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BRAF analysis by fine needle aspiration biopsy of thyroid nodules improves preoperative identification of papillary thyroid carcinoma and represents a prognostic factor. A mono-institutional experience

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Abstract

Background: The current preoperative diagnosis of a thyroid mass relies on microscopic evaluation of thyroid cells obtained by fine needle aspiration biopsy (FNAB). More recently, FNAB has been combined with molecular analysis to increase the accuracy of the cytological evaluation. In this mono-institutional prospective study, we evaluated whether the routine introduction of *BRAF* testing in thyroid FNAB could help ameliorate the preoperative recognition of papillary thyroid carcinoma (PTC) in ''suspended'' or malignant cytological categories. Moreover, we investigated the prognostic role of the BRAFV600E mutation in PTC.
Methods: BRAFV600E analysis was performed in thyroid FNAB from 270 patients classified into one of five cytological categories THY1, THY2, THY3, THY4, THY5. All subsequently underwent thyroidectomy ± node dissection, from October 2008 to September 2009 in our Department. For each cytological category, we considered the definitive histological diagnosis of PTC and the presence of the BRAFV600E mutation. In 141 patients with a final tissue diagnosis of PTC, we correlated the presence of BRAFV600E with gender, age, histotype, TNM, size of the lesion, extracapsular extension, node metastases and multifocality.
Results: The prevalence of the BRAFV600E mutation, among PTCs at final tissue diagnosis, was 69%. It improved the FNAB diagnostic accuracy from 88% to 91%. The BRAFV600E mutation was correlated with older age, classical variant of PTC, advanced stages in patients >45 years.
Conclusions: BRAFV600E testing could play a role in improving the diagnostic accuracy of FNAB for PTC, representing a useful adjuvant tool in presurgical characterization of thyroid nodes in particular cases. There is an association between the BRAFV600E mutation and some clinico-pathological characteristics of PTC.

Keywords: *BRAF*; cytology; papillary thyroid cancer.

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Introduction

The current preoperative standard diagnosis of a thyroid mass relies on microscopic evaluation of thyroid cells, obtained by fine needle aspiration biopsy (FNAB) and stained using May-Grunwald-Giemsa and/or Papanicolaou methods (1–4). However, cytological evaluation is not always simple, the interpretation of thyroid smears is a challenge and requires experience. Thus, the sensitivity and specificity of FNABs are variable, ranging from 70% to 98% and from 55% to 100%, respectively. Also, results are dependent on many factors, including sample collection and preparation, and the pathologist's skill. Indeed, in the presence of follicular growth pattern lesions, benign or malignant nature remains doubtful after cytology, and these cases are given the diagnostic label of ''follicular lesions'' or ''follicular neoplasia'', the incidence of which is 20%–25% of all cytological diagnoses. Most patients with ''follicular neoplasia'' undergo diagnostic/therapeutic surgery, and the incidence of malignant cases is no more than 20%. Such a low incidence of cancer requires more stringent criteria for selecting patients for surgery (5–7).

More recently, FNAB has been combined with molecular analysis, since cellular DNA and/or RNA in specimens are sufficient to demonstrate peculiar somatic point mutations or chromosomal rearrangements. Among different molecular

events characterizing thyroid cancer, point mutations in the *BRAF* gene represent a peculiar marker of papillary thyroid carcinoma (PTC), seen in up to 45% of cases (8–10). *BRAF* mutation testing is a highly specific procedure. It offers good sensitivity and therefore analysis for this mutation has been proposed as a diagnostic adjunctive tool, as well as in the evaluation of thyroid nodules with ''suspended'' cytological findings (11–13).

The aim of our study was to evaluate whether BRAFV600E mutation testing could increase the diagnostic accuracy of FNAB for PTC in thyroid nodules with a ''suspended'' or malignant cytological result, in a series of patients who underwent US-guided FNAB by the same experienced endocrinologist, cytological evaluation by the same pathologist and thyroidectomy by the same surgery team at our Department. Moreover, we investigated the prognostic role of the BRAFV600E mutation in PTC, comparing patients with a *BRAF* mutation versus patients carrying wild-type *BRAF*, according to some clinico-pathological characteristics.

