20 (13.9%)	10 (6.9%)	10 (6.9%)	
Moderate	69 (47.9%)	34 (23.6%)	35 (24.3%)	
Prominent	42 (29.2%)	26 (18.1%)	16 (11.1%)	
Exclusively follicular growth pattern	37 (25.7%)	12 (8.3%)	25 (17.4%)	0.004
Solid growth (at least focally)	22 (15.3%)	2 (1.4%)	20 (13.9%)	< 0.001
Oncocytic cell type and tall cell features (at least focally)	63 (43.8%)	51 (35.4%)	12 (8.3%)	< 0.001
Extrathyroidal invasion	40 (27.8%)	22 (15.3%)	18 (12.5%)	NS
Peritumoral invasion	116 (80.6%)	66 (45.8%)	50 (34.7%)	NS
Vascular invasion	39 (27.1%)	14 (9.7%)	25 (17.4%)	0.015
Lymphocytes in the tumor	52 (36.1%)	30 (20.8%)	22 (15.3%)	NS
Calcification	64 (44.4%)	29 (20.1%)	35 (24.3%)	NS
Inflammation in the preexisting thyroid tissue				
No	89 (61.8%)	47 (32.6%)	42 (29.2%)	NS
Little	21 (14.6%)	9 (6.3%)	12 (8.3%)	
Moderate/prominent	34 (23.6%)	20 (13.9%)	14 (9.7%)	
Nodular goiter	106 (73.6%)	55 (38.2%)	51 (35.4%)	NS

The Fisher exact test and the Mann-Whitney test; T-stage:* according to UICC 2009.
± indicates SD; NS, not significant.

TABLE 2. Subtypes of PTC and V600E-Mutated BRAF Protein Expression

	PTC Total	With Mutated BRAF	Without Mutated BRAF
Papillary microcarcinoma (< 1 cm)	72/144	42	30
Classical/mixed	40/72	26	14
Exclusively follicular	27/72	12	15
Exclusively tall cell features	3/72	2	1
Exclusively oncocytic cell feature	2/72	2	0
Papillary macrocarcinoma (> 1 cm)	72/144	34	38
Classical/mixed	45/144	27	18
Follicular variant	10/72	0	10
Encapsulated	7/10	0	7
Diffuse type	2/10	0	2
Macrofollicular	1/10	0	1
Diffuse sclerosing variant	6/72	0	6
Oncocytic variant	6/72	5	1
Warthin tumor like	4/6	4	0
Tall cell variant	3/72	2	1
Solid variant	2/72	0	2

Our protein expression analyses showed that mutated BRAF protein occurs early in carcinogenesis of PTC with no significant difference between microcarcinomas and macrocarcinomas. Similar results were previously shown by sequencing analyses showing BRAF mutation with a range of 20% to 52% in papillary microcarcinoma,[15,35] which generally has an indolent clinical course.[25] This is in concordance to the finding that BRAF mutations, which are common in malignant melanoma, are already evident in noninvasive melanocytic nevi.[28,39]

The expression of mutated BRAF was significantly associated with diverse subtypes of PTC. As mutational analyses reported previously, mutated BRAF expression was more often seen in tall cell variants and oncocytic variants (including all "warthin tumor-like" variants).[33,36] Moreover, in our study, the expression of mutated BRAF protein was more common in tumors showing even focal tall cell and/or oncocytic cell features. In contrast, an exclusively follicular growth pattern was predictive for the absence of mutated BRAF protein, confirming results from other studies using a DNA-based approach.[36] Only some of the papillary microcarcinomas with exclusively follicular

