The Role of Molecular Markers and Tumor Histological Type in Central Lymph Node Metastasis of Papillary Thyroid Carcinoma

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Objective: To look for genetic mutations that might predict central compartment lymph node metastasis (LNM) in papillary thyroid carcinoma (PTC) using strict criteria for N0 and N1 disease.

Design: We identified patients with PTC from our institution’s pathology archives. Strict criteria were used for assessing the presence or the absence of central neck LNM. Disease was classified as N0 only if a comprehensive ipsilateral and pretracheal central neck dissection was performed and if pathological analysis revealed no evidence of LNM. Primary tumor samples were analyzed for a panel of known or suspected PTC-associated molecular markers, including BRAF, RET-PTC, KRAS, NRAS, HRAS, PIK3CA, and their variants.

Setting: Academic medical center.

Patients: Three hundred eighty-nine patients with PTC.

Main Outcome Measure: Molecular mutations in tumors with and without LNM.

Results: Of 389 identified cases, 209 fit the inclusion criteria, with 158 classified as node positive (N1) and 51 as node negative (N0). The follicular variant histological type was present in 7 of 158 N1 tumors (4.4%) and 24 of 51 N0 tumors (47.1%) and thus was strongly associated with lack of central neck metastasis in this study (odds ratio, 0.05; 95% CI, 0.02-0.14). Predictive factors for central LNM included extracapsular extension, angiolymphatic invasion, and higher T stage (T3 and T4). The BRAF mutation was more prevalent in the classic PTC histological type than the follicular variant. None of the molecular marker mutations that were analyzed in this study, including the BRAF mutation, predicted LNM in classic PTC.

Conclusions: Positive risk factors for central LNM include male sex, extracapsular extension, angiolymphatic invasion, and advanced T stage. The follicular variant histological type has a significantly lower incidence of central neck metastasis. In contrast to recent studies, the BRAF mutation was not significantly associated with central neck LNM from PTC when using a strict definition of a central neck dissection.
These recommendations, however, are based on expert opinion. The ability to predict which tumors are at higher risk for central neck metastasis would help to stratify who should undergo elective central neck dissection (CND). If this prediction can be made accurately at the time of the initial surgery, the need for reoperation in the central neck and the potential resulting morbidity may be reduced.

Molecular markers may play a role in stratifying this risk. The use of molecular markers of genetic mutations expressed in cancer specimens in the diagnosis and management of papillary carcinoma is rapidly expanding. Molecular markers have the potential to not only improve our diagnostic accuracy but also tailor treatments according to patterns of tumor behavior.

Most identified mutations in PTC lie within the same mitogen-activated protein kinase pathway, and BRAF V600E is the most commonly identified mutation in PTC, with prevalence ranging from 29% to 83%. The genotype results from a point mutation causing constitutive activation in the BRAF gene (OMIM *164757) and consequently the mitogen-activated protein kinase cascade. An advantage of this mutation is that it is highly sensitive and specific for PTC and is not found in follicular carcinoma or benign thyroid tumors.

Many studies have evaluated BRAF with respect to tumor behavior. Several retrospective studies have demonstrated an association of BRAF mutations with aggressive tumor characteristics, such as extracapsular extension, nodal metastasis, and recurrence, although the results are controversial. A recent study by Elisei et al in 2008 also demonstrated increased mortality with BRAF V600E tumors with a median follow-up of 15 years. Arguably the largest study examining BRAF V600E and tumor characteristics is a 2007 meta-analysis by Xing that compiled data from 28 retrospective studies. Although most individual studies alone did not show a significant association, they demonstrated an overall association of the BRAF mutation with extrathyroidal extension, lymph node metastasis, and stages III/IV disease (odds ratios [ORs], 2.50, 1.83, and 2.14, respectively). However, a limitation of the meta-analysis is that many of the studies included categorized cases as node negative without central compartment dissection. Given the high incidence of occult nodes, failure to perform a standard dissection may result in a false-negative N0 classification.

