Pre-operative role of BRAF in the guidance of the surgical approach and prognosis of differentiated thyroid carcinoma

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Abstract

Objective: The p.V600E BRAF and RAS mutations are found in 30–80% of differentiated thyroid carcinoma (DTC). BRAF mutation has been associated with poor prognosis. This study investigated the role of molecular studies in preoperative diagnosis of DTC and the association of p.V600E mutation with prognostic factors.

Design: Prospective study.

Methods: A total of 202 patients with cytological diagnosis of Bethesda III–VI underwent preoperative molecular studies and subsequent thyroidectomy. p.V600E and RAS mutations were studied in the cytology smears, using real-time PCR genotyping technique. The BRAF mutation (BRAFV600E or BRAFV600K) was correlated with histological and clinical findings.

Results: Molecular study of 172 nodules with Bethesda III–V cytology improved negative predictive value and accuracy of Bethesda III and IV diagnosis. BRAF mutation was present in 65% of 94 DTC and p.Q61R NRAS in one. Except for age, BRAFV600E and BRAFV600K did not differ in sex, tumor size, histological subtype, multifocality, vascular invasion, extrathyroidal extension, or prognostic staging. Among papillary carcinomas, lymph node (LN) metastasis was diagnosed in 23% BRAFV600E and 37% BRAFV600K. Distant metastasis occurred in four BRAFV600E. Recurrent or persistent disease was more frequent in BRAFV600E (26.7 vs 3.3% BRAFV600E, P=0.002) along follow-up of 29.8±10 months. BRAFV600E patients without LN metastasis by pre-operative evaluation submitted to thyroidectomy with central neck dissection (CND) had more frequent LN metastasis (45 vs 5% no CND, P=0.002), but no difference in clinical outcome was observed.

Conclusions: Pre-operative identification of BRAF mutation improved cytological diagnosis of DTC, but it was not associated with poor prognostic factors. Prophylactic CND did not guarantee better outcome in BRAFV600E patients.

Introduction

Thyroid nodular disease is a common condition, particularly with the advent of higher resolution ultrasound scanners, reaching up to 69% of patients that undergo US examination (1, 2). A proper preoperative diagnosis would restrict most thyroid surgeries to thyroid carcinoma, which represent no more than 5–15% of the cases (1).

Fine-needle aspiration biopsy (FNAB) guided by ultrasound is considered the gold standard technique to pre-operative diagnosis of thyroid carcinomas. However, indeterminate cytological diagnosis still occurs in 15–30%, requiring unnecessary surgeries in up to 80% of them (3, 4).

Papillary thyroid carcinoma (PTC) corresponds to about 85% of differentiated thyroid carcinomas (DTC) (1). In an effort to improve pre-operative DTC diagnosis, molecular studies are proposed. PTC is associated with the
p.V600E mutation of the \( \text{BRAF} \) gene in 30–80% and less frequent to \( \text{RAS} \) mutations (5, 6, 7, 8, 9). Therefore, it has been proposed that their identification in cytological material would improve sensitivity and accuracy of FNAB, especially in indeterminate or suspicious for thyroid cancer (Bethesda III, IV, and V) (10). Besides, it has been suggested that the p.V600E mutation is associated with poor prognosis (11). Recently, a meta-analysis concluded that p.V600E mutation was correlated with male gender, larger tumor, extra-thyroid extension, multifocality, and greater incidence of cervical lymph nodes (LNs) metastasis, resulting in more frequent advanced clinical stages (odds ratio 1.82) (12). Presumably, pre-operative molecular evaluation of the \( \text{BRAF} \) p.V600E mutation in FNAB material could not only improve cytological diagnosis accuracy but also guide to a selective neck dissection of the central compartment, a more aggressive surgical therapy, to prevent poor outcome.

This study aims to analyze the benefits of molecular studies as a tool to improve pre-operative diagnosis in indeterminate or suspicious nodules in FNAB. In addition, we analyzed the correlation of p.V600E mutation with poor prognostic factors.

