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CLINICAL STUDY

BRAF V600E mutation analysis increases diagnostic accuracy for papillary thyroid carcinoma in fine-needle aspiration biopsies

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Abstract

Objective: Papillary thyroid carcinoma (PTC) represents the majority of differentiated thyroid cancers, presenting the V600E activating *BRAF* mutation in 29–83% of cases. The aim of our study is to analyze the influence of *BRAF* mutation analysis on the diagnostic accuracy of fine-needle aspiration biopsy (FNAB) in patients with suspected PTC.

Design and methods: Thyroid cytoaspirates from 469 nodules (size: 1.1 ± 0.8 cm) with ultrasonographic features suspicious of malignant lesion, performed in 374 patients, were submitted to cytological evaluation and to biomolecular analysis, carried out after somatic DNA isolation, specific PCR amplification, and subsequent automated direct sequencing. All PCR fragments were also processed by specific enzyme restriction analysis.

Results: *BRAF* V600E mutation was found in 48 samples, 41 of which were also cytologically diagnosed as PTC, with histologic confirmation after thyroidectomy. Total thyroidectomy was perfomed also in seven patients with negative cytology but positive *BRAF* mutation, with histological confirmation of PTC in all. Among the 429 *BRAF*-negative samples, 407 had negative cytology for PTC, while 22 were diagnosed as suspected PTC and underwent total thyroidectomy with histological diagnosis of PTC in 17 and benign lesion in five. The prevalence of *BRAF* V600E mutation among histologically diagnosed PTC patients was 64%. Biomolecular analysis significantly increased cytology sensitivity for PTC from 77.3 to 86.7% ($P < 0.01$).

Conclusions: These data indicate that *BRAF* V600E mutation analysis can significantly improve FNAB diagnostic accuracy. However, biomolecular analysis is complementary to cytology, which should always be performed.

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Introduction

Fine-needle aspiration biopsy (FNAB) represents the main pre-surgical tool in the diagnosis of thyroid nodules, with sensitivity and specificity reported to be 70–98% and 55–100% respectively (1). However, 15–20% of FNAB provides inconclusive results, being unable to discriminate between benign and malignant lesions because of inadequate sampling or because cytology findings cannot be diagnostic in some settings. In these cases, partial or total thyroidectomy is necessary for diagnostic purposes (2). More recently, FNAB has been combined to biomolecular analysis, since FNAB specimen is sufficient to isolate DNA in the search for somatic mutations, and/or RNA to perform rearrangement studies. Indeed, it has been recently demonstrated that molecular testing of thyroid nodules for a panel of mutations can be effectively performed in clinical settings, and that this approach can enhance the accuracy of FNAB cytology also in the case of indeterminate cytology (3). Point mutations in the *BRAF* gene, implicated in the neoplastic transformation of follicular cells, represent a specific molecular marker for papillary thyroid carcinoma (PTC) and PTC-derived cancers, characterizing up to 45% of PTCs. Previous studies have evaluated the presence of *BRAF* V600E mutation in FNAB from suspected thyroid nodules, with a prevalence of 62% mutated *BRAF* PTCs and variable sensitivity and specificity due to differences in the population studied and to the experimental methods employed (3–12). The aim of the present study is to evaluate whether *BRAF* V600E mutation analysis could increase the diagnostic accuracy of FNAB for PTC in suspicious thyroid nodules of patients referred to a single institution.

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Materials and methods

Subjects and FNAB procedure

Among the patients undergoing FNAB for diagnostic purposes at the Section of Endocrinology of the University of Ferrara from October 2007 to December 2008, 374 patients (262 women and 112 men, mean age 50.7±0.7, ranging from 14 to 88 years) showed one or multiple thyroid nodules with ultrasonographic characteristics that, according to the AACE/AME guidelines (13), were suspicious for malignancy (iso/hypoechoic nodules with shaded margins with or without microcalcifications). *BRAF* V600E mutation analysis was performed, in parallel to classic cytology, in the 469 ultrasound (US)-guided FNAB thyroid nodule samples detected in these 374 patients. According to FNAB cytological analysis, the nodules were classified as benign, follicular lesions (including follicular lesions of undetermined significance and follicular neoplasm/suspicious for follicular neoplasm), suspicious for malignancy, malignant, and non-diagnostic, following the guidelines of National Cancer Institute thyroid fine needle aspiration (FNA) state of the science conference (14). Patients gave written informed consent and the study was approved by the local ethical committee.

