Original Article

Is $BRAF^{V600E}$ mutation a marker for central nodal metastasis in small papillary thyroid carcinoma?

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ABSTRACT

Utilizing *BRAF*^{V600E} mutation as a marker may reduce unnecessary prophylactic central neck dissection (pCND) in clinically-nodal negative (cN0) neck for small (≤2cm) classical papillary thyroid carcinoma (PTC). We aimed to assess whether *BRAF* is a significant independent predictor of occult central nodal metastasis (CNM) and its contribution to the overall prediction after adjusting for other significant preoperative clinical factors in small PTC. Primary tumor tissue (paraffin-embedded) from 845 patients with small classical cN0 PTC who underwent pCND was tested for *BRAF* mutation. Clinicopathologic factors were compared between those with and without *BRAF*. *BRAF* was evaluated to see if it was an independent factor for CNM. Prediction scores were generated using logistic regression models and their predictability was measured by the area under the ROC curve (AUC). The prevalence of *BRAF* was 628/845 (74.3%) while the rate of CNM was 285/845 (33.7%). Male sex (OR=2.68,95%CI=1.71-4.20), large tumor size (OR=2.68,95%CI=1.80-4.00), multifocality (OR=1.49,95%CI=1.07-2.09), lymphovascular permeation (OR=10.40,95%CI=5.18-20.88) and *BRAF* (OR=1.65,95%CI=1.10-2.46) were significant independent predictors of CNM while coexisting Hashimoto's thyroiditis (OR=0.56,95%CI=0.40-0.80) was an independent protective factor. The AUC for prediction score based on tumor size and male sex was similar to that of prediction score based on tumor size, male sex and *BRAF* status (0.68 vs. 0.69,*p*=0.60). Although *BRAF* was an independent predictor of CNM, knowing its status did not substantially improve the overall prediction. A simpler prediction score based on male sex and tumor size might be sufficient.

INTRODUCTION

Papillary thyroid carcinoma (PTC) is the most common type of differentiated thyroid carcinoma with an adjusted incidence doubled over the last 20 years (Kilfoy *et al*., SEER 2013, HKCR 2013). Despite its relatively good prognosis, locoregional recurrence (LR) is common (Wong *et al.* 2012). With recognition of the concept of step-wise progression of lymph node metastasis originating from the central (level VI) to the lateral compartment (levels II-V) and the fact that preoperative ultrasonography (USG) only identifies approximately half of the central nodal metastasis (CNM), a growing number of surgeons have advocated routine prophylactic central neck dissection (pCND) at the time of the total thyroidectomy (TT) (Machens *et al.* 2009, Hwang *et al.* 2011, Roh *et al.* 2009). However, this remains controversial particularly in low-risk PTC as the American Thyroid Association (ATA) only recommends central neck dissection (CND) in clinically involved (cN1) neck lymph nodes or in T3 and T4 tumors (Cooper *et al.* 2009). Although a recent meta-analysis has found that those with cN0 neck who undergo pCND might have reduced risk of LR than those who undergo TT-alone in the short-term, the former group has higher risks for temporary hypoparathyroidism and overall morbidity (Lang *et al*. 2013a). Therefore, identification of predictive factors for occult CNM is crucial to reduce unnecessary pCND (Koo *et al*. 2009, Hartl *et al*. 2012, Zhao *et al*. 2013, Zhang *et al*. 2012).

In recent years, a T1799A point mutation in the v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) resulting in a valine-to-glutamic acid switch at codon 600 ($BRAF^{V600E}$) has emerged as a molecular marker for aggressive behavior in PTC (Xing *et al.* 2005, Xing *et al.* 2013a). Previous studies have found that *BRAF*+ve tumors are significantly larger in size, more frequent lymph node metastasis and extrathyroidal extension and also higher tumor stage, risk of LR and

disease-related mortality than *BRAF*-ve tumors (Li *et al.* 2012, Alzahrani & Xing. 2013, Frasca *et al.* 2008, Xing *et al.* 2009, Xing *et al.* 2013b, O'Neill *et al.* 2010, Kim *et al.* 2012). Therefore, in addition to the existing prognostic staging systems,(Lang *et al.* 2007a) *BRAF* mutation could be used as a potential marker for stratifying tumor risk (Xing *et al.* 2009, Yip *et al.* 2009, Howell *et al.* 2013). Previous studies have examined the utility of *BRAF* mutation testing in optimizing surgical management and suggested that *BRAF*+ve patients may benefit from more extensive initial surgery such as pCND (Xing *et al.* 2009, O'Neill *et al.* 2010, Yip *et al.* 2009, Joo *et al.* 2012). Joo *et al.* evaluated the utility of *BRAF* mutation by pyrosequencing on 148 preoperative fine needle aspiration (FNA) specimens and concluded that preoperative *BRAF* analysis by FNA could help to predict occult CNM (Joo *et al.* 2012). However, most studies only evaluated the association of *BRAF* with overall presence of lymph node metastasis rather than occult CNM alone (Frasca *et al*. 2008, Xing *et al.* 2009, O'Neill *et al.* 2010, Kim *et al,* 2012, Yip *et al.* 2009, So *et al.* 2011, Kim *et al.* 2006, Nam *et al.* 2012). In addition, there have been few studies adopting the strict definition of a pCND when examining the association between *BRAF* mutation and lymph node metastasis (Howell *et al*. 2013, Paulson *et al.* 2012, Lee *et al.* 2-12, Dutemhefner *et al* 2013). Furthermore, in some studies (Xing et al. 2005, Frasca et al. 2008, So *et al.* 2011, Kim *et al.* 2006, Nam *et al.* 2012), after adjusting for other significant clinicopathologic factors such as age, sex, multifocality, tumor size and extrathyroidal extension, *BRAF* became non-significant. Therefore, currently there is still insufficient data to support pCND on the basis of *BRAF* mutation status alone in low-risk PTC (Xing *et al.* 2013a). Given these controversies, our study aimed to assess whether *BRAF* mutation was a significant independent predictor of occult CNM in cN0 neck and also the role of *BRAF* mutation in