**Subjects and methods**

**Patients**

We prospectively recruited consecutive patients with thyroid nodules submitted to ultrasound-guided FNAB from 2009 to 2011 in the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo and Instituto do Câncer do Estado de São Paulo. Subjects with cytological diagnosis of atypia of undetermined significance (Bethesda III), follicular, or \( \text{Hürthle cell neoplasm} \) (Bethesda IV), suspicious for malignancy (Bethesda V) or \( \text{PTC} \) (Bethesda VI), were selected for molecular studies. \( \text{BRAF} \) and \( \text{RAS} \) genes mutations were investigated in cytological material and confirmed in tumor tissue. All FNAB diagnoses were revised by the same pathologist. Thyroid nodules with Bethesda III, IV, and V cytology were selected for analysis of the performance of cytological and molecular diagnosis.

Patients with cytological diagnosis of Bethesda III and IV were submitted to partial thyroidectomy (PT) or total thyroidectomy (TT). All patients with cytological diagnosis of Bethesda V and VI were submitted to total or near TT. Patients with pre-operative evidence of LNs metastasis underwent therapeutic neck dissection. A subgroup of patients without clinical or ultra sonographic evidence of cervical LN metastasis was submitted to prophylactic central neck dissection (CND), as a part of another institutional protocol (13). Dutenhefner et al. (13) selected prospectively 45 patients without known LN metastasis from a group of 52 consecutive patients with cytological diagnosis of Bethesda V and VI to be submitted to TT and CND. Twenty-three of them had been included in our study. Both patients’ physicians and surgeons were blinded to the result of the molecular study.

Postoperatively, patients with thyroid carcinoma were evaluated with serial cervical ultrasound and serum thyroglobulin. Functional sensitivity for the thyroglobulin assay was 0.2 ng/ml (Access, Beckman Coulter, Fullerton, CA, USA). Recurrent or persistent disease was defined in the presence of distant metastasis, loco-regional recurrence, and suppressed or stimulated serum thyroglobulin >2 ng/ml after >1 year of follow-up. The sixth edition of the tumor, LN, and metastasis classification was used for staging (TNM, American Joint Committee on Cancer classification) (14), as well as American Thyroid Association (ATA) classification for the risk of recurrence (1). Clinical characteristics, molecular analysis, cytological and histological patterns as well as initial surgical approach and recurrence of disease were analyzed.

The study was approved by the Institutional Research Ethics Committee and all subjects gave their informed written consent to participate.

**\( \text{BRAF} \) and \( \text{RAS} \) mutations**

DNA extraction from thyroid FNAB stained slides followed the standard phenol/chloroform/isoamyl alcohol protocol (15). Briefly, slides were placed in the bath with histological xylene for 24 h to remove the coverslip and the material was then scraped from the slide and transferred to a clean microtube for extraction using phenol/chloroform/isoamyl alcohol. DNA extraction from material conserved in paraffin was performed as previously described (13). One milliliter of xylene preheated in an incubator at 95 °C was added in a microtube of 1.5 ml with three sections equivalent to 10 \( \mu \)m of conserved tumor. They were agitated and placed in an incubator at 37 °C for 30 min, and then centrifuged for 5 min at 15,000 \( \text{g} \), after which the supernatant was discarded. For effective elimination of the paraffin, this step was repeated another two times. The samples were then submitted to two washings with 500 \( \mu \)l absolute ethanol to remove the organic solvent. After each addition of absolute ethanol, the microtubes were centrifuged at 13,000 \( \text{g} \) for 5 min at 4 °C and the supernatant was
showed the WT sequence of exon 15 of the BRAF gene.

For DNA extraction: 20 µl of proteinase K (10 mg/ml) and 480 µl of a solution of 2.5 ml of Tris–HCl 1 M (pH 8.0); 500 µl EDTA 0.5 M (pH 8.0); 250 µl of Tween 20; and 46.75 ml of deionized water were added to each microtube. The samples were incubated for 18 h at 37°C. Two extractions were carried out with 500 µl of phenol–chloroform. The tubes were carefully inverted and centrifuged at 13 000 g for 2 min at 4°C. The supernatants were transferred to new microtubes and a new extraction was carried out with phenol–chloroform. DNA extracted from NPA cells (a melanoma cell line containing a biallelic BRAF p.V600E mutation) was used as positive control (kindly provided by E T Kimura). As negative control, we used DNA extracted from normal thyroid tissue, which showed the WT sequence of exon 15 of the BRAF gene.

The pellets were resuspended in sterile water. Total nucleic acids were extracted and sample adequacy for molecular analysis was assessed based on the general quantity and quality of isolated nucleic acids.