PTC histological classification

According to previous reports (15), PTCs were defined as belonging to the conventional type when presenting with papillary architecture, stromal reaction, psammoma bodies, and, mainly, with typical PTC nuclei (enlarged, elongated, or irregular nuclei, appearing as clear, ground glass, empty nuclei with nuclear grooves, intranuclear pseudoinclusions, and multiple nucleoli). PTCs were defined as belonging to the follicular variant when composed of follicles with or without solid areas, where nuclear grooves and intranuclear pseudoinclusions were detected in >30% and in >20% of the observed nuclei in serial sections, respectively. Follicular variant of PTC also displayed enlarged, overlapping, ground glass, and irregularly shaped nuclei, dark colloid, irregular, and elongated follicles, intrafollicular multinucleated giant cells, and rare psammoma bodies, in keeping with previously published work (16, 17).

DNA isolation and BRAF mutation analysis

All US-guided FNAB procedures were performed by an experienced endocrinologist (G T) using a standardized protocol. Material from the needle pass through the nodule was used to prepare a smear for cytology and the needle was washed out with 5 ml of normal saline into a collection tube, obtaining enough material for both cytology and molecular analysis. Only one case was judged as inadequate to perform cytology. Samples were centrifuged for 5 min at 671 ***g***; the cell pellet was resuspended in 500 μl of normal saline and kept frozen. Samples were then submitted to overnight digestion at 56 °C with proteinase K (Qiagen) prior to DNA isolation, performed by using the QIAamp DNA Micro kit (Qiagen). PCR for *BRAF* exon 15 was then performed in the conditions described in Table 1 by using the Invitrogen Taq DNA polymerase following the manufacturer's instructions, with the Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). Each sample was processed in triplicate. The amplified products were purified with the QiaQuick PCR purification kit (Qiagen) and then submitted to sequencing reaction using the BigDye Terminator Cycle Sequencing Ready Reaction Kit 3.1 (Applied Biosystems), applying the following cycle profile: 96 °C for 10 s and 60 °C for 4 min (45 cycles). The samples were then purified using CentriSep Spin Columns and sequenced on an ABI PRISM Genetic Analyzer 3130 (Applied Biosystems).

To further confirm the presence or absence of *BRAF* V600E mutation, restriction length polymorphism analysis (RFLP) was performed using the restriction enzyme TspRI (New England Biolabs, Ipswich, MA, USA), which cuts the wild-type *BRAF* amplification product (215 bp) into two fragments of 120 and 95 bp.

Statistical analysis

Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy were calculated for each detection method and for combined methods. Kappa (k) analysis was performed to evaluate the agreement between the diagnostic methods and the final pathology diagnosis ($k=0$, poor; $k=0.01$–0.20, slight; $k=0.21$–0.40, fair; $k=0.41$–0.60, moderate; $k=0.61$–0.8, substantial; $k=0.81$–1.00, almost perfect). The categorical data were summarized using frequencies and percentages. The results are expressed as mean±S.E.M., as percentage, or as absolute values. The unpaired Student's t-test was used to evaluate individual differences between means. The diagnostic sensitivity of cytology was compared with that observed performing both cytology and *BRAF* V600E mutation analysis by McNemar test (with Yates correction). To measure the strength of association between pairs

Table 1 PCR conditions for *BRAF* exon 15 amplification.