89 contributing to the overall prediction after adjusting for other significant preoperative clinical
90 factors in a large cohort of small (≤2cm) PTC.

91

PATIENTS AND METHODS

Patients

The present study protocol was approved by the local institutional review board (IRB No:H-1305-020-486). All consecutive patients who underwent total thyroidectomy and CND at Seoul National University Hospital from December 2008 – November 2012 were retrospectively analyzed. All data were collected prospectively. Patients who were diagnosed preoperatively by FNA or intraoperatively on frozen section were included. Figure 1 shows the study flow chart. Altogether there were 1916 patients with small (≤2cm) classic PTC who underwent total thyroidectomy and CND. All tumors classified as histological variants of PTC (including follicular variant) (n=52) (see Table 1) or with pathologic size >2.0cm were excluded. Of the 1916 patients, 168 (8.8%) were excluded because *BRAF* testing was not done or available while 457 (23.9%) were excluded because they were suspicious of or cytologically-confirmed to have lymph node metastases detected on preoperative neck USG or intraoperative evaluation. Within this latter group, 363 patients subsequently underwent lateral selective neck dissection while the other 94 underwent therapeutic CND. Therefore, there were 1291 clinically nodal negative PTC patients who underwent TT + prophylactic CND (pCND) and had their tumor tissue tested for *BRAF* mutation. To ensure an adequate pCND specimen, those patients with less than 3 central lymph nodes (CLNs) harvested by pCND were excluded (n=446). Therefore, 845 patients were eligible for analysis. However, since a substantial proportion of patients were excluded, patient/tumor characteristics were compared between the two groups to look for possible selection bias on the basis of CLN yield.

Methods

DNA isolation from surgical specimen and FNA samples

B-type Raf Kinase V600E (*BRAF*^{V600E}) mutation analysis from surgical specimen was conducted prospectively and routinely for all patients with PTC after February 2009. From the surgical specimen, areas of tumor were identified on hematoxylin and eosin (H&E) stained slides, marked by pathologists and dissected using a fine needle from 10-μm-thick unstained sections. In patients with bilateral or multifocal tumors, only the largest focus was examined for the *BRAF*^{V600E} mutation. Genomic DNA was isolated by incubation with extraction buffer [1 M Tris-HCl, pH 7.4; 0.5 Methylenediaminetetraacetic acid (EDTA), pH 8.0, 5% Tween 20] and proteinase K at 60°C for 12–15 h, followed by standard phenol-chloroform extraction and ethanol precipitation.

To see correlation of *BRAF* between surgical specimen and FNA sample, the results of *BRAF* test from the two materials were compared in 19 patients who had *BRAF* mutation analysis from FNA samples before surgery. All FNAs were carried out under ultrasound guidance. All aspirations (usually 2 passes for each lesion) were obtained with 25-gauge or 27-gauge needles. The aspirated material was fixed with a hemolytic and preservative solution (Cytolit; Hologic Cytyc Company) after rinsing the needle into this solution. The resulting slide was fixed in 95% ethanol and stained with Papanicolaou. DNA extraction was performed on FNA samples using the ThinPrep 2000 system (Hologic Cytyc Company) using the QIAamp tissue kit (Qiagen, Hilden, Germany).

BRAF^{V600E} mutation analysis

The *BRAF* exon 15, which contains the most common *BRAF* mutation, a T1799A transversion (*BRAF*^{V600E}), was amplified by polymerase chain reaction (PCR) with genomic DNA. The primers and PCR conditions were as follows: forward, 5'-TCATAATGCTTGCTCTGATAGGA-3'; reverse 5'-GGCCAAAAATTTAATCAGTGGA-

3'; denaturation at 94°C for 10 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final extension step at 72°C for 10 min. After purification of the PCR products with the QIAGEN-QIAquick PCR purification kit (QIAGEN, Hilden, Germany), direct DNA bidirectional sequencing was done with an ABI 3130XL Genetic Analyzer BigDye Terminator (Applied Biosystems, Foster City, CA). Sequence data were analyzed manually by two independent pathologists

Management of PTC

A preoperative USG was routinely performed to examine both central and lateral neck compartments with any suspicious nodes aspirated for cytology. TT was the preferred procedure for all patients with a preoperative diagnosis of PTC. Once the diagnosis of PTC had been confirmed by frozen section, regardless of the tumor size or local extent, an ipsilateral pCND was performed for unifocal tumors while a bilateral pCND was performed for bilateral or isthmic tumors. All pCND were carried out in accordance to anatomical landmarks described by the ATA (Carty *et al*. 2009) and were performed immediately after the completion of the TT. It comprised the removal of all nodes and fibro-fatty tissue extending vertically from the hyoid bone to the thoracic inlet and laterally from the medial border of common carotid artery to the midline of the trachea. The ipsilateral recurrent laryngeal nerve (RLN) was mobilized and skeletonized along its entire cervical course.