Searching for p.V600E mutation in cytology smears was performed by real-time PCR genotyping with primers defined by Benlloch et al. (16). Samples were genotyped using TaqMan according to the manufacturer’s instruction (Applied Biosystems). The fluorescence data were analyzed with the allelic discrimination software of the StepOne version 2.0 Software (Applied Biosystems) and amplification plots produced in the PCR were also checked. In order to confirm genotyping findings, all samples were automatic sequenced using BigDye Terminator Kit on the ABI3100xl (Applied Biosystems).

Codons 12–13 and 61 of K-RAS, N-RAS, and H-RAS genes were directly sequenced only in samples without BRAF mutation as previously described (17).

Statistical analysis

Data were processed using PASW Statistics Software, version 17.0 (SPSS, Inc.) and MedCalc for Windows (version 12.0.3.0; MedCalc Software). Two-tailed P values were used and P values <0.05 were considered statistically significant.

Categorical variables are presented as absolute and relative (percentages) frequencies. Differences were evaluated by Pearson’s χ²-test and Fisher’s exact test when appropriate. Continuous variables are presented as mean ± s.d. Differences among studied subgroups were determined using Student’s t-test if presenting normal distribution, and Mann–Whitney U test for non-normal distributions.

Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy for the diagnosis of malignancy in the subgroups of Bethesda III, IV, and V biopsies were calculated for cytological and molecular diagnosis of p.V600E mutation, either alone or in combination. We defined the true-positive, true-negative, false-positive, and false-negative results based on final histological diagnosis (benign and malignant).

Results
Clinical characteristics and pre-operative diagnosis

Two hundred and two patients were submitted to FNAB to investigate 208 thyroid nodules. A 116 nodules had the diagnosis of Bethesda III (55.8%), 20 Bethesda IV (9.6%), 36 Bethesda V (17.3%), and 36 Bethesda VI (17.3%). Female patients were more frequent than males in both benign thyroid nodular diseases and thyroid carcinomas (88 vs 84%, P = 0.42); however, patients were older in the first group (53.6 ± 13.7 vs 46.9 ± 15.8 years old, P = 0.002). We selected 172 thyroid nodules with Bethesda III, IV, and V cytology for pre-operative molecular studies and analysis of the performance of cytological and molecular diagnosis.

According to cytological diagnosis, DTC was diagnosed in 19.8% of Bethesda III, 35% of Bethesda IV, and 80.6% of Bethesda V. Classical PTC were more frequent among Bethesda V and VI diagnoses. Forty-three out of 64 Bethesda V and VI patients (67%) were classical papillary carcinoma compared with 13 of 30 Bethesda III and IV (43%) (P = 0.028). The frequency of p.V600E mutation was 6.9% in Bethesda III, 20% in Bethesda IV, and 52.8% in Bethesda V. The p.Q61R NRAS mutation was identified in only one patient. All data were confirmed with the molecular analysis of surgically removed tumor.

Among benign nodules, 69.3% were adenomatous nodules, 21.9% were follicular adenomas, and 8.8% were thyroiditis. Size was statistically different between benign lesions and DTCs (26.2 ± 16.8 vs 21 ± 16.5 mm, P = 0.01). None of the benign nodules had a p.V600E or RAS mutations in cytological material and tissue analysis.

The diagnostic performance of cytology and molecular results are described in Table 1. The presence of the p.V600E BRAF mutation provided a specificity and PPV for diagnosis of DTC of 100%. The combination of cytological diagnosis and pre-operative molecular study improved the NPV and accuracy of both Bethesda III and IV specimens. On the other hand, in Bethesda V and VI cytology, the BRAF study was associated with a worse NPV and accuracy than the cytological diagnosis alone.
Table 1  Diagnostic performance of cytology and molecular test in identification of 94 differentiated thyroid carcinomas.

<table>
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<tr>
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<th>Sensitivity (%)</th>
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<th>Positive predictive value (%)</th>
<th>Negative predictive value (%)</th>
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<tr>
<td>Bethesda III</td>
<td>23</td>
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<td>18.4</td>
<td>19.8</td>
<td>22.8</td>
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<td>7</td>
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<td>88.6</td>
<td>35</td>
<td>53.7</td>
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<tr>
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<td>30.9</td>
<td>93.9</td>
<td>80.6</td>
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<td>65.4</td>
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<tr>
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<td>35</td>
<td>37.2</td>
<td>99.1</td>
<td>97.2</td>
<td>65.7</td>
<td>71.2</td>
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<tr>
<td>BRAF</td>
<td>61</td>
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<td>100</td>
<td>100</td>
<td>77.6</td>
<td>84.1</td>
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<tr>
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<td>100</td>
<td>100</td>
<td>86.1</td>
<td>87.1</td>
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<tr>
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<td>100</td>
<td>100</td>
<td>81.2</td>
<td>85</td>
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<td>100</td>
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n, number of cases with histological diagnosis of differentiated thyroid carcinoma; BRAF, presence of p.V600E BRAF gene mutation.

