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ORIGINAL SCIENTIFIC REPORT

$BRAF^{V600E}$ Mutation is Associated with Decreased Disease-Free Survival in Papillary Thyroid Cancer

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Abstract

Background The *$BRAF^{V600E}$* mutation is a recognised molecular marker in papillary thyroid cancer (PTC), reported incidence from 30 to 80 %. $BRAF^{V600E}$ aberrantly activates the MAPK pathway, a central regulator of cell growth and proliferation. Previous studies have reported conflicting data regarding the impact of $BRAF^{V600E}$ on clinicopathological features of PTC. The study aims to determine whether $BRAF^{V600E}$ is useful as a prognostic biomarker in PTC.

Methods A cohort study of patients undergoing surgery for PTC was undertaken. The primary outcome measure was disease-free survival. Secondary outcome measures were tumour size, nodal positivity and radioactive iodine ablation rate. All cases were re-examined to confirm PTC. Immunohistochemistry for $BRAF^{V600E}$ was performed on tissue microarrays. A single endocrine pathologist, blinded to clinicopathological data, interpreted staining.

Results 496 patients with PTC were included, and 309 (62 %) were $BRAF^{V600E}$ positive. Tumour size was similar for $BRAF^{V600E}$-positive and -negative tumours (21.3 vs. 23.2 mm, $p = 0.23$). $BRAF^{V600E}$-positive patients were significantly older at first operation (mean age 45 versus 49 years, $p = 0.003$). $BRAF^{V600E}$-positive PTCs had a higher rate of disease recurrence (12.9 vs. 5.6 %, $p = 0.004$), lymph node metastasis (44 vs. 29.4 %, $p = 0.004$) and extra-thyroidal extension (44 vs. 22 %, $p < 0.001$). Five-year disease-free survival was 89.6 % for $BRAF^{V600E}$ positive and 96.3 % for negative tumours, $p < 0.001$. There was no difference between groups for vascular invasion or multifocality. The mean follow-up was 57 months for both groups.

Conclusion $BRAF^{V600E}$ in PTC predicts an increased risk of lymph node metastasis, extra-thyroidal extension and reduced disease-free survival. It is an additional useful prognostic biomarker.

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Introduction

Thyroid cancer is the commonest endocrine malignancy, comprising 1 % of all cancers. 80–85 % of thyroid cancer cases are papillary thyroid cancer (PTC) [1]. Generally, PTC has a good long-term prognosis with a 10-year survival rate over 90 % [1]. However, a subgroup of patients develops more aggressive disease. These patients experience disease recurrence with associated morbidity and mortality, with an estimated 35,000 people dying annually from thyroid cancer worldwide [2]. Surgical options are more restricted and difficult when disease recurs,

particularly when tumours become refractory to radioactive iodine.

Clinical guidelines have been developed to stratify patients into different risk groups. This allows clinicians to tailor more aggressive treatment and closer follow-up to patients considered high risk. Currently, these guidelines are based on clinicopathological risk factors including patient age, gender, co-morbidities, tumour size, histological type, lymph node involvement and extra-thyroidal spread [3–6]. However, risk stratification is often inaccurate with 15 % of tumour recurrence and 10 % of thyroid cancer mortality accounted for by patients deemed "low risk" by such criteria [2, 7, 8]. Therefore, clinicians have endeavoured to develop better prognostic algorithms and recently molecular tumour markers have gained widespread interest.

Genetic alterations frequently occur in PTC, the most common within the gene encoding BRAF kinase, c.1799T > A, p.Val600Glu ($BRAF^{V600E}$), with a reported prevalence ranging from 29 to 83 % [6, 9]. BRAF activates the mitogen-activated protein kinase (MAPK) intracellular signal transduction pathway. The MAPK pathway has a central role in regulation of gene expression, cell growth, proliferation and survival [10, 11]. $BRAF^{V600E}$ accounts for over 95 % of BRAF mutations and is oncogenic by constitutive MAPK activation [9, 12]. $BRAF^{V600E}$ occurs almost exclusively in PTC and PTC-derived anaplastic carcinoma [6]. Consequently, BRAF has been investigated for its tumorigenic and prognostic value in clinical practice, although initial clinical results were mixed. Whereas many studies demonstrated $BRAF^{V600E}$ to be associated with parameters of tumour aggressiveness [6, 13–16], others found no association [17–19].