Postoperative assessment

All post-surgical patients were followed up within 1-2 weeks and then 2-3 monthly for the first year. Those taking calcium +/- calcitriol supplements were followed more frequently with an aim of gradually weaning off these supplements while maintaining normocalcemia. By definition, those who discontinued all supplements in the presence of normocalcemia ≤ 6 months after

surgery were regarded as temporary hypoparathyroidism whereas those who continued for >6 months were categorized as permanent hypoparathyroidism. Also both vocal cords were examined endoscopically 1-2 days before and within 2 weeks after thyroidectomy using flexible laryngoscope. Any reduction in cord movement was recorded as vocal cord palsy. Those with vocal cord palsy were examined every 3 months. The presence of cord palsy lasting > 6 months was regarded as permanent.

Follow-up protocol

All post-surgical patients were followed up within 2 weeks in a specialized oncology clinic. A follow-up visit was conducted at 3-month, 6-month and then annually thereafter. Clinical examination, neck USG and non-stimulated Tg level were done during follow-up visits. Stimulated thyroglobulin (sTg) was defined as a Tg level measured in the presence of TSH >30 mIU/L either by thyroxine withdrawal or recombinant TSH injections. Radioiodine (RAI) ablation and pre-ablation sTg level were done approximately 3 months after surgery (because most patients would have had a contrast CT before they were referred to us for neck USG and surgery) while the post-ablation sTg level was taken approximately 9 months after surgery (6-7 months after RAI ablation). Tg autoantibodies were measured at the same time. The decision for RAI was based on presence of ≥1 risk factors such as tumor size >1.5cm, lymph node metastasis, age >45 years old, extrathyroidal extension, macroscopic postoperative residual disease in the neck and distant metastasis. Thirty millicuries (mCi) I131 was the standard ablative dose for low-risk PTC. TSH suppression to <0.1 mIU/L was recommended for high- and intermediate-risk patients. All relevant clinical, laboratory, radiologic, and perioperative data were collected prospectively and follow-up data were regularly updated in a computerized database.

Statistical analysis

Continuous variables were expressed as mean±SD and groups were compared using the Mann-Whitney U test. Chi-square tests were used to compare categorical variables. Any clinicopathologic features which were statistically significantly associated with occult CNM in the univariate analysis were entered into multivariate analysis by logistic regression to determine independent factors and to formulate combined prediction scores based on the regression coefficients. The area under a receiver characteristic (ROC) curve (AUC) was used to measure the relative predictability of independent factors and combined prediction scores. AUC values close to 1.00 meant better predictability whereas close to 0.500 meant poorer predictability. A bootstrap approach with 1,000 resamples was used to compare AUCs and to estimate 95% confidence intervals for each AUC. All statistical analyses were conducted using SPSS version 18.0 (SPSS, Inc., Chicago, IL, USA) and R version 2.14.0 (R Foundation for Statistical Computing, Vienna, Austria). P values below 0.05 were considered statistically significant.

RESULTS

Our cohort was mostly females (86.7%). The mean (±SD) and median (range) age at operation were 45.7 ± 11.9 and 46.0 (12.0 – 77.0) years old, respectively. The mean (±SD) tumor size was 0.8 ± 0.4cm. The mean (±SD) number of CLNs and positives CLNs removed were 6.6 ± 3.8 and 0.9 ± 1.8, respectively. The overall rate of occult CNM was 285/845 (33.7%) while the rate of *BRAF*+ve mutation in primary tumors was 628/845 (74.3%).

Table 2 shows a comparison of patient characteristics between those with ≥3 CLNs and with <3 CLNs. There were no significant differences except for a higher concomitant Hashimoto's thyroiditis (HT) ($p<0.001$) and CNM ($p<0.001$) for those with ≥3 CLNs.

Table 3 shows a comparison of patient clinicopathological features, tumor characteristics and *TNM* tumor stages between *BRAF*+ve and *BRAF*–ve groups. Age and sex ratio were similar between the two groups. The *BRAF*+ve group had significantly larger sized tumors (0.8cm vs. 0.7cm, $p<0.001$) and higher incidence of extrathyroidal extension (61.0% vs. 43.3%, $p<0.001$) and occult CNM (37.4% vs. 23.0%, $p<0.001$) while the incidence of coexisting HT was significantly less (34.6% vs. 52.5%, $p<0.001$) than the *BRAF*-ve group. The number of CLNs harvested was similar between the two groups regardless of the extent of pCND but the overall number of metastatic CLNs excised and the central lymph node ratio (CLNR) in the *BRAF*+ve group were significantly higher than in the *BRAF*-ve group (1.0 vs. 0.7, $p<0.001$ and 16.1% vs. 10.6%, $p<0.001$). However, when stratified into unilateral and bilateral pCND, these significant differences were not observed with bilateral pCND. The *BRAF*+ve group had significantly higher proportion of stage III tumors and a corresponding lower proportion of stage I tumors than *BRAF*-ve group. As a result, RAI ablation was given more frequently in the *BRAF*+ve group (35.2% vs. 22.6%, $p=0.001$). After excluding those with elevated anti-Tg antibody, the pre-

ablation sTg level in the *BRAF*+ve group was significantly higher than the *BRAF*-ve group (2.4ug/L vs. 1.0ug/L, p=0.032) while the post-ablation sTg was similar (0.6ug/L vs. 0.2ug/L, p=0.473).