Primers	Amplicon	Cycles	Denaturation	Annealing	Extension
Forward: 5′-TCATAATGCTTGCTCTGATAGGA-3′ Reverse: 5′-GGCCAAAAATTTAATCAGTGGA-3′	215 bp	35	94 °C 30 s	51 °C 1 min	72 °C 30 s

of variables without specifying dependencies, Spearman order correlations were run. $P<0.05$ was considered significant in all tests.

Results

Cytological examination

Cytological examination of the 469 nodules showed that in total 308 (65.7%) nodules were cytologically benign. Moreover, 89 samples (19%) belonged to the group of follicular lesions (29 follicular lesions of undetermined significance and 60 follicular neoplasm/suspicious for follicular neoplasm), 22 (4.7%) were suspicious for malignancy (18 suspicious for PTC, two suspicious for medullary thyroid cancer (MTC), and two for metastases of breast cancer), and 49 (10.4%) were consistent with malignant lesions (45 with PTC and four with MTC). Only one sample (0.2%) was inadequate. Among the 166 patients submitted to thyroidectomy, histological evaluation detected 74 cases of PTC and one anaplastic carcinoma (AC), including 42 microcarcinomas, corresponding to 15.9% of all the nodules. In the other 91 cases, histology showed six MTC, seven follicular thyroid carcinomas (FTC), 74 follicular adenomas (FA), one follicular hyperplasia, one lymphocytic thyroiditis, and two were consistent with breast cancer metastases.

Detection of BRAF *V600E mutation in FNAB samples*

Direct DNA sequencing showed the presence of *BRAF* V600E mutation in 48 samples (10.2% of all nodules) also confirmed by RFLP, which displayed three bands (215, 120, and 95 bp) when the *BRAF* V600E mutation was present (heterozygote), since the TspRI restriction enzyme digested the PCR products from wild-type alleles. Two bands were evident on gel analysis when the *BRAF* V600E mutation was absent (120 and 95 bp). Representative cases are shown in Fig. 1.

Among the 48 samples displaying the *BRAF* V600E mutation, 41 had a cytological diagnosis consistent with PTC (10 suspicious and 31 diagnostic for PTC) and therefore the patients were submitted to thyroidectomy. Histological examination diagnosed the presence of PTC in 40 cases (34 PTC of conventional type and six follicular variant PTC) and of AC in one. The other seven nodules with positive *BRAF* V600E mutation had a negative cytology (six benign lesions and one follicular lesion). On the basis of the results of the biomolecular analysis, the patients were submitted to thyroidectomy and final histological examination was consistent with PTC of conventional type in all seven cases. Revision of cytological samples, performed after *BRAF* V600E mutation detection and final histology consistent with

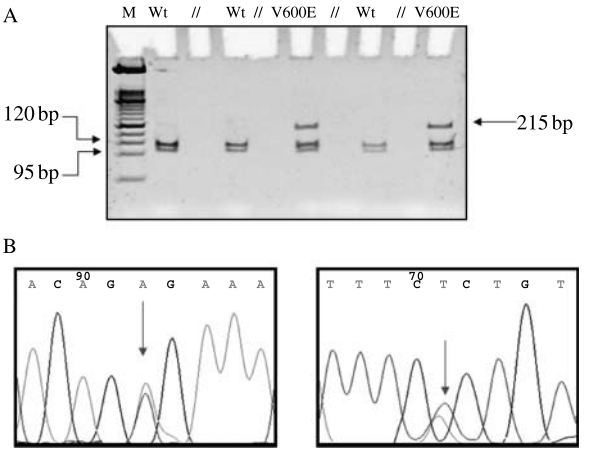

Figure 1 *BRAF* V600E evaluation. Representative analysis of thyroid aspirates. (A) Five samples were analyzed by PCR for *BRAF* and then submitted to RFLP by incubation with the restriction enzyme TspRI. Three samples displayed a wild-type (Wt) digestion pattern showing two fragments of 120 and 95 bp respectively, while two samples displayed a mutated (V600E) digestion pattern showing three fragments of 215 bp (undigested mutated allele), 120 and 95 bp respectively. M, molecular marker; //, empty lane. (B) Forward (left) and reverse (right) DNA-sequencing electropherograms of a V600E heterozygote mutation from a FNAB specimen.