This controversy has mainly been resolved by larger multi-centre studies and meta-analyses. These have confirmed association between $BRAF^{V600E}$ and advanced tumour characteristics, especially extra-thyroidal extension, lymph node involvement and stage III and IV disease [2, 10, 14–16, 20]. More recently, studies have investigated the association of $BRAF^{V600E}$ with recurrence, demonstrating $BRAF^{V600E}$-positive patients have an increased chance of recurrence. However, these reports were mostly single-institution studies with small patient series [13, 16, 21–24]. A recent international multi-centre study demonstrated that $BRAF^{V600E}$-positive patients have a higher chance of recurrence, even in conventionally low-risk stage 1 and 2 and micro-PTC [24]. The aim of this study was to evaluate BRAF mutation as a prognostic biomarker in PTC.

Materials and methods

This cohort study involved 525 consecutive patients with PTC who underwent surgery from 1990 to 2012 at the University of Sydney Endocrine Surgery Unit. Cases were identified from a prospectively maintained thyroid surgery database, after approval from the local institutional human research ethics committee. Paraffin-embedded tissue was available only from the Department of Anatomical Pathology at the Royal North Shore Hospital, with acquisition regulated by the New South Wales Human Tissue Act 1983. Hence, the number of patients in this study does not represent the full clinical workload of the unit during this time.

The primary outcome measure was disease-free survival, defined as the absence of structural PTC recurrence during follow-up. Structural recurrence was defined as disease that was visible on cross-sectional imaging (ultrasound, CT, MRI or PET/CT) and confirmed to be papillary thyroid carcinoma on cytology or histopathology, independent of serum thyroglobulin levels. This definition therefore excludes patients with slight elevations in serum thyroglobulin and no evidence of disease on imaging. Secondary outcome measures were tumour size, cervical lymph node positivity and rate of radioactive iodine ablation.

All cases were reviewed centrally to confirm diagnosis of PTC. Tissue microarrays (TMAs) were constructed from formalin-fixed paraffin-embedded (FFPE) tissue, containing 2 × 1 mm cores from each tumour. Sufficient material was present for immunohistochemistry (IHC) in the TMA sections of 496 patients. $BRAF^{V600E}$ mutation-specific IHC was performed using a commercially available mouse monoclonal antibody (clone VE1, Spring Bioscience, Pleasanton, CA) using the methods we previously described [25]. Briefly, VE1 IHC was performed using the Leica Bond-III autostainer (Leica Microsystems, Mount Waverley, VIC, Australia) used according to the manufacturer's protocol with alkaline antigen retrieval (solution ER2, VBS part no: AR 9640, Leica Microsystems) with the primary antibody used at a dilution of 1 in 80.

$BRAF^{V600E}$ staining was interpreted as positive if there was any positive cytoplasmic staining in neoplastic cells. Staining in colloid and non-neoplastic cells was disregarded. Staining was interpreted by a single experienced endocrine pathologist (AG), blinded to all clinicopathological data (including results of *BRAF* testing if performed using DNA-based methods). 101 cases from this cohort had previously undergone IHC for $BRAF^{V600E}$ on whole sections plus molecular testing by Sanger Sequencing and restriction fragment length polymorphism (RFLP) in a previous study, in which IHC appeared superior to Sanger sequencing and RFLP [25]. As a quality assurance measure, the results of cases scored blinded in TMA format were compared to the results of IHC previously performed on whole sections and compared to molecular testing in the Bullock et al. study [25].

Statistical analysis was undertaken using STATA software. Continuous variables were assessed by the independent samples t test or the Mann–Whitney U test. Categorical variables were compared using the Fisher exact test or Pearson's Chi-squared test where appropriate. Kaplan–Meier survival curves, log-rank tests and Cox proportional regression analysis, at time of last follow-up, were used to compare recurrence by $BRAF^{V600E}$ mutation status and other factors. A p value of <0.05 was considered significant.

Results

Of 525 patients identified in the thyroid cancer database, 29 were excluded due to an inadequate amount of tissue to assess BRAF status by IHC. There were 496 remaining patients: 309 (62 %) were $BRAF^{V600E}$ positive and 187 (38 %) were negative. There was complete concordance between the IHC results interpreted on the TMA when compared to that previously performed on whole sections [25] in the cases which appeared in both cohorts ($n = 101$).