Table 4 shows a comparison of patient clinicopathologic features, tumor characteristics and *BRAF* mutation status between those with (N1a group) and those without occult CNM (N0 group). Age was similar between the two groups but the proportion of males was significantly higher in the N1a group (22.5% vs. 8.6%, p=0.023). Also N1a group had significantly larger sized tumors (0.8cm vs. 0.7cm, p=0.001) and higher incidence of tumor multifocality (41.4% vs. 31.1%, p=0.003), extrathyroidal extension (69.1% vs. 50.0%, p<0.001), lymphovascular permeation (20.4% vs. 2.1%, p<0.001) and *BRAF*+ve mutation status (82.5% vs. 70.2%, p<0.001). However, N1A group had significantly lower incidence of coexisting HT than N0 group (26.7% vs. 45.5%, p<0.001)

Table 5 shows the multivariate analysis for occult CNM. Male sex (OR=2.681, 95%CI=1.709 4.202, p<0.001), large tumor size (OR=2.684, 95%CI=1.802 – 3.997, p<0.001), tumor multifocality (OR=1.491, 95%CI=1.065 – 2.087, p=0.020), lymphovascular permeation (OR=10.395, 95%CI=5.176 – 20.877), p<0.001), and *BRAF*+ve mutation (OR=1.647, 95%CI=1.101 – 2.463, p=0.015) were independent risk factors while coexisting HT (OR=0.560, 95%CI=0.396 – 0.792, p=0.001) was an independent protective factor for occult CNM.

Since only male sex, tumor size and *BRAF*+ve mutation are potentially known before operation (i.e without histopathology), these 3 factors were used to formulate a preoperative prediction score by logistic regression. Table 6a shows a comparison of predictability as measured by area under the receiver operating characteristic curve (AUC) between tumor size and two combined prediction scores. Although the AUC of the three prediction scores were not significantly

different, the most important was that the AUC for prediction score 3 (based on tumor size, male sex and *BRAF*) was not significantly higher than that of prediction score 2 (based on tumor size and male sex) (0.69 vs. 0.68, p=0.60). Therefore, despite being an independent predictor in the multivariate analysis (see Table 5), knowing the *BRAF* mutation status did not add substantially to the overall prediction of occult CNM. Table 6b shows a comparison of occult CNM rate between each quartile of prediction score 2 and 3. For both scores, the chance of occult CNM increased from <20% to 55% as the prediction score increased from the first to the fourth quartile. Table 7 shows the correlation of *BRAF* mutation status between FNA and surgical specimen. Of the 19 patients, 17 had matched *BRAF* results while 2 had mismatched results. For these 2 mismatched cases, both were *BRAF*+ve on FNA but *BRAF*-ve on surgical specimen. The correlation rate between FNA and surgical specimen was 89.5%.

Table 8a shows a 2x2 table between *BRAF* mutation and CNM. The sensitivity and specificity of *BRAF*+ve mutation status in predicting occult CNM were 235/285 (82.5%) and 167/560 (29.8%), respectively while the positive (PPV) and negative predictive values (NPV) were 235/628 (37.4%) and 167/217 (77.0%), respectively. To simulate what might happen with lower *BRAF* prevalences, Table 8b shows a 2x2 table between *BRAF* positivity and CNM when the *BRAF* prevalence was lowered to 40%. The sensitivity, specificity, PPV and NPV became 51.9%, 64.8%, 37.3% and 76.9%, respectively.

In terms of clinical outcomes, rate of temporary and permanent hypocalcemia were 32.7% and 1.9%, respectively while temporary and permanent RLN injury were 8.9% and 1.4%. After a mean follow-up of 9.4 ± 5.4 months, there was no LR detected.

DISCUSSION

The optimal initial surgical management for PTC patients without preoperative or intraoperative evidence of nodal involvement (i.e. cN0 PTC) remains controversial as the ATA currently only recommends CND for those with cN1 PTC. However, since pCND may reduce LR in the short-term (Lang *et al*, 2013a), a more selective approach to minimize overall surgical morbidity would seem sensible and perhaps, cost-saving in the long-term (Lang *et al*. 2013a, Lang & Wong 2013b). It is worth noting that despite our cohort comprised of patients with no evidence of clinical or ultrasound evidence of CNM, the presence of occult CNM was still 33.7%. This finding is of interest because of the recent discussions on whether pCND is justified and on whether RAI should be given more selectively (Cooper *et al*. 2009). In terms of surgical morbidity, our rates of hypocalcemia and RLN injury after pCND was not significantly higher or different from our previous series without pCND performed (Chung *et al*. 2007) and were comparable to the literature (Lang *et al.* 2013a).