The aim of this study was to characterize the risk of central nodal metastasis by using a rigorous definition of CND and N0 disease. To accomplish this, we studied 2 groups of PTC tumors, those with and those without central lymph node metastasis (LNM), and analyzed a panel of known PTC molecular markers and the specific histological characteristics of the primary tumor.

This retrospective study was approved by our institutional review board. We identified 389 patients with PTC from our institution’s pathology archives. We used strict criteria to assess the presence or the absence of central neck LNM. We included all patients who underwent a total thyroidectomy with adequate CND. The N1 group included patients with at least 1 positive lymph node in the central neck on final pathological examination. Patients were included in the N0 group only if a compartment-oriented CND was performed, including a minimum of an ipsilateral and pretracheal node dissection, and no metastatic disease was identified in the specimen. We excluded 180 patients for the following reasons: 77 for inadequate CND, 60 for recurrent disease, 15 for initial pathological results that were unavailable or at an outside hospital, 12 for insufficient tissue for processing, 6 for incomplete records, 5 for the patient opting out of the study, 4 for hemithyroidectomy alone, and 1 for unknown primary tumor.

All patient medical records were reviewed for age, sex, family history, imaging studies, gross nodal disease at surgery, tumor histological type, extracapsular extension, presence of lymphocytic thyroiditis, angiolympathic invasion, and LNM. All archived specimens were assessed for quality with hematoxylin–eosin–stained sections. Specimens with adequate cellularity underwent further processing. We performed mutational analysis of BRAF exons 11 and 15 and exons 1 and 2 of KRAS (OMIM *190070), NRAS (OMIM +164790), and HRAS (OMIM *190020) as previously reported.

**DNA EXTRACTION**

We prepared DNA from shavings taken from the area of the paraffin block corresponding to areas of tumor on the corresponding slides. The tumor specimens were deparaffinized by serial extraction with xylenes and ethanol and air dried at room temperature. The DNA samples were extracted using commercially available mini kits (Qiagen).

**POLYMERASE CHAIN REACTION AMPLIFICATION**

We amplified DNA by means of polymerase chain reaction (PCR) with initial denaturing of 500 ng of purified tumor DNA at 95°C for 60 seconds, then 42 cycles of PCR (94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds) followed by a final extension at 72°C for 7 minutes using a commercially available PCR system (Expand High Fidelity; Roche). Negative controls were used in each reaction to ensure quality control. Primer pairs used for BRAF, KRAS, NRAS, and HRAS amplification are shown in Table 1.

**RET PROTO-ONCOGENE REARRANGEMENT ANALYSIS**

We detected ret proto-oncogene (RET; OMIM +164761)–PTC1 and RET–PTC3 translocation products in a multiplexed 2-color reverse transcription–PCR assay with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal positive control. We used PTC1- and PTC3-specific forward PCR primers with a common RET reverse primer and 6-carboxyfluorescein (FAM)–labeled TaqMan probe (Applied Biosystems), whereas GAPDH was detected with a red fluorescent–labeled probe (Texas Red; Biosynthesis, Inc). The primer and probe sequences included H4 (PTC1) forward primer AAAGCCAGCGTGACCATC, ELE1 (PTC3) forward primer TGGCTTACCCAAA AGCAGAC,
Table 1. Primers Used for Gene Amplification