Patient demographics at baseline are shown in Table 1. Patients with $BRAF^{V600E}$-positive PTC were significantly older at the primary operation ($p = 0.003$). Clinicopathological characteristics at baseline are shown in Table 2.

$BRAF^{V600E}$-positive PTCs were associated with a significantly higher rate of extra-thyroidal extension—44 % compared to 22 % in $BRAF^{V600E}$-negative patients ($p < 0.001$). More $BRAF^{V600E}$-positive patients underwent a central lymph node dissection (CND) at their initial operation ($p < 0.001$). $BRAF^{V600E}$-positive patients who underwent a CND had more lymph nodes removed and a higher number of positive lymph nodes, 44 % compared to 29.4 % ($p = 0.004$). Routine CND is standard procedure in this unit for patients with PTC over 1 cm in maximum diameter. This has been incorporated into our protocols since 2003, and BRAF status was not known at the original surgery.

Tumour size was similar for $BRAF^{V600E}$-positive and -negative tumours (21.3 vs. 23.2 mm, $p = 023$). There was no significant association between $BRAF^{V600E}$ positivity and vascular invasion or multifocal disease.

Disease outcomes are shown in Table 3. Follow-up was equal for both groups, with a mean of 57 months from the primary operation, range 2–517 months for $BRAF^{V600E}$ negative and 2–528 months for positive patients. There was no difference between groups in the proportion of patients (87.5 % both groups) who received radioactive ablation (RAI) treatment. There was no difference in the number or overall doses of RAI received between groups.

The number of patients diagnosed with distant metastases was extremely low at diagnosis, only 0.02 % in each

Table 1 Demographic details of patients

	BRAF negative	BRAF positive	p value (CI)
Number of patients	187 (37.7 %)	309 (62.3 %)	
Male sex (%)	43 (30 %)	79 (26 %)	0.29
Age at first operation (mean ± SD)	45 ± 16.93	49 ± 15.99	0.003 (46.2–49.1)
Age range at first operation	10–88 years old	17–88 years old	

Table 2 Clinicopathological features from initial surgery

	BRAF negative	BRAF positive	p value (CI)
Vascular invasion	49 (27 %)	94 (31 %)	0.356
Extra-thyroidal spread	40 (22 %)	135 (44 %)	<0.001
Size of tumour (mean ± SD)	23.2 ± 18.14	21.3 ± 15.43	0.232 (20.5–23.5)
Initial LN dissection	88 (48 %)	199 (65 %)	<0.001
Number LN removed (mean)	6.3	8.1	0.003
% patients with positive LN	29.4 %	44 %	0.004
Multifocal disease	81 (44 %)	135 (44 %)	0.497
TNM stage	($n = 185$)	($n = 303$)	0.500
I	84 (45.41 %)	128 (42.24 %)	
II	45 (24.32 %)	40 (13.20 %)	
III	50 (27.03 %)	120 (39.60 %)	
IV	6 (3.24 %)	15 (4.95 %)	

Table 3 Long-term follow-up data

	BRAF negative	BRAF positive	*p* value
Follow-up time	56.9	56.8	0.98 (51.1–62.6)
Recurrence	10 (5.3 %)	40 (12.9 %)	0.004
Time to recurrence (months)	50.4	49.4	0.96 (33.0–66.2)
Received RAI ablation	160 (87 %)	264 (87 %)	1.0
Number RAI doses (mean)	1.11	1.16	0.505
No. I^{131} ablations	($n = 184$)	($n = 303$)	
0	23 (12.5 %)	38 (12.5 %)	
1	129 (70.1 %)	206 (68.0 %)	
2	26 (14.1 %)	40 (13.2 %)	
≥ 3	6 (3.3 %)	19 (6.3 %)	
Distant mets	3 (0.02 %)	6 (0.02 %)	0.535
Number dead	3 (0.02 %)	13 (0.04 %)	