PTC, highlighted the presence of very few cells displaying intranuclear pseudoinclusions. Consequently, cytological re-examination was consistent with samples suspicious for PTC.

Among the 421 samples that did not display the *BRAF* V600E mutation, 302 had a cytological diagnosis consistent with benign lesion and therefore the patients were not operated on. These patients underwent follow-up US examinations (up to 6 months) that did not show any change over time, and further FNAB reported negative cytology. Therefore, these nodules were considered as truly negative for malignancy, but the possible presence of malignancy could not be completely ruled out. In 88 nodules, cytology was consistent with follicular lesions (29 follicular lesions of undetermined significance and 59 follicular neoplasm/suspicious for follicular neoplasm). The patients underwent surgery and these nodules were histologically diagnosed as 10 follicular variant PTC, 7 FTC, and 71 FA. Among the 12 nodules cytologically diagnosed as suspicious for malignancy, eight nodules suspicious for PTC were shown to be three PTC of conventional type and five benign lesions by histology, while two nodules suspicious for MTC and two for breast cancer metastases were confirmed at final histology. Cytology was consistent with malignant lesions in 18 samples, and final histological examination showed the presence of PTC of conventional type in 14 and of MTC in four samples (Fig. 2). Only one case was not cytologically diagnosed due to sample inadequacy.

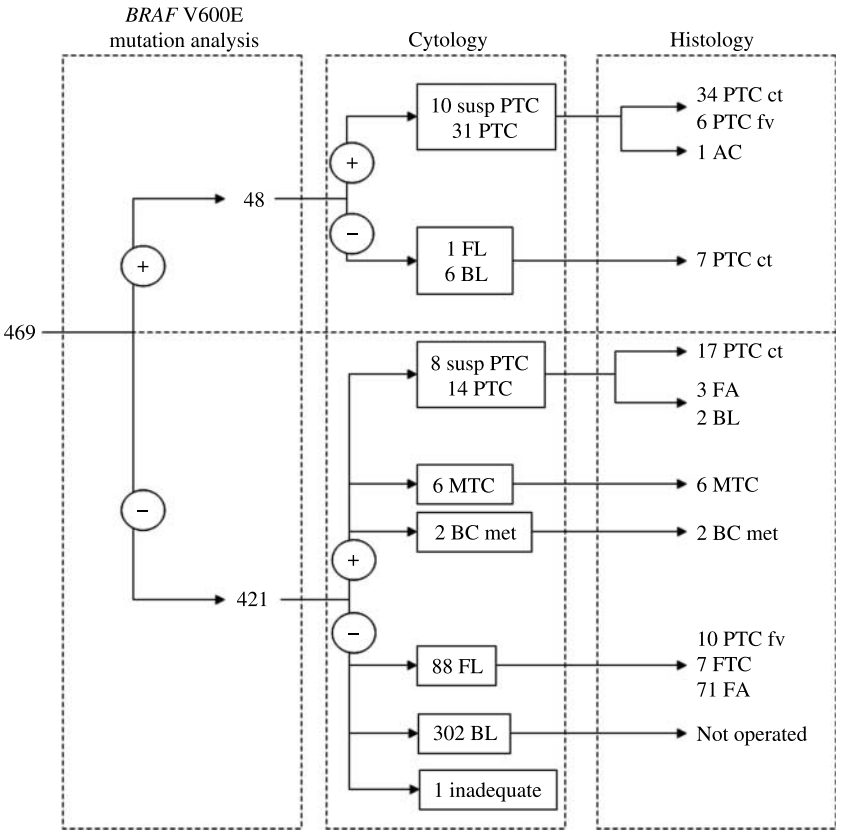

Figure 2 Detection of the *BRAF* V600E mutation in 469 FNAB specimens, also analyzed by cytology and compared with definitive histological evaluation. PTC, papillary thyroid carcinoma; PTC ct, papillary thyroid carcinoma conventional type; PTC fv, papillary thyroid carcinoma follicular variant; susp PTC, suspicious of PTC; AC, anaplastic carcinoma; FTC, follicular thyroid cancer; FA, follicular adenomas; MTC, medullary thyroid cancer; FL, follicular lesion; BL, benign lesion; BC met, metastases of breast cancer.