Molecular analysis and prognosis

From 94 patients with final histological diagnosis of DTC, 61 (65%) had pre-operative cytological identification of the p.V600E mutation. There was no significant difference in clinical and histological features between individuals with or without BRAF mutation, except for age (BRAF+ 49.6±15 vs BRAF− 41.8±16.3 years old, P=0.021; Table 2).

Among all PTC cases, LN metastasis was diagnosed in 23% BRAF+ and 37% BRAF− (P=0.168). Distant metastasis occurred in only four BRAF− patients. Recurrent or persistent disease was more frequent in BRAF− patients (26.7 vs 3.3% BRAF+, P=0.002) during follow-up of 29.8±10 and 28.5±6.6 months respectively.

Eighty-four (89%) DTCs corresponded to classical and follicular variants of papillary carcinoma. Subgroup analysis confirmed anterior findings. The only prognostic factor associated with BRAF mutation was age (Table 3). The frequency of LN metastasis did not significantly differ between BRAF+ (20%) and BRAF− (31%) patients. However, distant metastasis, diagnosed only in BRAF−, and recurrent or persistent disease (BRAF+ 3.6% vs BRAF− 24.1%) were also more frequent in BRAF− patients.

The N-RAS p.Q61R mutation was identified in a 30-year-old female patient, whose thyroid nodule was diagnosed as Bethesda V at FNAB. She presented with a multifocal follicular variant of PTC with the largest tumor measuring 1.9 cm. She was classified as TNM stage I and low risk of recurrence. She had no recurrence over a follow-up of 32 months.

Prophylactic CND and BRAF mutation

Thirteen patients had prior diagnosis of LNs metastasis, so they were excluded from subsequent analyses. Fifty-four patients BRAF+ without preoperative diagnosis of LNs metastasis were submitted to either TT or PT (n=43) or TT and prophylactic CND (TT+CND, n=11). LNs metastasis was significantly more frequent in subjects submitted to TT+CND (45 vs 5% TT/PT, P=0.002), resulting in more frequent TNM stages III and IV (45.5 vs 14% TT/PT, P=0.035) and ATA stages of intermediate and high risk of recurrences (63.6 vs 27.9% TT/PT, P=0.038). Despite a larger extent of local disease in patients submitted to prophylactic CND, no difference in clinical outcome was observed between subgroups, as no patient had recurrent or persistent disease even with a longer mean follow-up of patients submitted to TT+CND (33.5±7.2 vs 26.1±9.3 months in TT/PT, P=0.013).

Similar results were found in BRAF− patients. LNs metastasis was present in 6.7% of patients submitted to TT and 50% of patients submitted to TT+CD (P=0.024). Only one patient in the second subgroup had persistent distant metastatic disease (P=0.44) at final evaluation.

Discussion

It has been proposed that molecular studies would improve the accuracy of cancer detection in FNAB of thyroid nodules. Recent studies have demonstrated the feasibility of mutations detection in clinical FNAB samples, extensively BRAF and RAS mutations, and the improvement of accuracy of FNAB especially in indeterminate cytology. Detection of other molecular markers such as as RET/PTC and PAX8/PPARγ mutations does not contribute substantially to cancer diagnosis and in addition, they are technically more difficult to study (18).

Although only one case presented a mutation of RAS gene, the p.V600E BRAF mutation was detected in 65% of thyroid carcinomas in our institution. The molecular study of BRAF gene showed 100% specificity and PPV to diagnosis of DTC and an accuracy of 84.1%.
In our study, we could observe that the combination of BRAF and cytology analysis was especially useful in patients with a cytological diagnosis of Bethesda III and IV (Table 1), while in Bethesda V and VI specimens, the cytological diagnosis alone was associated with a high specificity (93.9 and 99.1% respectively) and high PPV (80.6 and 97.2% respectively) and the combination with BRAF analysis added no benefit but, in fact, was associated with a worse NPV (62.2–41.2% and 65.7–7.7% respectively). The BRAF study increased the NPV and accuracy of combined pre-operative diagnosis of Bethesda III and IV cytology possibly because of their low sensitivity for the diagnosis of DTC attributed to low prevalence of malignant disease in these groups (19.8 and 35% respectively).