Materials and methods

From October 2008 to September 2009, we prospectively enrolled 270 consecutive patients (66 males and 204 females, with a mean age of 47.8 years, range 11–74 years). All individuals had a sonographic single node and/or a node with suspected sonographic features (hypoechoic solid aspect and/or microcalcifications and/or irregular borders and/or intranodular hypervascularity) who underwent US-guided FNAB for cytological evaluation and *BRAF* mutations testing, and subsequent thyroidectomy at our Department. All patients came from north-eastern Italy, a borderline iodine sufficient area according to World Health Organization (WHO) criteria (14).

Nodes were classified according to the diagnostic categories of the British Thyroid Association as: non-diagnostic (Thy1), benign (Thy2), indeterminate (Thy3), suspicious of malignancy (Thy4), malignant (Thy5) (15). The final histology referred to the node in which cytological and molecular analysis were performed.

Moreover, among these 270 cases, we evaluated 141 patients with a final tissue diagnosis of PTC. We subdivided these patients into two groups: group 1 included patients with a *BRAF* mutation, and group 2 included patients carrying a wild-type *BRAF*. We compared the two groups according to the following clinico-pathological characteristics: gender, age, histotype, TNM staging, size of the lesion, extracapsular extention, node metastases and multifocality. All patients provided informed consent.

DNA isolation and *BRAF* mutation analysis

DNA from FNAB-material was isolated using the QIAamp DNA Micro kit (Qiagen, Italy), according to the manufacturer's protocol. *BRAF* status of exon 15 was evaluated by both direct sequencing and by mutant allele-specific PCR amplification (MASA) for the T to A substitution at nucleotide 1799 (V600E), following descriptions from the literature (16, 17). In cases with discrepant results, molecular analysis was also performed in the histological sample and the final result retained for statistical analysis.

Statistical analysis

Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy were calculated for each detection method and for combined methods. Univariate analysis, using χ^2- and Student's t-test was used to analyze clinical and pathological data in patients with or without the BRAFV600E mutation. A p-value <0.05 was considered statistically significant.

Results

Cytological evaluation and *BRAF* analysis in FNAB samples.

Of the 270 thyroid FNABs, nine cases were classified as Thy1 (3.3%), four as Thy2 (1.5%), 119 as Thy3 (44.1%), 45 as Thy4 (16.7%), and 93 as Thy5 (34.4%).

In Thy1, final tissue diagnosis detected three cases of goiter, three of thyroid adenoma, two of PTC and one case of follicular thyroid carcinoma (FTC); molecular analysis showed the BRAFV600E mutation in both cases of PTC.

In Thy2, final tissue diagnosis detected three cases of thyroid adenoma and one case of PTC, also carrying the BRAFV600E mutation in its FNAB as shown by molecular analysis.

In Thy3, final tissue diagnosis detected 89 cases of adenoma, nine of goiter, one of Hashimoto thyroiditis, five of PTC and 15 of FTC, the BRAFV600E mutation was detected in two cases of PTC.

In Thy4, the final tissue diagnosis detected three cases of adenoma, two of goiter and 40 of PTC, of which 28 carried the BRAFV600E mutation. In Thy5, among the 93 PTC, 65 showed the BRAFV600E mutation (Table 1).

Cytological evaluation showed a sensitivity of 85%, a specificity of 95%, a PPV of 96%, an NPV of 81% and a global accuracy of 88%. *BRAF* testing showed a sensitivity of 62%, specificity of 100%, PPV of 100%, NPV of 64% and a global accuracy of 76%. The combined methods

Table 1 Cytological, histological and molecular results. The prevalence of PTC at final histology and the prevalence of BRAFV600E according to cytological categories, and in PTC are shown.

Cytology	Cases	PTC at final histology	BRAFV600E in cytology, %	BRAFV600E in PTC, %
Thy1	9	2	2 PTC (22%)	100
Thy2	4	1	1 PTC (25%)	100
Thy3	119	5	2 PTC (1.7%)	40
Thy4	45	40	28 PTC (62%)	70
Thy5	93	93	65 PTC (70%)	70
Total	270	141	98 PTC (36.3%)	69

Table 2 PPV, NPV, sensitivity and specificity of cytology from FNAB, molecular analysis and both the methods.