To our knowledge, this is one of the largest studies examining the association between *BRAF* mutation and occult CNM in cN0 PTC. To ensure that *BRAF* was truly a preoperative rather than a postoperative predictor, a small proof of principle series of 19 FNA cases was conducted and showed an 89.5% correlation of *BRAF* between FNA samples and surgical specimens. Similar to previous studies (Li *et al*. 2012, Frasca *et al*. 2008, Xing *et al*. 2009), our data confirmed that the *BRAF*+ve group had significantly larger, more advanced and aggressive tumors than the *BRAF*-ve group. It was interesting to find that the *BRAF*+ve group had significantly less coexisting HT on histology (34.6% vs. 52.5%, $p<0.001$). This finding appeared to concur to previous studies which found reduced peritumoral lymphocytic infiltration in *BRAF*+ve PTCs (Virk *et al.* 2013,

Sargent *et al.* 2006). Although the precise reason for this remains unclear, a recent study demonstrated that tumors with coexisting HT behaved less aggressively and had a better prognosis than those without coexisting HT (Dvorkin *et al*. 2013). Therefore, this inverse association was in keeping with the concept that *BRAF*+ve tumor behaved more aggressively. Our data also showed that the pre-ablation sTg level was significantly higher in *BRAF*+ve group implying that the risk of microscopic residual disease after a total thyroidectomy with pCND might still have been higher in the *BRAF*+ve group. Nevertheless, the post-ablation sTg was similar and so, a longer follow-up was necessary to evaluate its true impact of *BRAF* on survival outcomes. However, unlike other studies, our study did not find significant association between age, sex, tumor bilaterality and multifocality with *BRAF* mutation (Li *et al*. 2012, Kim *et al* 2006, Nam *et al* 2012).

In terms of predicting occult CNM, male sex, tumor size, tumor multifocality, lymphovascular permeation, coexisting HT and *BRAF* mutation were independent risk factors by multivariate analysis. Although two large previous studies also reported similar findings, neither examined the role of *BRAF* in the context of other significant clinicopathological factors (So *et al*. 2011, Zhang *et al*. 2012). Paulson *et al.* reported their experience of 175 classic cN0 PTC but found no association between *BRAF* mutation and occult CNM (Paulson *et al.* 2012). Two similarly-designed but smaller studies also did not find any significant association between *BRAF* mutation and occult CNM (Lee *et al.* 2012, Dutenhefner *et al.* 2013). In fact, in one of the studies, the authors went further and concluded that it was premature in utilizing *BRAF* mutation status to decide whether or not to perform pCND in cN0 PTC (Lee *et al.* 2012). In contrast to these previous studies, although we did find that *BRAF* mutation status (OR=1.65, 95%CI=1.101 – 2.463) was an independent predictor of occult CNM in cN0 PTC, it did not

contribute significantly to the overall prediction. When formulating preoperative prediction scores using male sex, tumor size and BRAF+ve mutation, although the predictability (as measured by AUC) improved with each additional factor entered into the prediction score (i.e. from prediction score 1 to 3), the improvement in predicting occult CNM was not statistically significant. Our data found that using a simpler prediction score of tumor size and male sex alone, the prediction (as measured AUC) was similar to a more complicated prediction score of tumor size, male sex and *BRAF* mutation (0.68 vs. 0.69, p=0.60). Given the fact that *BRAF* testing is associated with extra cost, perhaps a simpler prediction score based on male and tumor size might be sufficient. Therefore, although *BRAF* mutation was an independent predictor for occult CNM, it did not substantially or significantly improve the overall prediction of occult CNM in cN0 patients. Despite the high pre-test probability (74.3%) of *BRAF* positivity, both the specificity (29.8%) and PPV (37.4%) were relatively low and so these further emphasized the fact that *BRAF* mutation was not useful in predicting CNM in small cN0 PTC.

However, it is worth noting that based on the adjusted OR, the *BRAF*+ve tumor in our study only had a 1.6 – 1.7 times greater chance of harboring occult CNM than a *BRAF*-ve tumor whereas to date, two other studies which found significant association had almost twice as high adjusted OR values (Howell *et al* 2013, Joo *et al*. 2012). Perhaps, in these studies, *BRAF* mutation might have a more significant impact on the overall prediction. Also we would like to acknowledge several shortcomings. Firstly, this was a retrospective analysis and so was prone to selection biases. Secondly, although our series of 19 FNA cases did show a 89.5% correlation between FNA samples and surgical specimens, our study was principally based on paraffin-embedded sections after thyroidectomy and so our results might be slightly different from studies which tested *BRAF* mutation primarily from FNA samples. Therefore, our study could not be strictly