BRAF V600E mutation analysis was performed on the surgical specimen from the 27 histologically diagnosed PTC that were negative at FNAB, sampling at least four different sites of the thyroid gland, but all cases were negative.

The presence of *BRAF* mutation in each cytological category is displayed in Table 2. Overall, *BRAF* V600E mutation was detected in 48 out of 74 PTC (58 PTC of conventional type and 16 follicular variant PTC) and one AC at final histological examination, corresponding to 64% of this group of cancers.

Diagnostic value of BRAF *V600E mutation in FNAB samples*

To assess the diagnostic value for PTC of *BRAF* V600E mutation detection method, we compared the results of the biomolecular analysis to the 'gold standard', i.e. the post-operative definitive histological diagnosis. The diagnostic accuracy for PTC of direct DNA sequencing analysis of the *BRAF* V600E mutation in FNAB was 94.2%. By classifying as true positive all the cases showing histology consistent with PTC and with AC after thyroidectomy, sensitivity was 64%, while specificity was 100%. Positive (PPV) and negative (NPV) predictive values were 100 and 93.6% respectively. Concerning cytology, diagnostic accuracy in detecting PTC was 95.3%, with a sensitivity of 77.3% and a specificity of 98.7%. PPV and NPV were 92.1 and 95.8% respectively. When examining the agreement between *BRAF* mutation analysis and histology, the Kappa value (k) of the direct DNA-sequencing analysis was 0.749 ± 0.045, indicating a substantial agreement between the two diagnostic procedures. On the other hand, when comparing cytology and histology results, k value was 0.813 ± 0.02, indicating an almost perfect agreement. Combining the results of both evaluations

Table 2 *BRAF* V600E mutation analysis in nodule samples according to cytological category.

Cytological category	Samples	BRAF+	BRAF−
Benign lesions	308	6	302
Follicular lesions (89)			
Follicular lesions of undetermined significance	29	0	29
Follicular neoplasm/ suspicious	60	1	59
Suspected malignant (18 PTC, 2 BC met, and 2 MTC)	22	10	12
Malignant (45 PTC and 4 MTC)	49	31	18
Inadequate	1	0	1
Total	469	48	421

PTC, papillary thyroid cancer; BC met, breast cancer metastases; MTC, medullary thyroid carcinoma.

Table 3 Statistical evaluation.

	PPV (%)	**NPV** (%)	**Sensitivity** (%)	**Specificity** (%)	**Accuracy** (%)	***k***	**Error**
Cytology	92.1	95.9	77.3	98.8	95.4	0.81	0.02
V600E *BRAF* mutation analysis	100.0	93.7	64.0	100.0	94.4	0.76	0.05
Both	92.9	97.5	86.7	98.8	96.9	0.88	0.01

performed on the FNAB (*BRAF* mutation analysis and cytology), diagnostic accuracy for PTC increased up to 96.8%, with a sensitivity of 86.7% and a specificity of 98.7%. PPV and NPV were 92.9 and 97.5% respectively, and k value was 0.817 ± 0.03 indicating an almost perfect agreement when comparing the results of both analyses and histology results (Table 3).

Statistical analysis with McNemar test (with Yates correction) showed that the sensitivity of combined methods was significantly higher ($P < 0.01$) as compared with the sensitivity of each method alone (+9.4% versus cytology and +22.7% versus *BRAF* mutation analysis).

BRAF *V600E mutation and clinocopathological characteristics*

To assess the pre-operative prognostic value of *BRAF* V600E mutation in PTCs, we compared the clinicopathological characteristics of PTC confirmed at post-operative definitive histology and the results of the biomolecular analysis.