	PPV, %	NPV, %	Sensitivity, %	Specificity, %	Accuracy, %
Cytology	96	81	85	95	88
BRAF	100	64	62	100	76
Boths	96	84	88	95	91

showed a sensitivity of 88%, specificity of 95%, PPV of 96%, NPV of 84% and a global accuracy of 91% (Table 2).

BRAF: demographic and pathological data

The 141 patients affected with PTC were subdivided into two groups according to the presence or absence of the BRAFV600E mutation in thyroid FNAB: group 1 included 98 cases (69.5%) with the BRAFV600E mutation and group 2 included 43 cases (30.5%) with wild-type *BRAF*. The two groups were compared to each other with respect to gender, age, histotype, TNM staging, size of the lesion, extracapsular extention, node metastases and multifocality. The results are reported in Table 3. Univariate analysis showed that age at diagnosis >45 years ($p<0.005$), histotype ($p=0.001$) and advanced stage in patients over 45 years ($p=0.03$) were significantly correlated with BRAFV600E mutation, whereas gender, size of the lesion, extracapsular extension, node metastases and multifocality were not.

Discussion

US-guided FNAB is the gold standard method for detecting malignancy in thyroid nodes. However, approximately 10%–30% of FNAB cytological results are not conclusive, and approximately 20%–30% turn out to be malignant after surgery (3–5). This situation leads patients and physicians into a diagnostic dilemma.

More recently, different studies evaluated if the application of molecular analysis to thyroid FNAB for genetic events characterizing the malignant phenotype could increase sensitivity and accuracy in the cytological setting (11–13). The BRAFV600E mutation is the most common genetic alteration observed in PTC in adults, seen in approximately 45% of cases (9, 10). As such, *BRAF* testing represents a suitable candidate, especially in routine practice, being a cost effective procedure with a high specificity for PTC with virtually no false-positive results. *BRAF* mutations are reported in half of PTC cases; subsequently routine analysis could not be particularly helpful in cases with ''indeterminate'' (Thy3) cytological results, where FTC and the follicular variant of PTC represents most malignancy cases at final histological evaluation. However, a previous report by Nikiforov et al. (13) showed that *BRAF* testing improves cytological diagnosis, also in indeterminate cases. However, in their report the authors considered in the ''indeterminate'' group ''suspicious'' cytological results. Thus, the prevalence of malignancy was particularly high (around 40%), and PTC was the most frequent thyroid cancer (13). In another study, Zattelli et al. (12) showed that *BRAF* mutation analysis could increase cytological accuracy, in particular by refining some benign cytological diagnosis. However, it was not able to

Table 3 Demographic and histological characteristics according to BRAFV600E mutation in 141 patient with PTC.

	Group 1 (n=98)	Group 2 (n=43)	p-Value
Gender	F 76 (78%) M 22 (22%)	F 33 (77%) M 10 (23%)	0.84
Age	Mean 51 years, 25–74	Mean 44 years, 11–72	0.005
Histology	PTC 96 (98%) FV PTC 0 TC PTC 2 (2%)	PTC 38 (88%) FV PTC 5 (12%) TC PTC 0	0.001
Size	≤5 mm, 4 (4%) ≤10 mm, 23 (23%) >10 mm, 71 (73%)	≤5 mm, 2 (5%) ≤10 mm, 6 (14%) >10 mm, 35 (81%)	0.62
TNM <45	I 32 (91%) II 3 (9%)	I 19 (95%) II 1 (5%)	0.92
TNM >45	I 19 (30%) II 0 III 39 (62%) IV 5 (8%)	I 11 (48%) II 1 (4%) III 8 (35%) IV 3 (13%)	0.03
Extracapsular extension	53 (54%)	24 (56%)	0.8
Node metastases	36 (37%)	18 (42%)	0.59
Multifocality	45 (46%)	18 (42%)	0.3