In clinical practice, the precise pre-operative diagnosis of thyroid nodule must be done to avoid unnecessary surgery in benign disorders. NPV around 84% in Bethesda III and IV (86.1 and 81.2% respectively) indicate that 16% of negative results in molecular study of these groups would still represent malignant cases. Therefore, the absence of BRAF mutation in Bethesda III and IV is not sufficient to defer surgery. As an alternative, Alexander et al. (19) proposed a panel of benign gene-expression classifier with 92% sensitivity and NPV of 95% to improve diagnosis and avoid surgery in most benign thyroid nodules. While this panel is not available worldwide, BRAF and RAS studies remain feasible methods that, despite not useful to indicate benign disease and defer surgery, can allow detection of malignant disease with indication for surgery. It has been suggested that the p.V600E BRAF mutation is associated with poor prognosis.

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Except for older age, we found no association of the p.V600E mutation with poor prognostic risk factors or with unfavorable outcome. The frequency of LN metastasis was not different between BRAF+ and BRAF− cases. In addition, distant metastasis was observed only in BRAF− tumors (Table 2) and recurrent and persistent disease were also observed more frequently in BRAF− patients (P=0.002), despite a short period of follow-up. Li et al. suggested the association of BRAF mutation with poor prognostic factors, but they recognized that most of the studies analysed were retrospective and there was no consensus on performance of prophylactic CND; different histological subtypes were also studied altogether, even unfavorable ones such as tall cell and diffuse sclerosing variants (12). In this study, only four out of 91 PTC had unfavorable solid variant and three PTCs had variants of undefined prognosis (oncocytic and Warthin-like). There was no tall cell variant frequently associated with p.V600E and known as more aggressive. The most prevalent subgroup of classical and follicular variants of PTC showed no association of BRAF mutation with poor prognostic factors or poor outcome. Our findings corroborated recent studies (20, 21, 22, 23) and also discarded the association of p.V600E with higher frequency of cervical LN and distant metastases (13, 24, 25, 26). In order to justify the lack of association, it has also been proposed that it is not the intratumoral presence of BRAF mutation that determines prognosis but the higher frequency of mutant BRAF alleles, identified in an innovatory analysis of pyrosequencing technique, that favors more frequent recurrence (27).

Nevertheless, it has been suggested that the identification of a possible unfavorable risk factor, such as the presence of BRAF mutation, before surgery would allow an aggressive initial approach, such as central LN dissection (28). We evaluated BRAF+ patients submitted either to thyroidectomy or thyroidectomy plus prophylactic CND and found no difference in outcome, despite more frequent LNs metastasis in subjects submitted to TT+CND (P=0.002). A recent study that investigated the prognostic significance of microscopic LNs metastasis detected with prophylactic neck dissection has proved they had no impact in the low loco regional recurrence rates (29). Dutenhofner et al. (13) studied PTC submitted to prophylactic CND and found no difference in the LNs metastasis presence between BRAF+ and BRAF−. Our prospective study confirmed the high frequency of microscopic LNs metastasis diagnosed in prophylactic CND, despite the presence of BRAF mutation. However, as it was not associated with a better outcome, we question its benefits, especially considering the higher risk of surgical comorbidities, such as permanent hypoparathyroidism.

This study has some limitations as we analyzed only 94 patients with DTC and partly submitted to thyroidectomy plus central node dissection (11/54 BRAF+). Besides, a short period of follow-up was analyzed (28.1 ± 9.9 months) and a longer period would be more revealing regarding DTC.

In summary, the identification of p.V600E BRAF mutation in cytology smears improved accuracy to preoperative diagnosis of DTC in Bethesda III and IV in which cytology alone is associated with low sensitivity. In accordance, the molecular study does not avoid surgery, but permits identification of BRAF+ PTC that requires surgery.

In this study, BRAF mutation was not associated with poor prognostic factors or unfavorable outcome. We conclude that the role of pre-operative molecular analysis is restricted to improving the diagnosis of indeterminate cytological specimens and is still controversial in guiding surgical extent and postoperative adjuvant treatment of PTC.

**Declaration of interest**
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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