group. Although the time to structural recurrence was similar between the groups, the percentage recurrence during follow-up was significantly higher in patients with $BRAF^{V600E}$-positive PTC; overall, 12.9 % of $BRAF^{V600E}$-positive patients had structural recurrence compared to only 5.3 % of $BRAF^{V600E}$ negative. Disease-free survival at five years was adversely affected by $BRAF^{V600E}$ status; 96.3 % of $BRAF^{V600E}$-negative patients were disease free, compared to 89.6 % of $BRAF^{V600E}$ positive. Locoregional structural recurrence was detected in patients on follow-up by either palpation and/or imaging. This includes ultrasound, CT, MRI or PET/CT and is independent of thyroglobulin levels. In multivariate analysis using Cox proportional hazards model, gender, tumour stage and $BRAF^{V600E}$ status were significantly associated with structural recurrence. The risk of local recurrence is increased by 2-fold on multivariate analysis for $BRAF^{V600E}$-positive patients, in contrast to $BRAF^{V600E}$ negative (Table 4).

Figure 1 demonstrates the significant reduction in disease-free survival with T stage, according to the tumour-node-metastasis (TNM) cancer staging system, developed by the American Joint Commission on Cancer. $BRAF^{V600E}$-positive patients were shown to have a significant reduction in disease-free survival (Fig. 2), compared to

Table 4 Cox regression multivariate analysis of clinicopathological factors associated with recurrence in PTC

	Hazard ratio	*p* value	95 % CI
Gender	2.52	0.004	1.33–4.77
Age (at primary surgery)	1.0	0.416	0.98–1.03
T stage	1.78	0.03	1.06–3.0
Extra-thyroidal extension	2.59	0.083	0.88–7.60
Primary lymph node dissection	0.68	0.24	0.36–1.30
BRAF positivity	2.44	0.0034	1.04–5.71

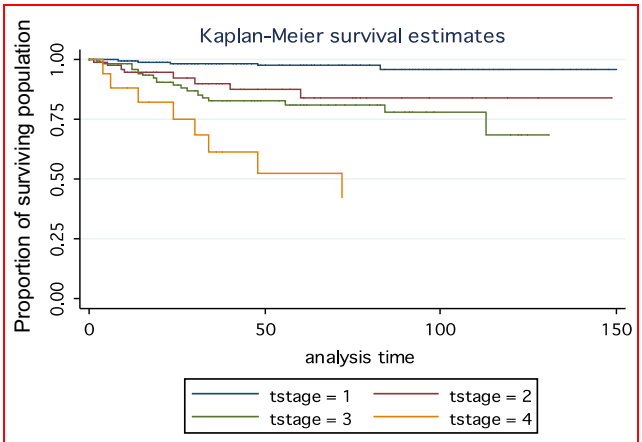

Fig. 1 Kaplan–Meier survival analysis of disease-free survival, stratified by tumour stage T1–T4

$BRAF^{V600E}$ negative. When outcome was analysed in subgroups, according to T stage, $BRAF^{V600E}$ positivity was established as an independent indicator for poor prognosis in T2, T3 and T4 tumours (Figs. 3, 4).

Discussion

This study documents cancer-related outcomes in a large cohort of patients with PTC and confirms a strong association between BRAF positivity and reduced disease-free survival. We believe these data support the incorporation of BRAF immunohistochemical staining into routine histopathological reporting of well-differentiated thyroid cancer, especially in units where routine central node dissection does not occur.

The incidence of thyroid cancer has been steadily rising worldwide. SEERS (Surveillance, Epidemiology and End

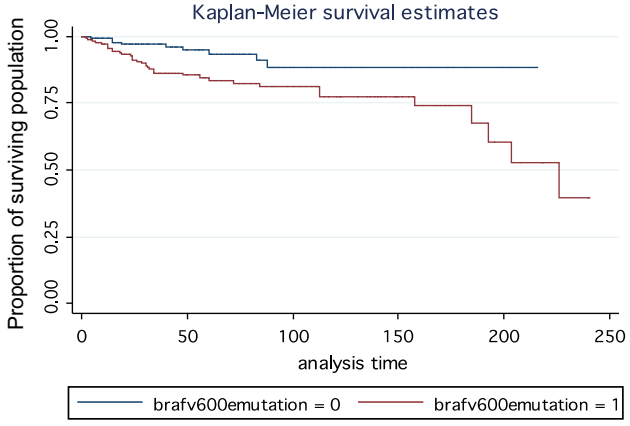

Fig. 2 Kaplan–Meier survival analysis of disease-free survival, stratified by $BRAF^{V600E}$status