Group 1 included 98 cases of PTC with a *BRAF* mutation and group 2 included 43 cases of PTC with wild-type *BRAF*. At histology: PTC, classical variant of PTC; FV PTC, follicular variant of PTC; TC PTC, tall cell PTC.

solve the diagnostic problem of follicular patterned lesions. Indeed, in this study the BRAFV600E mutation was demonstrated in only one of 88 indeterminate cases (12). In our study, we decided to perform molecular analysis only in nodes that were single or shared sonographic features of malignancy. Using this selection criteria, we could collect a large group of indeterminate, suspicious or clearly malignant cytological diagnoses. Our data agree with those of Zattelli et al. (12) demonstrating that the *BRAF* test is a highly specific test and is able to ameliorate, even if quite marginally, the accuracy of cytological evaluation, increasing its sensitivity from 85% to 88%. We consider that its assessment could be useful in particular cases: 1) in those malignant lesions where FNAB could hesitate in a ''benign'' (Thy2) or ''inadequate''/''suspended'' (Thy1/Thy4) result, because of the small node size or the small amount of cancer cells with evident atypical features; 2) in follicular thyroid lesions together with other molecular biomarkers, such as cytoplasmatic expression of galectine-3 as previously demonstrated by our group (18). In the last case, the application of a molecular panel, also in absence of events associated with thyroid malignancy, may support the surgeon's decision to perform, in a given follicular lesion, thyroid lobectomy rather than total thyroidectomy (18).

Moreover, many studies have reported that the BRAFV600E mutation is associated with more aggressive cancer features, comprising an extended disease at diagnosis and a high risk of recurrence and progression towards RAI-resistance during follow-up. Thus, the BRAFV600E mutation is a new predictor of poor prognosis (19–21). Consequently, different authors recommend performing *BRAF* testing in PTC prior to surgery to guide the initial extent of thyroid surgery and to personalize subsequent follow-up (22). In a previous report by our group, we demonstrated that thyroid recurrence in individuals that have lost the ability to concentrate radioiodine carried a high-frequency of *BRAF* mutation, and such genetic events may promote progression to major impairment in concentrating radioiodine (23). In the present study, we noted that the BRAFV600E mutation was statistically correlated with an age >45 years ($p=0.005$), histotype ($p=0.001$) and stage ($p=0.03$).

Another interesting observation is the particularly high frequency of BRAFV600E mutations in our PTC series – around 68% – comparable to that shown by Zattelli et al. (12) geographical influences could exert a putative role in determining such relevant prevalence.

In conclusion, the BRAFV600E mutation could represent a useful molecular marker for preoperative diagnosis of PTC, and improves diagnostic accuracy of FNAB, in particular in small malignant thyroid nodes or nodes with quite differentiated cellular aspects that could be associated with non-conclusive cytological results. Moreover, the BRAFV600E mutation should be considered a molecular factor for poor prognosis.

Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Research funding played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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References