<table>
<thead>
<tr>
<th>Gene</th>
<th>Exon</th>
<th>Primer Sequence</th>
<th>Orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRAF</td>
<td>11</td>
<td>5'-TCCTTTGCTGTCAGGACAGCT-3'</td>
<td>Sense</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-GAGATCTAGCCATCTGCTTCT-3'</td>
<td>Antisense</td>
</tr>
<tr>
<td>KRAS</td>
<td>1</td>
<td>5'-TTACAAGTATGTGACAGTCAGTCAGTCT-3'</td>
<td>Sense</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-TGGCATTCCCTGTGGTTTTT-3'</td>
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</tr>
<tr>
<td>HRAS</td>
<td>1</td>
<td>5'-GCCAGAGACCTGCTGAGAGG-3'</td>
<td>Sense</td>
</tr>
<tr>
<td></td>
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<td>5'-AGGCTCATCTGCTGCTTG-3'</td>
<td>Antisense</td>
</tr>
<tr>
<td>NRAS</td>
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<td>5'-GATCAGGCTGTGCTGCTG-3'</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>5'-GATGAAACACACACAGAAA-3'</td>
<td>Antisense</td>
</tr>
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RET reverse primer GTTGCCCTTG ACCACCTTTC, RET probe 5'-FAM-CCAAAGTGG GAATTCCTCCTGGA-3'1ABKFQ, GAPDH forward primer ACTGGCAGATCCTTCTCTTCC, GAPDH reverse primer GCCCAATACGACCAATCTC, and GAPDH probe 5'-5'Texrd-NN/TGG GGA AGG TGA AGG TCG GA/3IAbRQSp/-3'. The PCR reactions were performed in an instrument using a 20-µL reaction volume, with the product's probes master reaction mix (LightCycler 480 instrument and mix; Roche) and complementary DNA template derived from 40 to 200 ng total of RNA. The GAPDH probe was used at 0.15µM, and all other primers and probes were used at a final concentration of 0.3µM each. Cycling conditions included an initial 10-minute denaturing step at 95°C followed by 40 cycles of 95°C for 10 seconds and 60°C for 20 seconds. Samples that scored positive for the RET-PTC1 and RET-PTC3 multiplex were retested with each primer pair individually to determine the RET fusion partner.

ASSAY DESIGNER SOFTWARE was used to design assay multiplexes targeting mutations in known cancer genes, and assays were performed according to manufacturer’s instructions (TypePLEX; Sequenom, Inc). Initial PCR reactions used 10 ng of DNA per multiplex in a total volume of 5 µL, with 100nM primers, 2mM magnesium chloride, 500mM deoxynucleotide triphosphate, and 0.1 U Taq polymerase. Amplification included 1 cycle of 94°C for 4 minutes followed by 45 cycles of 94°C for 20 seconds, 56°C for 30 seconds, and 72°C for 1 minute and 1 final cycle of 72°C for 3 minutes. Unincorporated nucleotides were inactivated by addition of 0.3 U shrimp alkaline phosphatase and incubation at 37°C for 40 minutes, followed by heat inactivation of shrimp alkaline phosphatase at 85°C for 5 minutes. Single base primer extension reactions were performed with 0.625mM to 1.250mM extension primer and 1.35 U TypePLEX DNA polymerase (Thermo Sequenase; GE Healthcare). Extension cycling included 1 cycle of 94°C for 30 seconds, 40 cycles of 94°C for 5 seconds, 5 cycles of 52°C for 5 seconds and 80°C for 5 seconds, and 1 cycle of 72°C for 3 minutes. Extension products were purified with an ion exchange resin, and approximately 10nL of product was spotted on bioarray matrices (SpectroChip II; Sequenom, Inc). A matrix-assisted laser (Bruker Corporation) desorption/ionization time-of-flight mass spectrometer (MassARRAY Compact; Sequenom, Inc) was used to resolve extension products. Software from the manufacturer (MassARRAY Typer Analyzer software; Sequenom, Inc) was used for automated data analysis, accompanied by visual inspection of extension products.

We identified 389 patients with PTC from our institution’s pathology records. A total of 209 met the inclusion criteria; 158 had node-positive (N1) tumors and 51 had node-negative (N0) tumors.

STATISTICAL ANALYSIS
Statistical analyses were performed using the χ² test to evaluate for frequency differences on univariate analyses and the Fisher exact test when expected values were less than 5. Separate subanalyses were performed to control for tumor histological type. We calculated 2-tailed P values and considered P < .05 as statistically significant. Odds ratios were calculated using univariate logistic regression with confidence intervals set at 95%.

RESULTS
We identified 389 patients with PTC from our institution’s pathology records. A total of 209 met the inclusion criteria; 158 had node-positive (N1) tumors and 51 had node-negative (N0) tumors.