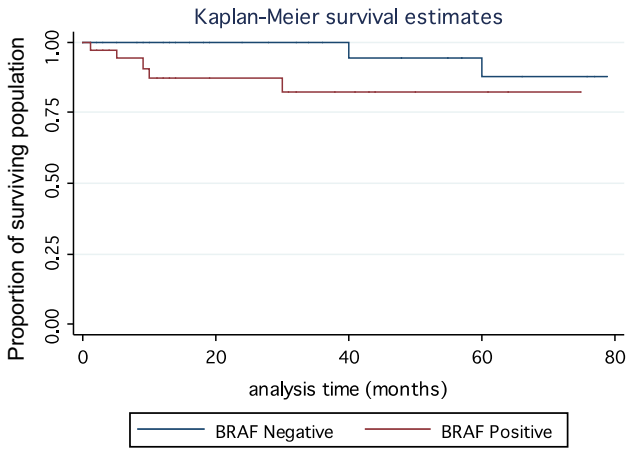

Fig. 3 Kaplan–Meier survival analysis of disease-free survival in T2 tumours, stratified by $BRAF^{V600E}$ status ($p = 0.04$)

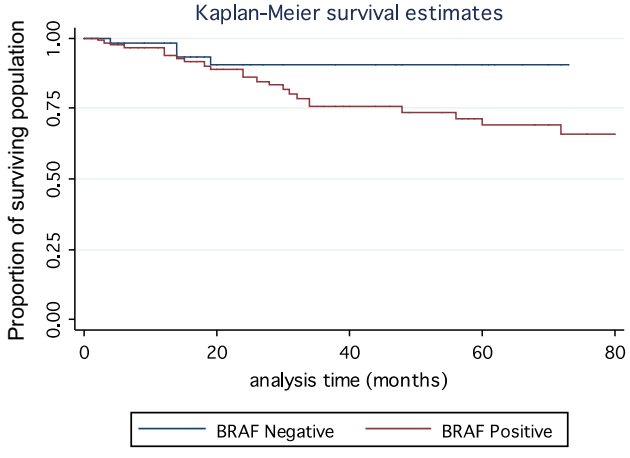

Fig. 4 Kaplan–Meier survival analysis of disease-free survival in T3 and T4 tumours, stratified by $BRAF^{V600E}$ status ($p = 0.011$)

Results) data from the USA have demonstrated that over the past ten years, the rates of new thyroid cancer cases have risen on average by 5.5 % each year, with an associated increased mortality by 0.8 % per annum. This rise is mirrored in other countries including the United Kingdom (Office for National Statistics) and Australia (Cancer Australia Statistics).

The rise in thyroid cancer is considered both an apparent and true increase. The escalating use of sensitive radiological investigations has identified a number of small, asymptomatic cancers, which may have remained clinically undetectable. However, the number of larger and more advanced thyroid cancers has also increased, notably only the PTC subtype [26, 27]. In contrast to most other cancers, including breast, colorectal, prostate and lung, where mortality has decreased over the past two decades, mortality from thyroid cancer has slightly risen [26, 28].

It is therefore increasingly important to differentiate which patients with PTC are higher risk for more aggressive disease and a greater chance of recurrence, compared to the majority with more benign disease. Molecular markers are being investigated for use as potential prognostic biomarkers, which could help in planning personalised treatment [29].

The most common genetic mutations associated with PTC are RET/PTC rearrangement and activating mutations in *BRAF*. RET/PTC is correlated with radiation exposure and childhood PTC. In contrast, $BRAF^{V600E}$ is usually seen in adults with a more aggressive phenotype [29]. $BRAF^{V600E}$ has a higher incidence in conventional PTC and tall cell variants, but is less common in follicular variant PTC [30].

This study assesses the impact of $BRAF^{V600E}$ in a large consecutive cohort of patients treated for thyroid cancer at a single-tertiary Australian institution. The proportion of $BRAF^{V600E}$-positive PTCs in our cohort is consistent with previous reports [14, 30].