1. Pelizzo MR, Boschin Merante I, Toniato A, Pagetta C, Casal Ide E, Mian C, et al. Diagnosis, treatment, prognostic factors and long term outcome in papillary thyroid carcinoma. Minerva Endocrinologica 2008;33:359–79.
2. Pacini F, Burroni L, Ciuoli C, Di Cairano G, Guarino E. Management of thyroid nodules: a clinicopathological, evidence-based approach. Eur J Nucl Med Mol Imaging 2004;31: 1443–9.
3. Cap J, Ryska A, Rehorkova P, Hovorkova E, Kerekes Z, Pohnetalova D. Sensitivity and specificity of the fine needle aspiration biopsy of the thyroid: clinical point of view. Clin Endocrinol (Oxf) 1999;51:509–15.
4. Braga M, Cavalcanti TC, Collaco LM, Graf H. Efficacy of ultrasound-guided fine-needle aspiration biopsy in the diagnosis of complex thyroid nodules. J Clin Endocrinol Metab 2001;86: 4089–91.
5. Poller DN, Ibrahim AK, Cummings MH, Mikel JJ, Boote D, Perry M. Fine needle aspiration of the thyroid. Cancer 2000;90: 239–44.
6. Mazzaferri EL, Jhiang SM. Long term impact of initial surgical and medical therapy on papillary and follicular thyroid cancer. Am J Med 1994;97:418–28.
7. Pelizzo MR, Boschin IM, Toniato A, Piotto A, Pagetta C, Gross MD, et al. Papillary thyroid carcinoma: 35-year outcome and prognostic factors in 1858 patients. Clin Nucl Med 2007;32: 440–4.
8. Delys L, Detours V, Franc B, Thomas G, Bogdanova T, Tronko M, et al. Gene expression and the biological phenotype of papillary thyroid carcinomas. Oncogene 2007;26:7894–903.
9. Xing M. BRAF mutation in papillary thyroid cancer: pathogenic role, molecular bases, and clinical implications. Endocr Rev 2007;28:742–62.
10. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, et al. Mutations of the BRAF gene in human cancer. Nature 2002;417:949–54.
11. Xing M, Tufano RP, Tufaro AP, Basaria S, Ewertz M, Rosenbaum E, et al. Detection of BRAF mutation on fine needle aspiration biopsy spicemens: a new diagnostic tool for papillary thyroid cancer. J Clin Endocrinol Metab 2004;89:2867–72.
12. Zattelli MC, Trasforini G, Leoni S, Frigato G, Buratto M, Tagliati F, et al. BRAFV600E mutation analysis increases diagnostic accuracy for papillary thyroid carcinoma in fine needle aspiration biopsies. Eur J Endocrinol 2009;161:467–73.
13. Nikiforov YE, Steward DL, Robinson-Smith TM, Haugen BR, Klopper JP, Zhu Z, et al. Molecular testing for mutations in improving the fine-needle aspiration diagnosis of thyroid nodules. J Clin Endocrinol Metab 2009;94:2092–8.
14. WHO/UNICEF/ICCIDD (2007) Assessment of iodine deficiency disorders and monitoring their elimination, 2nd ed. Geneva: World Health Organization.
15. British Thyroid Association. Guidelines for the management of

thyroid cancer. 2nd ed. London: British Thyroid Association; 2007.
16. Xu X, Quiros RM, Gattuso P, Ain KB, Prinz RA. High prevalence of BRAF gene mutation in papillary thyroid carcinomas and thyroid tumor cell lines. Cancer Res 2003;63:4561–7.
17. Sapio MR, Posca D, Troncone G, Pettinato G, Palombini L, Rossi G, et al. Detection of BRAF mutation in thyroid papillary carcinomas by mutant-allele-specific PCR amplification (MASA). Eur J Endocrinol 2006;154:341–8.
18. Pennelli G, Mian C, Pelizzo MR, Naccamulli D, Piotto A, Girelli ME, et al. Galectin-3 cytotest in thyroid follicular neoplasia: a prospective, monoinstitutional study. Acta Cytol 2009; 53:533–9.
19. Xing M, Westra WH, Tufano RP, Cohen Y, Rosenbaum E, Rhoden KJ, et al. BRAF mutation predicts a poorer clinical prognosis for papillary thyroid cancer. J Clin Endocrinol Metab 2005;90:6373–9.
20. Lupi C, Giannini R, Ugolini C, Proietti A, Berti P, Minuto M, et al. Association of BRAFV600E mutation with poor clinicopathological outcomes in 500 consecutive cases of papillary thyroid carcinoma. J Clin Endocrinol Metab 2007;92:4085–90.
21. Elisei R, Ugolini C, Viola D, Lupi C, Biagini A, Giannini R, et al. BRAF (V600E) mutation and outcome of patients with papillary thyroid carcinoma: a 15-year median follow-up study. J Clin Endocrinol Metab 2008;93:3943–9.
22. Yip L, Nikiforova MN, Carty SE, Yim JH, Stang MT, Tublin MJ, et al. Optimizing surgical treatment of papillary thyroid carcinoma associated with BRAF mutation. Surgery 2009;146: 1215–23.
23. Mian C, Barollo S, Pennelli G, Pavan N, Rugge M, Pelizzo MR, et al. Molecular characteristics in papillary thyroid cancers (PTCs) with no 131I uptake. Clin Endocrinol (Oxf) 2008;68: 108–16.