considered to be examining the association between preoperative *BRAF* mutation and occult CNM. Nevertheless, even assuming that our study was entirely based on FNA samples, our conclusion would not have changed because this would have further lowered the predictability of *BRAF* mutation due to the lower detection *BRAF* on FNA samples (Yip *et al.* 2009). Thirdly, due to the strict definition of pCND, over a third of patients with inadequate number of CLNs had to be excluded from analysis. Although by excluding such substantial number of patients may introduce selection bias, the comparison of patient/tumor characteristics between those with ≥3CLNs (n=845) and with <3CLNs (n=446) did not reveal significant differences (Table 2). The only differences were those with ≥3 CLNs had significantly higher percentages of coexisting HT and CNM than those with <3 CLNs. The former finding could be explained by the fact that HT tended to have larger-sized CLNs and that led to higher CLN yield (Hartl *et al.* 2012) while the latter finding was probably due to inadequate nodes sampled and nodal under-staging (Lang *et al*. 2007b, Lang *et al*. 2012). Lastly, we would like to highlight the fact that our overall prevalence of *BRAF* positivity was relatively high (74.3%) when compared to that of other studies when only classical PTC were considered (≈45%) (Lee *et al.* 2012, Xing *et al*. 2013b). This is particularly interesting given the fact that these patients had small cN0 PTC. Although by including only the classical subtype of PTC did increase the overall prevalence of *BRAF* positivity from 72.9% to 74.3%, this increase was small because these variants only accounted for 5.8% of the entire cohort (see Table 1). Therefore, the exact reason for such high prevalence of *BRAF* positivity in our cohort remains unclear and may be due to geographical, genetic or diet-linked factors, as suggested previously (Frasca *et al*. 2008). However, it is worth noting that in our locality, the prevalence of BRAF positivity has been reported to be much higher (60-70%) than other parts of the world (Chung *et al*. 2006, So *et al.* 2011) and so this was unlikely due to a

selection or institutional bias. When the prevalence of *BRAF* mutation was lowered, our data showed that only the sensitivity and specificity of *BRAF* reversed while PPV and NPV remained static (see Table 8A and 8B). Although the absolute risk predicted by our model (Table 6b) may differ slightly with lower *BRAF* mutation prevalence, we think that the increased risk of occult CNM associated with *BRAF* should be generalizable. However, we would acknowledge the applicability of *BRAF* mutation as a marker to reduce unnecessary pCND could be weakened due to the high prevalence of *BRAF* positivity in our cohort. Nevertheless, this was one of the largest studies aimed at examining the association between *BRAF* mutation and occult CNM in small cN0 PTC.

Conclusion

Among the cN0 PTC patients who underwent pCND, the *BRAF*+ve tumors were significantly larger in size, had more extrathyroidal extension, occult CNM, higher CLNR, pre-ablation sTg level but less coexisting HT than the *BRAF*-ve tumors. Male sex, large tumor size, tumor multifocality, LV permeation and *BRAF* mutation were significant independent predictors of occult CNM while coexisting HT was a significant independent protective factor. When *BRAF* mutation was entered into logistic regression to formulate a prediction score, that score was not significantly better than that of a prediction score based on male and tumor size only. Therefore, based on our analysis using primarily paraffin-embedded tissue, despite being an independent predictor of CNM, *BRAF* did not add substantially to the overall prediction of occult CNM. Given the extra cost associated with *BRAF* testing, a simpler prediction score based on male and tumor size might be sufficient.

Declaration of interest

The authors declare that they have no competing interests

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None

Authors contributions

Lang / Chai / Cowling / Kim / Lee / Min were involved in the review of literature, acquisition of data and drafting and completing the manuscript. Lang / Chai / Cowling / Kim / Lee / Min were also involved in the review of literature and drafting the manuscript. Lang / Chai / Cowling / Kim / Lee / Min conceived the study, participated in the co-ordination and the acquisition of data and helped to draft the manuscript. All authors read and approved the final manuscript.

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FIGURE LEGEND

Figure 1. The study flowchart

521 Table 1. Prevalence of *BRAF* mutation in the classic papillary thyroid carcinoma (n=845) and the
522 excluded histopathologic variants (n=52)

Variant of papillary thyroid carcinoma	*BRAF* mutation (%)
- Classic / conventional (n=845)	628 (74.3)
- Follicular variant (n=21)	7 (33.3)
- Tall cell (n=15)	14 (93.3)
- Oncocytic (n=11)	4 (36.4)
- Diffuse sclerosing (n=2)	1 (50.0)
- Solid cell (n=2)	0 (0.0)
- Clear cell (n=1)	0 (0.0)

523

524

525 Table 2. A comparison of patient/tumor characteristics between those with ≥3 central lymph
526 nodes (CLNs) harvested and those with <3CLNs harvested during prophylactic central neck
527 dissection

	Patients with ≥3CLNs harvested (n=845)	**Patients with <3CLNs harvested (n=446)**	***p*-value**
Age at operation (years)	45.7 ± 11.9	46.5 ± 11.7	0.218
Sex			0.116
- Male	112 (13.3)	76 (17.0)	
- Female	733 (86.7)	370 (83.0)	
Tumor characteristics			
- Tumor size (cm)	0.8 ± 0.4	0.8 ± 0.4	0.546
- Tumor bilaterality	171 (20.2)	73 (16.4)	0.087
- Tumor multifocality	292 (34.6)	133 (29.8)	0.085
- Extra-thyroidal extension	477 (56.4)	254 (57.0)	0.828
- LV permeation	70 (8.3)	30 (6.7)	0.780
- Coexisting HT	331 (39.2)	60 (13.5)	**<0.001**
- Occult CNM (pN1a)	285 (33.7)	66 (21.4)*	**<0.001**
BRAF mutation	628 (74.3)	338 (75.8)	0.564

528 Abbreviations: LV = lymphovascular; HT = Hashimoto's thyroiditis; CNM = central nodal
529 metastasis

530 *even after excluding those with no CLNs harvested (n=138)