growth pattern showed expression of mutated BRAF, but none of the follicular variants (macrocarcinoma with exclusively follicular growth) was scored as positive for BRAF V600E expression. As exclusively follicular growth pattern was more often seen in microcarcinoma as against macrocarcinomas, we speculate that PTCs with BRAF mutation may begin with an exclusively follicular growth pattern; however, during their enlargement of the tumor, they develop at least focal papillary enfoldings. In a previous study, none of the diffuse sclerosing variants of PTC showed BRAF mutations but more likely harbored different RET/PTC rearrangements.[29] We investigated 6 diffuse sclerosing variants, and none of these tumors expressed the mutated BRAF protein. In addition, none of the solid variants expressed mutated BRAF protein, and solid growth pattern, even if only focally seen, was inversely associated with expression of the BRAF protein. Case reports of solid variants with BRAF analysis showed mutations in exon 15 of the BRAF gene, which were different from the typical T1799A BRAF mutation and thus do not lead to expression of the V600E BRAF protein.[31,34,6]

Many studies have shown that BRAF mutation is associated with the age of patients.[21,36] In concordance with these findings, in our study as well the expression of the mutated V600E BRAF protein was strongly associated with the age of patients, with higher prevalence among the elderly.

Many authors have found an association between tumor aggressiveness and T1799A BRAF mutation, but some studies could not demonstrate this association.[2,11,24,37] In our cohort, no correlation of the expression of mutated BRAF protein with clinicopathologic signs of more aggressive tumor behavior such as peritumoral invasion, extrathyroidal extension, lymph node metastases, and tumor stage was seen. In addition, stromal parameters such as desmoplastic stromal reaction or calcification, both of which are known to be associated with lymph node metastases, were not associated with the expression of the mutated BRAF protein.[1,17–20] Moreover, vascular invasions were seen significantly more often in tumors that were scored negative for mutated BRAF protein. Neither T4 tumors according to UICC2009 (n = 2) nor tumors with distant metastases (n = 4) showed expression of the mutated BRAF protein. It has been suggested that the most likely reason for association of BRAF mutation and tumor aggressiveness is the fact that conventional PTCs (and especially the tall cell variant) have a more aggressive behavior compared with the follicular variant of PTC.[32,33,36,37] In our collective we investigated a relatively large number of variants of PTC (eg, diffuse sclerosing variant, solid variant, and follicular variant of diffuse type) that are known to have more aggressive behavior, but they were not associated with the T1799A BRAF mutation. In addition, in our study most tumors in young patients under the age of 20 years (n = 5) showed advanced tumor stage with lymph node metastasis in all tumors but without expression of mutated BRAF protein. The high prevalence of mutated BRAF protein in microcarcinoma argues against a direct association with aggressiveness, as these tumors are known for an excellent clinical prognosis.[25] Perhaps locoregional differences in the pathogenesis of PTC may lead to different incidence rates of the BRAF mutation and thus to different results regarding its association with tumor aggressiveness.[23,27,36] In a recent study investigating a large series of PTCs, Cheng and colleagues found that V600E BRAF mutation was associated with lymph node metastasis, extrathyroidal extension, and vascular invasion. Nevertheless, this finding might be attributed to the strong association of BRAF mutations with classical PTC, as, within morphologic subtypes, BRAF mutation status did not deliver additional prognostic information. Moreover, BRAF wild-type classic PTCs showed the most aggressive behavior of all tumors.[5]

In summary, our study showed that the immunohistochemical detection of the mutated BRAF V600E protein is a reliable, highly specific method for detection of the BRAF V600 mutation in PTC.

In addition, we could confirm the high prevalence of mutated BRAF in papillary microcarcinomas. Expression of mutated BRAF showed strong correlation with different growth patterns and cell types but not with parameters of clinicopathologic aggressiveness (eg, metastasis, tumor invasiveness, etc.). Nevertheless, the expression of BRAF protein might be of clinical interest, especially in therapy-resistant disease, as new therapeutics inhibiting the mutated V600E BRAF protein are clinically available.[12] It is noteworthy that a multicentric clinical trial investigating the FDA-approved BRAF inhibitor vemurafenib in BRAF V600E-mutated PTC is ongoing (NCT01286753, www.clinicaltrials.gov). In addition, immunostaining for BRAF V600 might be an interesting diagnostic tool in fine needle aspirations of suspect nodules of the thyroid, helping to identify PTC.

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