The mean age and the male to female ratio (M/F) of PTC patients with the *BRAF* V600E mutation (50.9 ± 2.3 years; M/F = 0.45) were not significantly different from those of PTC patients without the *BRAF* V600E mutation (49.8 ± 2.6 years; M/F = 0.44). Extracapsular extension (43.8 vs 31%) and nodal metastases (35.4 vs 36.6%) did not significantly differ among the two groups. On the other hand, microcalcifications and microcarcinomas were significantly more represented in PTC patients with *BRAF* V600E mutation compared with those without (54.2 and 64.6 vs 31 and 38% respectively; $P < 0.05$). In particular, all seven *BRAF* V600E-mutated samples diagnosed as benign lesions cytology-wise were microPTC of conventional type.

From the histological point of view, PTC of conventional type was significantly more represented in PTC patients with *BRAF* V600E mutation as compared with those without (85.4 vs 58%; $P < 0.01$), while we observed the opposite for the follicular variant of PTC (12.5 vs 46%; $P < 0.01$).

Discussion

US-guided FNAB is the most reliable diagnostic tool in the pre-surgical diagnosis of clinically suspicious nodules. Recently, a new molecular marker has been found to increase the accuracy of FNAB cytological examination. Indeed, *BRAF* mutation analysis of FNAB has been reported to have high diagnostic specificity to detect PTC in previous studies examining 1153 FNAB samples with different techniques, such as RFLP, direct sequencing, pyrosequencing, real-time allele-specific amplification, and mutant allele-specific PCR amplification (3–11). In the present study, 469 FNAB samples, obtained from suspicious thyroid nodules, were analyzed for *BRAF* V600E mutation by direct sequencing and RFLP. The results confirm that this approach significantly improves diagnostic FNAB sensitivity in detecting PTC. In our series, the combination of molecular analysis and cytological examination correctly diagnosed 65 out of 74 PTC and identified one AC at final histology, including seven samples that were diagnosed as benign cytology-wise, but displayed *BRAF* V600E mutation. Therefore, our results confirm the previously reported 100% PPV of *BRAF* V600E mutation in predicting the presence of PTC (3), and suggest that patients with nodules positive for *BRAF* V600E mutation are candidates for total thyroidectomy independently of cytological results. On the other hand, *BRAF* mutation analysis failed to identify 27 (36%) out of 74 PTCs and one AC. As expected, *BRAF* mutation was not always present in PTCs, being reported in 29–83% of the cases (18). As compared with the reported ~45% overall mean prevalence of *BRAF* mutation among PTCs (19–21), we observed a higher prevalence (64%), likely due to the selection criteria of nodules to be evaluated. In fact, we choose to study *BRAF* V600E mutation only in FNAB samples from nodules with US features suspicious for malignancy, according to the AACE/AME guidelines (13). Selecting nodules with solid hypoechoic US features has been reported to result in high sensitivity (87%) for the detection of thyroid malignancy (22), despite low specificity (15.6–27%) and low PPV (23). In our series, these US characteristics were present in 308 cytologically benign lesions (65.7% of the samples), similarly to a previous study (22). The application of the described selection criteria resulted in a lower sensitivity (77.3%) of cytology in detecting PTC in our series. However, FNAB diagnostic sensitivity for PTC was significantly increased to 86.7% when examining the samples both by cytology and by *BRAF* V600E mutation analysis. On the other hand, specificity and PPV of cytology were elevated (98.7 and 92.1% respectively) and were not significantly increased by adding *BRAF* V600E mutation analysis. We also found a lower rate of false-positive results for PTC of cytology (1.05%), as