Table 3. A comparison of patient clinicopathological features, tumor characteristics and postoperative stimulated thyroglobulin levels between those with a *BRAF* mutation (*BRAF* +ve group) and without a *BRAF* mutation (*BRAF* –ve group)

	***BRAF*+ve group (n=628)**	***BRAF*-ve group (n=217)**	***p*-value**
Age at operation (years)	45.8 ± 11.9	45.6 ± 11.8	0.802
Sex			0.116
- Male	90 (14.3)	22 (10.1)	
- Female	538 (85.7)	195 (89.9)	
Tumor characteristics			
- Tumor size (cm)	0.8 ± 0.4	0.7 ± 0.4	**<0.001**
- Microcarcinoma (<1cm)	460 (73.2)	177 (81.6)	**0.045**
- Tumor bilaterality	135 (21.5)	36 (16.6)	0.107
- Tumor multifocality	225 (35.8)	67 (30.9)	0.186
- Extra-thyroidal extension	383 (61.0)	94 (43.3)	**<0.001**
- LV permeation	53 (8.4)	17 (7.8)	0.780
- Coexisting HT	217 (34.6)	114 (52.5)	**<0.001**
- Occult CNM (pN1a)	235 (37.4)	50 (23.0)	**<0.001**
Extent of pCND			0.063
- Unilateral	483 (76.9)	179 (82.5)	
- Bilateral	145 (23.1)	38 (17.5)	
No. of CLNs harvested	6.5 ± 3.6	6.9 ± 4.3	0.144
- Unilateral pCND (n=662)	6.0 ± 3.2	6.1 ± 3.0	0.463
- Bilateral pCND (n=183)	8.3 ± 4.3	10.8 ± 6.7	0.105
No. of metastatic CLNs excised	1.0 ± 1.8	0.7 ± 1.7	**<0.001**
- Unilateral pCND (n=662)	0.8 ± 1.6	0.5 ± 1.4	**<0.001**

- Bilateral pCND (n=183)	1.4 ± 2.3	1.5 ± 2.4	0.886
Central LNR (%)	16.1 ± 26.7	10.6 ± 24.2	**<0.001**
- Unilateral pCND (n=662)	14.7 ± 25.6	8.8 ± 22.4	**<0.001**
- Bilateral pCND (n=183)	20.7 ± 29.4	18.3 ± 30.9	0.542
Stage of PTC by *TNM*			**0.008**
- Stage I	374 (59.6)	155 (71.4)	
- Stage II	3 (0.5)	1 (0.5)	
- Stage III	251 (40.0)	61 (28.1)	
Postsurgical RAI ablation	221 (35.2)	49 (22.6)	**0.001**
Pre-ablation			
- TSH (mIU/L)	99.3 ± 92.2	91.3 ± 59.1	0.539
- sTg level (ug/L)*	2.4 ± 12.7	1.0 ± 1.6	**0.032**
Post-ablation			
- TSH (mIU/L)	119.0 ± 56.1	107.4 ± 40.0	0.356
- sTg level (ug/L)*	0.6 ± 1.8	0.2 ± 0.1	0.473

534 Continuous variables are expressed as mean ± SD; categorical variables are expressed as number
535 (percentage)

536 Abbreviations: PTC = papillary thyroid carcinoma; HT = Hashimoto's thyroiditis; LV=
537 lymphovascular; CLN=central lymph node; CNM = central nodal metastasis; pCND =
538 prophylactic central neck dissection; LNR= lymph node ratio; *TNM* = 7th edition Tumor, Node
539 and Metastasis staging system; RAI = radioactive iodine; TSH=thyroid stimulating hormone;
540 sTg=stimulated thyroglobulin

541 *after excluding patients with elevated anti-thyroglobulin antibody

Table 4. A comparison of patient clinicopathologic features and *BRAF* mutation status between those with occult central nodal metastases (N1a group) and those without occult central nodal metastases (N0 group)

	N1a group (n=285)	**N0 group (n=560)**	***p*-value**
Age at operation (years)	45.8 ± 11.9	45.6 ± 11.8	0.285
Sex (Male : Female)	64 : 221	48 : 512	**0.023**
Tumor characteristics			
- Tumor size (cm)	0.8 ± 0.4	0.7 ± 0.4	**0.001**
- Tumor bilaterality	66 (23.2)	105 (18.8)	0.099
- Tumor multifocality	118 (41.4)	174 (31.1)	**0.003**
- Extra-thyroidal extension	197 (69.1)	280 (50.0)	**<0.001**
- LV permeation	58 (20.4)	12 (2.1)	**<0.001**
- Coexisting HT	76 (26.7)	255 (45.5)	**<0.001**
BRAF V600E mutation	235 (82.5)	393 (70.2)	**<0.001**

Abbreviations: HT = Hashimoto's thyroiditis; LV = lymphovascular

Table 5. A multivariable analysis of clinicopathological risk factors for occult central lymph node metastases (N1a)

Covariates	**ß-coefficient**	**Odds ratio (95% confidence interval)**	***p*-value**
Male sex	0.986	2.681 (1.709 – 4.202)	**<0.001**
Tumor size	0.987	2.684 (1.802 – 3.997)	**<0.001**
Tumor multifocality	0.399	1.491 (1.065 – 2.087)	**0.020**
Extrathyroidal extension	0.248	1.282 (0.898 – 1.829)	0.171
Lymphovascular permeation	2.341	10.395 (5.176 – 20.877)	**<0.001**
Coexisting Hashimoto's thyroiditis	0.580	0.560 (0.396 – 0.792)	**0.001**
BRAF V600E mutation	0.499	1.647 (1.101 – 2.463)	**0.015**

Table 6a. A comparison of predictability of central nodal metastasis as measured by area under the receiver operating characteristic curve (AUC) between tumor size and combined preoperative prediction scores.