Along with previous studies, we have demonstrated that $BRAF^{V600E}$ correlates with more aggressive clinicopathological features, including extra-thyroidal extension and cervical lymph node involvement. $BRAF^{V600E}$-positive patients had a higher rate of initial lymph node dissection, with a significantly greater percentage of metastatic lymph nodes. The association between $BRAF^{V600E}$-positive PTC and cervical lymph node metastases is clinically relevant as PTC typically recurs in the cervical lymph nodes [31]. Along with other studies, we did not demonstrate that positive $BRAF^{V600E}$ status had an impact on distant metastases or overall survival [32]. However, our most significant finding was that $BRAF^{V600E}$ is associated with reduced disease-free survival, independent of age, sex and tumour stage.

A recently published retrospective single-institution cohort study, also with just over 500 patients, did not

demonstrate that $BRAF^{V600E}$ had an adverse effect on recurrence. However, in keeping with our findings, $BRAF^{V600E}$ strongly correlated with cervical lymph node metastases, 75 % with lymph node involvement compared 25 % of $BRAF^{V600E}$-negative patients. The strongest predictor of recurrence in that series was cervical lymph node metastases [33].

Total thyroidectomy with prophylactic CND is routinely performed in our unit for patients with a pre-operative diagnosis of PTC. However, the role of prophylactic CND remains controversial. The latest British guidelines for the management of differentiated thyroid cancer do not advocate prophylactic CND for patients with small tumours and no high-risk features, whilst in high-risk patients it is left to the discretion of the individual surgeon [34]. The American Thyroid Association has a similar recommendation, with prophylactic CND only recommended in patients with advanced primary tumours [3]. On the basis of our results, we would advocate that $BRAF^{V600E}$-positive patients should undergo a prophylactic CND, due to a higher likelihood of lymph node metastases and reduced disease-free survival.

Radioactive iodine ablation (RAI) is current accepted practice after total thyroidectomy in the majority of patients with PTC. Several recent studies, however, suggest that RAI can be dose-reduced (or even avoided) in patients with low-risk disease. A recent multi-centre trial has demonstrated that in low-risk thyroid cancer, low-dose RAI is as effective as high doses, with less adverse effects [35].

Current clinical guidelines that stratify patients as low or high risk are based on clinicopathological features, not considering molecular markers. Whether BRAF status should be included in treatment algorithms requires further study to demonstrate that RAI specifically reduces recurrence risk in BRAF-positive cases.

Targeted molecular therapies are being considered in patients with recurrent or resistant PTC. Sorafenib is one example, with activity against multiple tyrosine kinases, including RAF, c-KIT, platelet-derived growth factor (PDGF) and vascular endothelial growth factor receptor (VEGFR) 2 and 3 [36]. A recent randomised double-blinded phase III trial assessed its efficacy in patients with RAI-refractory locally advanced or metastatic differentiated thyroid cancer. Whereas sorafenib improved progression-free survival, this was independent of BRAF mutation status [37], possibly due to relatively weak action against BRAF kinase [36].

Vemurafenib is a potent kinase inhibitor of $BRAF^{V600E}$ currently licensed for use in unresectable or metastatic melanoma. The first-phase I trial was in patients with metastatic melanoma and the $BRAF^{V600E}$ mutation. A clinical response was demonstrated in $BRAF^{V600E}$-positive patients only. A further phase I trial with vemurafenib was carried out in three $BRAF^{V600E}$-positive patients with metastatic PTC. A partial response was seen in one patient and prolonged stabilisation of disease in the other two patients. An international multi-centre phase II trial is now underway [38]. Initial results with 51 $BRAF^{V600E}$-positive patients are encouraging, with a median progression-free survival of 15.6 months in patients having not received previous TKI therapy.

Conclusion

$BRAF^{V600E}$ positivity in our cohort of patients was a predictor of worse disease-related outcomes in PTC, independent of traditional risk factors. Our data demonstrate that $BRAF^{V600E}$ is associated with more aggressive clinicopathological features, a higher risk of structural recurrence and reduced disease-free survival.

Clinical guidelines categorising patients into low- or high-risk groups do not yet consider $BRAF^{V600E}$ status. However, due to the ongoing controversy around the extent of initial surgery in PTC, RAI dosages and new emerging treatments targeted at the $BRAF^{V600E}$ mutation, $BRAF^{V600E}$ mutation testing by immunohistochemistry should be considered in the routine histological evaluation of papillary thyroid carcinoma.

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