DEMOGRAPHICS AND TUMOR CHARACTERISTICS
No significant difference was found in age between groups. A higher proportion of male patients were found in the N1 group (45 of 158 [28.5%] vs 7 of 51 [13.7%]); thus, male sex was found to be a predictor of metastasis (OR, 2.50; 95% CI, 1.05-5.97). The follicular variant PTC histological type was present in 4.4% of N1 and 47.1% of the N0 tumors and thus was strongly associated with lack of central neck metastasis in this study (OR, 0.05; 95% CI 0.02-0.14). In light of these findings, the data were then analyzed according to classic PTC histological type. Tumor size was significantly different between the 2 groups, with larger tumor size (>2.0 cm) predictive of increased risk of tumor metastasis in the overall group (OR, 2.60; 95% CI, 1.36-5.00). For pure classic PTC, although the same trend was preserved, it did not attain statistical significance (OR, 2.19; 95% CI, 0.95-5.10). The T3 and T4 tumors were associated with increased risk of metastasis overall (OR, 3.80; 95% CI, 1.74-8.35) and for classic PTC (OR, 3.00; 95% CI, 1.53-14.05).

Extracapsular extension was a predictor of LNM overall (OR, 5.30; 95% CI, 2.25-12.53) and for classic PTC.
type (OR, 4.94; 95% CI, 1.62-15.00). Angiolympathic invasion was also a predictor of LNM for all histological types (OR, 6.15; 95% CI, 2.22-17.01) and for classic PTC histological type (OR, 15.70; 95% CI, 2.02-122.12). Multifocality was not a predictor of LNM for all patients (OR, 1.66; 95% CI, 0.87-3.17) and for classic PTC (OR, 1.15; 95% CI, 0.50-2.65). The demographic and histological characteristics with their risk of LNM are summarized for classic PTC in Table 2.

### MUTATIONS

The results of mutations in N0 and N1 are summarized in Table 3 (all histological types) and Table 4 (classic PTC only). The BRAF mutation was more likely to be associated with the classic PTC histological type than the follicular variant in the N0 and N1 groups (P < .001). All molecular marker mutations, including BRAF, did not predict LNM for classic PTC (OR, 0.48; 95% CI, 0.15-1.49). Specifically, BRAF mutations were found in 113 of 158 N0 tumors of all histological types combined (71.5%), but they were found in 109 of 148 N0 tumors of the classic PTC histological type (73.6%). In N1 tumors, BRAF mutations represented 71.5% of mutations for all histological types and 73.6% for the classic histological type. These results are shown in the Figure.

### COMMENT

Elective CLND remains controversial in the management of PTC. Lymph node metastases have been shown to be associated with higher rates of local recurrence in multiple studies, and recent large retrospective studies suggest increased mortality with CLN involvement. Furthermore, a recent large retrospective study by Grant et al suggests that elective CND may add a survival benefit to patients with PTC.

In addition, routine ipsilateral dissection can improve staging accuracy and help guide decisions for adjuvant radioactive iodine treatment. For example, Bonnet et al demonstrated that routine CLND plus selective levels 3 and 4 lateral neck dissection in T1 tumors changed the indication for radioactive iodine treatment in 30.5% of their patients. Finally, CLND has been shown to decrease postoperative thyroglobulin levels with implications for postoperative thyroglobulin monitoring and surveillance, and this decrease may affect prognosis.

Arguments against routine CLND include the increased risk of injury to the parathyroid glands with resulting hypocalcemia and recurrent laryngeal nerve injury, although some series demonstrate no differences in morbidity. Although data are conflicting, most thyroidectomies (>70%) are not performed by surgeons who perform a large volume of procedures, and a higher risk of morbidity may result when surgeons who do not routinely operate in this region perform the paratracheal dissection.