	AUC (95% confidence interval)	**p-value score 1 vs. 2**	**p-value score 2 vs. 3**	**p-value score 1 vs. 3**
Prediction score 1 based on tumor size only	0.65 (0.61 – 0.69)	0.33	-	-
Prediction score 2 based on tumor size and male sex	0.68 (0.64 – 0.72)	-	0.60	-
Prediction score 3 based on tumor size, male sex and *BRAF* mutation	0.69 (0.65 – 0.73)	-	-	0.13

Calculated from logistic regression:

Prediction score 1 = -1.716 + 1.288 x (tumor size in cm)

Prediction score 2 = -1.873 + 1.102 (male=1; female=0) + 1.283 x (tumor size in cm)

Prediction score 3 = -2.278 + 1.084 (male=1; female=0) + 1.246 x (tumor size in cm) + 0.569 (*BRAF*+ve=1; *BRAF*-ve=0)

The higher the prediction score corresponds to higher risk of occult central nodal metastasis

Table 6b. A comparison of central nodal metastasis (CNM) rate for each quartile of prediction score 2 and 3.

	Prediction score 2*	**CNM (%)**	**Prediction score 3***	**CNM (%)**
1st quartile	0.00 – 0.51	48/249 (19.3)	0.00 – 0.94	40/228 (17.5)
2nd quartile	0.52 – 0.89	67/245 (27.3)	0.95 – 1.31	65/233 (27.9)
3rd quartile	0.90 – 1.41	62/160 (38.8)	1.32 – 1.81	64/177 (36.2)
4th quartile	>1.42	108/191 (56.5)	> 1.82	116/207 (56.0)

Prediction score 2 = -1.873 + 1.102 (male=1; female=0) + 1.283 x (tumor size in cm)

Prediction score 3 = -2.278 + 1.084 (male=1; female=0) + 1.246 x (tumor size in cm) + 0.569 (*BRAF*+ve=1; *BRAF*-ve=0)

*To avoid negative values and facilitate interpretation, +1.74 was added to each prediction score 2 while +2.15 was added to each prediction score 3. This makes no difference to the performance of the score.

569 Table 7. Correlation of *BRAF* mutation status between using fine-needle aspiration (FNA) materials and surgical specimen

Patient no.	**Age at operation (yrs)**	**Sex (M/F)**	**Tumor size (cm)**	**Occult CNM (pN1a)**	***BRAF* mutation**		
					On FNA	**On surgical specimen**	**Matching between FNA and surgical specimen**
1	37	F	0.5	Negative	Negative	Negative	Matched
2	46	F	1.0	Negative	Negative	Negative	Matched
3	39	F	0.6	Positive	Positive	Positive	Matched
4	46	F	0.5	Negative	Positive	Positive	Matched
5	50	F	0.9	Negative	Positive	Positive	Matched
6	73	F	0.6	Negative	Negative	Negative	Matched
7	54	F	0.9	Positive	Negative	Negative	Matched
8	68	F	0.4	Negative	Positive	Negative	Mismatched
9	31	F	1.2	Positive	Positive	Positive	Matched
10	50	M	0.5	Negative	Positive	Positive	Matched
11	39	F	0.4	Negative	Negative	Negative	Matched
12	55	F	0.6	Negative	Positive	Positive	Matched
13	57	F	0.3	Negative	Positive	Positive	Matched
14	63	F	0.3	Negative	Negative	Negative	Matched
15	30	F	0.5	Positive	Positive	Positive	Matched
16	34	F	0.6	Positive	Positive	Negative	Mismatched
17	55	F	0.3	Negative	Positive	Positive	Matched

18	44	F	0.3	Negative	Negative	Negative	Matched
19	50	F	2.0	Positive	Positive	Positive	Matched

570

Tables 8A. A 2x2 table between *BRAF* mutation and central nodal metastasis (CNM)

	CNM+ve	**CNM-ve**	**Total**
***BRAF*+ve**	235	393	628
***BRAF*-ve**	50	167	217
Total	285	560	845

Based on these data, the sensitivity, specificity, PPV and NPV of *BRAF* were 82.5%, 29.8%, 37.4% and 77.0%, respectively.

Table 8B. A 2x2 table between *BRAF* mutation and central nodal metastasis (CNM) when the *BRAF* prevalence was reduced to 40%.

	CNM+ve	**CNM-ve**	**Total**
***BRAF*+ve**	126	212	338
***BRAF*-ve**	117	390	507
Total	243	602	845

Based on these data, the sensitivity, specificity, PPV and NPV of *BRAF* became 51.9%, 64.8%, 37.3% and 76.9%, respectively.