compared with the reported 2% rate (24). However, cytology failed to correctly identify 17 PTCs (false negatives, 3.6%), seven of which were rescued by *BRAF* V600E mutation analysis, which was 100% specific, in keeping with literature data (3, 25). Taken together, in our series, an overall cytology–histology discrepancy rate of 4.7% was recorded, a much lower percentage as compared with the reported 15.3% (26), demonstrating a high accuracy (95.3%) of this approach. In addition, the discrepancy rate between pre-surgical (cytology and V600E mutation analysis) and post-surgical (histology) examination was reduced to 3.2% by adding the molecular analysis. Despite the great improvement in FNAB diagnostic sensitivity obtained by performing the molecular analysis, the latter was unable to solve one of the main diagnostic problems when evaluating thyroid nodules, i.e. follicular lesions. Indeed, only 1 out of 89 follicular lesions and follicular neoplasias displayed the *BRAF* V600E mutation, in agreement with previous reports (6). These results may be due to the employed selection criteria that restricted the analysis to samples with clinical and US suspicious features, in line with previous reports showing the lack of correlations between US/clinical features and malignancy in patients with indeterminate cytology (27). However, our results are not in line with the recently published report by Nikiforov *et al*. (3), reporting a high sensitivity for FNAB molecular testing in detecting malignancy in the category of indeterminate cytology. This discrepancy is mainly due to the different criteria applied in selecting the samples to be analyzed with molecular tools, which in the latter study included the search for several genetic alterations besides *BRAF* V600E mutation. Moreover, Nikiforov *et al*. include in the indeterminate group also FNAB-displaying cytology suspected for malignancy, while in our study this group includes only samples cytologically consistent with follicular lesions (follicular lesions of undetermined significance and follicular neoplasm/suspicious for follicular neoplasm), according to the guidelines of the National Cancer Institute Thyroid Fine-Needle Aspiration State of the Science Conference (14). On the other hand, our data confirm previous evidence reporting that *BRAF* V600E mutation can be detected also in anaplastic thyroid cancers (28).

Our series confirms that PTC with the *BRAF* V600E mutation is mainly of the conventional type, but, at variance with previous reports (29), they also included six follicular variant PTC (12.5%). Moreover, we failed to find the reported difference in age (29) or any difference in gender between PTC patients with *BRAF* V600E mutations and those without. On the other hand, our data confirm that *BRAF* V600E mutation does not predict the presence of extra-capsular extension or nodal metastases, as previously reported (10, 29, 30). Therefore, in our hands, pre-operative *BRAF* V600E mutation does not associate with clinicopathological characteristics that predict a poorer outcome. Interestingly, in our series, *BRAF* V600E mutation correlates with microcalcifications at US examination, increasing the value of this characteristic for suspected malignant lesions. Last but not least, *BRAF* V600E mutation is significantly more represented among microPTC, further underlining the importance of FNAB evaluation in nodules <1 cm. In these settings, biomolecular analysis gains more importance, since it is capable of rescuing possible cytological false-negative samples. Cytological re-examination of the seven PTCs with BRAF mutation, but initial negative cytology, was consistent with samples suspicious for PTC, since very few cells displaying intranuclear pseudoinclusions were identified. This evidence might indicate that these lesions were indeed PTC at a very initial stage, before clonal expansion of a few mutated cells.

Taken together, our results show that cytology can be greatly improved by implementing *BRAF* V600E genetic analysis, but 13% of PTC still escape both detection methods. Better results could be achieved by extending molecular analysis in the search for other cancer-related mutations, such as *RAS*, *RET/PTC*, and *PAX8/PPARγ* mutations (3).

Preclinical *in vitro* studies demonstrate that selective MAP kinase kinase inhibitors preferentially inhibit growth of thyroid cancer cell lines with *BRAF* mutations (31), suggesting that in the future *BRAF* patients with recurrent disease may take advantage of such drugs. This evidence indicates that *BRAF* mutation analysis may improve diagnostic capacity for PTC, consequently addressing therapeutic decision in PTC patients, as already suggested (3, 30).

In conclusion, our results demonstrate that *BRAF* V600E mutation analysis significantly increases PTC diagnostic accuracy of cytology in thyroid FNAB of clinically and US-suspicious nodules and may be added to pre-surgical risk evaluation of PTCs.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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