Given this controversy, the purpose of our study was to evaluate whether analysis of mutations in tumors might assist in the selection of patients for elective CLND. Our results showed that positive risk factors for central LNM include male sex, the classic variant of PTC, extracapsular extension, angiolympathic invasion, and advanced T stage. The presence of a BRAF mutation was...
not associated with increased metastasis when controlling for tumor histological type. We found a significant difference in LNM according to tumor histological type. Nearly half of our N0 group was found to have the follicular variant histological type (47.1%) compared with only 4.4% in the N1 group. Therefore, the follicular variant tumors were far less likely to be associated with central neck metastasis. This finding is consistent with those of several studies. In one of the first studies on this topic, Jain et al28 demonstrated that the follicular variant was associated with a significantly lower incidence of metastatic nodes compared with classic PTC (5.6% vs 35.7%; P < .001). This finding highlights the importance of stratifying by tumor type when evaluating the impact of mutations and could also explain the inconsistencies in results of other reports that have evaluated the relationship between BRAF and LNM. In contrast to many recent studies, we found that BRAF mutation was not significantly associated with LNM when only the classic PTC histological type was analyzed and a rigorous definition of the N0 status was used. These results are consistent with the study by Jung et al29 that demonstrated no difference in LNM according to BRAF status in a population of 210 patients when performing a standardized neck dissection and controlling for tumor type.

One factor that may explain the difference between our findings and others is the fact that our study used a strict definition of a complete ipsilateral CLND for inclusion in the study. One cannot be certain that the lymph node status is truly N0 unless all nodes in the at-risk basin are analyzed. An analysis based on review of the pathology report that indicates N0 status may not be accurate because it is not uncommon for perithyroid lymph nodes to be removed with thyroidectomy, and the fact that a few of these nodes did not contain metastatic disease does not necessarily indicate a true N0 status. Inclusion of such patients who underwent an incomplete neck dissection may result in a high rate of false-negative findings. A commonly cited study by Lupi et al30 from Italy investigating the role of BRAF in tumor aggressiveness reported a relatively low prevalence of lymph node metastases of only 11% in their cohort; however, the completeness of CND in their patient population is unclear. Furthermore, although their univariate analysis suggested a strong correlation of LNM with BRAF mutation, this was not a significant predictor in their multivariate analysis, in which only absence of a tumor capsule was correlated. In the meta-analysis published by Xing9 in 2007, the study by Lupi et al30 was one of the largest studies included but only the univariate data were used, potentially skewing the results of that meta-analysis. A second large study in the meta-analysis also lacked a standardized definition of CND.31 Therefore, we included in our N0 group only those patients who underwent a standardized central compartment dissection, consisting of removal of the pretracheal nodes and ipsilateral paratracheal nodes inferiorly from the level of the innominate artery to the hyoid superiorly and to the carotid artery laterally.

A second reason for the difference in our findings about mutations and lymph node metastases may be the relationship between the BRAF gene and PTC histological type. The BRAF mutation appears to be prevalent in tumors with classic histopathological features but is far less prevalent in the follicular variant type.32 Because follicular variant tumors tend to be less likely to result in LNM, failure to stratify by tumor histological type is a possible confounder in many studies.33,34 Although the numbers were limited, we observed that RAS mutations were more prevalent in the follicular variant of PTC, which is also consistent with the findings of several studies. Zhu et al35 examined the clinicopathological characteristics of 30 cases with the follicular variant histological type and revealed a prevalence of RAS mutations of 13 of 30 (43%) compared with 0 of 46 (0%) of the classic histological type. In addition, the follicular variant PTC cases in that study also demonstrated a lower rate of LNM.

In conclusion, the results of the present study support a correlation between the follicular variant PTC histological type and lack of LNM. The results did not support the notion that BRAF V600E mutations are associated with increased LNM when only the classic PTC histological type group is analyzed and when using a rigorous definition of the N0 status. Further research is needed to identify molecular markers that may be associated with the risk of LNM.

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Author Contributions: Drs Paulson, Schuff, and Corless had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Paulson, Shindo, Schuff, and Corless. Acquisition of data: Paulson and Schuff. Analysis and interpretation of data: Paulson, Shindo, Schuff, and Corless. Drafting of the manuscript: Paulson. Critical revision of the manuscript for important intellectual content: Shindo, Schuff, and Corless. Obtained funding: Corless. Administrative, technical, and material support: Shindo, Schuff, and Corless. Study supervision: Shindo and Schuff.

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REFERENCES


4. American Thyroid Association (ATA) Guidelines Taskforce on Thyroid Nodules and Differentiated Thyroid Cancer; Cooper DS, Doherty GM, Haugen BR, et al. Revised American Thyroid Association management guidelines for patients with


